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Study of the molecular mechanism involved in recycling of matrix protein receptor, PEX5, during glycosome biogenesis in *Trypanosoma brucei*

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Table of contents

Summary IX

Résumé XII

Abbreviations XV

Chapter 1: Introduction 1

1. Human African Trypanosomiasis 1
2. *Trypanosoma brucei* life cycle 7
3. Cell biology of *T. brucei* 9
4. Glycosome and peroxisome biogenesis 13
   - 4.1.1. Peroxisome membrane formation 14
   - 4.1.2. Peroxisome membrane protein import 16
   - 4.1.3. Peroxisome matrix protein import 16
     - 4.1.3.1.1. Targeting signal-dependent cargo recognition 17
     - 4.1.3.1.2. Docking of the receptor-cargo at the peroxisomal membrane 18
     - 4.1.3.1.3. Translocation and cargo release 19
     - 4.1.3.1.4. Receptor ubiquitination and recycling 21
5. The discovery of PEX4 and its role in peroxisome biogenesis 26
6. The ubiquitin-conjugating enzyme PEX4: structural features 35
7. Peroxisomal matrix protein receptor PEX5 40
8. State of the art in the research on glycosomal matrix protein import 43
9. The objective of the research described in this thesis 49

Chapter 2: Invited review. When, how and why glycolysis became compartmentalized in the Kinetoplastea. A new look at an ancient organelle 53

Chapter 3: The function of glycosomes in the metabolism of trypanosomatid parasites and the promise of glycosomal proteins as drug targets, Chapter in the book "Drug discovery for trypanosomatid diseases" 75

Chapter 4: General methodologies 121

1. Trypanosomes *in vitro* cultures 123
2. Transfections 123
3. Growth measurements 124
4. Molecular biological techniques 125
   - 4.1. Bacterial strains 125
   - 4.2. Yeast strains 126
   - 4.3. Gene cloning procedures 126
   - 4.4. Genome integration analysis 132
   - 4.5. Semi-quantitative RT-PCR and qPCR 132
5. Immunological techniques 134
   - 5.1. Antibodies production 134
   - 5.2. Western blot analysis 134
   - 5.3. Immunoprecipitation 135
     - 5.3.1.1. Native immunoprecipitation 135

5.3.1.2. Denaturing immunoprecipitation 135
5.4. Immunofluorescence 136

6. Biochemical techniques 137
6.1. Differential centrifugation 138
6.2. Isopycnic centrifugation 138
6.3. Selective permeabilization with digitonin 139
6.4. Bacterial recombinant protein expression and purification 139
6.4.1.1. Native purification 140
6.4.1.2. Inclusion bodies purification 141
6.5. TbPEX5 antibodies purification 142
6.6. TAP-tag purification of TbPEX22 142
6.7. Protease protection assay 143
6.8. PTS-1 *in vitro* binding assay 143
6.9. Protease susceptibility studies 144
6.10. Protein turnover analysis 145
6.11. *In vitro* import and export of TbPEX5 146
6.12. Pulse-chase experiments 146
6.13. Proteasome inhibition and cellular fractionation 147
6.14. RNAi experiments 147
7. Live cell imaging 148
8. Transmission electron-microscopy 148
9. Scanning electron-microscopy 149
10. Mass spectrometry 149
11. Yeast two-hybrid experiments 151

Chapter 5: Results and discussion 153

1. Determination of the nature of different TbPEX5 forms in wild-type *T. brucei* 154
1.1. PEX5 antibody purification and western blot analysis 155
1.2. TbPEX5 expression silencing by RNA interference 156
1.3. Mass spectrometry analysis of immunoprecipitated TbPEX5 158
1.4. Behaviour of recombinant TbPEX5 in SDS-PAGE 159
1.5. TbPEX5 susceptibility to parasite endogenous proteases 161
1.6. Subcellular distribution of TbPEX5 and its association with glycosomes 162
1.7. Functional study of the different molecular weight forms of TbPEX5 by PTS-1 *in vitro* binding assays 164
1.8. Turnover analysis of different molecular weight bands of TbPEX5 165
1.9. Metabolic labelling of TbPEX5 168
2. PEX5 Ubiquitination and its role in the receptor recycling 169
2.1. Ubiquitination studies in wild-type bloodstream and procyclic form *T. brucei*. 177
3. Identification and characterization of additional components involved in PEX5 recycling 177
3.1. Sequence homology searching: TbPEX4 and TbPEX22 180
3.2. *In silico* interaction studies of TbPEX4-TbPEX22 184
3.3. Protein-protein interaction studies 187
3.4. Expression and purification of recombinant peroxins TbPEX4 and TbPEX22. Production of antisera 188
3.5. Subcellular localization: Differential and isopycnic centrifugation and confocal fluorescence microscopy 197
3.6. Functional analysis 197
3.6.1.1. Construction of cell lines for RNAi against PEX4 and PEX22 and

phenotypic analysis. 197
3.6.1.2. Yeast mutant complementation 201
3.6.1.3. Knock-out of the TbPEX4 gene 206
3.6.1.3.1.1. Glycosome-independent morphology phenotype 225
4. Ubiquitination of TbPEX5 in mutants of the recycling complex 232

Chapter 6: Research paper. Channel-forming activities in the glycosomal fraction from the bloodstream form of *Trypanosoma brucei* 239

Chapter 7: General discussion: Conclusions, discussions and perspectives 255

1. Glycosomal matrix protein import 257
2. Summary of surprising features of the procyclic ΔPEX4 mutant – possible explanations 268
3. Channel-forming activities in glycosomes 271
4. Drug discovery aspects 276
   4.1. Glycosome biogenesis as drug target 276
   4.2. Solute translocation across the glycosomal membrane as drug target 280
   4.3. Glycosomal proteins as drug targets 281
   4.4. How drugs can reach their targets in the glycosome; their import into trypanosomes and import into glycosomes 282
5. The origin and evolution of peroxisome and glycosomes 285

References 287

Curriculum Vitae 297

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Summary

Trypanosoma brucei is the parasitic protist that is responsible for human sleeping sickness in Africa, a disease for which no adequate, affordable and harmless treatment is available. The parasite lives as a so-called procyclic-form trypanosome in the midgut of the tsetse fly, the vector that transmits the trypanosomes between people. It has been previously established that glycolysis is essential for the bloodstream form of the parasite, its life-cycle stage in humans, hence representing a promising drug target. Glycolysis in trypanosomatids is compartmentalized in peroxisome-like organelles called glycosomes, a unique feature not found in cells of other eukaryotes. Biogenesis of peroxisomes, organelles found in most eukaryotes, and of glycosomes in trypanosomatids are mediated by homologous proteins called peroxins (acronym PEX). The peroxins of trypanosomes are considered potentially good drug targets not only because glycolysis is essential for the parasites, but also because trypanosomes die when proper compartmentalization of glycolytic enzymes within glycosomes is disrupted.

The import of proteins into the glycosomal matrix involves a cytosolic receptor, PEX5, which recognizes the peroxisomal-targeting signal type 1 (PTS-1) present at the C-terminus of the majority of these proteins. In yeasts and mammalian cells it has previously been shown that the cargo-loaded PEX5 associates with the peroxisomal membrane, delivers its cargo and is then ubiquitinated, a modification that serves as a signal for retrieval of PEX5 from the organelle to be used for further cycles of import (monoubiquitination) or, when recycling is impaired, for its proteasome-dependent degradation (polyubiquitination). We have found stable monoubiquitinated PEX5 in cytosolic fractions of wild-type bloodstream- and procyclic-form *T. brucei*. This modification appeared to be resistant to DTT, suggesting the conjugation of an ubiquitin moiety to a lysine residue of PEX5. We reason that this modified PEX5 species represents recycled molecules that have been efficiently exported by the recycling peroxin complex and that are in transit to be deubiquitinated, as a physiological step in the receptor cycle. We have identified the *T. brucei* orthologue of PEX4, the

ubiquitin-conjugating (UBC) enzyme responsible for PEX5 monoubiquitination in yeast. This protein is expressed in both bloodstream and procyclic forms and is associated with the cytosolic face of the glycosomal membrane, probably via its interaction with the putative TbPEX22 that we also identified. Creation of a ΔPEX4 procyclic cell line by deletion of both alleles of the *TbPEX4* gene enabled us to demonstrate that this peroxin is involved in TbPEX5 monoubiquitination. Surprisingly, after transfection of this mutant with a construct for expression of the Green Fluorescent Protein with a PTS-1 followed by subcellular localization studies by live cell imaging and fluorescence microscopy, only a minor defect in glycosomal matrix protein import was observed. Analysis of the ΔPEX4 mutant by qPCR showed that other enzymes of the putative UBC repertoire were upregulated. We assume that another trypanosome-specific UBC protein has taken over the function of PEX4 in the monoubiquitination of PEX5, but with less efficiency. Interestingly, important defects in morphology and motility were found in the majority of the ΔPEX4 cells. We hypothesize that these seemingly glycosome-biogenesis unrelated defects resulting from the knockout of the *TbPEX4* gene could be explained by a disregulation of the expression of UBCs that are orthologous to yeast and human UBCs involved in cell-cycle control.

We have also observed that PEX5 ubiquitination was affected in procyclic cell lines in which TbPEX12 and TbPEX6 expression was decreased by RNA interference. These data thus indicate the involvement of these proteins in TbPEX5 cycling, comparable to what has previously been demonstrated for their counterparts in yeasts and mammalian cells, but nonetheless with some differences as suggested by our studies. TbPEX12 and TbPEX6 were shown to be necessary for correct glycosomal matrix protein import and for cell viability, proving that the TbPEX5 recycling process is also essential.

In addition, we have investigated the presence of channel-forming activities in glycosomal membrane preparations of *T. brucei* bloodstream forms. We found that glycosomal membrane proteins, when reconstituted in planar lipid bilayers, gave rise to the formation of three main kinds of channels. These were revealed by electrophysiological techniques. Currents were measured with amplitudes of

70-80, 20-25 and 8-11 pA (at a holding potential of +10 mV and with 3.0 M KCl as an electrolyte). The channels were in a fully open state over the membrane potential range +150 to -150 mV and showed no sub-conductance transitions. The channel with current amplitude of 20-25 pA is anion-selective while the other two types are slightly selective for cations. The anion-selective channel showed an intrinsic current rectification that may suggest a functional asymmetry of the channel's pore. We discuss the relevance of these findings for the transport of glycolytic intermediates and other metabolites between the glycosomal lumen and cytosol. We propose different explanations for the apparent paradox of on one hand the presence of non-selective channels in the glycosomal membrane and on the other hand the experimentally proved low exchange of metabolites between the glycosomal matrix and cytosol. Importantly, the discovery of these non-selective channels in the glycosomal membrane of *T. brucei* may set new criteria for the size and physicochemical properties of inhibitors targeted to glycolytic and other enzymes inside glycosomes that will be designed as potential drugs for sleeping sickness.

Résumé

Trypanosoma brucei est le protiste parasite responsable de la maladie du sommeil en Afrique ; maladie pour laquelle il n'existe aucun traitement adéquat, abordable et inoffensif. Le parasite vit sous la forme procyclique dans l'intestin de la mouche tsé-tsé, le vecteur qui transmet les trypanosomes entre les hommes. Il a été établi précédemment que la glycolyse est essentielle pour la forme sanguicole du parasite, stade du cycle de vie chez l'homme, et représente donc un cible thérapeutique prometteuse. La glycolyse des trypanosomatidés est compartimentée dans des organites de la famille des peroxisomes appelés glycosomes, une caractéristique unique que l'on ne trouve pas dans les cellules des autres eucaryotes. La biogenèse des peroxisomes, organites trouvés chez la plupart des eucaryotes, et celle des glycosomes des trypanosomatidés sont médiées par des protéines homologues appelées peroxines (PEX en abrégé). Les peroxines des trypanosomes sont considérées comme de bonnes cibles thérapeutiques potentielles, non seulement car la glycolyse est essentielle pour les parasites, mais également parce que les parasites meurent quand la compartimentation des enzymes glycolytiques dans les glycosomes est perturbée.

L'importation des protéines dans la matrice glycosomale implique un récepteur cytosolique, PEX5, qui reconnaît le signal de ciblage vers les peroxisomes de type 1 (PTS1). Dans les levures et les cellules mammifères, il a déjà été démontré que le récepteur chargé de sa protéine s'associe avec la membrane peroxisomale, livre son chargement et est ensuite ubiquitylé, une modification qui sert de signal pour la sortie de PEX5 de l'organite afin d'être réutilisé dans d'autres cycles d'importation (monoubiquitination) ou quand le recyclage n'est pas possible, pour sa dégradation dépendante du protéasome (polyubiquitination). Nous avons trouvé une PEX5 monoubiquitinée stable dans les fractions cytosoliques des formes sanguicol et procyclique sauvages de *T. brucei*. Cette modification semble être résistante au DTT, suggérant la liaison du groupement ubiquitine à une lysine de PEX5. Nous estimons que cette forme de PEX5 modifiée représente des molécules recyclées qui ont été exportées efficacement

pour le recyclage du complexe de peroxines et qui sont en transit vers une désubiquitination, comme une étape physiologique dans le cycle du récepteur. Nous avons identifié chez *T. brucei*, l'orthologue de PEX4, l'enzyme de liaison de l'ubiquitine (UBC) responsable de la monoubiquitination de PEX5 chez la levure. Cette protéine est exprimée dans les formes procyclique et sanguicole et est associée à la face cytosolique de la membrane glycosomale, probablement via une interaction avec la probable TbPEX22 que nous avons également identifiée. La création d'une lignée cellulaire procyclique ΔPEX4 par délétion des deux allèles du gène *TbPEX4* nous a permis de démontrer que cette peroxine est impliquée dans la monoubiquitination de TbPEX5. Etonnament, après la transfection de ce mutant avec une construction exprimant la « Green Fluorescent Protein » munie d'un PTS-1 et des études de localisation subcellulaire par des images de cellules vivantes et de la microscopie à fluorescence, seule une petite diminution de l'importation des protéines matricielles dans les glycosomes est observée. L'analyse du mutant ΔPEX4 par qPCR a montré que d'autres enzymes du répertoire probable des protéines UBC sont sur-régulées. Nous supposons qu'une autre protéine UBC a repris la fonction de PEX4 dans la monoubiquitination de PEX5, mais de manière moins efficace. Il est intéressant de noter que des défauts importants dans la morphologie et la mobilité ont été trouvés dans la majorité des cellules ΔPEX4. Nous supposons que ces défectuosités, qui résultent de l'élimination du gène *TbPEX4 et* qui ne semblent pas être liées à la biogenèse des glycosomes, peuvent être expliquées par une dérégulation de l'expression d'UBCs qui sont des orthologues des UBCs humaines et de levures impliquées dans le contrôle du cycle cellulaire.

Nous avons également observé que l'ubiquitination de PEX5 était affectée dans des lignées cellulaires procycliques dont l'expression de TbPEX12 et TbPEX6 avaient été réduites par interférence à l'ARN. Ces résultats indiquent donc un rôle de ces protéines dans le cycle de TbPEX5, comparable à ce qui avait déjà été démontré pour les protéines équivalentes dans la levure et les cellules mammifères, mais cependant avec quelques différences comme le suggèrent nos études. Il a été montré que TbPEX12 et TbPEX6 sont nécessaires pour l'importation correcte des protéines de la matrice glycosomale et pour la

viabilité cellulaire, prouvant que le recyclage de TbPEX5 est également essentiel.

De plus, nous avons étudié la présence d'activités de formation de canaux dans des préparations de membranes glycosomales des formes sanguicoles de *T. brucei*. Nous avons trouvé que les protéines de la membrane glycosomale, quand elles sont reconstituées dans des doubles couches lipidiques planes, permettaient la formation de trois types de canaux. Ils ont été révélés par des techniques d'électrophysiologie. Des courants d'amplitudes de 70-80, 20-25 et 8-11 pA (à un potentiel fixe de +10 mV et avec 3.0 M KCl comme électrolyte) ont été mesurés. Les canaux étaient dans un état complètement ouvert dans une gamme de potentiels de membrane de +150 à –150 mV et ne montraient pas de transition de sous-conductance. Le canal, avec un courant d'amplitude de 20-25 pA, est sélectif pour les anions, tandis que les deux autres sont légèrement sélectifs pour les cations. Le canal sélectif pour les anions a montré un courant de rectification intrinsèque qui pourrait suggérer une asymétrie fonctionnelle du pore du canal. Nous discutons la pertinence de ces découvertes pour le transport des intermédiaires glycolytiques et d'autres métabolites entre la lumière du glycosome et le cytosol. Nous proposons différentes explications pour le paradoxe apparent où d'un côté on a la présence de canaux non sélectifs dans la membrane glycosomale et de l'autre côté de faibles échanges de métabolites entre la matrice du glycosome et le cytosol prouvés expérimentalement. Il est important de noter que la découverte de ces canaux non sélectifs dans la membrane glycosomale de *T. brucei* met en place de nouveaux critères pour la taille et les propriétés physicochimiques des inhibiteurs dirigés contre la glycolyse et d'autres enzymes à l'intérieur des glycosomes qui seront modélisés comme médicaments potentiels pour la maladie du sommeil.

List of Abbreviations

AAA	ATPases associated with diverse cellular activities
ABC	ATP-binding cassette
ADH	Alanine dehydrogenase
ADP	Adenosine diphosphate
AEBSF	4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride
ALD	Aldolase
AMP	Adenosine monophosphate
AO	Alternative oxidase
APC/c	Anaphase-promoting complex/cyclosome
ATG	Autophagy-related gene or protein
ATP	Adenosine triphosphate
bp	Base pairs
BLAST	Basic Local Alignment Search Tool
BSA	Bovine serum albumin
CATT	Card agglutination test for trypanosomiasis
CDC	Centers for Disease Control and Prevention
CHAPS	3-[(3-Cholamidopropyl)dimethylammonio]-1- propanesulfonate
CNBr	Cyanogen bromide
CoA	Coenzyme A
Da	Dalton
DAPI	4',6-diamidino-2-phenylindole
DEAE	Diethylaminoethyl
DHAP	Dihydroxyacetone phosphate
DNDi	Drugs for Neglected Diseases initiative
OD	Optical density
DTT	Dithiothreitol
E1	Ubiquitin-activating enzyme
E2	Ubiquitin-conjugating enzyme
E3	Ubiquitin-ligase enzyme
ED_{50}	Effective dose (giving 50% effect)
EDTA	Ethylene diamine tetraacetic acid
EGFP	Enhanced green fluorescent protein
EGTA	Ethylene glycol tetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ENO	Enolase
ER	Endoplasmic reticulum
ERAD	Endoplasmic reticulum degradation pathway
FAZ	Flagellum attachment zone
FH	Fumarate hydratase
FRD	NAD-dependent fumarate reductase
FBPase	Fructose-1,6-bisphosphatase
G3PDH	Glycerol-3-phosphate dehydrogenase
G3P	Glycerol 3-phosphate
G6P	Glucose 6-phosphate
α-G6P	α-Anomer of glucose 6-phosphate
G6PDH	Glucose-6-phosphate dehydrogenase
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase

cGAPDH	Cytosolic glyceraldehyde-3-phosphate dehydrogenase
gGAPDH	Glycosomal glyceraldehyde-3-phosphate dehydrogenase
GDP	Guanosine diphosphate
GFP	Green fluorescent protein
GK	Glycerol kinase
GlcK	Glucokinase
GMD	GDP-mannose dehydratase
GNA	Glucosamine 6-phosphate N-acetyltransferase
GPO	Glycerol-3-phosphate oxidase
HAT	Human African Trypanosomiasis
His-tag	6X-histidine-tag
HGPRT	Hypoxanthine-guanine phosphoribosyltransferase
HXK	Hexokinase
HK1	Hexokinase isoform 1
HK2	Hexokinase isoform 2
HTS	High-throughput screening
IC_{50}	Inhibitory concentration (giving 50% inhibition)
IgG	Immunoglobulin G
IPTG	isopropyl β-D-thiogalactanopyranoside
I-PTS	Internal peroxisomal targeting signal
kDa	Kilodalton
Ki	Inhibition constant
LECA	Last Eukaryotic Common Ancestor
LB	Luria Bertani medium
MCF	Mitochondrial carrier family
MDH	Malate dehydrogenase
MK	Mevalonate kinase
Myc	Myelocytomatosis viral oncogene
MTOC	Microtubule organizing center
NAD	Nicotinamide adenine dinucleotide
NADH	Reduced Nicotinamide adenine dinucleotide
NADP	Nicotinamide adenine dinucleotide phosphate,
NADPH	Reduced Nicotinamide adenine dinucleotide phosphate,
NEM	N-ethylmaleimide
PCR	Polymerase Chain Reaction
PBS	Phosphate buffer saline
PEP	Phosphoenolpyruvate
PEPCK	PEP carboxykinase
PEX	Peroxisome (-related gene/protein)
PFK	Phosphofructokinase
1,3PGA	1,3-Bisphosphoglycerate
3PGA	3-Phosphoglycerate
PGAM	Phosphoglycerate mutase
6PGDH	6-phosphogluconate dehydrogenase
PGK	Phosphoglycerate kinase
Pi	Inorganic phosphate
pI	Isoelectric point
PMP(s)	Peroxisomal membrane protein(s)
PMSF	Phenylmethanesulfonylfluoride

PARP	Procyclic acidic repetitive protein
PPDK	Pyruvate phosphate dikinase
PPi	Inorganic pyrophosphate
PPP	Pentose-phosphate pathway
PRF	Paraflagellar rod
PTS-1	Peroxisomal targeting signal type 1
PTS-2	Peroxisomal targeting signal type 2
PYK	Pyruvate kinase
qPCR	Real-time polymerase chain reaction
RING	Really interesting new gene
RIPA	Radioimmunoprecipitation
RNAi	RNA interference
rpm	Rotations per minute
RT-PCR	Reverse transcription PCR
SAR	Structure-activity relationship
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SH3	Src homology 3
SHAM	Salicylhydroxamic acid
SIF	Stumpy induction factor
SQS	Squalene synthase
TAP	Tandem affinity purification
TCA	Trichloroacetic acid
TCA cycle	Tricarboxylic acid cycle
TEA	Triethanolamine
TEV	Tobacco etch virus
Tet	Tetracycline
TPI	Triosephosphate isomerase
TPR	Tetratricopeptide repeat
TR	Trypanothione reductase
UAP	UDP-N-acetylglucosamine pyrophosphorylase
UBC	Ubiquitin-conjugating enzyme family member
UDP	Uridine diphosphate
UGE	UDP-glucose 4'-epimerase
UGP	UDP-glucose pyrophosphorylase
UMP	Uridine 5-monophosphate
UTP	Glucose-1-phosphate uridylyltransferase
UTR	Untranslated region
VSG	Variant surface glycoprotein
WD	Tryptophan/Aspartic
WHO	World Health Organization
XPRT	Xanthine phosphoribosyltransferase
YPD	Yeast Extract Peptone Dextrose
ZPFM	Zimmerman post-fusion medium

Throughout the text we have chosen to use the consensus nomenclature for the Kinetoplastea, also used for many other protists, where the protein is indicated as uppercase and the gene is italicized uppercase (Clayton *et al.*, 1998). Also the

nomenclature used in this thesis for the peroxin genes and proteins of all organisms has, for convenience, been adapted to the kinetoplastid system.
The classification and nomenclature for kinetoplastids is used as proposed by Moreira *et al*. (2004).

Chapter 1

Introduction

Human African Trypanosomiasis

Sleeping sickness or Human African Trypanosomiasis (HAT) is an infectious disease caused by protist parasites belonging to the genus *Trypanosoma* and transmitted by the bites of tsetse flies. The subspecies *Trypanosoma brucei rhodesiense* causes an acute form of the disease affecting people in countries of east and southern Africa, while *T. b. gambiense* is responsible for the chronic form in west and central Africa (Fig. 1). A third subspecies, *T. b. brucei* is the causative agent of 'Nagana' in cattle, but does not affect humans. The vectors are tsetse flies belonging to the genus *Glossina* (Fig. 2A) (Williams, 1996). The geographic distribution of sleeping sickness is linked to the suitable habitats for the tsetse fly.

It was Sir David Bruce (Fig. 3) who identified in 1895 trypanosomes as the cause of sleeping sickness in humans and Nagana in cattle. Sleeping sickness threatens millions of people in sub-Saharan Africa. This disease was until recently endemic in 36 countries of which the 7 most affected ones represented 97% of all reported cases with the Democratic Republic of the Congo (DRC) alone accounting for 2/3 of all reported cases (DNDi, 2012). For the first time in 50 years, the number of new cases reported in 2009 and 2010 has dropped to less than 10,000 cases per year. However this drop is being observed with cautious optimism since the disease has in the past been almost brought under control, but has subsequently regained strength again (Kennedy, 2008). The number of people at risk remains 60 million in most of Africa south of the Sahara. It is estimated that currently there are some 30,000 infected people. Many of the affected populations live in remote areas with limited access to adequate health services, which debilitates the surveillance and therefore the diagnosis and treatment of cases. In addition, displacement of populations, war and poverty are important factors leading to increased transmission and this may alter the distribution of the disease due to weakened or non-existent health systems (WHO, 2012).

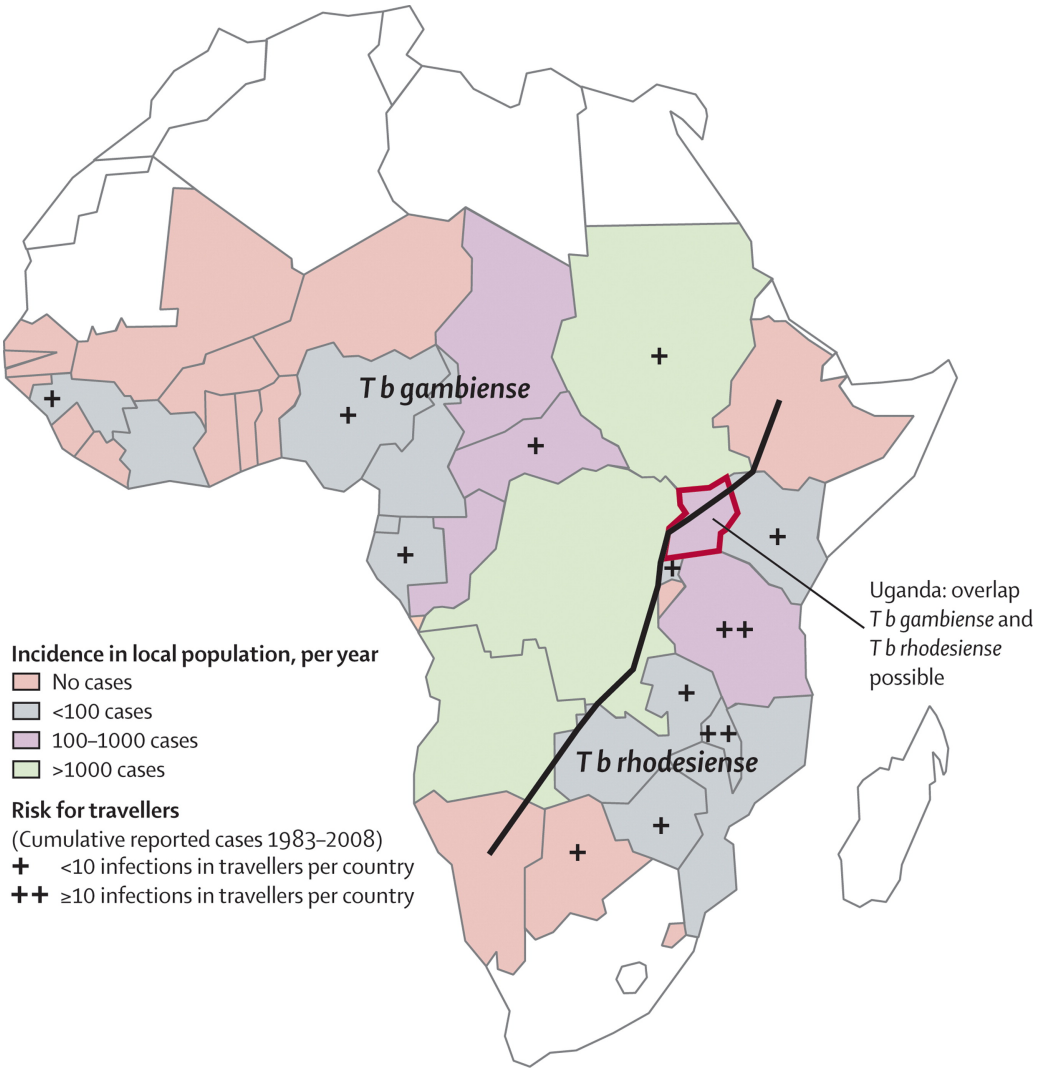

Figure 1. ***Distribution of human African trypanosomiasis.*** The black line divides the areas in which *Trypanosoma brucei gambiense* prevails and those in which *Trypanosoma brucei rhodesiense* predominates. The incidence of risk for travelers is shown. (Taken from Brun *et al.,* 2010)

There are two distinct stages during the course of sleeping sickness. The first or early stage of the disease, also known as the haemolymphatic phase, is defined by the restriction of the trypanosomes to the blood and lymph system. The symptoms of this stage are fever, headaches, joint pains and itching. The second or late stage of the disease, also known as the neurological phase, occurs when the parasites cross the blood-brain barrier and reach the central nervous system and is characterized by the presence of the parasites in the cerebrospinal fluid. In general, this is when the typical signs of the disease occur: changes in behaviour, confusion, alterations of the sleep patterns, sensory disturbances,

extreme lethargy, poor condition and coma (Fig. 2D). If left untreated, sleeping sickness patients die within months when infected with *T. b. rhodesiense* or within years when infected with *T. b. gambiense*. Wild and domestic animals may play a major role as parasite reservoirs for human infections with trypanosomes (Steverding, 2008).

The diagnosis of sleeping sickness is mainly based on the initial screening of a potential infection via indirect evidence by a serological test, the card agglutination test for trypanosomiasis (CATT), available for *T. b. gambiense*, by the clinical signs of the disease, such as swollen cervical glands, and by molecular methods (PCR). PCR-based diagnosis has proved to be a non-invasive tool to properly diagnose HAT and has been available for many years. However, it has difficulties for the determination of the stage of the disease (Truc *et al.,* 2012) and has not been recommended to predict the outcome after treatment (Deborggraeve *et al.,* 2011). Clinical evidence of HAT, however, still depends on visualization of parasites in blood, lymph or cerebrospinal fluid. Due to the toxicity of the drugs, the presence of parasites must be confirmed before treatment. Determination of the state of the disease's progression is made by examination of the cerebrospinal fluid obtained by lumbar puncture where the number of white blood cells and the presence of parasites are used to determine the course of treatment (WHO, 2012; CDC, 2012). Diagnosis must be made as early as possible and before the neurological stage in order to avoid complicated, difficult and risky treatment procedures.

The long, relatively asymptomatic first stage of *T. b. gambiense* sleeping sickness is one of the reasons why an exhaustive, active screening of the population at risk is required, in order to identify patients at an early stage and reduce transmission. Exhaustive screenings require a major investment in human and material resources. In Africa such resources are often scarce, particularly in remote areas where the disease is mostly found. As a result, many infected individuals may die before they can ever be diagnosed and treated (WHO, 2012).

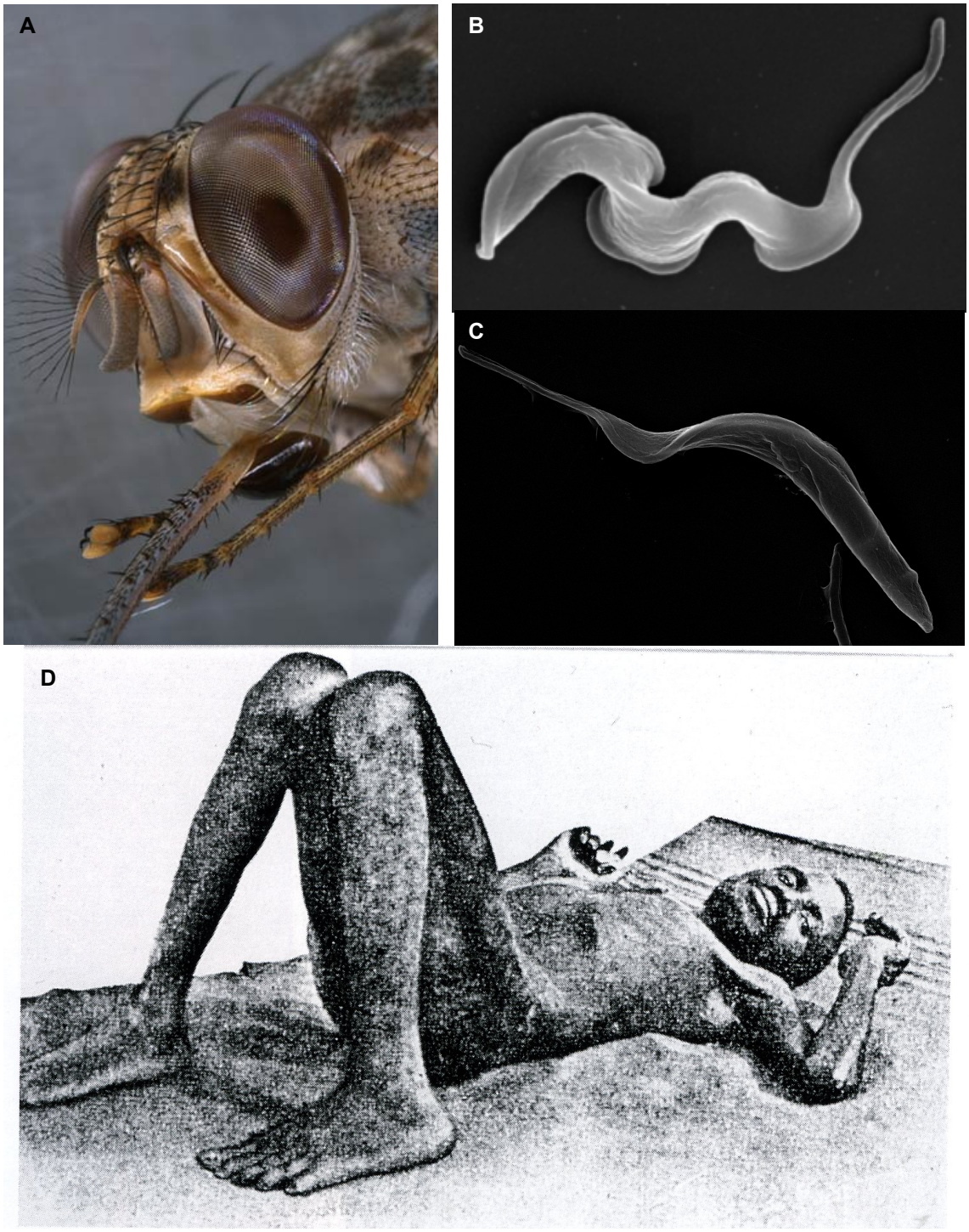

Figure 2. ***The sleeping sickness components***. **A**. Insect vector: female tsetse fly (picture of *Glossina morsitans morsitans* from the Serap Aksoy lab colony at the Yale University School of Public Health). **B.** *T. brucei* human stage: long-slender bloodstream-form trypanosome (scanning electron micrograph of bloodstream-form *T. brucei* from Susan Vaughan and Keith Gull). **C**. *T. brucei* insect stage: procyclic-form trypanosome (scanning electron micrograph of procyclic *T. brucei* from Melisa Gualdrón-López). **D.** Late stage of sleeping sickness (Source: The Wellcome Trust. Illustrated History of Tropical Diseases).

Figure 3. ***The discoverer of sleeping sickness and "nagana".*** David Bruce (1855-1931) on the veranda of his house at Ubombo in Zululand where he began his work on the relationship between the presence of trypanosomes in the blood and the cattle "fly fever" or "nagana". (Source: The Wellcome Trust. Illustrated History of Tropical Diseases).

The treatment of sleeping sickness depends on the stage of the disease. The first stage is treated with two existing drugs that were developed more than 50 years ago: Pentamidine is used to treat the first stage of *T. b. gambiense* and although the side effects cannot be negligible, it is well tolerated by most patients. It is administered as 7 daily intramuscular injections. Suramin is used since 1920 against *T. b rhodesiense*. It has severe side effects on the urinary tract as well as by eliciting an allergic response. The treatment course requires 5 weeks to be completed. Due to the complicated administration of these drugs, treatment of the first stage of sleeping sickness requires several analyses before, during and after the treatment and often needs hospitalization. This implies the use of material and qualified personal that are in most of the cases not available. Since these drugs do not work in the advanced stages of the disease, it is considered that the earlier the diagnosis is done, the better the prospect for a cure (WHO, 2012; Steverding, 2010).

The second stage (neurological phase) has been classically treated with Melarsoprol, a derivative of an arsenical introduced in 1949. This extremely painful drug is used against both subspecies (*T. b gambiense* and *T. b rhodesiense*) and it causes severe undesirable side effects. Indeed 1 out of 20 patients treated with Melarsoprol is killed by the side effects of the drug, with the encephalopathic syndrome being the most dramatic one. In addition, the efficacy of this drug is decreasing, especially in central Africa, where up to 30% of the treated population did not respond to the treatment. Eflornithine, introduced in 1990, is the most recent drug to treat second stage of *T. b. gambiense* sleeping sickness. Originally developed as an anti-cancer drug, it has been shown to work efficaciously against late-stage sleeping sickness. It is a specific and irreversible suicide inhibitor of ornithine decarboxylase, the first enzyme involved in polyamine biosynthesis. Despite the efficacy of this drug as a trypanocide, its administration is difficult since 4 intravenous infusions per day during 14 days are required for total cure. Obviously, this renders the treatment inaccessible to the majority of people in the affected areas. More recently (2009), a combination treatment with Eflornithine and Nifurtimox (the latter a drug used for American trypanosomiasis or Chagas disease, caused by *Trypanosoma cruzi* in South and Central America) has been implemented reducing the complication of the monotherapy with Eflornithine. Nevertheless, this treatment does not work for infections caused by *T. b. rhodesiense*. The WHO has included the Eflornithine/Nifurtimox combination therapy in the list of Essential Medicines and provides it free of charge for the treatment of the late stage of sleeping sickness caused by *T. b. gambiense* (WHO, 2012; Steverding, 2010).

Taking into account the current situation of the treatment and management of sleeping sickness in Africa, it becomes clear that there is a desperate need for new effective drugs that can target specifically essential cellular functions of *T. b. gambienese* and *T. b rhodesiense* without harming the human host. The lack of interest of the pharmaceutical industry to devote research and resources for the development of drugs that can cure this disease, stems from the fact that the affected people, living in the poorest regions of the world, cannot afford such

drugs, making sleeping sickness a neglected tropical disease as many others.

Fortunately, the creation of organizations like DNDi (Drugs for Neglected Diseases initiative), which is a collaborative, patients' needs-driven, non-profit drug research and development (R&D) organization committed to develop new treatments for Neglected Diseases, offers some hope for the future of a cure for diseases such as sleeping sickness.

Prospects for a vaccine against sleeping sickness are bleak, because of a remarkable aspect of *T. brucei*'s cell biology. In its mammalian host stage, each *T. brucei* is coated with a dense sheath of tightly packed identical variant surface glycoprotein (VSG) homodimers, analogous to a fur coat, which protects against complement-mediated lysis. An infected host eventually mounts an effective antibody response against the predominant VSG variants leading to antibody-mediated lysis. However, trypanosomes continually arise that have switched to the expression of antigenically different VSGs (Taylor *et al.,* 2006). This phenomenon leads to repeated peaks of parasitaemia, because one population of trypanosomes, expressing only a limited number of particular VSGs, is killed but a few parasites, each having switched to the expression of another VSG, will survive and proliferate to create a new peak of parasitaemia.

Trypanosoma brucei life cycle

The *Trypanosoma brucei* life cycle involves several successive developmental changes occurring while the parasite alternate living extracellularly in the mammalian host and in the insect vector (Fig. 4). This differentiation, which involves physiological, morphological and metabolic remodelling, allows the parasite to adapt to the different environments encountered during its life cycle. When an infected tsetse fly takes a blood meal from a mammalian host, it injects infective metacyclic trypomastigotes into the skin. From there, these trypomastigotes migrate to the lympathic system reaching the blood where they differentiate into bloodstream trypomastigotes (Fig. 2B), a developmental stage characterized by a long-slender shape and commonly named as bloodstream

form. Cells of this stage proliferate by binary fission and are transported by the bloodstream throughout the whole body. When the cell population reaches the highest peak of parasitemia, the long-slender trypanosomes differentiate in response to the secretion of the "stumpy induction factor" (SIF) (Vasella *et al.*, 1997) into the non-proliferative short-stumpy form, which is pre-adapted to the environmental conditions present in the midgut of the tsetse fly.

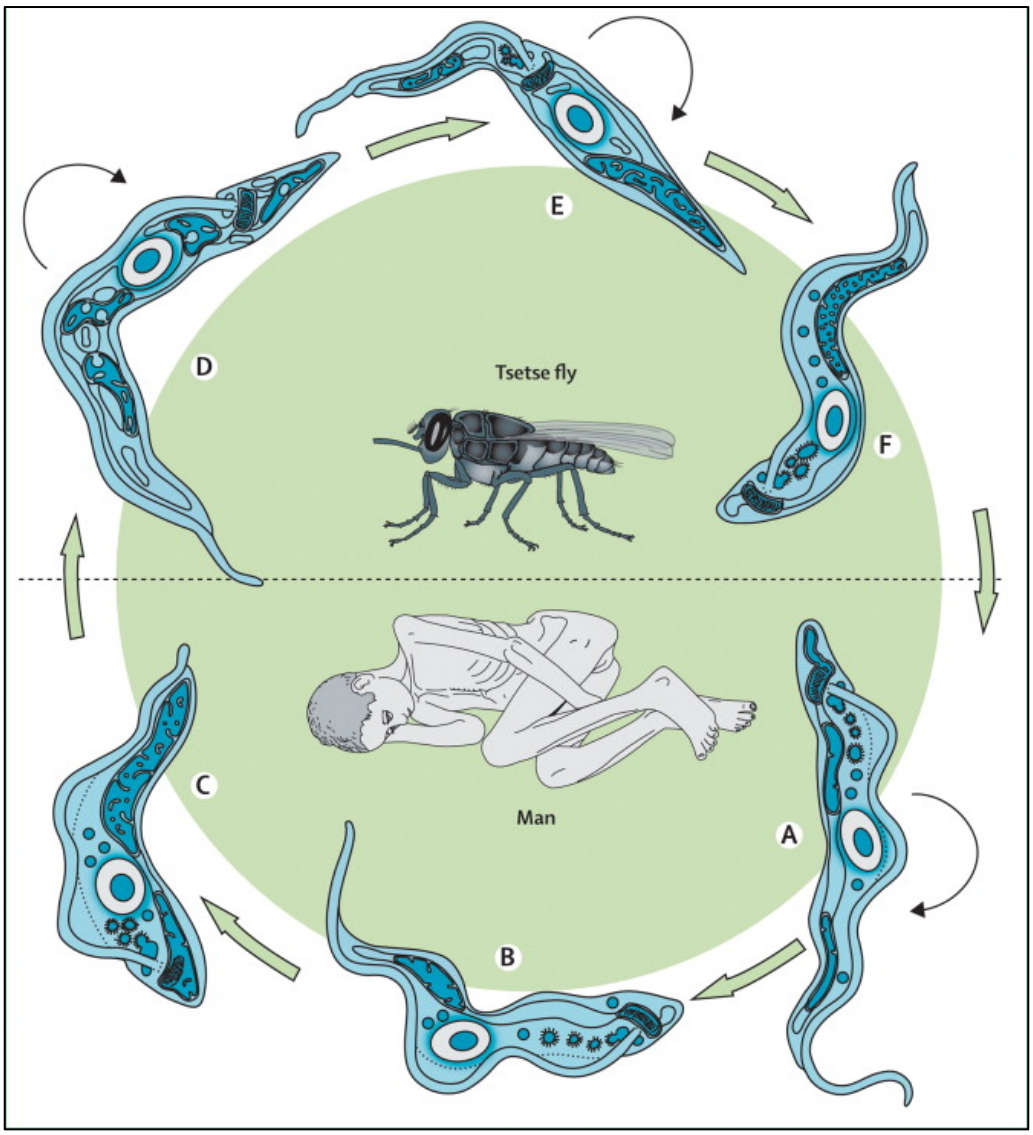

Figure 4. ***The life cycle of Trypanosoma brucei.*** In humans the bloodstream forms show a polymorphism with (**A**) dividing long-slender forms, (**B**) intermediate forms, and (**C**) stumpy forms. In the tsetse fly, stumpy forms transform to (**D**) dividing procyclic trypomastigotes in the midgut, then to (**E**) the migrating epimastigote forms, which develop in the salivary glands to (**F**) the infective metacyclic trypomastigotes, which are injected during the next blood meal into the mammalian host (reproduced from Brun *et al.*, 2010).

In reality, the transition between the morphological extremes of the slender and stumpy forms involves a progression from proliferation into cell-cycle arrest, accompanied by a series of biochemical and morphological transformations. For this reason, the bloodstream population is described as pleomorphic, with the transitional forms between slender and stumpy defined as intermediate forms (Fig. 4) (Matthews *et al.,* 2004). The life cycle continues when a non-infected fly, while feeding, takes up the infective stumpy forms. Inside the fly, trypanosomes undergo several differentiation steps while traveling from the lumen of the midgut to the salivary glands. First, the stumpies differentiate into proliferative procyclic trypomastigotes (Fig. 2C), a process that takes 24 hours and that occurs in the lumen of the midgut. Procyclic trypomastigotes that colonize the proventriculus differentiate into long-shaped non-dividing mesocyclic trypomastigotes. Asymmetric cell division of these proventricular cells gives rise to the short epimastigotes, which are believed to colonialize the salivary glands. In the salivary glands four different stages are found: attached dividing epimastigotes, premetacyclics and non-dividing nascent metacyclics as well as mature free metacyclic trypomastigotes, which are ready for the transmission to a new mammalian host (Sharma *et al.,* 2009). The totality of developmental stages that *T. brucei* undergoes during its life cycle, are characterized by cell size changes and a re-arrangement of relative positions of the kinetoplast, nucleus and flagellum within the cell.

Cell biology of *T. brucei*

T. brucei is a protist parasite that belongs to the Kinetoplastea class and to the Trypanosomatida order (Moreira *et al.,* 2004). All organisms assembled in this order are parasites of which several infect humans including also *T. cruzi*, the causative agent of Chagas Disease or American trypanosomiasis in Latin America, and *Leishmania* species which are responsible for several forms of leishmaniasis in many tropical and subtropical areas of the world.

Kinetoplastids are flagellated protists that are characterized by the presence of a kinetoplast, a unique structure containing a massive non-nuclear DNA

arrangement present in the single mitochondrion of these organisms (Moreira *et al.,* 2004). Additionally, other unique features distinguish this group of organisms from all other eukaryotes: an unusual, often highly complex form of editing of the mitochondrial RNA; the transcription of nuclear genes as very long multicistronic RNA molecules which are then processed to monocistronic mRNAs by trans-splicing; the apparent absence of specific RNA polymerase II promoters; and a unique way of metabolic compartmentalization. The most peculiar aspect in the kinetoplastids' metabolic compartmentalization is the sequestering of the majority of glycolytic enzymes and some other metabolic pathways within organelles called glycosomes (Opperdoes and Borst, 1977; Hannaert *et al.,* 2003).

Trypanosomatids are highly motile cells, approximately 18–20 μm long and 3 μm thick (Fig. 5). They adopt a wide range of morphologies during their life cycles, but all feature a subpellicular microtubule array that serves to maintain an elongated spindle shape. The flagellum of these cells emerges from the parasite body through a structure termed the flagellar pocket, which is a small invagination of the surface whose membrane is maintained in a distinct domain. In some trypanosomatids, such as *T. brucei* and *T. cruzi*, the flagellum is attached to the cell body through a unique structure called the flagellum attachment zone (FAZ), while in *Leishmania* spp. the flagellum extends free from the pocket into the environment. Membrane connection, filaments and specialized microtubules along the length of the cell body compose the FAZ. In addition to the classical "9 + 2" microtubule axoneme, the flagellum possesses a lattice-like structure called the paraflagellar rod (PFR) which exits from the flagellar pocket and runs alongside the axoneme and whose function is associated to motility (Gull *et al.,* 1999).

In trypanosomatids the kinetoplast is physically connected to the cytoplasmic basal body though a series of filaments expanding from the basal body to a specialized region of the mitochondrial membrane and from there to the kinetoplast. The basal body complex is the only easily recognizable microtubule organizing center (MTOC) in *T. brucei*. This complex appears to be responsible

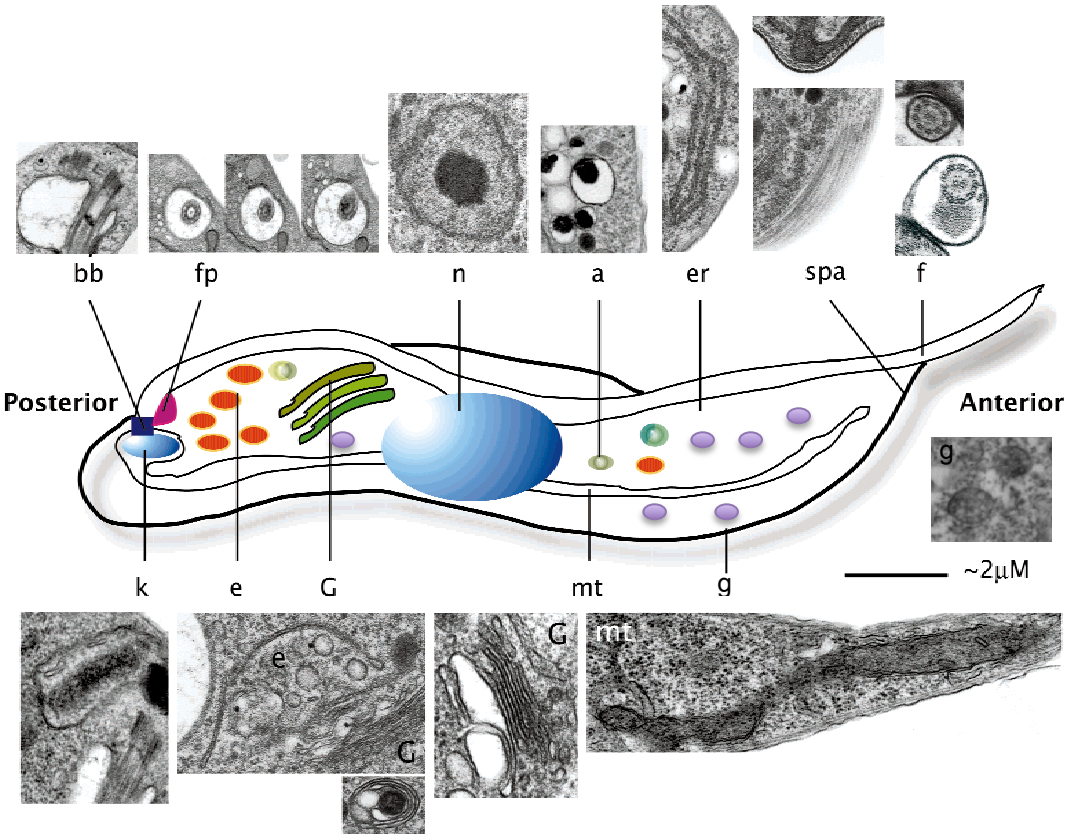

Figure 5. ***Schematic of the trypanosome cell.*** The central image is a cartoon showing the locations of the major organelles and structures. The endoplasmic reticulum (er) has been omitted for clarity as this structure extends throughout the cell and also forms the nuclear envelope. Structures shown are: a: acidocalcisome; fp: flagellar pocket; bb: basal body; n: nucleus; spa: subpellicular microtubule array; f: flagellum; k: kinetoplast (mtDNA); e: endosomes; G: Golgi complex; mt: mitochondrion; and er: endoplasmic reticulum. The insets show examples of these structures as seen by thin-section transmission electron microscopy. For the basal body, note the presence of the structure as it passes through the flagellar pocket membrane. Three serial section images of the fp are shown; at this level the flagellum lacks the paraflagellar rod, which normally supports the axoneme. The image of the nucleus (n) illustrates the double membrane surrounding the organelle, together with the single prominent electron-dense nucleolus. Acidocalcisomes are observed as electron-dense structures within the cytoplasm, but in thin-section mounts the luminal contents are often partially or fully lost, resulting in apparently empty structures. Three closely apposed rough ER membranes are shown. The subpellicular microtubule array (spa) is shown in both transverse (top) and glancing (bottom) section; note the highly ordered periodicity of this cytoskeletal component that is responsible for maintaining the overall shape of the organism. Two transverse sections through the flagellum are shown, one at the level of the flagellar pocket (top) and lacking a paraflagellar rod, and a second higher up, where the flagellum contains the axoneme and paraflagellar rod. Note the presence of the electron-dense membrane associated with the flagellum-contact zone on the flagellar pocket. The kinetoplast (k) is observed as a thick electron-dense disc of material (in this example in transverse section). The kinetoplast is enclosed in the mitochondrial membrane and is in close association with the base of the flagellum. The endocytic and secretory apparatus of the Golgi complex (G) and endosomes (e) are also closely associated; endosomes close to the flagellar pocket are indicated, as is a Golgi stack (G). A second prominent Golgi complex is also shown. At the bottom a multivesicular endosome is shown. A section through a portion of the single mitochondrion (mt) in the anterior portion of a procyclic cell is shown. Finally glycosomes (g) are observed as electron-dense particles surrounded by a single lipid bilayer. Approximately 60 glycosomes are present per cell. The posterior and anterior ends of the cell are also indicated. (Adapted from Field *et al.,* 2004)

for the nucleation of the flagellar axoneme microtubules in the classical manner (Gull *et al.,* 1999). It is believed that the kinetoplast is connected to the basal body to ensure correct segregation at mitosis (Field *et al.,* 2004).

The nucleus occupies the central region of the cell. The relative positions of the kinetoplast and nucleus alters, dependent on the developmental stage of the cell; Ex: in bloodstream forms, the kinetoplast lies close to the posterior end of the cell. By contrast, the kinetoplast lies midway between the cell nucleus and posterior end in procyclic forms while in epimastigote forms the kinetoplast is anterior to the central nucleus (Matthews, 2005).

The majority of the canonical endomembrane system has been identified including the endoplasmic reticulum (ER), a stacked Golgi apparatus and several endosomal compartments. A peculiarity of trypanosomatids is the restriction of the endocytic and secretion processes through the flagellar pocket. Additionally, the exocytic and endocytic compartments (the single lysosome and the endosomes) are polarized in the cell, being located between the nucleus and the kinetoplast (Field *et al.,* 2007). By contrast, the ER, the mitochondrion, the glycosomes and acidocalcisomes are all dispersed throughout the cell volume.

Glycosomes are microbodies that belong to the organelle family of peroxisomes, together with the peroxisomes found in representatives of all eukaryotic lineages, and glyoxysomes of plants and the Woronin bodies of some fungi (Fig. 6). They are bounded by a single phospholipid bilayer membrane, have an electron-dense matrix, and do not contain any detectable DNA. Glycosomal matrix and membrane proteins are synthesized in the cytosol and subsequently imported into the organelles. Although glycolytic enzymes are the most prominent proteins in glycosomes (they may comprise up to 90% of the content in bloodstream-form *T. brucei*), some other enzymes are also present and are shared with peroxisomes: *e.g.* enzymes of peroxide metabolism, fatty-acid oxidation and ether-lipid biosynthesis among others (Hannaert *et al.,* 2003).

Remarkably, it has been found that correct compartmentalization of glycolysis of

bloodstream form of *T. brucei* is absolutely essential for the survival of parasites. (Moyersoen *et al*., 2004)

For an extensive description of the metabolic content and evolutionary aspects of glycosomes in each of the Trypanosomatida parasites, see Chapter 2.

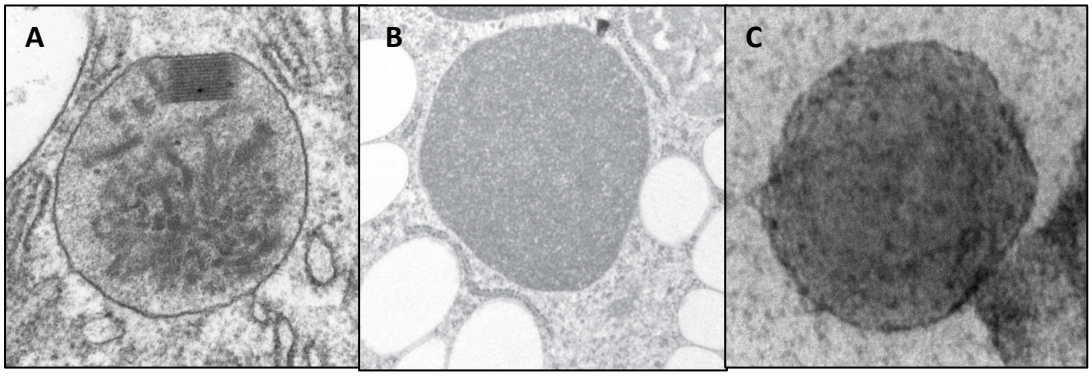

Figure 6. ***The peroxisome family***. Transmission electron micrograph of: **A.** Peroxisome from the marine mollusc *Lepidochitona cinerea (*taken from Alexandre Lobo-da-Cunha's laboratory website). **B**. Glyoxysome of *Arabidopsis thaliana* (taken from Grahan, 2008). **C**. Glycosomes from bloodstream form *T. brucei* (from the TROP Unit laboratory).

Glycosome and peroxisome biogenesis

In recent years intensive research on notably peroxisomes from different yeast species and mammalian cells has yielded a great number of data concerning the biogenesis of peroxisomes that have helped to understand the molecular mechanisms that are responsible for the formation of these organelles.

The biogenesis of peroxisomes is substantially different from that of other cellular compartments such as mitochondria and chloroplasts, which have had evolved from an endosymbiotic bacterium, an evolutionary origin that is considered unlikely for peroxisomes and glycosomes (see Chapter 2). Peroxisome formation involves different proteins (so far a non-redundant set of 33 proteins have been identified in different organisms) commonly named as peroxins, which accomplish the different steps of the process: peroxisome membrane formation, peroxisome proliferation and compartmentalization of peroxisomal matrix proteins (Platta *et al.,* 2007b; Wolf *et al.,* 2010; Rucktäschel *et al.,* 2011).

Peroxisome membrane formation

There has been a long-lasting controversy about the origin of peroxisomes, but it is now widely accepted that the ER in eukaryotic cells is involved in the formation of peroxisomes, strongly suggesting an evolutionary relationship between the organelles and that membrane system. A strong argument in favour of an evolutionary origin of peroxisomes from the ER comes from the exclusive eukaryotic character of peroxisomal membrane proteins as well as the astonishing mechanistic similarities of the peroxisomal matrix-protein import machinery and the endoplasmic reticulum degradation pathway (ERAD) (Schlüter *et al.,* 2006; Schliebs *et al.,* 2010). Several additional independent lines of evidence for the link between ER and peroxisomes are: the double localization of some peroxisomal membrane proteins (PMPs) (Ex: PEX2, PEX16 in yeast) in the ER and peroxisomes at steady-state levels; the presence of ER-specific modifications in certain PMPs (Ex: glycosylations in yeast); the physical connection between ER and peroxisomes (Ex: mouse dendritic cells); the formation of peroxisomes in a ΔPEX3 peroxisome-deficient mutant after reintroduction of the wild-type YFP-tagged PEX3 gene (Ex: yeast and human fibroblasts) where by real-time fluorescence it was observed first inserted into the ER, concentrated in foci that bud off to give vesicles that develop into fully functional peroxisomes. The latter result also supports the notion of *de novo* formation of peroxisomes and, in conjunction with the other data, is suggestive of the involvement of the ER in this process. Still, controversy exists with respect to the localization of some PMPs under wild-type conditions (thus excluding effects that might be attributed to overexpression of specific genes). On the other hand, a large number of data, collected from studies of the peroxisome fission machinery (Dynamin-like proteins, Tail-anchored proteins and proteins of the PEX11 family) strongly indicate that peroxisomes of yeast multiply most likely by growth and division. In mammalian cells both types of multiplication seem to occur simultaneously (Nuttal *et al.,* 2011; Nagotu *et al.,* 2010). This has led to the proposal of two models for peroxisome formation. In the growth and division model, the ER provides existing peroxisomes with membranes allowing them to autonomously grow and divide. The peroxisomal matrix proteins are

imported into the pre-existing organelles as occurs for some PMPs, while other PMPs are targeted via the ER (Fig. 7). The maturation model implies that ER-derived vesicles fuse to form pre-peroxisome structures. Once the PMPs required for matrix-protein import are inserted into these structures, the peroxisomal matrix-protein import starts. In this model some PMPs traffic via the ER while others are inserted during the maturation process (Fig. 7) (Ma *et al.,* 2011). However, recent data about PMP import in *S. cerevisiae* may indicate that PMPs exclusively traffic via the ER, opening once more essential questions about peroxisome formation through the ER (Nuttal *et al.,* 2011).

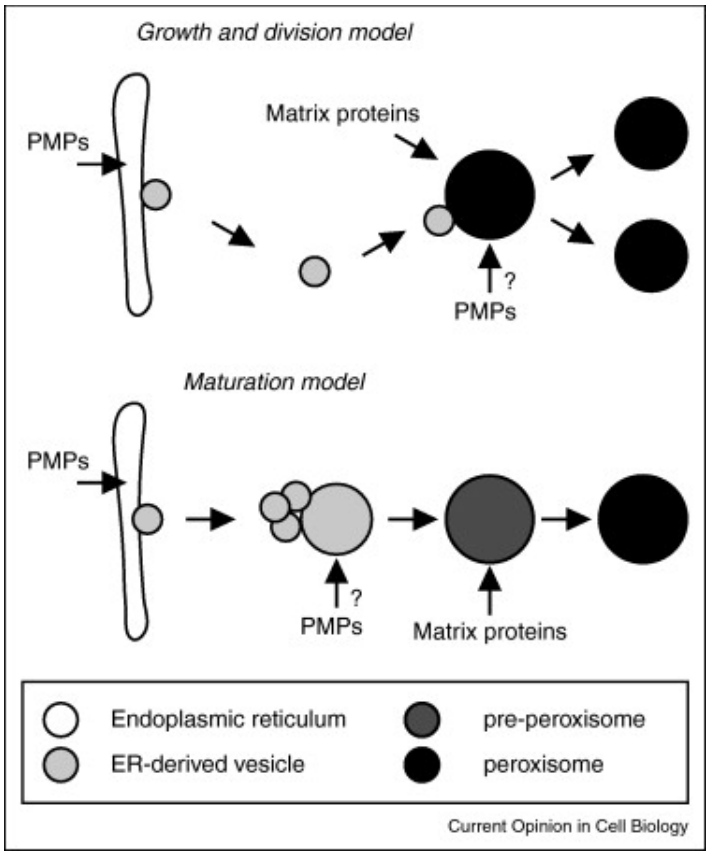

Figure 7. ***Peroxisome formation models***. Top: Growth and division model. This model predicts that a specialized region of the ER provides existing peroxisomes with membranes allowing them to grow and divide. The peroxisomal matrix proteins are imported into pre-existing peroxisomes. Some peroxisomal membrane proteins (PMPs) originate directly from the ER, while others are imported into peroxisomes. Bottom: Maturation model. This model foresees that peroxisomes can form from the ER when ER-derived vesicles fuse with pre-existing peroxisomes. When all the PMPs required for matrix-protein import are inserted in the pre-peroxisomes, then the matrix protein import starts. In this model some PMPs are imported via the vesicles from the ER, while others are imported during the maturation process. (Reproduced from Nuttall *et al.,* 2011)

Peroxisomal membrane protein import

The import of peroxisomal membrane proteins (PMPs) requires PEX3, PEX19 and in some organisms PEX16 (Fig. 8). PEX19 is a soluble, mostly cytosolic sometimes farnesylated protein that assists to bring newly synthesized proteins from their site of synthesis on free polyribosomes in the cytosol to the peroxisomal membrane (Lazarow, 2003). The PMP receptor function of PEX19 is based on the finding that most PMPs contain conserved PEX19-binding sites, which are almost always required for their peroxisomal localization as well. This targeting signal comprises a PEX19-binding site plus a membrane-anchoring sequence which can be a transmembrane segment (peroxisomal integral membrane proteins) or an anchor-protein binding site (peripheral membrane proteins). The PMP proteins that depend on PEX19 for their targeting to the peroxisomes are named class I (Fig. 8B). A PEX19-independent pathway has been proposed for PEX3 and PEX16 defining the class II PMPs. These two integral peroxisomal membrane proteins are involved in the insertion of new membrane proteins into the peroxisomal membrane. It is believed that these proteins travel from a specialized region of the ER to the peroxisomes. The function proposed for PEX3 is the binding of PEX19 at the peroxisomal membrane thus functioning as membrane docking site for the cargo-loaded PEX19 (Platta and Erdmann, 2007). On the other hand, PEX16 is thought to serve as a recruitment factor for PEX3. This peroxin is present in mammalian cells but absent in yeast. The recent finding that, in *S. cerevisiae*, many class I PMPs travel to the peroxisome through the ER, suggests a new role for PEX3 and PEX19 in the budding of peroxisomal vesicles from the ER (Nuttal *et al.,* 2011).

Peroxisomal matrix-protein import

The peroxisomal matrix-protein import is a multistep process that involves interaction between several peroxins in the cytosol and at the peroxisomal membrane. This process can be conceptually dived in five steps (Figure 9):

Targeting signal-dependent cargo recognition: Peroxisomal matrix proteins are targeted to the organelles through their peroxisomal-targeting signals: PTS-1 consisting of a tripeptide, SKL or its conserved variants located at the extreme C-terminus of the great majority of matrix proteins; PTS-2 is a nonapeptide (R/K)(L/V/I/Q/)XX(L/V/I/H/Q)(L/S/G/A/K)X(H/Q)(L/A/F) near the N-terminus of a smaller subset of peroxisomal matrix proteins (Ma *et al.,* 2011; Rucktäschel *et al.,* 2011).

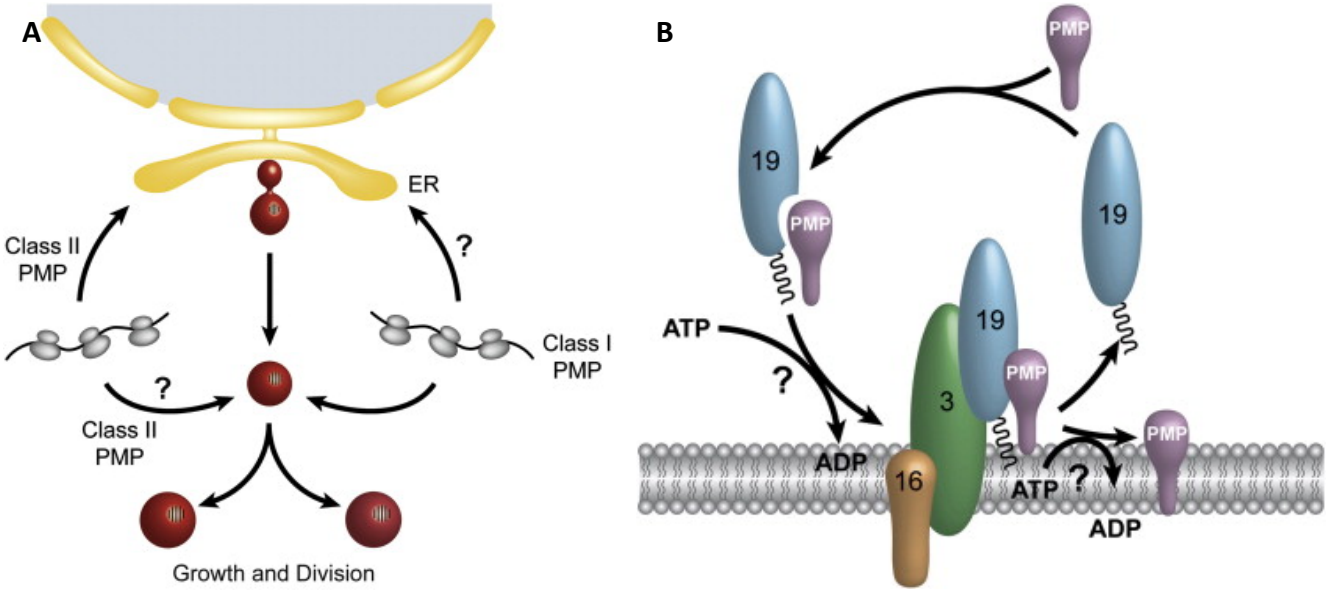

Figure 8. ***Topogenesis of peroxisomal membrane proteins.*** **A.** PMPs of Class I are imported into existing peroxisomes in a PEX19-dependent manner. Class II PMPs are first transported to the ER from where they concentrate in pre-peroxisomal vesicles that are either targeted to existing peroxisomes or function for *de novo* formation of peroxisomes. Examples of Class II PMPs are PEX3 and PEX16. The questions marks represent the still open debate about the route of transit of some PMPs. **B.** PEX19-dependent import of PMPs. PMPs of Class I harbour a peroxisomal membrane targeting signal that is recognized by the cytosolic PMP receptor PEX19. After recognition, the receptor-cargo docks at the peroxisomal membrane through the interaction with the docking factor PEX3. The PMPs are inserted into the membrane by a poorly understood process but with the possible assistance of PEX19, PEX3 and in some organisms (Ex: mammalian cells) of PEX16. After insertion of these Class I PMPs, the receptor PEX19 shuttles back to the cytosol presumably to perform new rounds of import. (Reproduced form Rucktäschel *et al.,* 2011)

The two targeting signals PTS-1 and PTS-2 are recognized in the cytosol by two peroxisomal matrix protein receptors, PEX5 and PEX7, respectively. PEX5 is a predominately cytosolic protein that possesses two domains: a C-terminal domain composed of 6-7 tetratricopeptide repeat (TPR) motifs which interact with the PTS-1 of the peroxisomal cargo and a structurally disordered N-

terminal domain that functions in the receptor's docking [through its conserved pentapeptide (WxxxF/Y) motifs] and its recycling. PEX7 is a cytosolic protein belonging to the WD protein family. WD domains are known to participate in transient protein-protein interactions. PEX7 is composed of six 40-60 amino-acid long repeats, each with a central tryptophan/aspartic (WD) pair, distributed throughout almost the entire protein and preceded by a distinct N-terminal region. Unlike the PTS-1 protein import, the PEX7-mediated protein import requires species-specific auxiliary proteins also known as co-receptors: PEX18 and PEX21 in *S. cerevisiae*, PEX20 in *Yarrowia lipolytica*, *Pichia pastoris*, *H. polymorpha* and *Neurospora crassa* or a longer splice variant of the PTS-1 receptor PEX5 in plants and mammals. The co-receptor forms a ternary complex with the cargo-loaded PEX7 in the cytosol and directs the complex to the peroxisomal membrane. Peroxisomal matrix proteins which do not contain a PTS-1 or PTS-2 reach the peroxisomal matrix by different mechanisms which may be piggy-back transport in which the proteins hijack onto the peroxisomal targeting pathways by interaction with consensus PTS-containing proteins. Some other proteins containing non-identified PTS-targeting signals (commonly named as I-PTS) interact with non-canonical domains of the receptor PEX5 (Rucktäschel *et al.,* 2011).

Docking of the receptor-cargo at the peroxisomal membrane: The next step in matrix protein import is the association of the receptor-cargo complexes, formed in the cytosol, with the peroxisomal membrane. A docking complex comprised by the peroxins PEX14, PEX13 and in baker's yeast also PEX17, are responsible for this step. PEX14 is a membrane-associated protein composed of a N-terminal region with a conserved core, a PxxP motif, a transmembrane segment, a coil-coiled region followed by a barely conserved C-terminal region (Galland and Michels, 2010). PEX13 is an integral membrane protein with its N- and C-termini exposed to the cytosol. A Src homology 3 (SH3) domain present in the cytosolically exposed C-terminus, is crucial for the interaction with PEX5, while the N-terminal part is involved in the binding of PEX7. PEX14 forms a complex with PEX13 that also binds to both receptors. In baker's yeast PEX14 forms a binding platform for PEX17, whose function is still unknown. It has been found

that PEX17 does not contribute to the integrity of the docking complex and is absent from higher eukaryotes. The docking subcomplex has been shown to physically associate with another subcomplex of the peroxisomal membrane: the RING (really interesting new gene)-finger subcomplex, comprising the PEX2-PEX10-PEX12 peroxins, in order to form an import-competent complex, called the importomer (Ma *et al.,* 2011; Rucktäschel *et al.,* 2011). The docking and RING subcomplexes are bridged by the peroxin PEX8 in *S. cerevisiae* and by PEX3 in *P. pastoris* (Deckers *et al.,* 2010).

Translocation and cargo release: Different from mitochondrial and chloroplast protein import, where proteins are imported unfolded, peroxisomes are able to import folded proteins and also large oligomeric proteins into the peroxisomal matrix. Remarkably, even gold particles with a diameter of 9 nm when decorated with PTS-1 are successfully imported. The mechanism by which folded and oligomeric peroxisomal proteins translocate through the membrane after the docking events has been a puzzle for many years. In 2005, Erdmann and co-workers proposed a model in which a transient aqueous pore could be formed in the peroxisomal membrane after the docking of the cargo-loaded receptor, by which matrix proteins could be translocated and that disassembles after the translocation is completed. In this model a role for the import receptor PEX5 as an integral part of the translocation pore was assigned based on experimental evidence that the membrane-bound fraction of PEX5 can behave as an intrinsic membrane protein and can form stable interaction with the docking complex (Erdmann *et al.,* 2005).

Very recently, Meinecke *et al.* (2010) solved a major part of the puzzle of the peroxisomal matrix translocation in yeast. These authors have elegantly reconstituted the peroxisomal importomer into liposomes and shown the activity of a gated ion-conducting channel as a result of the formation of a highly dynamic pore. They demonstrated that membrane-associated import receptor PEX5 together with the docking protein PEX14 forms a dynamic channel that is opened upon docking of the cytosolic receptor–cargo complex with a diameter up to 9 nM. This remarkable discovery explains how peroxisomes are able to

import completely folded and even oligomeric proteins of different size and gives account of an extremely important role of PEX5, additional to its function as PTS-1 protein receptor, in the peroxisomal matrix-protein import process.

The next step in the matrix-protein import is the cargo release in the peroxisomal matrix. A role for PEX8 has been proposed based on the presence of PTS-1 and PTS-2 signals in this protein. Nevertheless, the fact that mutations on the targeting signals of this peroxin do not affect protein import and that this protein has not been yet identified in higher eukaryotes, makes that the mechanism of cargo release remains an open question.

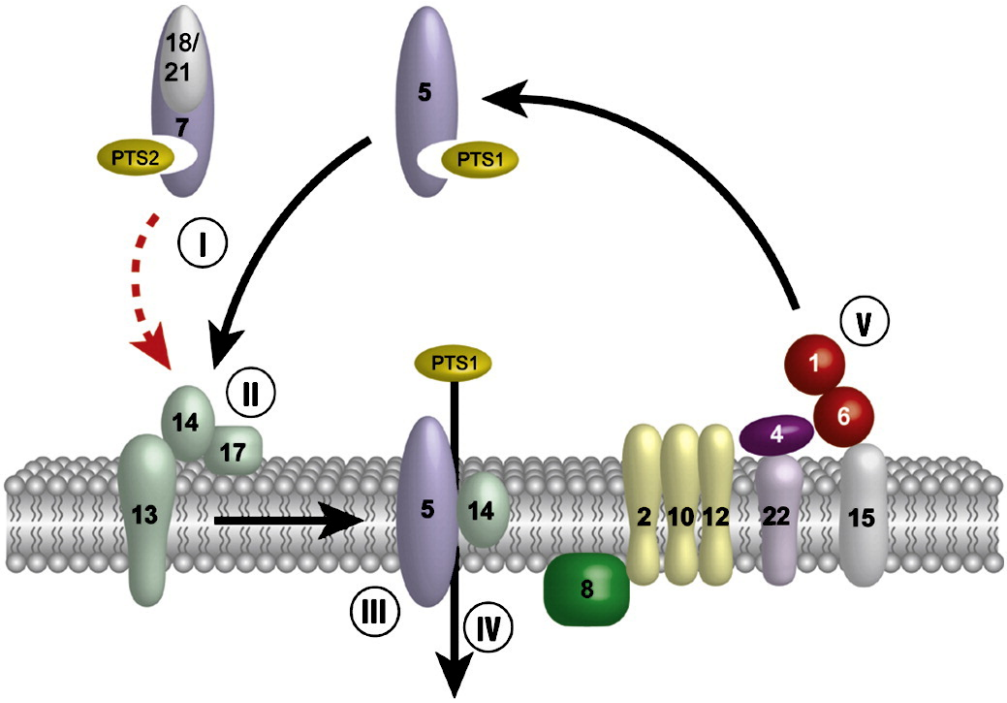

Figure 9. ***Peroxisomal matrix protein import in yeast cells.*** The peroxisomal protein import process can conceptually be divided in five steps: (**I**) cargo recognition in the cytosol; (**II**) docking of the receptor–cargo complexes at the peroxisomal membrane; (**III**) cargo-translocation into the peroxisomal matrix; (**IV**) disassembly of the receptor–cargo complex and (**V**) export of the receptor back to the cytosol. PTS-1 containing proteins are recognized by the soluble import receptor PEX5 in the cytosol. Proteins harbouring the PTS-2 are recognized by PEX7 and the cofactors PEX18 and PEX21 in *S. cerevisiae*, the orthologous PEX20 in other fungi or PEX5L in plants, kinetoplastids and mammals. After this step, the receptor–cargo complex targets to and associates with the peroxisomal membrane via the docking complex consisting of PEX14, PEX13 and PEX17. The transport of PTS1-proteins across the membrane is facilitated by formation of a pore mainly consisting of PEX14 and PEX5. PEX8 connects the RING-complex to the docking complex. The three ubiquitin ligases PEX2, PEX10 and PEX12 form the RING-complex and together with ubiquitin-conjugating enzymes like PEX4 are responsible for receptor ubiquitination. In the last step of the cycle, the receptor PEX5 is exported back to the cytosol by the two AAA$^+$-ATPases, peroxins PEX1 and PEX6, and is enabled for the next round of import. (Taken from Rucktäschel *et al.*, 2011)

Receptor ubiquitination and recycling: After the cargo release, the two receptors, PEX5 and PEX7, must be retrieved from the peroxisomes. PEX7 has been shown to be fully internalized into the matrix (Nair *et al*., 2004), whereas PEX5 has to be removed from the membrane (Fig. 10). It was first demonstrated in human cells that PEX5 is a cycling protein that translocates to the peroxisomal membrane and is recycled to the cytosol (Dammai *et al.,* 2001). The molecular machinery that is involved in this process was defined as the recycling complex. It comprises two cytosolic AAA$^+$-ATPases (the family of ATPases associated with diverse cellular activities), PEX1 and PEX6, which anchor to the peroxisomal membrane through PEX15 in yeast cells or PEX26 in mammalian cells and which have been identified as the translocases responsible for the removal of PEX5 from the peroxisomal membrane (Platta *et al.,* 2005). Also part of the recycling complex is PEX4 (in yeast/plants), a homologue of E2 ubiquitin-conjugating enzymes together with its membrane anchor protein PEX22. In mammalian cells PEX4's function is accomplished by the cytosolic ubiquitin-conjugating enzymes UbcH5a/b/c. Additionally involved in the receptor recycling are the peroxins of the RING-finger subcomplex which have E3-ubiquitin ligase activity. Contrary to the docking events, which have been shown to be ATP independent, the receptor recycling requires the free energy from the hydrolysis ATP by the AAA$^+$-ATPases and by the ubiquitination machinery.

The initial identification of a homologue of ubiquitin-conjugating enzymes by Wiebel *et al.* (1992) and Wiemer *et al.* (1996) as a protein (called pPAS2, later renamed PEX4) required for proper peroxisomal matrix protein import, raised the question whether ubiquitination was an important event during peroxisome formation. Intensive research on this subject, first in several yeast species and later also in mammalian cells, has clarified the role of ubiquitination in peroxisomal matrix-protein import.

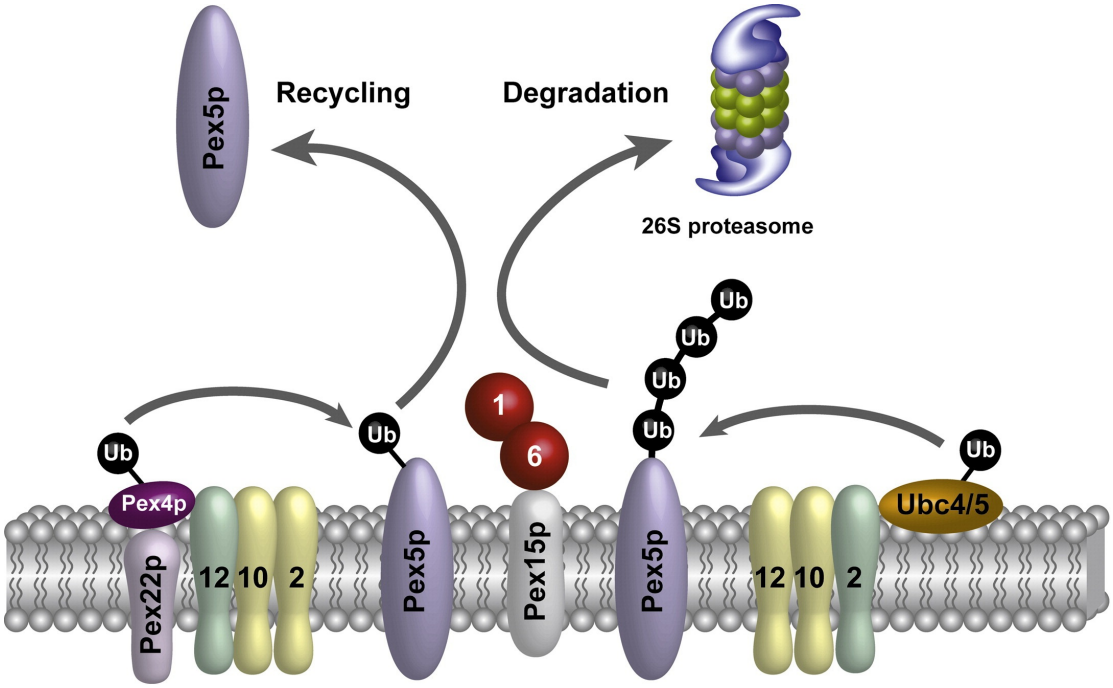

Figure 10. ***Model of ubiquitination of S. cerevisiae PTS1-receptor PEX5***. *S. cerevisiae* PEX5 has been proved to be mono- or polyubiquitinated at the peroxisomal membrane. The peroxisomal bound ubiquitin-conjugating enzyme PEX4, catalyzes the cysteine-dependent monoubiquitination of PEX5 together with the ubiquitin-ligase enzyme PEX12, a member of the RING finger complex. This represents the signal for the ATP-dependent translocases PEX1/PEX6 to export PEX5 from the membrane to the cytosol in order to perform new rounds of import. PEX5 polyubiquitination occurs when receptor recycling is impaired, functioning as a signal for proteasome-dependent degradation. The lysine-dependent polyubiquitination of PEX5 depends on the cytosolic ubiquitin-conjugating enzymes UBC4/UBC5 which act together with the RING-complex ubiquitin-ligase PEX2. Polyubiquitinated PEX5 is supposed to be also recognized and exported by the AAA^{+}-ATPase complex. This forms part of a quality-control mechanism. (Taken from Girzalsky *et al.,* 2010).

Ubiquitination is a posttranslational modification that plays an essential role in the physiology of cells and organisms. Among the first functions of ubiquitination to be discovered was its role in the targeting of proteins for degradation in the proteasome (Pickard, 2001). However, recent research revealed – and continues to do so – an increasing number of functions associated with ubiquitination, like control of protein activity, organellar targeting, ribosomal function, post-replicational DNA repair, the initiation of the inflammatory response, and the regulation of the function of certain transcription factors (Hochstrasser, 2009).

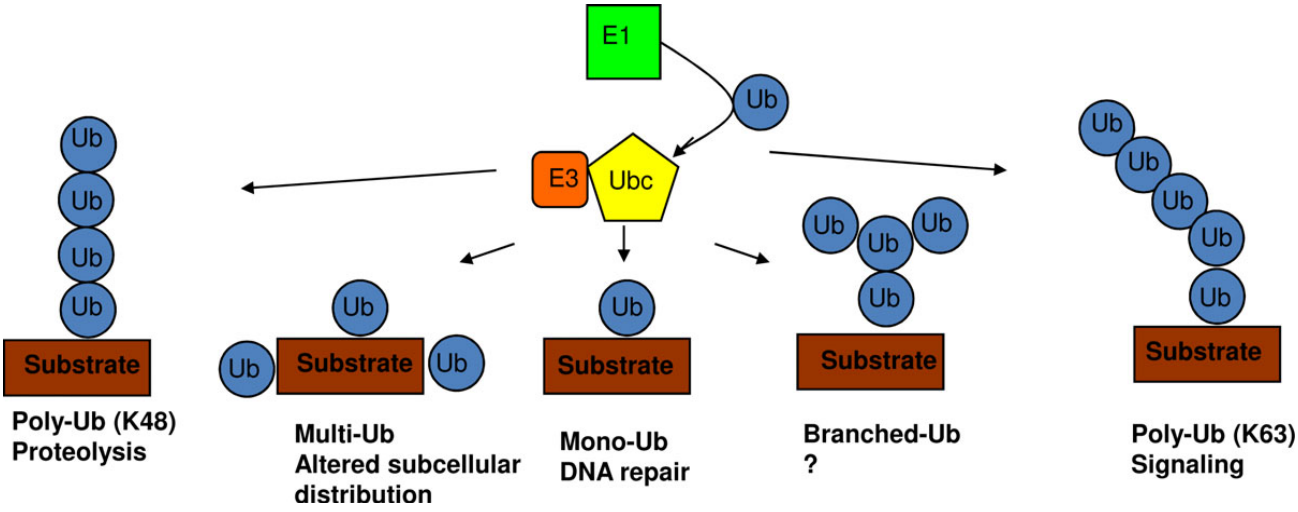

Figure 11. ***Cellular functions associated to different types of ubiquitination***. Different modes of ubiquitination lead to different substrate fates. The versatility of Ub in regulating different processes is derived from its ability to be conjugated as a monomer on one (monoubiquitination) or more substrate lysines (multiubiquitination) or as a polymer (polyubiquitination) by the sequential addition of further Ubs to each other through Ub lysines. Since Ub contains seven lysines, polyubiquitination can generate linear or branched chains with different topologies. Monoubiquitination can regulate DNA repair, viral budding and gene expression, while polyubiquitination through K48 of Ub generally results in proteasomal degradation, and K63-linked Ub chains can function in signalling and endocytosis. (Taken from Sadowski *et al.,* 2010)

The mechanism of ubiquitination involves three steps: first, the C-terminus of ubiquitin (a small protein of 76 residues) forms a thio-ester bond with the catalytic cysteine of the ubiquitin-conjugating enzyme (E1) in an ATP-dependent manner. Ubiquitin is then transferred from the E1 to the catalytic cysteine of the ubiquitin-conjugating enzyme (E2). Finally, an ubiquitin-ligase (E3) binds both the ubiquitin-charged E2 and substrate to catalyze the transfer of the C-terminus of ubiquitin to a substrate lysine to form an isopeptide bond, resulting in substrate monoubiquitination (Fig. 12). Substrates can be ubiquitinated on numerous lysines, resulting in multiubiquitination. In addition, some E2/E3 combinations can then utilize lysines on the substrate-conjugated ubiquitin, to catalyze further cycles of ubiquitination, resulting in substrate polyubiquitination (Fig. 12) (Sadowski *et al.,* 2010). This three-step mechanism initiates all known ubiquitination reactions, irrespective as to whether the substrate-bound ubiquitin(s) will signal proteasomal proteolysis, endocytosis, or some other fates for its substrates. In general, proteins targeted by

monoubiquitination can regulate DNA repair, gene expression, formation of multivesicular bodies in the endocytic pathway, nuclear-protein export among other functions. Polyubiquitination through successive ubiquitins linked by K48-G76 isopeptide bonds generally targets proteins for proteasomal degradation, while K63-linked ubiquitin chains can regulate kinase activation, DNA damage tolerance, signal transduction and endocytosis (Fig. 11) (Sadowski *et al*., 2010).

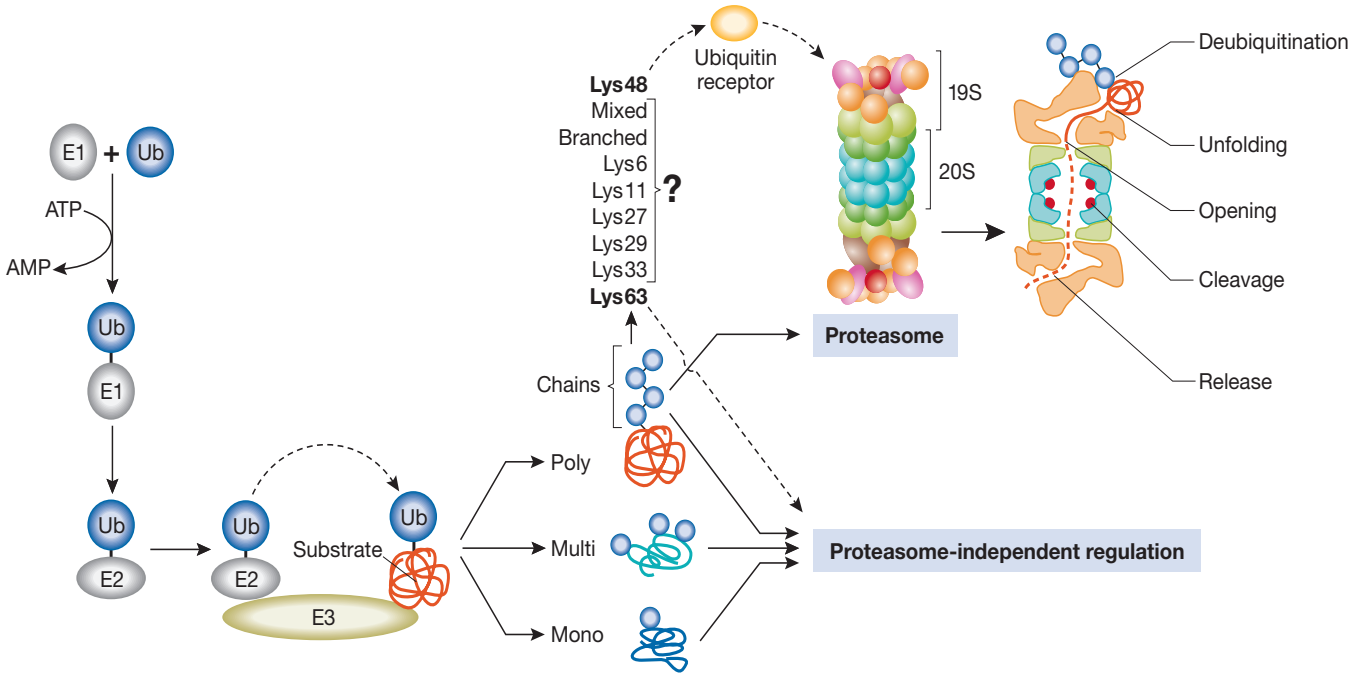

Figure 12. ***Ubiquitination cascade.*** The ubiquitin conjugation to a specific substrate implies several coordinated enzymatic reactions. First ubiquitin (Ub) is activated by an ubiquitin-activating enzyme (E1), transferred to a ubiquitin-conjugating enzyme (E2), which binds to a ubiquitin ligase (E3) for conjugation of ubiquitin predominantly onto lysine residues in substrates. E3s confer substrate specificity to the system. Additional rounds of Ub conjugation onto substrate-attached Ubs lead to the formation of Ub chains. Different lysines in Ub can be used for chain formation. (Taken from Keiser and Fon., 2007)

The organization of the enzymatic conjugating cascade is hierarchical: There is usually one E1; a significant but limited number of E2s, each of which may serve several E3s; and a much larger number of E3s. Each E3 recognizes a set of substrates that share one or more ubiquitination signals (Fig. 13).

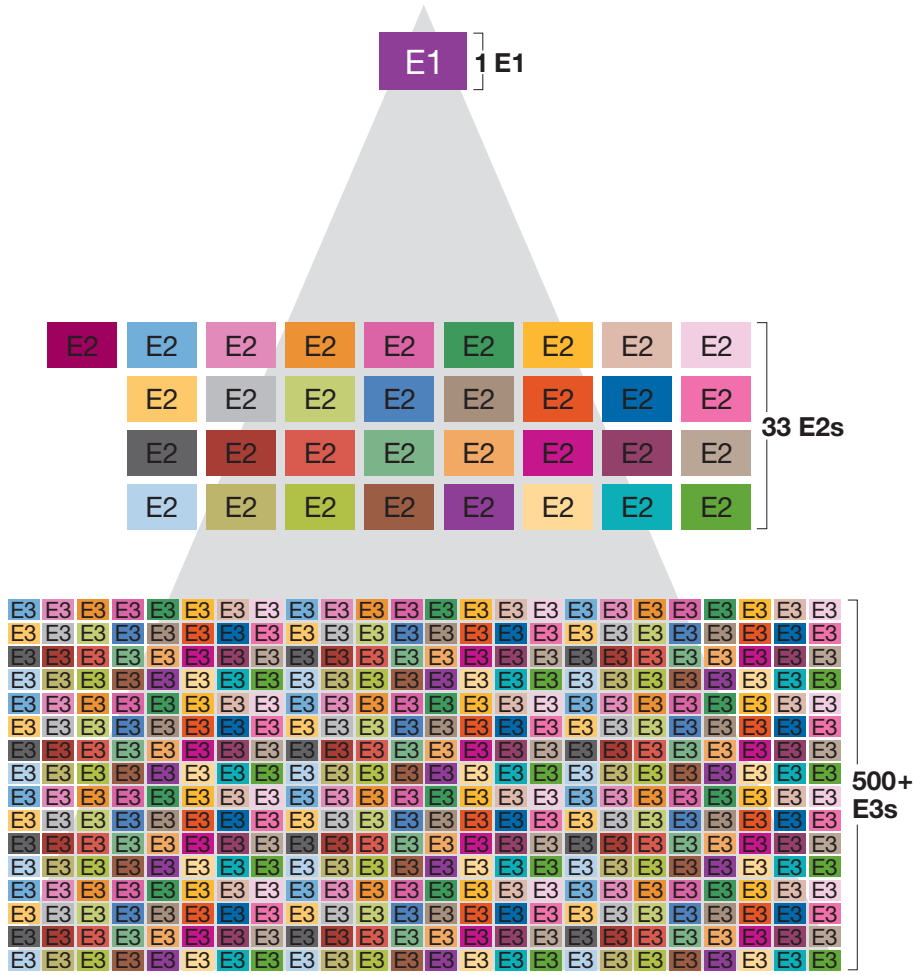

Figure 13. ***Complexity of the mammalian ubiquitin-conjugating system.*** A single E1 is thought to activate ubiquitin (Ub). At least 33 different E2s function as carriers of activated Ub and combine with more than 500 E3s for substrate-specific ubiquitination. The different combinations affect substrate selection and Ub-chain topology. (Taken from Kaiser and Fon, 2007)

It should be mentioned that after the first association of ubiquitination with peroxisome biogenesis in 1992, controversial data about the process have been accumulating during years, until very recently. These data, obtained for different yeast species, concerned the target, type of ubiquitination and its function in the formation of peroxisomes. However, intensive research by several groups has helped to create recently a clear picture of the function of this modification and the proteins involved.

Since ubiquitination during glycosome biogenesis plays a predominant role in the work described in this thesis, we find it relevant to provide here a

description of the historical frame of the unravelling of the role of ubiquitination in peroxisome biogenesis.

The discovery of PEX4 and its role in peroxisome biogenesis

Initially, Van der Klei *et al.* (1998) characterized the function of PEX4 in *H. polymorpha.* They observed that this ubiquitin-conjugating enzyme was required for the proper function of the PTS-1 import machinery and postulated a link between ubiquitination and PEX5 recycling.

Shortly afterwards, Koller and coworkers demonstrated in 1999 that PEX4 of *P. pastoris* was anchored to the peroxisomal membrane through a new peroxin, PEX22 (Koller *et al*., 1999). They provided data which implicated that the PEX4-PEX22 complex has a function in matrix protein import. A remarkable observation was that upon knockout of the PEX4 and PEX22 genes, the protein levels of the receptor PEX5 were drastically decreased. To explain the unstable levels of PEX5 in the ΔPEX4 and ΔPEX22 mutants, they proposed that the PEX4–PEX22 complex might function similar to the Cue1p–UBC7p complex, regulating the proper assembly and/or correct stoichiometry of protein import complexes at the peroxisomal membrane. Under this assumption the function of PEX4 at the membrane would be to ubiquitinate and therefore target misfolded membrane proteins or nonstoichiometric subunits of the complex, leading to their degradation by the 26S proteasome in the cytosol.

Collins *et al.* (2000), who performed an epistasis analysis of peroxins involved in peroxisomal matrix protein import in *P. pastoris*, found that PEX4, PEX22, PEX1 and PEX6 were factors acting in the terminal steps of the import process, after the matrix-protein translocation events. In this report they also characterized a ΔPEX4 mutant and found that loss of PEX4 resulted in reduced steady-state levels of PEX5. Interestingly, ultrastructural analysis showed that peroxisomes in this mutant were much smaller and consequently significant less of peroxisomal matrix proteins (PTS-1 and PTS-2) were found associated with the peroxisomes after cell fractionation. Accordingly, they observed that in the

ΔPEX4 mutant the majority of PEX5 was associated with an organellar pellet, suggesting a function of PEX4, and hence for ubiquitination, in PEX5 recycling.

Erdmann and coworkers (Platta *et al*., 2004) demonstrated later that the matrix-protein receptor PEX5 was ubiquitinated in *S. cerevisiae* mutants lacking PEX4, PEX22, PEX1 and PEX6, as well as in cells affected in proteasomal degradation. More precisely they defined that the monoubiquitination of the receptor occurs in cells lacking PEX4-PEX22, while polyubiquitination takes place in cells not having PEX1/PEX6. They observed that this occurs exclusively at the peroxisomal membrane and that this form of ubiquitination is dependent on UBC4, an ubiquitin-conjugating enzyme involved in proteasomal degradation. Notably, these authors did not find a decrease in the steady steady-levels of PEX5 in the mentioned *S. cerevisiae* mutants, which is in disagreement with the previous report about PEX5 in the *P. pastoris* mutants. The fact that they found PEX5 ubiquitination in cells affected in the proteasome function but with an intact import machinery, was an indication that this modification may reflect a genuine stage in the PEX5 receptor cycle.

Kiel *et al*. (2005a) also found ubiquitinated PEX5 in the pellets of ΔPEX4, ΔPEX22, ΔPEX6, ΔPEX1 and ΔPEX15 *S. cerevisiae* cells, once more an ubiquitination process that depended on UBC4. These authors reported similar, enhanced steady-state levels of the PEX5 receptor in each of these mutants which are affected in the recycling of the receptor, compared to the wild type. Based on their data they proposed that ubiquitination of PEX5 in *S. cerevisiae* is part of a quality control mechanism, that operates at a basal level during peroxisomal matrix protein import. This ubiquitination would serve to target PEX5 molecules that did not recycle properly for removal by proteasomal degradation. Interestingly, the authors interpreted the previous report on the reduced steady-state levels of PEX5 in *P. pastoris* recycling mutants as a species-specific effect of a much faster PEX5 turnover by the proteasome due to the obstruction of the recycling in the respective mutants. They reasoned that in *S. cerevisiae* a different balance between the recycling and proteasomal

degradation must exist, causing already at optimal conditions the turnover of a significant number of receptor molecules. However only under certain circumstances, as in the ΔPEX1 and ΔPEX4 mutants, the quality control mechanism generates more ubiquitinated species of PEX5 than can be degraded quickly enough by the proteasome and therefore allows them to be readily visualized.

In the same year, Kiel *et al*. (2005b) also reported that in *H. polymorpha* polyubiquitination of PEX5 is important for peroxisomal matrix import. Interestingly, the obstruction of the polyubiquitination by expression of a K48R mutated version of ubiquitin caused a mislocalization of peroxisomal matrix proteins, a similar phenotype as found for ΔPEX4 mutant cells. They found that in both the K48R ubiquitin and the ΔPEX4 mutant cells a significant proportion of PEX5 was ubiquitinated and subsequently degraded. Moreover, they proved that this modification occurred at the Lys21 of PEX5. They demonstrated that the ΔPEX4 mutant cells of *H. polymorpha* have similar low steady-state levels of PEX5 as those reported for *P. pastoris* recycling mutants, and that this dramatic decrease in PEX5 levels is due to degradation by the proteasome. Remarkably the authors concluded the polyubiquitination of PEX5 in *H. polymorpha* does not require the function of PEX4.

Shortly afterwards Kragt *et al.* (2005) discovered that the receptor PEX5 was monoubiquitinated in *S. cerevisiae* wild-type cells. In this work, that involved immunoprecipitation techniques, it was proved that PEX5 was monoubiquitinated at two different lysine residues and that this modification requires the peroxisomal membrane. It was established that this PEX5 modification is dependent on functional docking and RING complexes, thus identifying the monoubiquitination as a late event in the peroxisomal matrix import process. More importantly, the authors reported that in PEX4/PEX22 mutants a more pronounced PEX5 ubiquitination still takes place, but with a slightly different migration pattern on gels, indicating polyubiquitinated chains as described by others in previous reports. Similar ubiquitination profiles were

found for PEX1 and PEX6 mutants. The authors could determine that the monoubiquitination of PEX5 did not depend on UBC4, while they could confirm once more that, in contrast, the PEX5 polyubiquitination found in mutants of the recycling complex is dependent on UBC4. Based on their data and in the previous reports, they proposed a model in which the PEX4/PEX22 complex plays a role in the monoubiquitination of PEX5 in wild-type cells, in conjunction with the RING-finger complex, which likely acts as E3 ligases and that this modification represents the signal for receptor recycling. It was postulated that this signal must be recognized by the PEX6/PEX1/PEX15 complex, which pulls the receptor from the membrane into the cytosol, where it is quickly deubiquitinated. On the other hand, they agreed with the model in which proteasomal degradation of PEX5 is dependent on its polyubiquitination by UBC4 and probably the same RING-finger complex when recycling is impaired. It should be noted that, in this work, the steady-state levels of monoubiquitinated PEX5 appeared to be rather low, making its detection difficult without addition of inhibitors of the deubiquitinating enzyme during the lysate preparation to stabilize this PEX5 form. Importantly, these authors discussed the fact that in *S. cerevisiae* a decrease of non-modified PEX5 was never observed in the recycling mutants, contrary to what has been proved for *P. pastoris* and *H. polymorpha*. They attributed this phenomenon to the fact that less than four ubiquitin moieties may be insufficient for proteasomal recognition and further degradation.

At the end of 2005, Zolman and co-workers reported the identification of the *Arabidopsis thaliana* PEX4/PEX22 homologues (Zolman *et al.,* 2005). They found peroxisomal malfunction features in the single ΔPEX4 mutant and enhanced peroxisomal defects in the double ΔPEX4/ΔPEX22 mutant. Remarkably, these two plant proteins were able to rescue the matrix-protein import defect of the yeast PEX4 or PEX22 *S. cerevisiae* mutants after their coexpression in the yeast, confirming them as the functional *A. thaliana* orthologues for the import process. Unfortunately, they could not attribute their function in *A. thaliana* PEX5 ubiquitination because PEX5 immunoblot analysis did not lead to the identification of slower migrating forms of the matrix-protein receptor.

Interestingly, they observed stabilized steady-state levels of PEX5 in the mutants indicating similarities with *S. cerevisiae*.

It was in 2007 when an optimized system to study PEX5 monoubiquitination was published by Platta *et al.* (2007b). These authors claimed that the polyubiquitination of the receptor in *S. cerevisiae* interfered with the proper detection of monoubiquitinated molecules. To solve this inconvenience, they created functional PEX5 mutants with either a 3x-myc tag added to its N-terminus (which in other proteins had proved to avoid polyubiquitination) or with two mutations introduced at adjacent lysine residues that were predicted to function as a polyubiquitin acceptor sites. In these cell lines they could detect stable monoubiquitination of the mutated PEX5. Moreover, work with these mutants allowed them subsequently to distinguish PEX4 from others UBCs as the protein responsible for this modification, establishing that PEX5 is the physiological substrate of the peroxisome-bound active PEX4. Furthermore they established a cell-free ubiquitination system that proved the molecular interaction between PEX4 and PEX5. It was elegantly demonstrated that PEX5 monoubiquitination occurs independently of the AAA^+-ATPases and that both mono- and polyubiquitination are sufficient to prepare PEX5 for the AAA^+-ATPase dependent release to the cytosol. This work solved the mystery of the function of PEX4, the only ubiquitin-conjugating enzyme associated with peroxisome biogenesis.

Soon after the proof of PEX4 function provided by Platta *et al*., Williams *et al.* (2007) reported that the PEX4-mediated monoubiquitination of PEX5 occurs at a conserved cysteine residue in the N-terminal part of the *S. cerevisiae* peroxin, while the UBC4 dependent ubiquitination occurs at two different lysine residues. This represents one of the few examples reported where a SH-group of a cysteine residue is used as an acceptor site for the attachment of ubiquitin to a target protein.

In the same year, the group of Azevedo (Carvalho *et al*., 2007) reported for the first time the ubiquitination of mammalian PEX5. In an *in vitro* import/export

system they could detect also two types of ubiquitin-modifications. A slow thiol-resistant PEX5 modification of which the physiological relevance was not further discussed and a thiol-sensitive one that involved a cysteine residue (C11) of PEX5. The function for the cysteine-dependent ubiquitination of yeast PEX5 proposed by Williams *et al.* (2007) was experimentally corroborated by the group of Azevedo who found that also in mammalian cells it is required for the receptor's recycling. This remarkably shows the conserved nature of the PEX5-mediated protein transport pathway in distantly related organisms. However, at this stage of the research the nature of the UBC protein responsible for PEX5 monoubiquitination in mammalian cells was still not known, since the most similar protein to yeast and plant PEX4s, identified in the human proteome, was localized in the nucleus, giving no hint in the possible identity of the enigmatic UBC protein involved in peroxisome biogenesis.

In 2008, the same team (Grou *et al.,* 2008) described the identification of the mammalian counterpart of PEX4 responsible for the cysteine-dependent ubiquitination of PEX5. They made use of a previously developed *in vitro* import system with purified peroxisomes in which PEX5 efficiently docks at the peroxisomal membrane but is not able to be recycled back, probably due the lack of export factors (ubiquitin, E1, etc.). They explored the factors present in the cytosol required for the ubiquitination of PEX5. By adding partially purified UBC enzymes they found that three cytosolic proteins belonging to the E2D family (UBCH5a/b/c) efficiently ubiquitinated PEX5 in their *in vitro* system. The modification was done at the predicted cysteine residue (C11), generating export competent species of PEX5. The finding that cytosolic and redundant UBC proteins perform the PEX4 function in mammals, and probably also in human cells, explains why for so many years the mammalian homologue of yeast/plant PEX4 was not identified. UBCH5s are also involved in other cellular functions such as the control of the cellular levels of p53 or Iκβα, meaning that they are not devoted exclusively to the ubiquitination of PEX5. In line with this is the evidence for a wide capability of interactions with many E3 proteins that has been reported for the members of the E2D family. These results show an important mechanistic difference between the PEX5 ubiquitination in

yeasts/plants compared to mammalian cells, where the cytosolic UBCH5 proteins must be temporarily recruited to the peroxisomal membrane to interact with the E3 ligases and the ubiquitin's substrate PEX5.

It was only several years later, in 2011, when Okumoto *et al.,* presented for the first time evidence for the *in vivo* cysteine-ubiquitination of PEX5 in mammalian cells, thereby elegantly proving that it regulates the receptor's recycling (Okumoto *et al.*, 2011).

During long time speculations have been made about the identity of the E3 ligases involved in PEX5 ubiquitination. It was already proposed early that the members of the RING-finger complex (PEX2/PEX10/PEX12) might be responsible, based on several facts: they are an essential part of the importomer and hence required for matrix-protein import; and they each possess a RING-finger domain, a structure recognized for having E3s' ubiquitin-ligase activity. In addition, early studies indicating a physical interaction of PEX10 and PEX4 in *S. cerevisiae* supported this hypothesis (Eckert *et al.,* 2003).

In 2009, Erdmann and coworkers (Platta *et al*., 2009) finally could confirm that indeed PEX2 and PEX12 are the E3 peroxins responsible for the *in vivo* poly- and monoubiquitination of PEX5, respectively. They elegantly demonstrated that the ligase activity of these peroxins requires the binding of zinc, that the presence of each of the three RING-finger proteins is required for the complex formation and that they can be autoubiquitinated in an E2-dependent manner, with the pair PEX4-PEX12 being in charge of monoubiquitination, the signal for PEX5 recycling, and UBC4-PEX2 as the E2-E3 pair responsible for PEX5 polyubiquitination in the signalling of PEX5 for proteasomal degradation. These findings disagree with a previous report by Williams *et al.* (2008), which also showed dependence of PEX10 for the UBC4-polyubiquitination of PEX5 *in vivo*. However, Platta *et al.* (2009) argued that the fact that PEX18 from *S. cerevisiae* and PEX20 from *P. pastoris* are also ubiquitinated for their recycling, opened the possibility that the PEX10-ligase activity could be required for the ubiquitination of other substrates than PEX5. On the other hand, Miyata *et al.* (2012) proved

that in mammalian cells PEX10 is rather the RING-finger peroxin responsible for cysteine-monoubiquitination of PEX5.

It has been postulated that after export of PEX5 from the membrane to the cytosol the ubiquitin moiety has to be removed. The first evidence for this process was obtained by *in vitro* studies with rat cells where the cysteine-monoubiquitin moiety of PEX5 was shown to be removed by two distinctive mechanisms: an enzymatic-independent mechanism by which a minor fraction of monoubiquinated PEX5 is deubiquitinated as the result of a nucleophilic attack by glutathione and an enzymatic-dependent mechanism where an unknown deubiquitinating enzyme was involved (Grou *et al.,* 2009). The identity of such a deubiquitinating enzyme was very recently published by the same authors (Grou *et al*., 2012), who found that USP9X (ubiquitin-specific protease 9X) is the most active deubiquitinating enzyme acting on cysteine-ubiquitinated PEX5. The authors of this work corroborated the previous data regarding the physical separation of the Ub-PEX5 export and deubiquitination events, which they assume to occur independently at the peroxisomal membrane and in the cytosol, respectively.

The identity of the deubiquitinating enzyme from *S. cerevisiae* has also been confirmed very recently. Debelyy *et al.* (2011) found that UBP15 functions as the ubiquitin hydrolase in the deubiquination of PEX5 and, very importantly, that this activity is associated with the export complex comprised by PEX1/PEX6. This discovery implies that PEX5 is deubiquitinated during or shortly after the export event. The authors argue that although UBP15 is not required for peroxisomal biogenesis under normal conditions, the combined evidence from all experimental data indicates that UBP15 might regulate the specific detachment of the ubiquitin moiety, making it a vital step in the receptor cycle. Their data also indicate a difference with regard to the subcellular localization where the PEX5-deubiquitination step takes place between mammals and baker's yeast.

The group of Fujiki (Miyata *et al*., 2012) has recently found a new co-factor of

PEX6 involved in the cysteine-monoubiquitinated PEX5 export from peroxisomes in mammalian cells. They identified AWP1/ZFAND6, an ubiquitin binding NFκB modulator. This cytosolic protein contains two zinc-finger domains, one of which is involved in the regulation of NFκB and the recognition of polyubiquitinated protein. They observed that AWP1 interacts with PEX6 through its zinc-finger domain and via the Walker motifs of PEX6, indicating that the ATPase activity modulates the interaction. This protein was shown to interact with both mono- and polyubiquitin via its A20-type finger domain. Moreover this protein was able to interact *in vitro* with cysteine-ubiquitinated PEX5 as well as, at a basal level, with un-modified PEX5. AWP1 stimulates PEX5 export in an *in vitro* export system. The functional characterization of AWP1 places this protein as an essential player in the normal PTS-1 protein import pathway. Reduced PEX5 levels found in an AWP1 RNAi mutant are in agreement with a similar phenotype observed in human fibroblasts from patients affected in either PEX1, PEX6 or PEX15, supporting a coordinated function for this new factor and the export complex.

It is worth mentioning here that it is quite relevant for our work, to be reported in this thesis, that the Fujiki team has found that ubiquitinated PEX5 in cellular fractions was present as both a DTT-sensitive and a DTT-insensitive form, representing Cys-linked ubiquitinated and lysine-ε-amino-linked ubiquitinated PEX5, respectively, in both Fao and HeLa cells (Okumoto *et al.,* 2011; Miyata *et al.,* 2012). The two forms of modified PEX5 were distinguishable by their subcellular distribution as a peroxisome associated Cys-ubiquitinated PEX5 and a cytosolic DTT-insensitive PEX5. These two forms were however indistinguishable by molecular weight. No comments on the nature of the cytosolic lysine-linked ubiquitinated PEX5 were made in this publication.

The ubiquitin-conjugating enzyme PEX4: structural features

The E2 protein family of ubiquitin-conjugating (UBC) enzymes is structurally well characterized thanks to the large number of structural data accumulated throughout the years (100 structures of E2 enzymes are available in the Protein Data Bank (PDB), over half of which are human E2 crystal structures) (Wenzel *et al.,* 2011). The available structural evidence reveals that E2 UBC domain structures from yeast to human are remarkably similar. However, a great functional diversity within this enzyme family is indicative of distinctive features in the E2s and the wide capacity of these E2s to interact with a large variety of other proteins. It is believed that the specificity of the functional ubiquitination is given mainly by the specificity of E2 interaction with E3 enzymes (Pickart, 2001).

There are 14 E2s in *S. cerevisiae*, and even more in higher organisms, with the highest number found in mammals and plants, 35 and 48 respectively, reflecting multiple isoforms of some E2s as well as the evolution of new E2s (Michelle *et al.,* 2009).

Figure 14 shows the general aspects of the structure of a canonical E2 enzyme and how it interacts with the other components of the ubiquitination machinery. The structural features of this family, as well as the enzymatic mechanism of the reactions in which these proteins participate have been very well reviewed by Capilli *et al.* (2007) and Wenzel *et al.* (2011). E2 enzymes are easily recognized from their evolutionarily conserved ~150-residue catalytic UBC domains. UBCs have a compact structure, shaped essentially like a prolate ellipsoid, comprising four α-helices, a short 3_{10}-helix near the active site and a four-stranded anti-parallel β-sheet. On the 'bottom' surface (in the view shown in Figure 14), the active-site cysteine residue to which Ubiquitin becomes conjugated is placed in a catalytic groove surrounded by highly conserved amino acids that mediate both the thio-ester formation (reception of Ubiquitin from the E1 Ub-activating enzyme) and substrate ubiquitination (isopeptide bond formation with a substrate lysine residue). Most notable is a trio of residues known as the 'HPN'

motif, the general motif of this superfamily, usually found 10 residues to the N-terminal side of the active-site cysteine residue. The histidine residue is thought to play a structural role in the formation of the E2 active site, whereas the asparagine residue is important for the catalysis of the isopeptide bond formation between Ubiquitin and a substrate lysine residue. In addition, several domains are highly conserved and play an important role in the function of these enzymes.

As the general mechanism for ubiquitination suggests, an E2 must interact with both an E1 and an E3; both interactions use partly overlapping surfaces on one pole of the UBC ellipsoid (Figure 14). The E1/E3 interaction site comprises residues on the N-terminal helix, or helix 1 (H1), and loops 4 and 7 (L4 and L7). The shared surface implies that an E2 must be free of the E3 before becoming re-loaded with Ubiquitin.

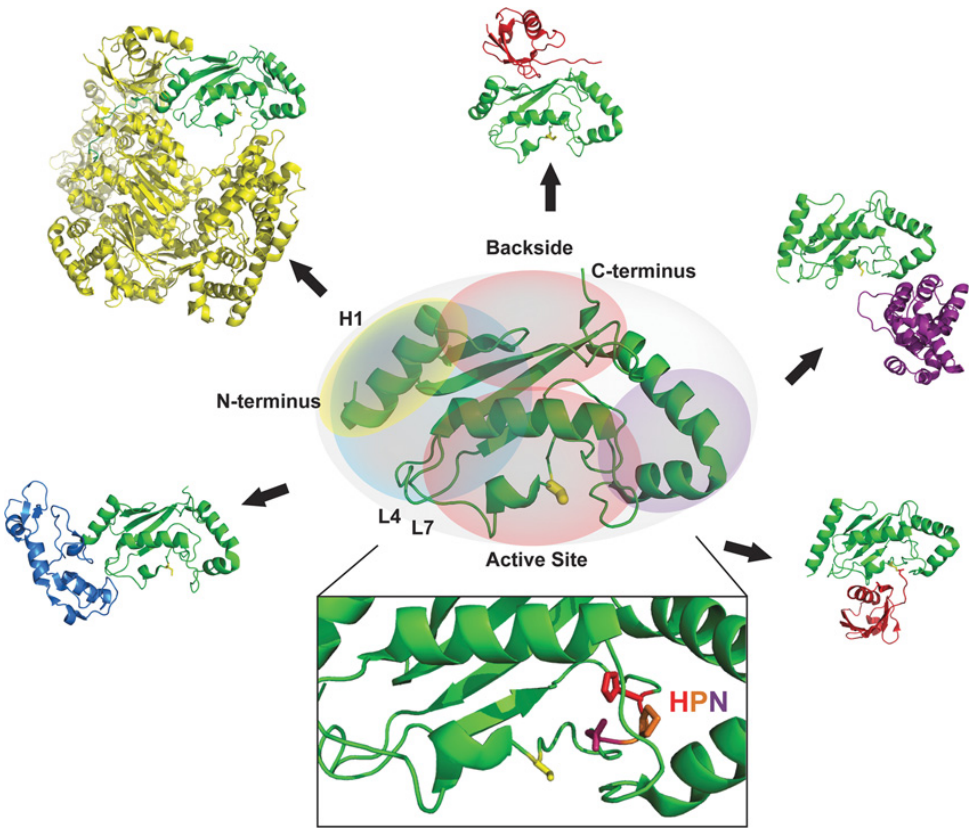

Figure 14. ***Structure of an UBC domain and its interaction surfaces.*** An example of the structure of a typical E2 enzyme (UBC13) (PDB code 2GMI) is shown in the center panel highlighting its interaction surfaces. Examples of structures of E2-E1, E2-E3, E2-free-ubiquitin, E2-bound-ubiquitin, E2-substrate complexes are also displayed. The E2 in each interacting pair is shown in green. Clockwise from the lower-left corner: the E1/E3-binding surface as seen in complex of UBC13 with the RING E3 TRAF6 (blue) (PDB code 3HCT) and in UBC12 in complex with the NEDD8-activating E1 (yellow) (PDB code 2NVU); the backside binding surface as seen in the E2-Ub complex UBCH5c-Ub (red) (PDB code 2FUH); the substrate-binding surface as seen in the SUMO E2 UBC9 in complex with its substrate RanGAP1 (purple) (PDB code 1Z5S); the activated Ub/Ubl surface as seen in the UBCH5b~Ub complex (PDB code 3A33). The inset at the bottom of the Figure shows the highly conserved HPN motif at the active site, with the active-site cysteine residue shown in yellow stick representation. (Taken from Wenzel *et al.,* 2011).

Many E2s are characterized by additional segments located within and flanking the UBC domain. Most common are E2s that contain variable N- or C-terminal extensions appended to the UBC domain. Historically, the E2 family has been divided into four classes according to the presence of these additional protein segments: class I, UBC domain only; class II, UBC domain plus a C-terminal extension; class III, UBC plus an N-terminal extension; and class IV, UBC plus both N- and C-terminal extensions. Although the topology of the UBC domain is highly conserved, the N- and C-terminal extensions are widely variable in both size and structure. Although this classification seems a fair way to group E2s, the different classes are not predictive for functionality, such as which E3s a given E2 will interact with, or whether an E2 transfers monoUb or is capable of building a polyUb chain (Wenzel *et al.,* 2011). In a recent effort to develop a more appropriate E2 classification, a phylogenetic analysis of similarities within the UBC domains yielded 17 different families of E2s (Michel *et al.,* 2009). However, assigning functional significance to each family may have limited utility when there are half as many families as E2s, and additional biochemical and functional characterization will be required to lend predictive value to this fine-grained classification (Wenzel *et al.,* 2011).

Recently, Williams *et al*. (2011) published the crystal structure of the *S. cerevisiae* PEX4-PEX22 complex, reporting a novel and an unusual function for ScPEX22. The ScPEX4 structure was shown to adopt a typical UBC fold (Fig. 15). As in other E2 structures, the active site cysteine (Cys115) is situated in a cleft formed by the 3_{10}-helix and the loop region between α2 and α3. Examination of the residue-average B-factors in the co-crystal structure, suggests the presence of

some highly flexible 'hotspots' in ScPEX4 which could indicate that these flexible loops segment ScPEX4 into three, loosely associated subdomains, a 'cap' comprising α1, a 'core' domain consisting of β1–β2–β3–β4–3_{10}–α2 and an interaction motif formed by α-helices α3 and α4.

ScPEX22 also comprises a mixed β-sheet/α-helical fold, consisting of five parallel β-strands in the order β3–β2–β1–β4–β5, which form a central β-sheet that is tightly sandwiched on both sides by eight α-helices (Figure 15A). Despite the apparent similarity with a Rossmann-like fold, the authors concluded that the overall fold of PEX22 was novel.

The ScPEX4:PEX22 binding site is established by the insertion of helix α5 from ScPEX22 into the V-shaped arrangement of the C-terminal helices α3 and α4 of ScPEX4 (Fig. 15B). Additional interactions are formed by the exposed surface of helix α4 from ScPEX4 and strand β3, helix α4 and the joining loop region from ScPEX22, effectively burying around 750 $Å^2$ of the solvent-accessible interface area. In ScPEX4, a total of 19 residues participate in the interface formation involving both polar and hydrophobic contacts (Figures 15B-C). Accordingly, a number of interfacing residues, specifically Asp151, Ala165, Gly168, Ile169 and Tyr172, are conserved in homologous PEX4 sequences, but not in other UBC enzymes. Particularly, the contribution of Tyr172 to the interaction, beyond the hydrogen bond to Lys133 from ScPEX22, extends into extensive Van der Waals interactions, involving Val86, His113, Lys114, Leu116 and Leu131 from ScPEX22, residues that are for a large part conserved within the PEX22 family (Fig. 15B). Remarkably, the ScPEX22-interaction surface in ScPEX4 does not overlap with any other predicted interacting region supporting the prediction of a totally functional ubiquitin activating ScPEX4 when attached to the peroxisomal membrane.

Very interestingly, Williams *et al.* (2011) found that the function of ScPEX22 was not only the mere targeting of ScPEX4 to the peroxisomal membrane. By biochemical methods they showed that *in vitro* association with ScPEX22

enhances ScPEX4's ability to perform self-ubiquitination, but more importantly that ScPEX22 binding-deficient forms of ScPEX4 were unable to ubiquitinate the peroxisomal import receptor PEX5 *in vivo*. They concluded that the ScPEX4:PEX22 assembly, and not ScPEX4 alone, functions as the E2 enzyme required for PEX5 ubiquitination, establishing a novel mechanism of E2 enzyme regulation.

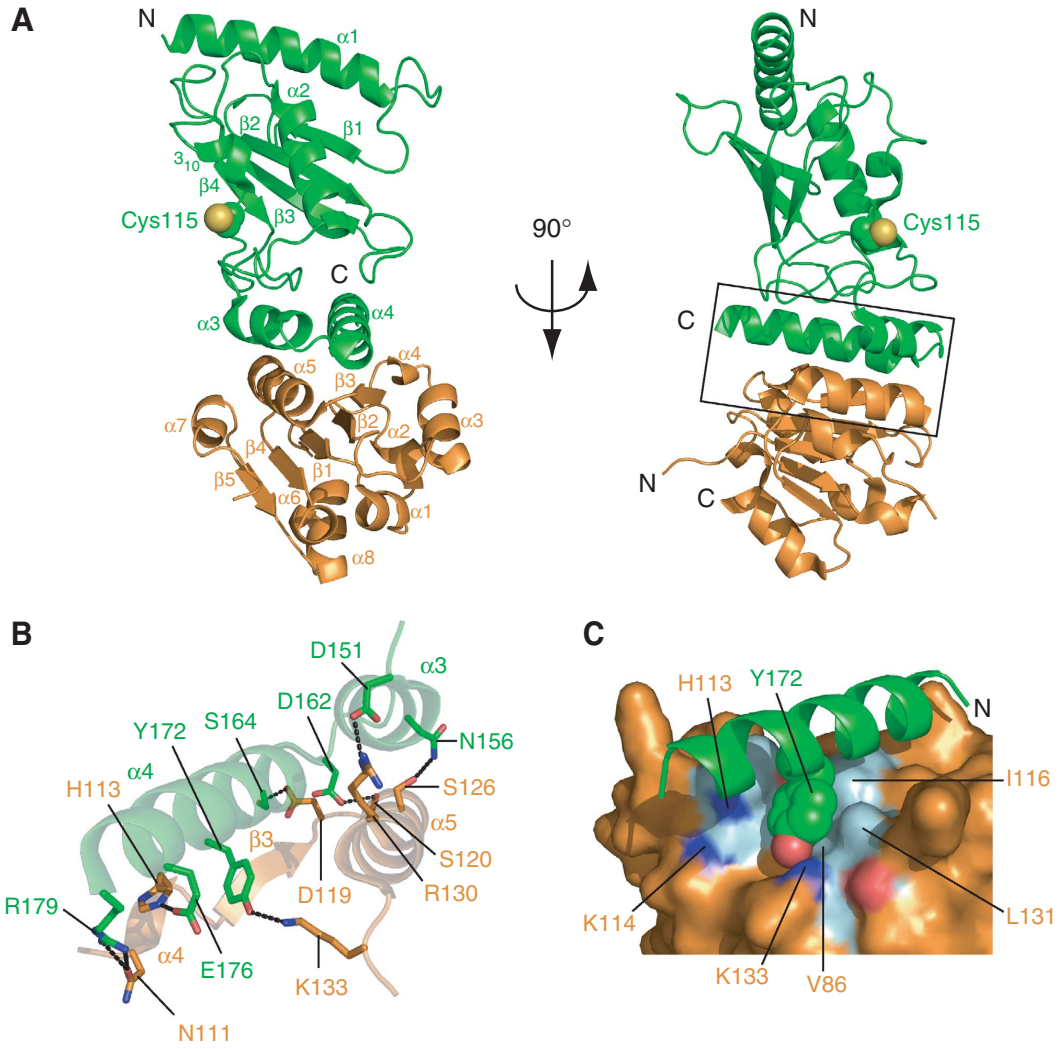

Figure 15. ***Molecular basis of PEX:PEX22 complex formation.*** (**A**) Overall structure of PEX4 (green) in complex with PEX22 (orange) in two different orientations. The interface is formed by the C-terminal helices α3 and α4 from PEX4 and helices α4 and α5 and β-strand β3 from PEX22 (boxed). PEX4's active site Cys115 is indicated in sphere presentation. (**B**) Specific residue interactions found in the PEX4:PEX22 interface. Residues that participate in hydrogen bonding (dotted lines) are shown in stick representation and labelled. Nitrogen and oxygen atoms are coloured blue and red, respectively, with carbon atoms coloured in green. (**C**) Surface representation of PEX22 (orange) showing residues involved in hydrophobic contacts (cyan) with Tyr172 of PEX4 (green). Nitrogen and oxygen atoms are coloured blue and red, respectively. (Reproduced from Williams *et al.,* 2011)

Peroxisomal matrix protein receptor PEX5

The study of PEX5 recycling in *T. brucei* has been a major topic of the research performed during this project. Therefore, we will introduce important structure-function aspects of PEX5 as well as describe relevant species-specific features of the receptor.

In plants and kinetoplastids, the peroxisomal matrix-protein receptor PEX5 is present as only one isoform that is directly involved in PTS-1 import and together with PEX7, also in PTS-2 protein import (Galland *et al*., 2007). In yeast also only one isoform of PEX5 exists, which is exclusively devoted to PTS-1 protein import. By contrast, in mammals two isoforms exist as a result of alternative splicing: the shorter PEX5S and a longer PEX5L, differing in an internal segment of 37 amino acids which contributes to the formation of the PEX7 binding site. PEX5S only binds PTS-1 containing-proteins, the long isoform PEX5L interacts with PEX7 via the so-called PEX7-binding box, which is also found in the yeast co-receptors PEX18, PEX20 and PEX21 which assist PEX7 in PTS-2 protein import (Stanley *et al*., 2006; Wolf *et al.,* 2010). The consensus sequence of the PEX7-binding box is also present in PEX5 from plants and kinetoplastids.

As mentioned previously, PEX5 is a protein composed of two separate domains: The N-terminal half and the C-terminal TPR domain. The poorly conserved N-terminal region is non-globular and intrinsically unstructured, primarily functioning in the docking of PEX5 to the peroxisomal membrane (Stanley *et al.,* 2006; Williams *et al.,* 2010). A seven-fold repeated pentapeptide WxxxF/Y motif is widely distributed in this domain of mammalian PEX5 and some of these motifs have been proved to be involved in the binding of PEX13 and PEX14. Nevertheless important differences between taxonomic groups exist (for a detailed review see Galland and Michels, 2010) (Fig. 16).

The C-terminal domain is globular and highly conserved. It is almost entirely composed of tetratricopeptide repeats (TPRs), which despite of a low sequence

similarity, adopts regular conserved secondary structures. These are helix-turn-helix motifs, tandemly repeated to generate regular solenoid proteins with a general function in protein-protein interactions. In PEX5 the PTS-1 recognition is done through the TPR domain. PEX5 has been also involved in the recognition of some peroxisomal proteins without canonical PTS-1 motifs. It is assumed that this mechanism of binding does not involve the TPR domain, since a construct of a PEX5 receptor composed of only the N-terminal part was still functional (Stanley *et al.,* 2006) (Fig. 16).

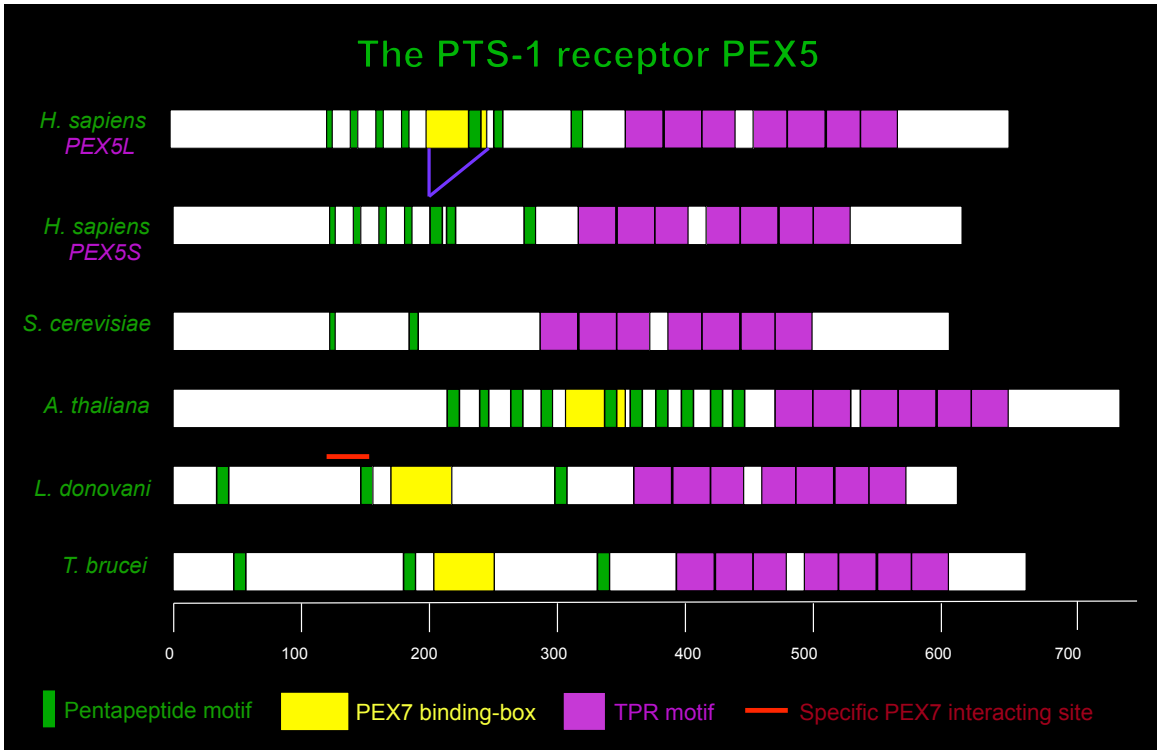

Figure 16. ***Schematic representation of PEX5's structure organization for several species***. Pentapeptide motifs defined as WXXX(F/Y) are represented in green, the PEX7-binding box is presented in yellow and the C-terminal TPR motifs are shown in fuchsia. The specific PEX7 interacting site found in *Leishmania donovani* PEX5 is shown as a red line.

Early studies on the quaternary structure of PEX5 of several organisms led to the proposal of a model, the so-called "pre-implex model", in which it was considered that the recruitment and import of peroxisomal matrix-protein assemblies occurred by oligomerization of the PEX5 receptor, more precisely by forming a tetrameric structure. Since peroxisomal matrix proteins are often oligomers, it was assumed that each monomer of PEX5 would bind an oligomer

of a PTS-1 protein resulting in the formation of a huge protein complex at the peroxisomal membrane. This would cause a high local concentration of PTS-1 proteins – and through the interaction between PEX7 or its co-receptors also PTS-2 proteins – at the docking complex which, in turn, could facilitate protein translocation through the membrane. This model was abandoned when Costa-Rodriguez *et al.* (2005) proved by studying hydrodynamic properties of the N-terminal part of PEX5 that PEX5 is a monomeric non-globular protein that behaves abnormally in size-exclusion chromatography, explaining the previous experimental data obtained for the oligomeric states of PEX5.

Within the topic of this thesis, it is relevant to mention that Kiel *et al.* (2005b) pointed out that PEX5 is a highly unstable protein *in vitro* and that especially the cytosolic pool of PEX5 is subject to proteolysis. This has also been suggested earlier by Gould *et al.* (1996), who observed that the cytosolic form of the PTS-1 receptor exhibits pronounced protease sensitivity and that it could only be inhibited by addition of NaF to the lysis and fractionation buffers. They refer to a proteolytic degradation also observed for the PTS-1 receptor from human, *S. cerevisiae* and *H. polymorpha*. Kiel *et al.* (2005b) mentioned that protein precipitation by treatment of whole cells with trichloroacetic acid (TCA) prior to cell breakage prevented PEX5 from any proteolytic degradation. When reviewing the literature it became clear to us that such proteolytic degradation is commonly observed in many western blot analyses performed with lysates from yeast cells (Platta *et al.,* 2007: Fig 1C; Platta *et al.,* 2009: Fig. 1B, 2A and 7A; Williams *et al.,* 2007: Fig. 1A and 2A; Meinecke *et al.,* 2010: Suppl. Fig. S3) as well as from mammalian cells (Okumoto *et al.,* 2011 Fig. 1D, 3B and 4A). Although faster migrating forms of PEX5 are usually detectable, they are not mentioned and discussed, probably because of the assumption that they result from an unspecific *in vitro* proteolytic process. Although it seems that this proteolysis occurs apparently exclusively on PEX5 even in the presence of protease inhibitor cocktails that prevent degradation of other cytosolic proteins, and that in some cases it also occurs in TCA lysates (as in Platta *et al.,* 2007a: Fig. 1C), to our knowledge no further studies have been undertaken to acquire insight into the putative protease(s) responsible for it neither in a possible physiological

function for such a process.

State of the art in the research on glycosomal matrix-protein import

Glycosomes are organelles that belong to the peroxisome family. They share metabolic functions, characteristics of morphology and also their route of biogenesis, as revealed by morphological studies. Thus, a prediction was, when molecular studies on glycosome biogenesis started, that glycosomes are formed by a similar mechanism involving proteins homologues to peroxins. This prediction has been amply confirmed, as described below.

The study of glycosome biogenesis has gained a great interest in the scientific community because these organelles compartmentalize the great majority of glycolytic enzymes. This metabolic pathway has been validated as an excellent drug target for the design of new drugs against trypanosomatids that cause human diseases such as sleeping sickness, Chagas disease and leishmaniases. This unique compartmentalization had provided glycolytic enzymes of trypanosomatids with unique kinetic and structural features that can be exploited for the structure-based drug design of specific inhibitors that affects the parasites but do not harm the human host. For a detailed description of current stage of the research on the trypanosomatids' glycolytic pathway as a drug target see Chapter 3 of this thesis. In addition, it has been proved by different experimental approaches that at least for the human stage of *T. brucei,* the correct compartmentalization of this pathway inside the glycosomes is absolutely essential for the parasite survival. This fact and the low level of sequence conservation between trypanosomatids peroxins and their counterparts in humans, make the glycosomal matrix-protein import process and the proteins involved interesting as drug targets (Moyersoen *et al.,* 2004; Galland and Michels, 2010).

As mentioned before, glycosomal matrix proteins are imported post-translationally from the cytosol into glycosomes by a similar mechanism as operative in peroxisomal matrix-protein import. It is assumed that the half-life

of the glycolytic enzymes in the cytosol of trypanosomatids is very short (Ex: for procyclic forms between 1.0 and 3.4 min), indicating that the import is a quite fast process as is the case for peroxisomal matrix-protein import in mammalian and yeast cells. As in peroxisomes, glycosomal matrix proteins possess PTS motifs. The trypanosomatid genomes contain about 250 proteins with a predicted PTS (approximately two-third have a putative PTS-1 while just one-third a PTS-2). This group of predicted glycosomal proteins include enzymes of all known glycosomal metabolic processes including glycolysis, the pentose-phosphate pathway, purine salvage, pyrimidine biosynthesis, β-oxidation of fatty acids, ether-lipid biosynthesis, several reactions of the isoprenoid biosynthetic pathway and proteins involved in protection against oxidative stress, as well as proteins with unknown function (Moyersoen *et al.,* 2004; Opperdoes and Szikora, 2006; Colasante *et al*., 2006; for more details, see Chapters 2 and 3).

So far, various homologues of mammalian and yeast peroxins have been identified in genomes of the trypanosomatids (Table 1). All of them have been molecularly characterized, some in considerable detail, others less extensively: *Leishmania donovani* PEX2 (Flaspohler *et al.,* 1997), *T. brucei* PEX2 (Guerra-Giraldez *et al.,* 2002), *T. brucei* PEX11 (and GIM5A and GIM5B, isoforms of PEX11)(Lorenz *et al.,* 1998; Maier *et al.,* 2001; Voncken *et al.,* 2003), *L. donovani* PEX5 (Jardin *et al.,* 2000), *T. brucei* PEX5 (de Walque *et al.,* 1999), *T. brucei* PEX7 (Galland *et al.,* 2007), *L. donovani* PEX7 (Pilar *et al.,* 2008), *T. brucei* PEX13 (Verplaetse *et al.,* 2009), *T. brucei* PEX14 (Moyersoen *et al.,* 2004), *L. major* PEX14 (Jardin *et al.,* 2002), *T. brucei* PEX10, *T. brucei* PEX12, (Krazy and Michels, 2006; Saveria *et al.,* 2006) *T. brucei* PEX6 (Krazy and Michels, 2006) and *T. brucei* PEX19 (Banerjee *et al.,* 2005; Yernaux *et al*., 2006), proving that besides the general low overall sequence conservation with its counterpart in other organisms (between less than 10% to maximally 34%), mechanistically trypanosomatid glycosome biogenesis is similar to that of peroxisomes in yeast and mammalian cells (Table 1).

Depletion of all so far identified *T. brucei* peroxins, either involved in glycosomal division such as PEX11, GIM5A and GIM5B, in PMP protein import as PEX19 or in

the glycosomal matrix protein import such as PEX5, PEX7, PEX14, PEX13, PEX2, PEX10, PEX12 and PEX6 by RNAi, has a large effect on the growth of the trypanosomatids, essentially always resulting in death of the parasites, although for some of them faster than for others (for an extensive review, see Moyersoen *et al.,* 2004).

When comparing trypanosomatid peroxins involved in the docking events with their counterparts in yeast, plant and mammalian cells, remarkable differences have been found in the interactions between the different peroxins.

In mammalian cells, a long isoform of PEX5 acts as a co-receptor of PEX7 for proper PTS-2 protein import. This involves on PEX5L a region that is called the PEX7-binding box and that is also found in plant PEX5 and in PEX7 co-receptors in yeasts. The PTS-2 import process in trypanosomes was predicted to occur like in mammalian cells because their PEX5s also have conserved the sequence of the binding box for PEX7 in its N-terminal domain. Indeed, the interaction of TbPEX5 and TbPEX7 has been experimentally proved (Galland *et al.,* 2007), however the region of PEX5 responsible for it has not been identified yet. Neither has the binding region of PEX7 been identified. It should be mentioned that *T. brucei* PEX7 is unique because of the presence of an approximately 40 amino acids long C-terminal extension rich in proline residues, but the function of it is not known yet. For *L. donovani* PEX5 it was surprisingly found that the interaction with PEX7 occurs via a region between the first and second of the three pentapeptide motifs present in the N-terminal half, but outside the predicted PEX7-binding box. Whether *T. brucei* PEX5 binds to PEX7 like its orthologue in *L. donovani* is still an open question.

Surprisingly, was also the report that *L. donovani* PEX7 interacts with *L. donovani* PEX14 through the PxxP motif of PEX14 (Pilar *et al.,* 2008), quite different from the PEX7-PEX14 interaction in other organisms. Moreover the PxxP motif usually acts as an SH3 ligand and indeed, this has been reported for PEX14-PEX13 interactions.

Table 1. ***Summary of peroxins identified in trypanosomatids***. A. Homologue identification of putative peroxins based on sequence similarity searches in the trypanosomatid genomes. Experimentally confirmed identity and essentiality are shown for each species. B. Percentages of identity between peroxin amino-acid sequences. (Adapted from Moyersoen *et al.,* 2004)

A	***T. brucei***			***Leishmania* spp.**			***T. cruzi***		
	Homologue identified	Peroxin identity confirmed	Essentiality confirmed	Homologue identified	Peroxin identity confirmed	Essentiality confirmed	Homologue identified	Peroxin identity confirmed	Essentiality confirmed
PEX1	X			X			X		
PEX2	X	X	X	X	X	X	X		
PEX5	X	X	X	X	X	X	X		
PEX6	X	X	X	X			X		
PEX7	X	X	X	X	X	X	X		
PEX10	X	X	X	X			X		
PEX11	X	X	X	X			X		
PEX11 (GIM5A)	X	X	X						
PEX11 (GIM5B)	X	X	X	X			X		
PEX12	X	X	X	X			X		
PEX13	X	X	X	X			X		
PEX14	X	X	X	X	X		X		
PEX19	X	X	X	X			X		
B	***T. brucei***			***Leishmania* spp.**			***T. cruzi***		
	T.b-T.c	*T.b-L. spp*	*T.c-L.* spp.	*T.b-Human*	*T.c-Human*	*L.* spp.-*Human*	*T.b- Sc*	*T.c- S.c*	*L.* spp.-*S.c*
PEX1	60	49	49	27	28	27	23	23	23
PEX2	65	51	56	24	26	26	15	18	16
PEX5	64	52	52	26	27	26	22	22	21
PEX6	76	65	68	32	34	33	34	36	34
PEX7	76	65	68	32	34	33	34	36	34
PEX10	65	53	54	27	26	28	25	25	26
PEX11	61	59	62						
PEX11 (GIM5A)	66[a]	56[a]	56[a]	12-17[b]	12-16[b]	12-19[b]	11-13[b]	12-15[b]	12-13[b]
PEX11 (GIM5B)									
PEX12	57	40	43	20	20	18	17	18	13
PEX13	51	30	33	7	7	9	15	12	11
PEX14	45	36	34	16	15	14	13	17	14
PEX19	36	27	26	19	13	17	17	17	17

[a] average of identities of GIM5A and GIM5B homologues in the trypanosomatid species indicated.
[b] range of the identities between the different trypanosomatid PEX11 isoforms (PEX11, GIM5A, GIM5B) and the three isoforms of human PEX11:PEX11α, PEX11β, PEX11γ.

Trypanosomatid PEX13s distinguish themselves through their longer YGx-repeat region in the N-terminal half and the absence of the Pro-rich motif (PPPKPWE) located N-terminally of the Tyr-Gly repeat and of which the function is still unknown. A most remarkable feature of all trypanosomatid PEX13s is the presence of a predicted PTS-1 motif at its C-terminus, the tripeptide TKL (Verplaetse *et al.,* 2009). So far, the function of this candidate targeting signal is unknown. Since PEX13 is a peroxisomal membrane protein, its insertion into the

membrane is independent of a PTS, so this function can be excluded. The mapping of the interaction between TbPEX13 and TbPEX5 indicates that it is done via the SH3 domain and the third pentapeptide motif in TbPEX5. The same motif was also shown to be involved in the interaction with full-length TbPEX14 (Choe *et al*., 2003). It is worth mentioning that *T. brucei* PEX5, like in other trypanosomatids, possesses just three pentapeptide motifs, considerable less than its mammalian (seven) and plant (9 – 11) orthologues and more similar to the situation in yeasts.

Trypanosomatid PEX14s have been proved to interact with PEX5s, however the regions of PEX5 involved seem to differ between the two species. The N-terminal region of *T. brucei* PEX14 interacts efficiently with the first and third pentapeptide motifs in PEX5 (Choe *et al*., 2003; Moyersoen *et al.,* 2004), while in *L. donovani* this interaction is done by a different region of PEX5, not involving any of the pentapeptide motifs but a downstream region close to the start of the C-terminal TPR domain (Jardin *et al.,* 2002). The situation found for *T. brucei* is similar to that in other organisms, while that reported for *L. donovani* is drastically different.

Concerning the trypanosomatids' recycling complex, several studies accounted for the molecular characterization of the members of the RING-finger complex in *T. brucei* (PEX2: Guerra-Giraldez *et al.,* 2002; PEX10 and PEX12: Krazy and Michels, 2006 and Saveria *et al*., 2006) and for PEX2 in *L. donovani* (Flaspohler *et al.,* 1997). Depletion of each of the RING-finger peroxins leads to the death of *L. donovani* promastigotes, bloodstream and procyclic *T. brucei* cells. This was attributed to the mislocalization of glycolytic enzymes (Flaspohler *et al.,* 1997; Krazy and Michels, 2006). It was also shown that PEX10 and PEX12 of *T. brucei* localize in the glycosomes and more precisely that the two proteins behave as integral membrane proteins, each with its N-terminus and C-terminus (containing the C3HC4 type RING domain, a cysteine-rich region that coordinates the binding of two zinc ions) exposed to the cytosolic face of the glycosomes, a topology also found in its counterparts in yeast cells (Saveria *et al.,* 2006). Functional mutants of components of the RING-finger complex in

trypanosomatids exhibit lethal phenotypes, demonstrating their essentiality. However these studies explored for, none of them, the possible role in the ubiquitination of trypanosomatid PEX5, leaving this still an open question.

Another characterized member of the putative recycling complex in trypanosomatids is the dislocase *T. brucei* PEX6. This AAA^{+}-ATPase was also described by Krazy and Michels (2006), who showed that depletion of this peroxin has a strong effect on the growth of bloodstream and procyclic cells and also produces a mislocalization of glycolytic enzymes from the glycosomes to the cytosol. However, also in this case no evidence for a role in the PEX5 export was presented.

The low sequence conservation between the peroxins of trypanosomatids and humans, in addition to the considerable number of species-specific features of the interactions that occur between these proteins during the formation of these essential organelles, are valid arguments to consider them as promising drug targets in the pathogens kinetoplastids. This justifies a further study of the structures of the peroxins but also of the mechanistic details how they operate during the biogenesis of the glycosomes. Such studies are expected to discover important functional aspects and significant differences compared to peroxisome biogenesis in human cells. These could then be exploited for the design of inhibitors that block the formation of glycosomes, disrupting not only the glycolytic pathway, but the whole metabolic network occurring inside the organelle.

From the perspective of drug discovery, it should be mentioned that resolution of the structure of the C-terminal domain of *T. brucei* PEX5 in complex with several PTS-1 peptides, has already allowed the identification of several differences between the trypanosome and human PTS-1 receptor that could be exploited. The PTS-1 binding occurs in a site created between two halves of the TPR domain, composed respectively of the subdomains TPR1-3 and TPR4-7. Although the overall site is highly similar between *T. brucei* and human PEX5, some pockets are created in the interface between the two subdomains upon the

hinge motion between the two subdomains upon cargo binding. In human PEX5 one of these pocket contains among other residues a Met and Cys, while in *T. brucei* two Ala residues are found at the corresponding positions. This makes the pocket in the trypanosome receptor larger and more hydrophobic. The design of an inhibitor fitting in the trypanosome PEX5 pocket but not the human one could obstruct the PTS-1 protein release from *T. brucei* receptor without interfering with the function of the human PEX5. This could represent an excellent way of interfering with the organelle biogenesis (Sampathkumar *et al.,* 2008). This is just one example of important structural differences found in peroxins that sustain our efforts in the understanding of this essential process in the biology of the parasites with the hope of identifying an interesting drug target that can be used for screening of possible inhibitors as well as for structural-based drug design.

The objective of the research described in this thesis

The study presented in this thesis has comprised two lines of research concerning glycosomes of *T. brucei*:

The first research line concerned glycosome biogenesis. As it has been explained in the general introduction, glycosome biogenesis in *T. brucei* has been validated as a potential drug target against sleeping sickness. First, a large amount of experimental evidence indicates that correct compartmentalization of glycolytic enzymes inside glycosomes is essential for proper functioning of glycolysis – thus ATP production – in the human-stage form of the parasite and secondly the peroxins involved in the glycosomal matrix-protein import have been shown to possess a low degree of similarity compared to their counterparts in mammalian cells. Therefore, a good understanding of the molecular mechanism by which proteins are imported into glycosomes – and identification of differences with the mechanism in the host cells – is a crucial step forward in the fundamental research leading to the rational development of new drugs for African sleeping sickness that target the glycosome biogenesis process. This formed a major objective of this PhD research project. At its start, some proteins involved in the

biogenesis of peroxisomes in yeast and mammalian cells had not been identified yet in trypanosomatids. This was the case for PEX8, PEX3, PEX15/26 and the PEX4/PEX22 complex. Equally relevant was the lack of molecular data concerning the steps performed by the receptor TbPEX5, during its recycling after PTS-1 protein import. Initially the objective of this thesis project has been the unravelling of the molecular events involved in the recycling of *T. brucei* PEX5 and the discovery of new, essential factors implicated in this process. Our scientific approach has been based on a biochemical study of TbPEX5 in which the posttranslational modification by ubiquitination has been a focus of study. During the early stages of this project, bioinformatics analysis of the *T. brucei* genome allowed the identification of two candidate new peroxins, TbPEX4 and its anchor membrane TbPEX22, homologues of proteins involved in the recycling of PEX5 receptor in other organisms such as yeast and plant cells. We have used several biochemical and cell biology approaches for the subcellular localization and functional studies of these new peroxins. Chapter 5 contains still unpublished data where we described first the *in vitro* proteolytic processing of TbPEX5 which leads to the formation of a shorter form of the protein and that has been the basis for our further study of the receptor's ubiquitination. Furthermore, we present the first experimental evidence for ubiquitination of *T. brucei* PEX5, as well as, the confirmation and characterization of the candidate *T. brucei* PEX4 as the ubiquitin-conjugating enzyme responsible for this posttranslational modification. Additionally a functional analysis of some earlier identified peroxins, TbPEX6 and TbPEX12 has been addressed in this thesis to unravel their role in TbPEX5 ubiquitination and recycling.

The second research line described in this thesis addresses channel-forming activities in the glycosomal membrane. This research is presented in Chapter 6 as a research publication. This work has been part of a collaborative project with Prof. Vasily Antonenkov, from Oulu University, Finland, whose expertise is the transport of small solutes through the peroxisomal membrane in mammalian cells. By research previously performed in our laboratory, it was established that the glycosomal membrane represents an apparent impermeability barrier for co-factors and metabolites, which are mostly negatively-charged molecules.

The objective of our study was to acquire an understanding how small solutes, as some glycolytic intermediates, can cross the glycosomal membrane in order to sustain the several metabolic pathways present in the organelles. For this investigation we have established a methodology to isolate ultrapure glycosomal membranes from *T. brucei* and use the solubilized membrane proteins in electrophysiological experiments. This study has shown that glycosomal membrane posseses different channel-forming activities, which differ in their conductance (high, low and very low) and ions selectivity. The characterization of these activities by multiple-channel recording and single-channel analysis demonstrated that the channels were in an open state under all conditions tested, suggesting that they represent water-filled pores. The implications of this discovery for glycosomal metabolism, as well as for the design of drugs that target glycosomal enzymes are discussed.

Chapter 2

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Invited Review

When, how and why glycolysis became compartmentalised in the Kinetoplastea. A new look at an ancient organelle ☆

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ABSTRACT

A characteristic, well-studied feature of the pathogenic protists belonging to the family Trypanosomatidae is the compartmentalisation of the major part of the glycolytic pathway in peroxisome-like organelles, hence designated glycosomes. Such organelles containing glycolytic enzymes appear to be present in all members of the Kinetoplastea studied, and have recently also been detected in a representative of the Diplonemida, but they are absent from the Euglenida. Glycosomes therefore probably originated in a free-living, common ancestor of the Kinetoplastea and Diplonemida. The initial sequestering of glycolytic enzymes inside peroxisomes may have been the result of a minor mistargeting of proteins, as generally observed in eukaryotic cells, followed by preservation and its further expansion due to the selective advantage of this specific form of metabolic compartmentalisation. This selective advantage may have been a largely increased metabolic flexibility, allowing the organisms to adapt more readily and efficiently to different environmental conditions. Further evolution of glycosomes involved, in different taxonomic lineages, the acquisition of additional enzymes and pathways – often participating in core metabolic processes – as well as the loss of others. The acquisitions may have been promoted by the sharing of cofactors and crucial metabolites between different pathways, thus coupling different redox processes and catabolic and anabolic pathways within the organelle. A notable loss from the Trypanosomatidae concerned a major part of the typical peroxisomal H_2O_2-linked metabolism. We propose that the compartmentalisation of major parts of the enzyme repertoire involved in energy, carbohydrate and lipid metabolism has contributed to the multiple development of parasitism, and its elaboration to complicated life cycles involving consecutive different hosts, in the protists of the Kinetoplastea clade.

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1. Introduction

In 1977 Opperdoes and Borst reported the sequestering of the first seven enzymes of the glycolytic pathway of *Trypanosoma brucei*, responsible for the conversion of glucose into 3-phosphoglycerate, within microbodies (Fig. 1) which they called 'glycosomes' (Opperdoes and Borst, 1977). 'Microbodies' was a generic name for several organelles whose origins were uncertain, with variable morphology, although often spherical with a dense granular matrix and functions that were not well defined, but a common feature was that they did not contain any detectable genome (Müller, 1975). This group of organelles also included the so-called 'peroxisome-family of organelles', a family by itself heterogeneous in morphology and function, with small and large vesicles, and sometimes even visible in mammalian cells as a peroxisomal reticulum, but with the common aspects of containing H_2O_2-producing oxidases and catalase as well as other peroxidases for the decomposition of this reactive oxygen species, and bounded by a single lipid-bilayer membrane. The family included the peroxisomes of mammalian cells, peroxisomes and microbodies of various yeasts, peroxisomes of plant cells and glyoxysomes of germinating plant seeds.

Initially the assignment of glycosomes, which lack nucleic acids and possess a single bounding membrane, to the peroxisome family was not obvious because no H_2O_2-producing oxidases or catalase could be detected. However, the affiliation was later established when catalase activity was found in glycolytic enzyme-containing microbodies, thus glycosomes, of other members of the clade Kinetoplastea (Deschamps et al., 2011) such as the parabodonid *Trypanoplasma borelli* (Opperdoes et al., 1988).

☆ Note: In this paper the classification and nomenclature for kinetoplastids is used as proposed by Moreira et al. (2004).

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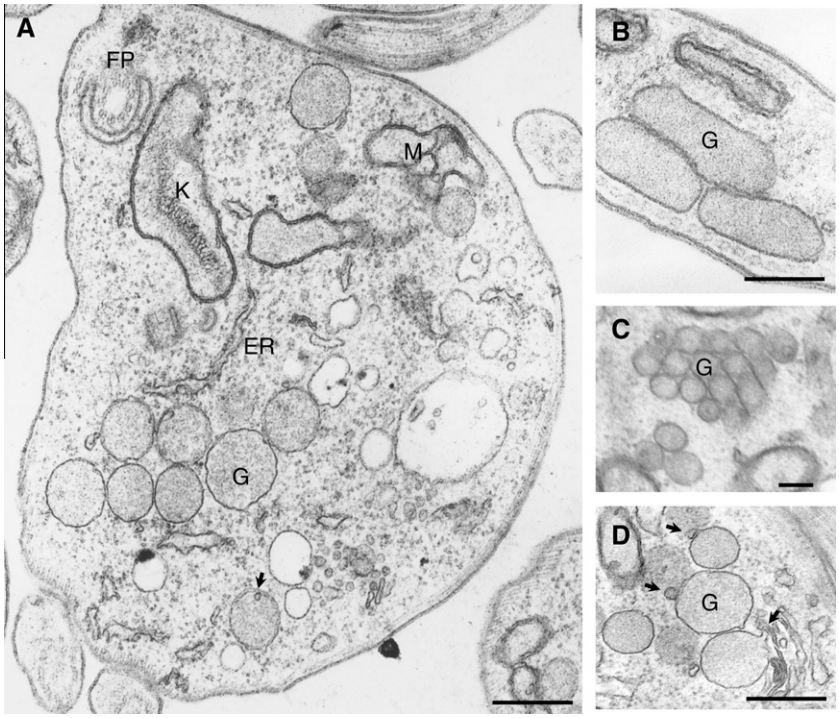

Fig. 1. Electron micrographic pictures of sections of trypanosomes showing the glycosome ultrastructure. (A) A picture of a bloodstream-form *Trypanosoma brucei* cell showing the abundance of glycosomes which are predominantly spherical. (B) In procyclic forms, the glycosomes are predominantly elongated organelles. (C) Bloodstream-form *T. brucei*; many glycosomes can cluster and adopt a very regular organisation. (D) Bloodstream-form *T. brucei*; many pictures show membrane appendices (indicated by arrows, also in A) facing glycosomes. Bars are 500 nm. G, glycosome; ER, endoplasmic reticulum; FP, flagelar pocket; M, mitochondrion; K, kinetoplast. EM pictures by courtesy of Dr. Isabelle Coppens, Johns Hopkins University, Baltimore, USA.

Moreover, other typical peroxisomal functions were located in glycosomes such as enzymes of ether–lipid biosynthesis (Opperdoes, 1984). In the 1990s, when the mechanism of biogenesis of peroxisomes and glycosomes was unravelled, the classification of glycosomes as peroxisomes became indisputable, because the same kind of topogenic signals are used and the proteins involved in the import of matrix proteins and insertion of membrane proteins in the organelles of trypanosomatids, mammals, plants and yeasts are homologous (see Section 5) (Moyersoen et al., 2004; Galland and Michels, 2010).

As described above, the enzyme content of peroxisomes can be very heterogeneous, with variations between the organelles of different organisms or cell types, but also large variations within the organelles of an organism or cell type in time, depending on its differentiation state, nutritional or hormonal conditions (Kunau, 2006 and references therein). Collectively, the various reactions are involved in the oxidative metabolism of carbohydrates, lipids, amino acids and purines, and the synthesis of certain lipids. The heterogeneity of enzymes and pathways in peroxisomes has made one wonder what could be the function of these organelles. A dominant opinion in the 1980s in this regard was that nature had recruited these organelles in order to sequester certain enzymes when that would be advantageous for a cell, for example (i) putting enzymatic reactions that produce toxic or reactive metabolites such as H_2O_2 in a separate compartment to protect the remainder of the cell from damage; (ii) creating reaction conditions for enzymes that could never be maintained in the cytosol, because they are incompatible with essential functions; or (iii) bundling a complex pathway to increase its efficiency by shortening diffusion times of metabolites between enzymes (Borst, 1986; Opperdoes, 1987). It was the latter explanation that was invoked as the rationale for the existence of glycosomes.

Bloodstream-form *T. brucei* is entirely dependent on glycolysis for its ATP supply: glucose, taken up from the blood, is converted into pyruvate that is excreted (Fig. 2A). The mitochondrion is largely repressed in this life-cycle stage; tricarboxylic acid (TCA) cycle enzymes and respiratory chain components are not present. It was reasoned that the observed high glycolytic flux, required for the production of sufficient ATP to sustain all cellular functions, including the cell's high motility and high turnover of the coat of variable surface glycoproteins (VSG), is made possible by sequestering the glycolytic proteins at high density in the approximately 65 glycosomes (Tetley and Vickerman, 1991) of the bloodstream-form parasites that together constitute approximately 4–5% of the cellular volume (Opperdoes et al., 1984). Glycolytic enzymes constitute over 90% of the total protein content of bloodstream-form glycosomes (Aman et al., 1985; Misset et al., 1986). In contrast, in the procyclic insect stage, where the metabolic repertoire is much larger due to the presence of a well-developed mitochondrion with TCA cycle enzymes and the respiratory chain is de-repressed (Fig. 2B), the glycolytic enzymes make up only 40–50% of the glycosomal protein (Hart et al., 1984). Putting the glycolytic enzymes at high concentrations inside glycosomes, together with two enzymes of glycerol metabolism, glycerol-3-phosphate dehydrogenase (G3PDH) and glycerol kinase (GK) (Fig. 2A), offered trypanosomes the additional possibility of creating specific reaction conditions (see above – argument ii) to synthesise ATP under anaerobic conditions, by allowing GK working in the thermodynamically unfavourable reverse direction, as a result of a low ATP/ADP ratio and the accumulation of glycerol 3-phosphate (G3P) within the organelles (Opperdoes and Borst, 1977; Hammond et al., 1985; Borst, 1986). Such conditions cannot easily be created in the cytosol where a high ATP/ADP ratio is needed for biosynthetic reactions.

2. The origin of peroxisomes and glycosomes – early ideas

In the early 1960s, Christian de Duve and coworkers identified, in rat liver cells, organelles that they called 'peroxisomes' after

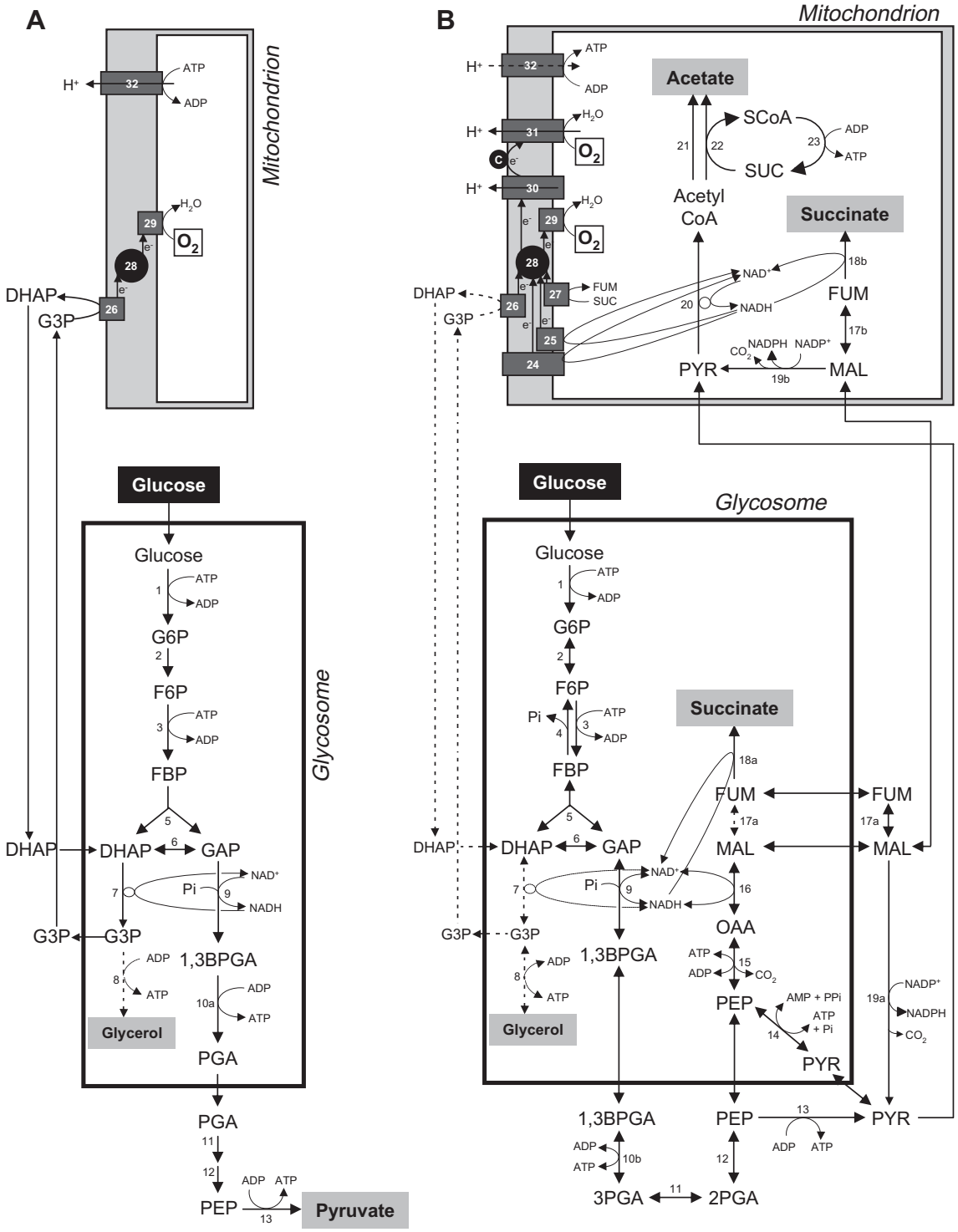

Fig. 2. Scheme of glycosomal and mitochondrial metabolism of bloodstream- (A) and procyclic-form *Trypanosoma brucei* (B). Excreted end-products from glucose metabolism are in black characters on a grey rectangle as background. Dashed arrows indicate steps which have not been shown to significantly occur, or which are supposed to occur at a background level or not at all, under standard growth conditions (glucose-rich medium). Abbreviations: 1,3BPGA, 1,3-bisphosphoglycerate; c, cytochrome c; DHAP, dihydroxyacetone phosphate; e^-, electrons; F6P, fructose 6-phosphate; FBP, fructose 1,6-bisphosphate; FUM, fumarate; GAP, glyceraldehyde 3-phosphate; G6P, glucose 6-phosphate; MAL, malate; OAA, oxaloacetate; PEP, phosphoenolpyruvate; 2PGA, 2-phosphoglycerate; 3PGA, 3-phosphoglycerate; PYR, pyruvate; SCoA, succinyl-CoA; SUC, succinate. Enzymes are: 1, hexokinase (HXK); 2, 6-phosphoglucose isomerase; 3, phosphofructokinase (PFK); 4, fructose-1,6-bisphosphatase (FBPase); 5, aldolase; 6, triosephosphate isomerase; 7, glycerol-3-phosphate dehydrogenase (G3PDH); 8, glycerol kinase (GK); 9, glyceraldehyde-3-phosphate dehydrogenase (GAPDH); 10a, glycosomal phosphoglycerate kinase; 10b, cytosolic phosphoglycerate kinase; 11, phosphoglycerate mutase; 12, enolase; 13, pyruvate kinase (PYK); 14, pyruvate phosphate dikinase (PPDK); 15, phosphoenolpyruvate carboxykinase (PEPCK); 16, glycosomal malate dehydrogenase; 17a, cytosolic fumarase; 17b, mitochondrial fumarase; 18a, glycosomal NADH-dependent fumarate reductase (NADH-FRD); 17b, mitochondrial NADH-dependent fumarate reductase (NADH-FRD); 19a, cytosolic malic enzyme; 19b, mitochondrial malic enzyme; 20, pyruvate dehydrogenase complex (PDH); 21, unknown enzyme; 22, acetate:succinate CoA-transferase; 23, succinyl-CoA synthetase; 24, rotenone-sensitive NADH dehydrogenase (complex I of the respiratory chain); 25, rotenone-insensitive NADH dehydrogenase; 26, FAD-dependent glycerol-3-phosphate dehydrogenase; 27, succinate dehydrogenase (complex II of the respiratory chain); 28, ubiquinone; 29, SHAM-sensitive alternative oxidase; 30, complex III of the respiratory chain; 31, complex IV of the respiratory chain; 32, F_0F_1-ATP synthase.

demonstrating that these organelles possess oxidases that reduce oxygen to H_2O_2 at the expense of a substrate RH_2 and large amounts of enzyme capable of reducing this H_2O_2 by either of two distinct mechanisms (i) a peroxidatic mechanism requiring an electron donor $R'H_2$ ('peroxidase') or (ii) the catalatic mechanism in which a second molecule of H_2O_2 serves as electron donor ('catalase') (de Duve and Baudhuin, 1966). Earlier, the organelles had already been observed in various mammalian tissues by electron microscopy as a special kind of cytoplasmic body with a single membrane and a fine, granular matrix (Rhodin, J., 1954. Correlation of ultrastructural organisation and function in normal and experimentally changed proximal tubule cells of the mouse kidney. PhD Thesis. Aktiebolaget Godvil, Karolinska Instituet, Stockholm.) and called a 'microbody'.

After their discovery in mammalian liver cells, peroxisomes with similar and additional biochemical properties were found in other tissues and other organisms, including yeasts. de Duve then proposed a scenario for their origin and evolution based on three premises (de Duve, 1969). First, the presence of the common properties suggested a phylogenetic relation, i.e. they might be traced back to a common origin. The second premise was that the origin must go back to a very early stage in evolution, possibly the primeval eukaryote. One should realise that only in later years were similar organelles (but with quite distinct enzymatic capacities and lacking all or almost all of the H_2O_2-dependent processes) found in some protists such as the kinetoplastids. The third premise was that the enzymatic diversity of the organelles is the result of progressive deletion, rather than acquisition of new enzymes. de Duve (1969) further proposed that some of the prokaryotes inhabiting the early anaerobic earth had developed this simple peroxidatic or catalatic mechanism involving the sequence $O_2 \rightarrow H_2O_2 \rightarrow H_2O$ as an adaptation to the rising concentration of oxygen in the atmosphere. This was thought to be a protection mechanism against the toxic oxygen, with low affinity for it and not coupled to oxidative phosphorylation. This is a type of respiration found today in many peroxisomes. This primitive aerobic prokaryote would, according to de Duve's hypothesis, have been endocytosed by a primitive phagocytotic eukaryote and over time evolved into peroxisomes. Based on the three premises, the scenario was proposed in which the peroxisome was derived from an endosymbiont with a H_2O_2-dependent respiratory activity and, in the course of evolution, irreversibly lost many of its enzymes (and its genome), particularly after many of its functions were taken over by a subsequent endosymbiont that gave rise to the mitochondrion and performed many of these functions with far greater kinetic and thermodynamic efficiency. Interestingly, those enzymes found in peroxisomes are not present in mitochondria and must have been lost from the latter. Except for a core repertoire that is present in peroxisomes of most organisms, such as the H_2O_2-linked enzymes (although the H_2O_2-producing oxidases vary in number and specificity from one type of peroxisome to the other) and some enzymes involved in ether–lipid biosynthesis, different sets of enzymes were retained in peroxisomes of distinct taxonomic lineages, such as enzymes of the glyoxylate cycle, α- and β-oxidation of fatty acids, plasmalogen biosynthesis, purine catabolism, alcohol oxidation, etc. (de Duve, 1969; Lazarow and Fujiki, 1985; Kunau, 2006). Other functions may subsequently have been acquired by peroxisomes of different lineages. Unlike mitochondria and plastids, the genome and transcription/translation machinery were completely lost from peroxisomes.

The common origin of peroxisomes of fungi, plants and animals was widely accepted, not only due to the general presence of the H_2O_2-dependent oxidation enzymes, but also due to the fact that these organelles share a special β-oxidation pathway with a remarkably uniform organisation and subunit structure, clearly different from the mitochondrial β-oxidation system (Borst, 1986). However, it was less obvious to imagine a common origin of peroxisomes and glycosomes, because how would an almost entire glycolytic pathway have ended up in the peroxisome of an ancestor of trypanosomes (see Section 7 for details)? Therefore, an independent origin of the glycosome through an endosymbiont was envisaged, and the glycolytic pathway and some typical peroxisomal functions were thought to have been selectively retained (Borst, 1986; Michels and Opperdoes, 1991; Michels and Hannaert, 1994). In contrast, in all organisms with 'real' peroxisomes, the glycolytic functions of the organelles' endosymbiotic ancestor would have been lost.

3. When did the glycosome evolve?

Initially, glycosomes were discovered in *T. brucei* (Opperdoes and Borst, 1977) and subsequently in other members of the order Trypanosomatida (Simpson et al., 2006; Deschamps et al., 2011), *Trypanosoma cruzi* and *Crithidia fasciculata* (Taylor et al., 1980), *Leishmania* spp. (Hart and Opperdoes, 1984), a *Phytomonas* sp. (Sanchez-Moreno et al., 1992) and in the free-living Parabodonina *T. borelli* (Opperdoes et al., 1988) and *Cryptobia salmositica* (Ardelli et al., 2000). The phylogenetic relationships of these organisms (based on the trees presented by Simpson et al., 2006 and Deschamps et al., 2011) are given in Fig. 3. In contrast, no compartmentalisation of glycolytic enzymes within organelles was detected in *Euglena gracilis*. *Euglena* does possess peroxisomes (Müller, 1975), however without detectable levels of glycolytic enzymes (Opperdoes et al., 1988). There are no indications that these organelles may ever have possessed such enzymes and lost them secondarily. Interestingly, an organellar localisation was recently reported for the glycolytic enzyme aldolase in *Diplonema papillatum* (Makiuchi et al., 2011); this observation, together with the presence of a type 2 peroxisomal-targeting signal (PTS) in this enzyme – similar to the aldolases of trypanosomatids – as revealed by sequence analysis, suggests that diplonemids also contain glycosomes. These data show that glycosomes must have evolved within the phylum Euglenozoa, in a common ancestor of the Diplonemida and Kinetoplastea, but after their separation from the Euglenida.

4. Evolution of ideas about the possible function(s) of glycosomes

Since the discovery of glycosomes in 1977, several possible functions have been proposed for the compartmentalisation of glycolysis and related pathways that will be presented here in a historical context. The initial notion that glycosomes originated because they provided an ancestral organism with the possibility to speed up its glycolytic flux became very unlikely when it was realised that glycosomes were also present in other kinetoplastid organisms which do not have a high flux. The high glucose consumption rates are only found in those life-cycle stages of some trypanosomatid species that live in sugar-rich environments such as *T. brucei* in the mammalian bloodstream and *Phytomonas* spp. that can be isolated from the phloem of plants (Cazzulo, 1992; Sanchez-Moreno et al., 1992). It is also unlikely that the common ancestor of the Diplonemida and Kinetoplastea that acquired glycosomes lived in environments where sugars were abundant, allowing or necessitating a high glycolytic flux.

This ancestor was almost certainly a free-living protist, as may be concluded from the estimation that it may have lived around 600 million years ago (Mya) (Fig. 3), well before most of the early representatives of the lineages that became hosts of its parasitic descendants developed: the first vertebrates and fish evolved in the Ordovician period, 440 Mya; the land plants in the Silurian

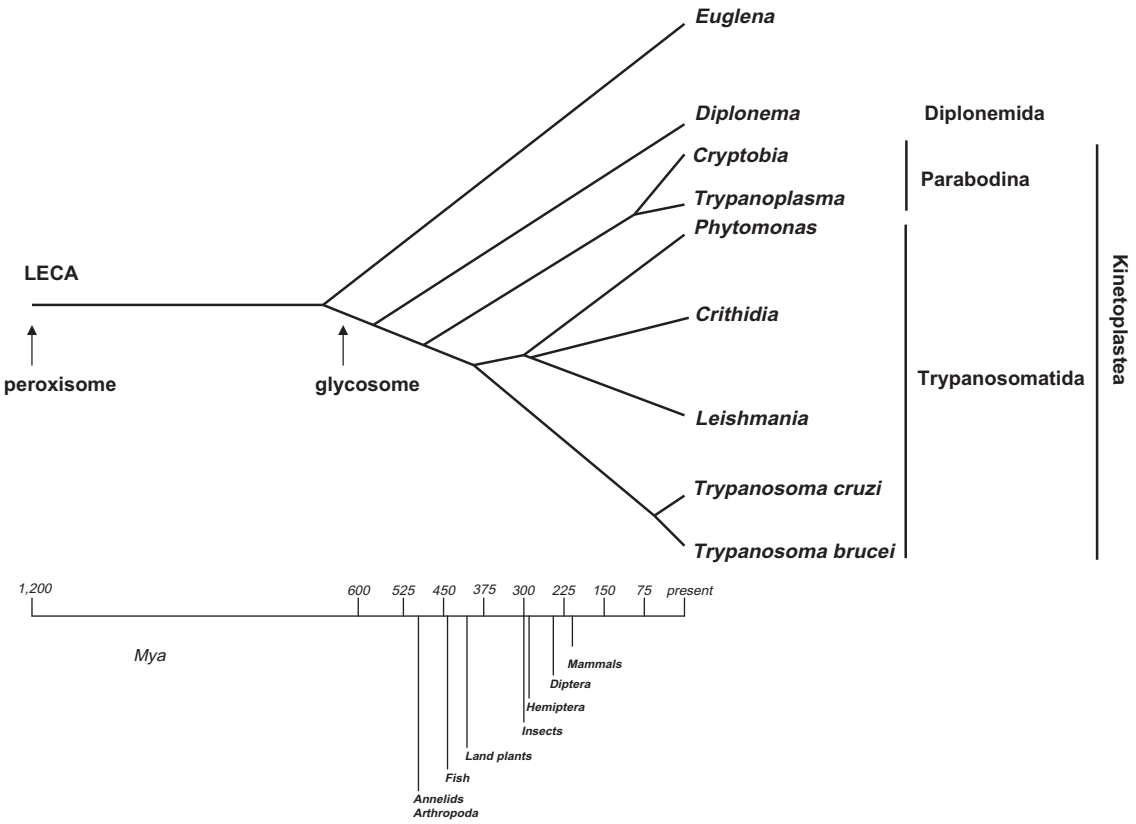

Fig. 3. Phylogenetic relationship of the Euglenozoa. The evolutionary time scale with the development of major taxons forming hosts to extant kinetoplastid parasites is given underneath, as adapted from data published previously (Kerr, 2006). The branching order of all depicted lineages is according to known evolutionary relationships, together with the time of branches within the Trypanosomatida, corresponding to the bar underneath, taken from Kerr (2006). However, the time of branching of the Euglenozoa into Euglenida, Diplonemida and Kinetoplastea, and the latter lineage into Parabodonida and Trypanosomatida has not been calibrated. Peroxisomes must have already been present in the last eukaryotic common ancestor (LECA); the ancestral organism that has acquired the glycosome is indicated. Mya, million years ago.

period, 400 Mya; and the insects currently involved in the transmission of trypanosomatid parasites, the Hemiptera and Diptera, 285 and 245 Mya, respectively (Kerr, 2006; Simpson et al., 2006). Arthropods and annelids – sister groups to insects and leeches, some of which are known as hosts of some extant kinetoplastids –already existed in the Cambrium period (>600 Mya) and may have been hosts to early parasitic ancestors of these protists. However, extant diplonematids are free-living organisms, although occasionally also reported as facultative parasites of plants, and among the kinetoplastids many species are also free-living, notably among the bodonids. All known trypanosomatid species are parasites, but a recent phylogenomic analysis showed that the trypanosomatids arose from within the bodonids (Deschamps et al., 2011), providing strong support that parasitism arose as a late event within the kinetoplastids. A detailed analysis of host–parasite relationships strongly suggests that parasitism evolved independently many times within kinetoplastids – from free-living organisms to monogenetic parasites involving a single host and from monogenetic to digenetic parasites that complete their life-cycle with two different hosts (Kerr, 2006; Simpson et al., 2006). Together, all of these data strongly support the notion that the common ancestor of these organisms that acquired the glycosome had been free-living.

Other arguments also render it very unlikely that the sequestering of the glycolytic enzymes at high density within an organelle serves to accelerate the glycolytic flux. Calculations showed that the flux would not be limited by diffusion of the metabolites even if the enzymes were not compartmentalised but distributed throughout the cytosol (Bakker et al., 1995). Moreover, it was realised that the glycolytic flux of bloodstream-form *T. brucei* is high, but not exceptionally, and other cells, both microorganisms and from tissues in multicellular organisms, may sustain a considerably higher glucose consumption rate without any form of glycolytic enzyme compartmentalisation (Bakker et al., 1995, 2000).

A mathematical model of glycolysis in bloodstream-form *T. brucei* was developed on the basis of experimentally determined kinetic data of all enzymes (Bakker et al., 1997). Simulations using this model correctly reproduced the steady-state process, and predicted fluxes and metabolite concentrations that had previously been determined under various conditions. Removal of the glycosomal membrane in the model, allowing the enzymes, cofactors and intermediates to distribute over the entire cytosol, had little effect on the steady-state flux, thus providing another argument that enzyme sequestering does not serve to accelerate the flux (Bakker et al., 2000).

If glycosomes do not serve to increase the glycolytic flux, what could their role be? A possibility considered was the prevention of unwanted interference of reverse or parallel pathways in the cell by their physical separation, such as glycolysis, gluconeogenesis and the pentosephosphate pathway (PPP) (Michels and Hannaert, 1994). These pathways share intermediates and some enzymes, but their simultaneous activity should be prevented or at least controlled to avoid futile cycles that may lead, for example, to loss of ATP. Most cells, both prokaryotes and eukaryotes, control these processes by kinetic regulation of the activity of each pathway's specific, non-shared key enzymes that catalyse reactions in opposite directions and involve large changes in free energy, such as the enzyme pairs hexokinase (HXK)/glucose-6-phosphatase (G6Pase), phosphofructokinase (PFK)/fructose-1,6-bisphosphatase (FBPase) and pyruvate kinase (PYK)/pyruvate carboxylase plus

phosphoenolpyruvate carboxykinase (PEPCK). The activities of these enzymes are regulated by feedback mechanisms by their products and/or by allosteric effectors. However, in trypanosomatids the glycosomal enzymes HXK and PFK are essentially unregulated; no product inhibition is observed and hardly any effectors are known (Opperdoes and Michels, 2001; reviewed by Verlinde et al., 2001).

We proposed, therefore, that trypanosomes might prevent interference, not by reciprocal activity regulation of the key enzymes, but through separation by a membrane: glycolysis (with PFK) in the glycosome and gluconeogenesis (with FBPase) and the PPP in the cytosol (Michels and Hannaert, 1994). The last three enzymes of the glycolytic pathway of all trypanosomatids, including PYK, are cytosolic (Fig. 2A). In contrast to the glycosomal HXK and PFK, the activity of this PYK is highly controlled. However, the hypothesis was invalidated because various PPP enzymes could be detected both in the cytosol and glycosomes (Heise and Opperdoes, 1999; Duffieux et al., 2000) and the FBPase appeared to be constitutively expressed in the glycosomes of *Leishmania major* (Naderer et al., 2006) and *T. brucei* (V. Hannaert and P. Michels, unpublished data). Interestingly, when expressed in *Escherichia coli* and purified, the *T. brucei* enzyme was active and no regular activity regulation mechanisms were found. However, in both bloodstream and procyclic glucose-grown *T. brucei*, which possess an active PFK, FBPase was found to be present but inactive as a result of a still unknown mechanism, probably covalent modification (V. Hannaert and P. Michels, unpublished data).

The activity regulation of HXK and PFK in essentially all cells not only serves to prevent interference of glycolysis and gluconeogenesis and thus wastage of ATP by futile cycling, but also protects the cells from the dangerous 'turbo-design' of the glycolytic pathway (Teusink et al., 1998). The pathway is organised such that ATP is invested first, before its net production. This ATP investment at the level of HXK and PFK makes these reactions virtually irreversible. However, this design, with net ATP production, while ATP serves as a substrate in the first reactions, brings the risk that these first reactions are boosted above the capacity of the downstream part of the pathway and thus of unrestricted accumulation of the intermediates. This is prevented by a tight regulation of HXK and PFK, notably through feedback inhibition by their products and other downstream metabolites. Indeed, yeast mutant cells lacking feedback regulation of HXK by trehalose 6-phosphate due to a lesion in the gene for trehalose-phosphate synthase and therefore incapable of synthesising this disaccharide from glucose 6-phosphate (G6P), could not grow on high concentrations of glucose; they accumulated hexose-phosphates while consuming ATP and inorganic phosphate rapidly until all phosphate had been incorporated (reviewed by Teusink et al., 1998).

The simulation of glycolysis in bloodstream-form *T. brucei* with the computer model mentioned above showed that under normal conditions a steady-state flux is reached with concentrations of intermediates similar to those that had been measured experimentally (Visser and Opperdoes, 1980). However, the in silico removal of the glycosomal membrane caused the uncontrolled rise of sugar–phosphate intermediates (Bakker et al., 2000). Apparently, the compartmentalisation of the first seven enzymes of the pathway compensates for the lack of activity regulation of HXK and PFK. This was explained by the fact that no net ATP is produced within the glycosome. Two molecules of ATP are invested per molecule of glucose at the level of HXK and PFK, and retrieved again in the reaction catalysed by phosphoglycerate kinase (reactions 1, 3 and 10a, respectively in Fig. 2A). Net ATP, two molecules per molecule of glucose consumed, is produced in the cytosol at the level of PYK (Fig. 2, reaction 13). This extraglycosomal net ATP is not available for boosting the first enzymes of the pathway present in the organelle.

Strong experimental support has been obtained for this hypothesis (Blattner et al., 1998; Furuya et al., 2001; Moyersoen et al., 2003; Kessler and Parsons, 2005; Haanstra et al., 2008). Both bloodstream- and procyclic-form *T. brucei* died as a result of partial mislocalisation of glycolytic enzymes caused by either coexpressing a glycosomal enzyme in the cytosol or by preventing the import of newly synthesised proteins into the glycosomal matrix by RNA interference (RNAi)-dependent knockdown of peroxins, proteins involved in the biogenesis of organelles, such as PEX14 (Blattner et al., 1998; Moyersoen et al., 2003). Interestingly, procyclic cells, which in contrast to bloodstream forms can also grow with proline as the energy source, were only killed by PEX14 depletion when cultured in the presence of glucose or fructose (Furuya et al., 2001; Kessler and Parsons, 2005). Proline is metabolised in the mitochondrion, not in glycosomes. Knockdown of HXK rescued the PEX14-depleted cells from glucose toxicity. These observations showed that the mislocalisation is not detrimental per se, but only when glucose is metabolised by glycolytic enzymes outside the organelles. Similarly, glycerol that is also metabolised via glycosomes in a 'turbo-designed' pathway with its ATP-dependent phosphorylation by GK – another enzyme for which no feed-back regulation has been found – as the first step (see Fig. 2) became toxic when PEX14 expression was knocked down (Kessler and Parsons, 2005). Indeed, it could be shown that the intracellular G6P concentration increases nearly 10-fold, up to about 50 mM, upon glucose addition to proline-grown PEX14-deficient procyclic trypanosomes (Haanstra et al., 2008). Loss of glycosomal compartmentalisation in these cells led to an even more dramatic accumulation for G3P (up to approximately 300 mM) upon glycerol addition. Depletion of GK rescued the PEX14-deficient cells from glycerol toxicity. These data provided strong experimental support for the hypothesis that pathway compartmentalisation is an alternative to activity regulation.

These results may suggest that the 'raison d'être' of the glycosome is that it offers kinetoplastids compartmentalisation of glycolytic enzymes as a means to compensate for the lack of feed-back regulation of HXK, PFK and GK and thereby protects these organisms against the toxic effect of glucose and glycerol. However, we consider this rather as a derived function, operating in present-day kinetoplastids, not as the original function of glycosomes when they were acquired by the ancestral protist. HXKs and PFKs are almost generally regulated, in both prokaryotic and eukaryotic organisms; several of the activity mechanisms and effectors are shared, thus it is likely that these mechanisms were already present very early in evolution. It seems thus much more probable that the kinetoplastid enzymes have lost the activity regulation mechanisms because they became redundant after their sequestration inside the organelles with the consequence that compartmentalisation is now required.

5. New insights into the origin of peroxisomes and the relationship between peroxisomes and glycosomes

The view that peroxisome origin and biogenesis are independent of the endoplasmic reticulum (ER) dominated the field for about 20 years (1985–2005). However, when the biogenesis of peroxisomes was studied in more detail, several data obtained appeared to be in conflict with this view. First, a basic set of proteins involved in peroxisome biogenesis, so-called peroxins, were found to be conserved throughout different phyla, albeit sometimes with very low sequence identities, including organisms with rather different types of peroxisomes such as the glycosomes of trypanosomatids (Moyersoen et al., 2004; Galland and Michels, 2010). Importantly, several peroxins possess domains only found in eukaryotes and no counterparts of any of the peroxins have ever been found in prokaryotes (Michels et al., 2005; Gabaldón et al.,

2006; Schlüter et al., 2006). Second, the mechanism of import of proteins into the peroxisomal matrix shows striking similarities with that of the retrieval of malformed proteins from the lumen of the ER for subsequent degradation by cytosolic proteasomes and indeed both processes are mediated by homologous proteins, suggesting a common origin within the eukaryotic cell (Erdmann and Schliebs, 2005; Gabaldón et al., 2006; Schlüter et al., 2006; Schliebs et al., 2010). Third, the majority (39–58%) of yeast and rat proteins with a known peroxisomal localisation or function are of eukaryotic origin. A smaller fraction (13–18%) has an α-proteobacterial origin and appear to be the result of recruitment of proteins originally targeted to mitochondria (Gabaldón et al., 2006). Fourth, experiments in several laboratories suggested that de novo synthesis of peroxisomes should be possible; introduction of wild-type genes in yeast mutants grown for several generations without detectable peroxisomes as a result of the incapability to synthesise them due to the lack of essential peroxins such as the integral membrane protein PEX3 or the cytosolic PEX19, restored the wild-type phenotype, suggesting the involvement of an endomembrane system in peroxisome biogenesis.

A direct involvement of the ER was first demonstrated by Tabak and coworkers (Geuze et al., 2003; Hoepfner et al., 2005) and subsequently by other researchers for peroxisomes of both mammalian cells and different yeasts. By using immuno-electron microscopy and electron tomography it was unambiguously shown in mouse dendritic cells that the peroxisomal membrane is derived from special lamellar sections of the ER (Geuze et al., 2003). Subsequently, by real-time fluorescence microscopy and biochemical techniques applied to *Saccharomyces cerevisiae pex3* and *pex19* mutants transfected with yellow fluorescent protein (YFP)-tagged PEX3 and PEX19 constructs, it was observed that PEX3 is first inserted into the ER, concentrated in foci that bud off in a PEX19-dependent manner to give vesicles that develop into fully functional peroxisomes (Hoepfner et al., 2005). The corollary of the observation that peroxisomes can be formed de novo, starting from the ER, in cells which did not have peroxisomes, is that an endosymbiotic origin of peroxisomes is very unlikely.

Studies in recent decades of cells from different organisms such as mammals and plants, fungi and trypanosomes have shown that the biogenesis mechanism of peroxisomes and peroxisome-like organelles is homologous and remarkably similar in different taxa. Two rather simple consensus motifs are being used to target proteins, newly synthesised in the cytosol, to the matrix of all these organelles: the type 1 PTS (PTS1) being a C-terminal tripeptide comprising the residues SKL or conservative variants thereof ((S/C/A)-(K/R/H)-(L/M)) and the PTS2, a nonapeptide located close to the N-terminus with the consensus motif (R/K-(L/V/I/Q)-xx-(L/V/I/H)-(L/S/G/A)-x-(H/Q)-(L/A)). Moreover, in each case a core set of homologous peroxins, although varying greatly in sequence, are being used in the different steps of the biogenesis process.

It should be realised that these different organisms being analysed belong to very different major taxonomic groups. The recently increased availability of genome sequences for a large variety of organisms and advances in molecular phylogenetics have allowed the classification of most eukaryotes within six 'suprakingdom-level'- or 'super'-groups, each containing protists, all with a common ancestor, the so-called last eukaryotic common ancestor or LECA. Mammals, fungi and some protists belong to the supergroup Opisthokonta, while plants and some groups of photosynthetic protists (e.g. *Chlamydomonas*) belong to the Archaeplastida. In the other supergroups (called Amoebozoa, Chromalveolata, Rhizaria and Excavata) only protist organisms are found (Simpson and Roger, 2004; Keeling et al., 2005). The Euglenozoa (including Kinetoplastea) belong to the Excavata, the apicomplexans and ciliates to the Chromoalveolata, and *Dictyostelium* to the Amoebozoa. The presence of a nucleus, ER, Golgi, the occurrence of mitosis and autophagy (Duszenko et al., 2011), and the possession of mitochondria or relic mitochondrial organelles in each of these major groups, indicate that all of these features were already present in the LECA. The fact that several features of peroxisomal biogenesis are conserved in the supergroups Opistokontha, Archaeplastida and Excavata means that peroxisomes must also have had a single origin in the LECA. This is further supported by the fact that indications for the existence of peroxisomes are also found in the other representatives of these three supergroups as well in the three others (Gabaldón, 2010). The apparent absence of peroxisomes in some of the lineages within supergroups, such as is the case for the apicomplexans, microsporidia, trichomonads and diplomonads (Gabaldón, 2010) has thus to be attributed to secondary loss. One may also conclude from the conservation of the biogenesis machinery and mechanism, and the presence of peroxisomes in all supergroups, that glycosomes had a common origin with peroxisomes within the ancestral common eukaryotic cell. Therefore, it seems logical to hypothesise that glycosomes must have evolved from authentic peroxisomes, which were originally derived from the ER, when these organelles in the common ancestor of the Diplonemida and Kinetoplastea acquired glycolytic enzymes. How this may have happened will be discussed in Section 7.

6. Recent ideas about the original function of glycosomes

To understand the function of glycosomes, other than the possible functions discussed in Sections 1 and 4 which should now be considered unlikely, one has to take into account (i) the time at which peroxisomes acquired glycolytic enzymes and the selective forces which may have played a role in this acquisition for the earliest organisms having glycosomes; (ii) properties of peroxisomes which may initially have been overlooked but could be relevant for (ancestral) kinetoplastids with peroxisomes containing glycolytic enzymes; (iii) not yet considered functions which may pertain to all kinetoplastids, not only to the best-studied representatives such as bloodstream-form *T. brucei*.

A clue about the possible function of glycosomes may come from properties that indeed may have initially been overlooked and most prominently emerged from recent studies of peroxisomes in the methylotrophic yeasts. Peroxisomes differ from other organelles such as mitochondria in the fact that they are much more versatile. Only a few functions, supposedly derived from the ancestral organelle, are shared by all members of the peroxisome family, and these functions may even play a quantitatively very minor role in the organelles of some organisms, such as peroxide and lipid metabolism in kinetoplastids. In contrast, some apparently acquired functions may play a qualitatively and quantitatively important, if not essential, role in the peroxisomes of organisms only in specific lineages, such as methanol metabolism in methylotrophic yeasts, the glyoxylate cycle in plant glyoxysomes and glycolysis and other pathways of carbohydrate metabolism in kinetoplastid glycosomes. Furthermore, the enzyme content often varies considerably within the peroxisomes of an organism when environmental conditions change, such as the cells' nutrients, or when cells are stimulated to differentiate by hormonal or other signals. This latter property is most prominent in peroxisomes of the methylotrophic yeasts *Hansenula polymorpha* and *Pichia pastoris* but appears to be shared by those of other yeasts and fungal, plant and mammalian cells (for recent reviews, see Manjithaya et al., 2010; Yoshimoto et al., 2010). Apparently, changes in enzyme content of the organelles are inducible. Glucose-grown methylotrophic yeasts contain only one or a few very small peroxisomes, whereas cells grown in a medium with methanol as a sole carbohydrate and energy source have multiple large

organelles that may occupy a major part of the cell volume (Fig. 4). When glucose-grown cells are shifted to methanol, the small peroxisomes increase in size as a result of the import of newly synthesised enzymes involved in methanol metabolism and new organelles are formed by fission.

The adaptation of these yeasts to new growth conditions not only involves the induction of a new set of peroxisomes with a different enzyme content, but also a massive, active degradation of the old, redundant organelles by autophagy (Fig. 4). This mechanism of selective induction and degradation of peroxisomes has been shown to endow the methylotrophic yeast cells with the capability of adapting rapidly and in an efficient manner to new growth substrates. Similar observations of inducible synthesis of peroxisomes with a new enzyme content and degradation of redundant ones by pexophagy have subsequently been made for peroxisomes of other yeasts (*S. cerevisiae*) and mammalian cells (Manjithaya et al., 2010). In view of this large versatility of all members of the peroxisome family, we hypothesise that providing metabolic flexibility to organisms is a general function of these organelles and is also highly relevant to understand the function of glycosomes in kinetoplastids.

Present-day trypanosomatid parasites have a complicated life-cycle during which they encounter considerable, sometimes even dramatic, environmental changes, particularly when the life-cycle involves different hosts such as insects and mammals (Fig. 5). During these changes, the parasites' morphology and metabolism are altered considerably. For several parasites it has been shown that autophagy plays a major role in the stage-dependent differentiation (reviewed in: Brennand et al., 2011; Duszenko et al., 2011). It is conceivable that, as in the methylotrophic yeasts, particularly stage-specific, important metabolic enzymes have been sequestered within glycosomes. Degradation of old glycosomes with a content for one stage by pexophagy and induction of biogenesis of glycosomes, with enzymes important for the next stage, could then play a crucial role in the efficient and rapid adaptation of the parasites to the new host or a new location in a host. The rapid adaptation may be essential to withstand the defence mechanisms of the host. Information about stage-specific metabolism, and specifically about the role of glycosomes in it, is only available for a few kinetoplastids, notably for bloodstream-form versus procyclic African trypanosomes (Colasante et al., 2006; Michels et al., 2006; Vertommen et al., 2008) and to a lesser extent for *Leishmania* spp. amastigotes versus promastigotes and different life-cycle stages of *T. cruzi* (Quiñones et al., 2004; Atwood et al., 2005; Paape et al., 2008; Rosenzweig et al., 2008). Indeed, important differences in carbohydrate, lipid and energy metabolism can be found between the different life-cycle stages and glycosomes figure prominently in these differences by considerable changes of their enzyme repertoire. Morphological studies of *T. brucei* differentiating from long-slender to short-stumpy bloodstream forms and stumpy trypanosomes to cultured procyclic forms have provided strong indications that a highly increased glycosome turnover involving autophagy (Fig. 6) occurs during these transitions and that the process is notably very important and fast during the formation of the procyclic forms (Herman et al., 2008).

The lack of information about the changes of enzyme content of glycosomes in non-trypanosomatid kinetoplastids and the possible role of induction of their biogenesis and degradation by pexophagy precludes drawing definitive conclusions about whether an ancient, or even the original, function of the organelles was to increase the adaptability of the organisms to changing conditions. However we consider it likely that is the case, since this function has also been observed for peroxisomes in organisms as different as mammals, plants, fungi and yeasts, thus representatives of

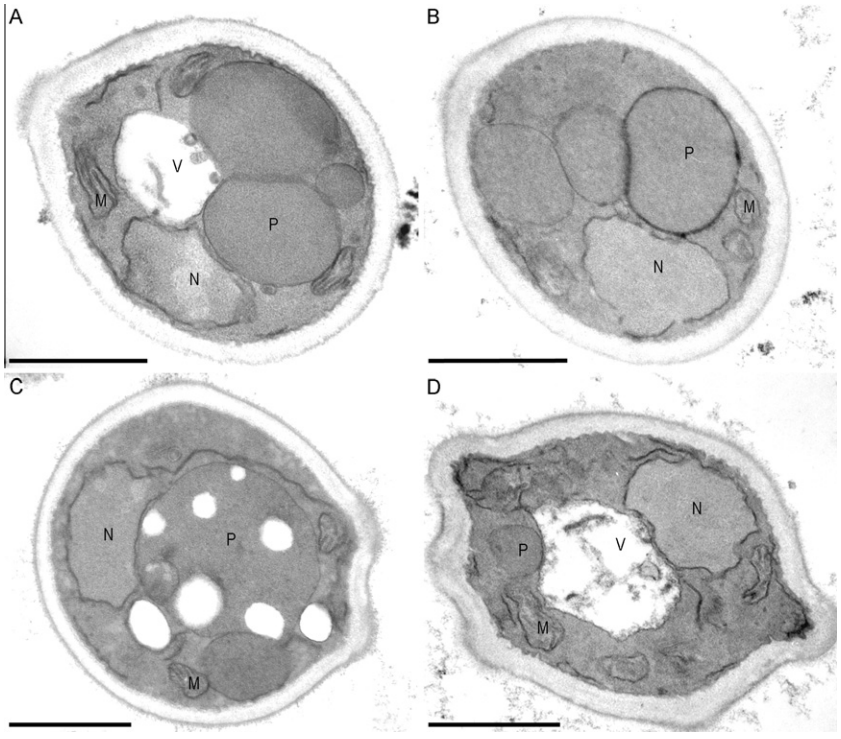

Fig. 4. Electron micrograph of a methylotrophic yeast to demonstrate the role of pexophagy in adapting cells to changing nutritional conditions. The picture shows the morphological events of glucose-induced peroxisome degradation in methanol-grown *Hansenula polymorpha*. (A) Control wild-type methanol-grown cells, showing three large peroxisomes. (B) Thirty min after the shift of cells to glucose an individual organelle becomes sequestered by electron dense membranes to form an autophagosome. (C) After fusion of the autophagosome with the vacuole, degradation of the organelle content is initiated. (D) After completion of degradation, invariably a single small organelle remains and escapes from degradation. The bar represents 0.5 μm. V, vacuole; P, peroxisome; N, nucleus; M, mitochondrion. Figure by courtesy of Prof. Ida van der Klei and Prof. Marten Veenhuis, University of Groningen, The Netherlands.

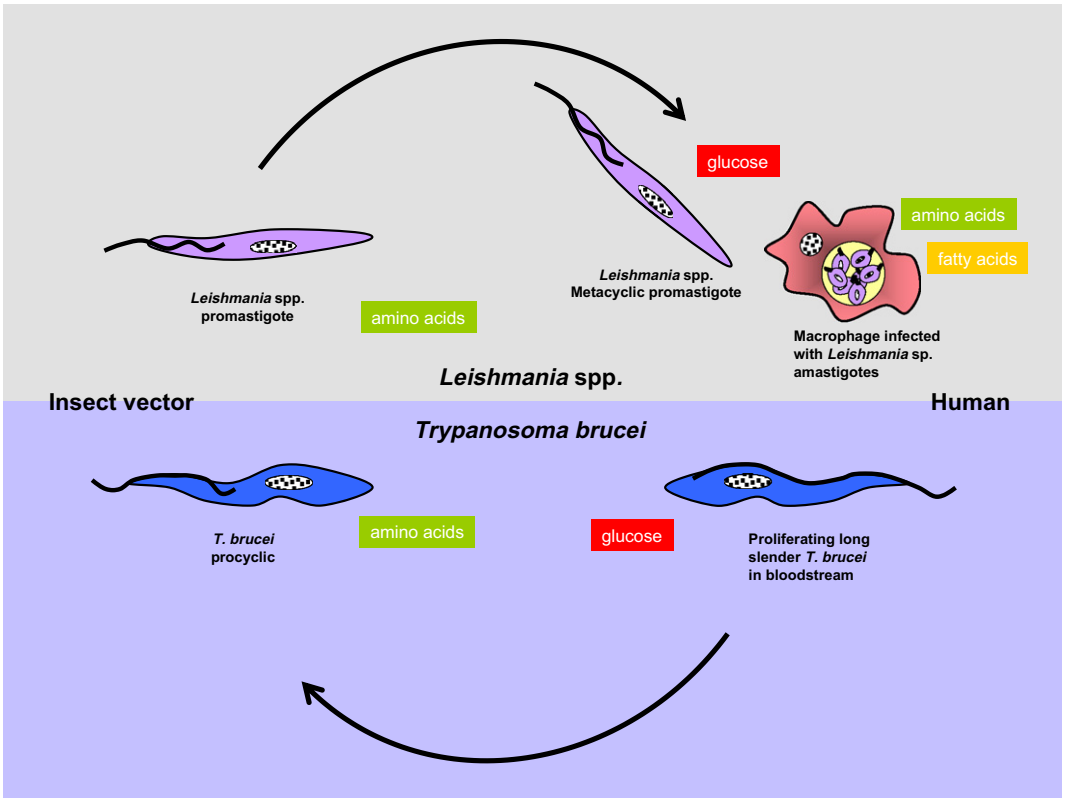

Fig. 5. Trypanosomatid parasites reside successively in environments with different nutrients during their life cycle. Part of the life cycles of *Leishmania* spp. and *Trypanosoma brucei* are shown with an indication of the major nutrients used for their free energy supply in the various differential stages.

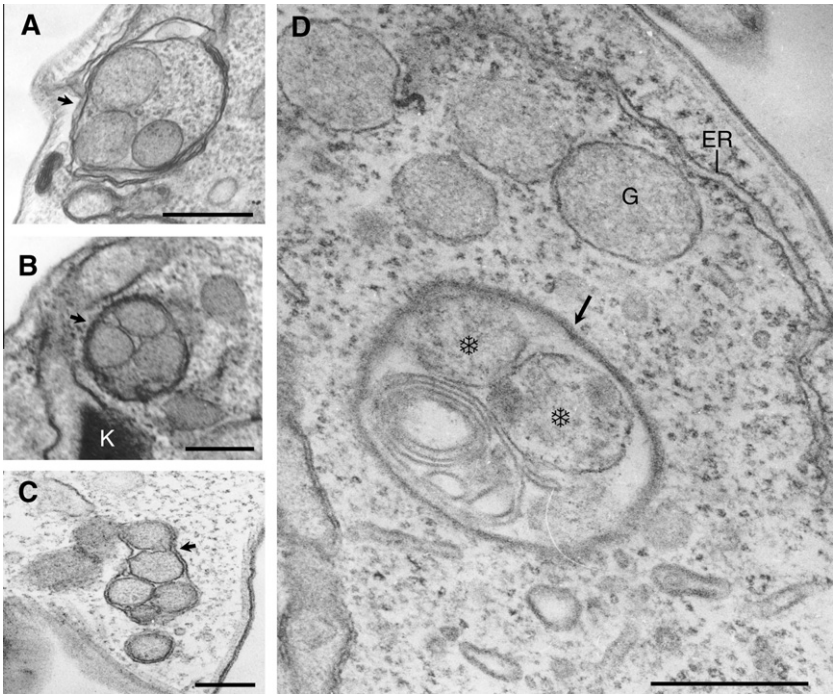

Fig. 6. Electron micrographs (EM) showing pexophagy in trypanosomatids. (A, B, C) Three examples of apparent pexophagy, where several glycosomes are sequestered within an organelle resembling an autophagosome. Arrows indicate the autophagosomal structures containing glycosomes as concluded from the size, shape and electron density of the organelles. (D) An example of apparent non-selective autophagy. The arrow shows a structure containing material in degradation that may include glycosomes (asterisks). Bars represent 500 nm. G, glycosome; ER, endoplasmic reticulum; K, kinetoplast. EM pictures by courtesy of Dr. Isabelle Coppens.

different supergroups. It therefore seems reasonable to assume that the capacity of induced peroxisome turnover by pexophagy was already an advantage for the environmental adaptability of the LECA. One can only speculate about the environmental changes

to which the common ancestor of the Diplonemida and Kinetoplastea, of which the peroxisomes acquired glycolytic enzymes, have been exposed and why sequestering glycolytic enzymes within their peroxisomes has been a selective advantage for metabolic reprogramming and adaptability of this organism, almost certainly a free-living one as discussed in Section 4. The organism may have fluctuated between environments with either fermentable sugars or with lipids, amino acids and/or other non-glycolytic substrates. Or alternatively, between environments with different oxygen tension, for example when the organism migrated up or down sediments. The differential availability of electron acceptors – oxygen on the one hand and anaerobic ones, either exogenous inorganic acceptors or endogenous organic ones on the other hand – would have necessitated important changes not only in mitochondrial metabolism but also intermediary, carbohydrate metabolism. The compartmentalisation of the latter may have facilitated these drastic metabolic adaptations. Future studies of the function and turnover of glycosomes in free-living kinetoplastids and diplonemids may be informative in this regard. Nonetheless, the fact that the property of inducible glycosome turnover existed in the early kinetoplastids may have been a major advantage for development of a parasitic life style by their evolutionary descendants, as will be argued in Section 9.

7. How did glycolytic enzymes become compartmentalised inside glycosomes of non-euglenid Euglenozoa?

As described in Section 2, initially an endosymbiotic origin had been proposed, both for peroxisomes and glycosomes, to explain not only the (later proved incorrect notion of an) autonomous existence of the organelle independent of other membrane systems in the cell, but also how they could contain metabolic pathways. It was difficult to imagine how a pathway could be compartmentalised if it had not been there from the beginning. The one-by-one transfer of individual enzymes of a pathway to the organelle was considered unlikely; intermediate stages would not provide a selective advantage but rather a burden. And transfer of all successive enzymes simultaneously, while also finding at once, inside the organelle, their substrates and cofactors, seemed unlikely as well. Endosymbiosis followed by subsequent loss of the overall majority of the enzymes seemed to be the preferable explanation. Such a scenario was successful for the development of mitochondria and chloroplasts from their respective original endosymbionts (Timmis et al., 2004; Martin, 2010).

Once the evidence of an origin of peroxisomes from the ER had been established and the origin of glycosomes was understood as the acquisition of glycolytic enzymes by peroxisomes of an ancestral organism within the Euglenozoa lineage, the problem was reformulated as to how an entire glycolytic pathway ended up in these peroxisomes. To avoid the problems formulated above, we hypothesised that the peroxisome of the common ancestor of the Diplonemida and Kinetoplastea contained a plastid, derived from an algal symbiont, that fused with the peroxisome (Hannaert et al., 2003a). The fused organelle subsequently lost its entire genome but retained some enzymes, such as those of the glycolytic pathway, of which the genes had been copied to the nuclear genome or replaced by nuclear genes. The import machinery for proteins in this organelle was then derived from the peroxisome that participated in this fusion. This scenario, despite the complicated membrane fusion events it invoked, was in accordance with the observed phylogenetic affiliation of a considerable number of trypanosomatid enzymes with those of algae, plants and cyanobacteria (Hannaert et al., 2003b). However, several other data have been reported that argued against such an ancestral plastid endosymbiont (Leander, 2004; Waller et al., 2004) and the genome sequencing projects did not reveal an indication for relicts of it in the DNA of trypanosomatids (El-Sayed et al., 2005).

Another possibility as to how an entire pathway may be re-routed from one compartment to another has recently been proposed by Martin (2010). This proposal was based on the observation that targeting of proteins to a specific compartment of a eukaryotic cell is never 100% specific. According to data in the literature, it is very common that proteins, despite having an apparent compartment-specific routing signal, are detected in multiple compartments; Martin (2010) quotes examples of the distribution, in different organisms, of single enzymes over two or more subcellular compartments such as cytosol, mitochondria, chloroplasts, ER, nucleus, peroxisomes and hydrogenosomes. In some cases, the dual targeting may be deliberate, i.e. when a protein possesses specific routing signals for two compartments, but in many cases a protein seems to be destined only for a single compartment, where indeed the majority arrives, while some 'mistargeting' occurs to other compartments. Dual localisation of enzymes of the glycolytic and PPPs has also been observed for trypanosomatids; a part of several enzymes was found in the glycosomes, another part in the cytosol (reviewed by Hannaert et al., 1999, 2003a). In some cases, notably for the PPP, the dual localisation seemed physiologically relevant but for glycolysis this was less likely to be the case (Bakker et al., 2000) and possible causes of the non-deliberate miscompartmentalisation have been discussed (Hannaert et al., 1999). Martin (2010) proposed that the small amount of mistargeting that happens all the time in a eukaryotic cell with all proteins has provided nature with the possibility of re-routing enzymes and entire pathways. In this way, a small amount of an entire pathway could arrive in an incorrect compartment, such as a glycolytic pathway inside a peroxisome. If that is the case, and it appears to provide a small selective advantage to the cell, the situation may be optimised further by the evolutionary processes of natural variation and natural selection. One could imagine that the glycosome has arisen in this way from peroxisomes.

The mechanism proposed by Martin (2010) provides also an explanation as to how other enzymes or pathways became sequestered in different organelles of the eukaryotic cell. Another relevant example in this regard is the considerable number of proteins from α-proteobacterial origin found in peroxisomes of mammals and yeast (see Section 5) (Gabaldón et al., 2006) and trypanosomatid glycosomes (Opperdoes and Michels, 2007) which have probably been re-routed to these organelles from the protomitochondrial endosymbiont when it developed into the mitochondrion and copied some of its genes to the nucleus.

As argued above, a functional glycolytic pathway would only develop inside peroxisomes if its substrates and cofactors would also be accessible inside the new compartment, and its products made available to the cytosol. This would require the presence of transporters or channels. Martin (2010) proposed that there may also have been 'slack' with regard to the specificity of the metabolite transporters in the membranes of different compartments to make this possible. Indeed, it seems likely that this has been the case for the development of glycosomes from peroxisomes. It has been a matter of debate for approximately 40 years whether the peroxisomal membrane is a true permeability barrier. Two opposite views have been defended: (i) peroxisomes are surrounded by an essentially impermeable lipid bilayer membrane that contains specific transporters for the exchange of solutes, and (ii) the membrane is permeable to small solutes due to the presence of non-selective or partially selective pores. Purification of peroxisomes and glycosomes usually results in organelles showing an apparent lower latency (ratio of an enzymatic activity before and after permeabilization of the organellar membrane) compared with mitochondria and lysosomes, when used in assays of their matrix enzymes. This was often attributed to fragility of the former organelles and damage resulting from their purification.

The identification of two classes of solute transporter molecules in the peroxisomal membrane – mitochondrial carrier family (MCF) proteins and ATP-binding cassette (ABC) transporters – reinforced the notion that the membrane was impermeable (reviewed by Theodoulou et al., 2006; Visser et al., 2007). ABC proteins have also been identified in the *T. brucei* glycosomal membrane (Yernaux et al., 2006: Igoillo-Esteve et al., 2011). Moreover, measurements of transmembrane pH gradients and electrical potentials supported this view, despite the conflicting results reported. In contrast, solute transport studies with isolated mammalian peroxisomes led, in 1987, to the proposal that the membrane contains non-selective pores (Van Veldhoven et al., 1987). The same conclusion was reached for plant peroxisomes based on biochemical and electrophysiological research performed in the 1990s. It appeared that compartmentalisation of plant peroxisomal metabolism is not caused by a boundary function of the membrane but primarily due to a tight association of the matrix enzymes, forming a multienzyme complex that permits a direct channelling of products from one enzyme to the next in the reaction sequence without the release of the intermediates into the bulk phase (reviewed by Reumann, 2000). Subsequent electrophysiological work on mammalian and yeast peroxisomes confirmed the presence of pore-forming activities in the boundary membrane, allowing the permeation of small metabolites, but restricting the exchange of more bulky compounds such as NAD(H), NADP(H), ADP, ATP, CoA and acetyl/acyl derivatives, etc. (Antonenkov and Hiltunen, 2006; Grunau et al., 2009; Rokka et al., 2009).

A protein responsible for pore formation in mammalian peroxisomes has been identified (Rokka et al., 2009). Recently, we showed the capacity to form similar current-conducting pores for glycosomal membrane proteins upon their solubilisation from membranes of highly purified glycosomes and subsequent incorporation into planar lipid bilayers (M. Gualdrón-López, P. Michels, V. Antonenkov, unpublished data). Concerning the transmembrane movement of large molecules, ABC transporters have been shown to be involved in uptake of medium- and long-chain fatty acids in both peroxisomes and glycosomes (Theodoulou et al., 2006; Visser et al., 2007; Igoillo-Esteve et al., 2011) and ATP/AMP exchange may be mediated by a MCF protein, similar to ATP/ADP antiport in mitochondria. Indirect evidence for the existence of a transmembrane adenine nucleotide antiporter has been obtained for glycosomes of *T. cruzi* (Sanz-Rodriguez et al., 2007). Nevertheless, of the 24 MCF proteins identified in the *T. brucei* genome database, to date none has been unambiguously located in the glycosomal membrane; 18 were found in the mitochondrial membrane while the localisation of the other six could not yet be established (Colasante et al., 2009).

Initially, after the discovery of the glycosome, a low permeability of its boundary membrane was invoked from the results obtained by experiments in which a pulse of radiolabelled glucose was given to the trypanosomes. A biphasic labelling of glycolytic intermediates was observed. The fast phase, within 15 s, was considered to represent the rapid formation of radioactive intermediates present in a small compartment, the glycosomes; the second phase, approximately 60 times slower, was interpreted as the appearance of glycolytic intermediates in the cytosol as a result of slow translocation across the glycosomal membrane (Visser et al., 1981). However, our recent indications for the existence of pore-forming activities in the glycosomal membrane should lead to the reinterpretation of these results: the fast phase may represent the rapid channelling through a glycolytic multienzyme complex and the slow phase the limited exchange of intermediates between the putative reaction highway within this complex and the bulk glycosomal/cytosolic environment.

The presence of pore-forming proteins and homologous MCF and ABC transporters in peroxisomes from organisms as different as mammals, plants and yeasts and glycosomes from trypanosomes renders it likely that these pores and transporters already existed in the ancestral organelles and therefore facilitated the relocation of enzymes from other compartments to these organelles. The enzymes could immediately form functional pathways because they would also have access to their substrates in their new compartments. It remains however to be determined how, despite this permeability of the boundary membrane, functional metabolic compartmentalisation is established within the peroxisomes and glycosomes. Specific questions related to this membrane permeability are: How is the remainder of the cell protected against the peroxides in the peroxisomes? How are metabolic networks with conserved stoichiometries maintained in the glycosomes (Bakker et al., 1997)? How can the thermodynamically-unfavourable reversal of the GK reaction occur in glycosomes of *T. brucei*? Is this all due to a specific structure of the organelles' protein matrix? The protein density inside these organelles is usually high. Do they form multienzyme complexes in which metabolite channelling occurs? Biochemical studies of glycosomes from bloodstream-form *T. brucei* indicated the presence of a multienzyme complex but its precise structure and the occurrence of channelling remains to be established (Aman et al., 1985; Misset et al., 1986; Bakker et al., 1995).

8. Evolution of glycosomes in different kinetoplastid lineages; losses and acquisitions

From a comparison of features shared between peroxisomes of some present-day organisms and the glycosomes of at least some kinetoplastids, conclusions may be drawn about features that must have been present in the original glycosome as developed in the common ancestor of the Diplonemida and Kinetoplastea (Fig. 7, Table 1 and Supplementary Fig. S1). These features are (i) a typical biogenesis process mediated by peroxins; (ii) degradation of the organelles by pexophagy; (iii) membranes containing pores allowing a rather non-selective permeation of small metabolites; (iv) involvement in H_2O_2 metabolism; (v) ether–lipid metabolism; (vi) fatty-acid β-oxidation and transport. With regard to solute transport, typical half-size ABC transporters, all belonging to subgroup D of this transporter family, are involved in uptake of long-chain fatty acids in all organelles of the peroxisome family. Furthermore, enzymes of the PPP, at least of its oxidative branch, may also have been present in the ancestral peroxisome, because these enzymes have a dual localisation in cells of many organisms, in the cytosol and peroxisomes; they have been found in peroxisomes of mammalian cells (Antonenkov, 1989; Wanders and Waterham, 2006), plants (Reumann, 2011) and trypanosomatids.

8.1. Carbohydrate metabolism: glycolysis, succinate production pathway, gluconeogenesis and glycerol pathway

The name 'glycosomes' could be taken to imply that glycolytic enzymes were the first enzymes newly acquired at the origin of these organelles. However, it seems likely that the glucose catabolism pathway of the original glycosomes was not the glycolytic pathway sensu stricto, responsible for the conversion of glucose to pyruvate, but rather an extended one, the succinate production pathway (Fig. 2B, reactions 15–18a). Whereas in classical glycolysis the two glycolytically produced NADH molecules are reoxidised by formation of two lactates, via pyruvate, from the two phosphoenolpyruvate (PEP) molecules produced from glucose, this succinate production pathway is different in that the reduction of only one PEP to succinate is sufficient for the oxidation of the two NADH (Fig. 2B, reactions 16 and 18a). The advantage of this latter process over classical glycolysis is that, while the ATP yield per molecule of glucose consumed remains at two, not all PEP is converted into the endproduct lactate, but that 50% of it can

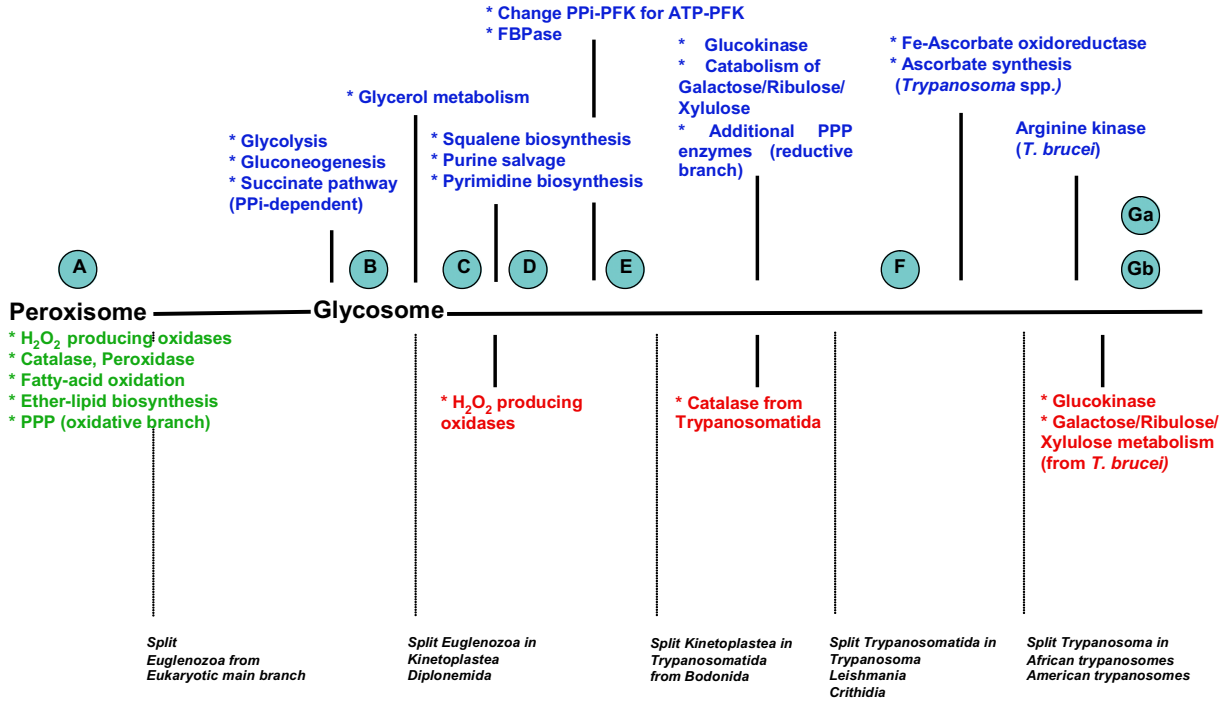

Fig. 7. Evolution of the glycosome. A timeline of the evolution of glycosome is shown, with indications of the probable times of its major acquisitions and losses of enzymes and pathways. Enzymes and pathways that were probably present in the ancestral peroxisomes are indicated in green. The acquisition of the succinate pathway, which may have operated in either the glycolytic or gluconeogenic direction and in which inorganic pyrophosphate (PPi) probably played a major role, was responsible for converting the peroxisome into a glycosome in a common ancestor of the Kinetoplastea and Diplonemida. Subsequent major modifications in the enzyme content of glycosomes of kinetoplastids are indicated; acquisitions are in blue above the timeline, losses are in red underneath. The positions of major branchpoints of lineages during the evolution of the Euglenozoa are indicated at the bottom of the figure, but are not precisely according to an evolutionary time scale (for which we refer to Fig. 3). Circles labelled (A) to (Ga) and (Gb) refer to pictures of the major metabolic pathways and enzymes that potentially have been present within the peroxisomes of the ancestral organism (A) and within glycosomes of Euglenozoan organisms at different stages of their evolution as indicated above the timeline. Those pictures are shown in Supplementary Fig. S1. For a discussion of the details, see Section 8 of the manuscript. PPP, pentosephosphate pathway; PFK, phosphofructokinase.

Table 1
Different scenarios demonstrating how glycolytic enzymes might have become compartmentalized in peroxisomes, leading to the formation of glycosomes.

Scenario	1st Acquisition		2nd Acquisition	
	Pathway acquired	Selective force	Pathway acquired	Selective force
1	Glycolytic enzymes responsible for glucose → PGA	Compartmentalisation to increase efficacy metabolic reprogramming	Succinate producing enzymes responsible for PEP → succinate	Re-oxidise glycolytically produced NADH
2	Gluconeogenic enzymes responsible for PGA → glucose 6-phosphate[a]	Compartmentalisation to increase efficacy metabolic reprogramming	Succinate consuming enzymes responsible for succinate → PEP	Re-reduce gluconeogenically produced NAD and provision of alternative gluconeogenic substrates[b]
3[c]	Enzymes of glycolysis and succinate production glucose → succinate [a]	Compartmentalisation to increase efficacy metabolic reprogramming	Key enzymes of gluconeogenesis (FBPase)	Compartmentalisation to increase efficacy metabolic reprogramming
4[c]	Enzymes of gluconeogenesis and succinate consumption succinate → glucose 6-phosphate	Compartmentalisation to increase efficacy metabolic reprogramming	Key enzymes of glycolysis (PFK)	Compartmentalisation to increase efficacy metabolic reprogramming
5	Enzymes of succinate production PEP → succinate	Oxidation of NADH produced by peroxisomal dehydrogenases	Enzymes of glycolysis responsible for glucose → PGA	Alternative way for NAD reduction, allowing loss of O_2-dependent H_2O_2 oxidases and (facultative) anaerobic growth

PGA, phosphoglycerate; PEP, phosphoenolpyruvate.
[a] Glycerol 3-phosphate and glycerol – after acquisition of glycerol kinase (GK) – might also have been early substrates for gluconeogenesis or ATP production via the lower branch of glycolysis (and downstream pathways).
[b] Substrates (amino acids, succinate, malate) fed into the gluconeogenic process via the succinate branch.
[c] Scenarios 3 and 4 may have occurred simultaneously if the fructose 6-P → fructose 1,6-P_2 and fructose 1,6-P_2 → fructose 6-P reactions were catalyzed by a single, inorganic pyrophosphate-dependent phosphofructokinase (PPi-PFK) and later have been separated when the PPi-PFK, catalyzing a reversible reaction, was replaced by an ATP-PFK and fructose-1,6-bisphosphatase (FBPase), each catalyzing an irreversible reaction.

be further utilised for production of essential metabolites or, via pyruvate, be directed to the mitochondrion for additional oxidation and ATP production. This succinate production pathway involves a soluble, NADH-dependent fumarate reductase (NADH-FRD, Fig. 2B, reaction 18a) different from and only distantly related to the more common membrane-bound quinol-dependent FRD/succinate

dehydrogenase widely present in mitochondria and bacteria. This glycosomal NADH-FRD has been found in all extant trypanosomatids studied to date, and an isoform of it is also present in the trypanosomatid mitochondrion (Fig. 2B, reaction 18b) (Besteiro et al., 2002; Coustou et al., 2005). The presence of this glycosomal succinate production pathway remains to be demonstrated in other kinetoplastid lineages in order to provide firm support for the notion that it was already operative in the ancestral glycosome. It should be noted that as early as 1980 the hypothesis was proposed for anaerobic prokaryotes that the succinate production pathway, involving a soluble FRD, evolved from the classical glycolytic, lactate producing pathway to increase the efficiency of sugar fermentation (Gest, 1980).

Although the genes for the enzymes of the succinate production pathway are present in all trypanosomatids studied, not all enzymes are expressed in all life-cycle stages. In bloodstream-form African trypanosomes – except *Trypanosoma congolense* and *Trypanosoma lewisi* – only the enzymes of the classical glycolytic route to pyruvate are expressed (Fig. 2A). In these organisms, all NADH formed during glucose catabolism by glyceraldehyde-3-phosphate dehydrogenase (GAPDH, Fig. 2, reaction 9) is reoxidised not via the succinate pathway, but by a glycosomal G3PDH (Fig. 2, reaction 7) which is part of the G3P shuttle routing the electrons to the mitochondrial O_2-dependent G3P oxidase system (Fig. 2, reactions 26, 28 and 29). Under anaerobic conditions, the G3P is converted into glycerol, with concomitant ATP synthesis, by GK operating in its reverse direction and thus balancing ATP consumption and production within the glycosomes. No pyruvate-dependent lactate dehydrogenase for the oxidation of glycolytic NADH has been detected in trypanosomatids. The use of the shortened glucose catabolic pathway in the bloodstream-form African trypanosomes is probably related to the abundance of available nutrients and therefore the much smaller need to spare glucose derived PEP for biosynthetic purposes or further oxidation.

One could also consider the possibility that initially only the succinate-producing branch, from PEP to succinate, was acquired to reoxidise NADH produced by dehydrogenases already present in the ancestral peroxisome (Table 1). In peroxisomes NADH, and possibly also some cytosolic NADH, is eventually oxidised by O_2, via the regeneration of the oxidation products of ethanol, α-hydroxy acids etc., the substrates of the H_2O_2-producing oxidases (de Duve and Baudhuin, 1966). The acquisition of a new NADH oxidation pathway such as the succinate branch by peroxisomes may thus have been the reason that the H_2O_2-producing oxidases became redundant. Particularly under anaerobic conditions, this may have been relevant. Having the NADH oxidation process established inside these organelles, the NADH producing process of glycolysis could then subsequently have been acquired.

Alternatively, one may wonder whether the pathway that was initially acquired by peroxisomes, resulting in the formation of glycosomes, was involved in succinate oxidation and/or gluconeogenesis instead of glycolysis and succinate production (Table 1). As discussed in Section 4, the ancestral organism that acquired glycosomes was most probably a protist living freely in an aquatic environment, maybe 600 Mya. Would this environment have contained sugars or rather less reduced substrates? Were the nutrients not instead amino acids, fatty acids or glycerol and glycerolipids? Such nutrients – amino acids upon their conversion into succinate or malate, fatty acids after their breakdown to acetyl-CoA – could then be used, other than for ATP synthesis, for sugar–phosphate formation by gluconeogenesis necessary for glucoconjugates and nucleotides. The succinate pathway would have been a succinate-consumption pathway (Fig. 2B, reactions 18a, 17a, 16 and 15) that may also have served to regenerate NADH and ADP used by GAPDH and FBPase (Fig. 2B, reactions 9 and 4), respectively, during gluconeogenesis. Only later, when the organisms encountered more sugar-rich environments, might the pathway have started to operate in the glycolytic/succinate production mode. No evidence is available that supports one or the other evolutionary scenario, except that thermodynamically the GK reaction is strongly favored in the glycerol consumption direction and the kinetics of the trypanosomatid GKs suggest that this is the principal purpose of this enzyme. The G3P thus produced could then be used both for catabolism via the lower part of the glycolytic pathway as well as for gluconeogenesis.

Present-day trypanosomatids readily consume glycerol and the reversal of the reaction may occur only under special conditions when special intraglycosomal metabolite concentrations are created. Therefore, we hypothesise that the glycerol metabolism branch (G3PDH and GK, Fig. 2, reactions 7 and 8) was only secondarily exploited for participation in the maintenance of the redox and ATP/ADP balance in the glycosome. Initially it may have merely contributed to increasing the flexibility of maintaining these balances, when the succinate production pathway between PEP and succinate was not used because conversion of PEP into pyruvate via pyruvate phosphate dikinase (PPDK, Fig. 2B, reaction 14) was preferred (either to spare ADP, by using inorganic pyrophosphate (PPi) (see Section 8.2) or to produce more pyruvate for its conversion into other essential metabolites or to be used in mitochondrial oxidation). Only later, in the unique situation of the bloodstream-form African trypanosomes with merely the classical glycolytic part of the pathway expressed, the G3PDH (and under anaerobic conditions also GK) became essential for maintaining the balances; this branch is not essential for glucose catabolism of procyclic-stage African trypanosomes expressing the full succinate production pathway (Ebikeme et al., 2010).

8.2. PPi-dependent metabolism

Another question is whether the originally acquired glycolytic/gluconeogenic pathway relied more on PPi and less on ATP than the pathway in extant trypanosomatids. PPi-dependent glycolytic enzymes, notably a PPi-dependent PFK instead of ATP-PFK and a PPDK instead of PYK are often found in (facultative) anaerobic bacteria and protists. PPDK catalyses the reversible reaction PEP + Pi + AMP ↔ ATP + Pi + pyruvate. The use of these reactions provides the cells with a larger ATP yield per molecule of glucose consumed, because the PPi produced in various biosynthetic processes is not hydrolysed by a pyrophosphatase but its free energy is used for glycolytic reactions (Mertens, 1993). Trypanosomatids possess a PPDK (Bringaud et al., 1998; Acosta et al., 2004) and interestingly, they contain an ATP-PFK (Fig. 2, reaction 3) that has all the features of a PPi-PFK (Michels et al., 1997). The sequences of PPi- and ATP-PFK have a very low similarity but yet have a common evolutionary origin (McNae et al., 2009). By mutagenesis it has been shown that the nature of the preferred phosphodonor can be readily changed (Chi and Kemp, 2000) and strong evidence exists for the notion that this has happened repeatedly during evolution, probably also early in the kinetoplastid lineage (Hannaert et al., 2003a; McNae et al., 2009). Importantly, the PPi-PFK catalysed reaction is reversible under physiological conditions, whereas the ATP-PFK reaction is not; the reverse reaction of the latter is catalysed by FBPase (Fig. 2B, reaction 4). It therefore seems quite possible that the initial pathway acquired by glycosomes contained a PPi-PFK that may have functioned in either direction. At some stage, the PFK changed its preference for phosphodonor from PPi to ATP, thus becoming a specific glycolytic enzyme, whereas the gluconeogenic function must then have been taken over by the acquisition of a FBPase, maybe by lateral gene transfer (Fig. 7). One could speculate whether the presence of a PPDK, as well as initially also PPi-PFK, indicates that the ancestral organisms had a (facultative) anaerobic life style.

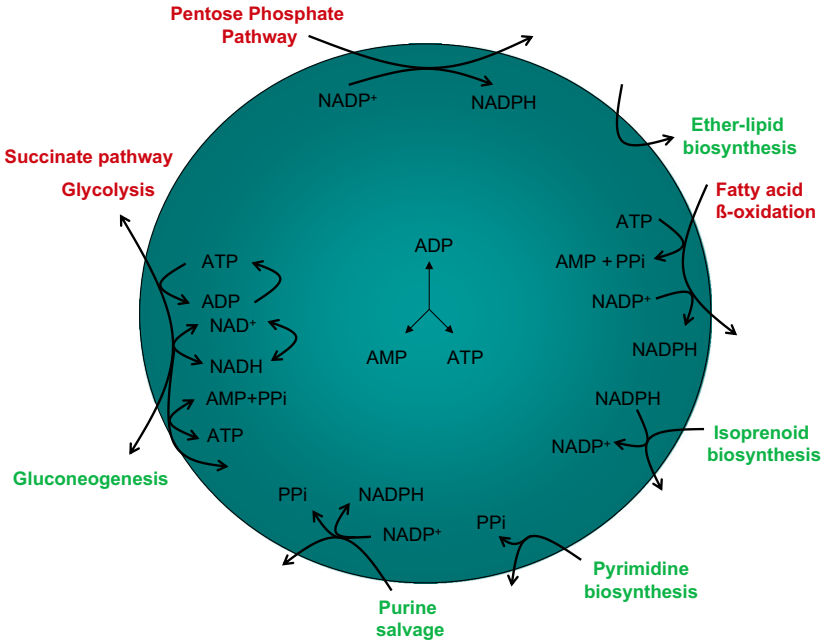

Fig. 8. Glycosomes form a crossroad of metabolic routes. Metabolic processes which are almost entirely or in part present within glycosomes are functionally linked by the respective production and use of PPi, ATP, ADP, AMP, NAD(H) and NADP(H). It is hypothesised that this linkage played an important role in the evolution of the glycosome as a distinct unit in the metabolism of the Kinetoplastea (see Section 8.6). Catabolic processes are indicated in red, anabolic processes are in green.

8.3. Glycosomes link pathways sharing cofactors

We thus infer that PPi played an important role in the metabolism in ancestral glycosomes. The consumption of PPi in the (extended) glycolysis may have been important for the simultaneous or subsequent recruitment of biosynthetic processes that produce this energy-rich phosphate. The same holds true for cofactors: NAD(H), NADP(H), ATP, ADP and AMP. The glycosomal membrane is impermeable for these compounds and it has been shown experimentally and by computer simulation that trypanosomatid metabolism needs to balance the intraorganellar production and consumption rates. Therefore, (parts of) biosynthetic routes involved in purine salvage, pyrimidine biosynthesis, squalene synthesis (see Section 8.6) and the PPP, all requiring the involvement of one or more of these cofactors and/or PPi, could become compartmentalised and functionally linked with the glycolytic/gluconeogenic pathways and with each other by sharing the cofactors and PPi (Fig. 8). In addition, the acquisition of an adenylate kinase made it possible to create or adjust the balance between the different adenine nucleosides. As a result, the kinetoplastids acquired the possibility to link important catabolic and anabolic pathways in the small volume of these organelles. One could imagine that this could be advantageous for the organism for the following reasons: (i) an increase in the efficiency of the metabolism by bringing (parts of) pathways –both catabolic and anabolic– sharing cofactors and other substrates together in a small volume, and maybe even in a multienzyme complex; (ii) a coordinated and rapid adjustment of an entire metabolic network during differentiation or adaptation to different nutritional conditions, by 'en bloc' synthesis and degradation of the pathways or core components of pathways, by turnover of the organelles, involving their induced biogenesis and autophagy. Induction by differentiation signals or by cues from the altered environment leads to drastic removal of old glycosomes, as well as proliferation and growth of new glycosomes together with alterations in the synthesis of enzymes to be imported in the new glycosomes (and the synthesis of other proteins in the cell) required for use under the new conditions. Accordingly, a population of new glycosomes with a different repertoire of enzymes efficiently and rapidly replaces the old population.

8.4. Other enzymes of carbohydrate metabolism

Glycosomes of all *Leishmania* spp., *T. cruzi* strains examined and of *C. fasciculata* contain a glucokinase (GlcK), i.e. a sugar-phosphorylating enzyme with specificity only for glucose, different from HXK that has a broad substrate specificity (Cáceres et al., 2007; F. Opperdoes, personal communication). No *GlcK* gene could be detected in the TriTrypDB database for any of the African trypanosomes. The GlcK and HXK are paralogous but very different and are classified into different groups of the hexokinase family (Kawai et al., 2005). It was shown that the two enzymes have a different specificity for the glucose anomer: HXK for α-glucose, GlcK for β-glucose (Cordeiro et al., 2007). This different preference may indicate different physiological functions, for example the HXK preferentially for glycolysis, where the second enzyme, 6-phosphoglucose isomerase, has a preference for α-G6P and GlcK for the PPP, where G6PDH is highly specific for β-G6P. When GlcK was acquired by glycosomes remains to be determined by analysis of other kinetoplastids. Nonetheless, it is probable that its absence from African trypanosomes is due to recent loss (Fig. 7 and Supplementary Fig. S1). Trypanosomatids, including *T. brucei*, contain aldose-1-epimerases in their glycosomes for reversibly converting the α- and β-forms of sugars (Colasante et al., 2006; Opperdoes and Szikora, 2006) as well a G6P epimerase that can anomerise both forms of G6P (A. Cordeiro, S. Gupta and P. Michels, unpublished data), rendering the GlcK redundant if its mere function were to provide the β-anomer of the phosphorylated sugar for the PPP.

Some other enzymes of carbohydrate metabolism are also present in glycosomes of *Leishmania* spp., *T. cruzi* and *C. fasciculata,* but not *T. brucei,* as inferred from the presence of their genes in the genome databases of the former three trypanosomatids but not the latter one, and each encoding a protein with a PTS: a galactokinase, ribulosekinase, xylulosekinase (the latter not in *T. cruzi*), as well as a ribulokinase, phosphomannomutase and glucosamine-6-phosphate

isomerase (Opperdoes and Szikora, 2006). Interestingly, all of these sugarkinases show a phylogenetic affiliation with bacteria and may thus have been acquired during the kinetoplastid evolution by horizontal gene transfer (F. Opperdoes, personal communication) and directed to the glycosomes, but lost again in *T. brucei* and in some other *Trypanosoma* spp. (Fig. 7 and Supplementary Fig. S1). Nevertheless, glycosomes of *T. brucei* do contain a glycosomal uridine diphosphate (UDP)-galactose-4'-epimerase, similar to the other trypanosomatids (Shaw et al., 2003). *T. brucei* cannot take up galactose because their hexose transporters do not have affinity for galactose, in contrast to those of many other trypanosomatids (Tetaud et al., 1997) and therefore this enzyme probably serves to synthesise UDP-galactose from UDP-glucose.

8.5. Arginine kinase

Trypanosomatids contain multiple isoforms for arginine kinase. Proteome analysis revealed the presence of two isoforms associated with glycosomes of bloodstream-form *T. brucei* (Colasante et al., 2006; Vertommen et al., 2008). Moreover, GFP-fusion data support such a localisation (M. Ginger, personal communication). No evidence for a glycosomal arginine kinase was found for *T. cruzi,* although in this species one of the isoforms was, by immunofluorescence analysis, located in non-identified punctate structures that were seemingly different from glycosomes (Miranda et al., 2009). The enzyme catalyses the ATP-dependent reversible transphosphorylation of arginine. Phosphoarginine is considered an energy buffer for the cell; it can accumulate much higher intracellular concentrations than ATP. In *T. cruzi* epimastigotes the arginine kinases have been shown to be related to parasite density and important to resist stress imposed by pH, reactive oxygen species and nutrient non-availability (Pereira et al., 2011). Arginine kinases are widely distributed in eukaryotes and thus were probably present already in the cells before the glycosomes originated. Glycosomes may have recruited the enzyme from the cytosol to function in ATP buffering of their matrix when the ATP threatens to drop to a too low level. However, the finding to date of arginine kinases in glycosomes of only *T. brucei* suggests that this recruitment was a late event that may have only occurred in African trypanosomes (Fig. 7 and Supplementary Fig. S1).

8.6. PPP, purine salvage, pyrimidine and squalene biosynthesis

At some stage, glycosomes also acquired parts of the pathways for purine salvage and biosynthesis of pyrimidines and squalene and perhaps at least part of some additional enzymes of the PPP (Fig. 7 and Supplementary Fig. S1), resulting in the dual localisation of the latter process in both cytosol and glycosomes. As mentioned at the beginning of Section 8, some enzymes of the oxidative branch of the PPP, such as G6PDH and phosphogluconate dehydrogenase, may have already been present in the ancestral organelle, but in trypanosomatids not only these enzymes, but also 6-phosphogluconolactonase, transketolase, transaldolase, ribokinase and ribose-5-phosphate isomerase contain a PTS and have been found in both compartments, although the extent of this compartmentalisation may differ considerably between species and their life-cycle stages (Duffieux et al., 2000; Maugeri and Cazzulo, 2004; Colasante et al., 2006; Opperdoes and Szikora, 2006). The need for intraglycosomal production of NADPH (Fig. 8), as well as for other phosphorylated intermediates, may have been the reason why a larger part of the PPP activity became compartmentalised in the trypanosomatids. An alternative scenario is also conceivable, i.e. that the ancestral peroxisome did not yet possess any PPP enzymes and that the organelles in both mammalian and plant cells and trypanosomatids acquired them – although to a different extent – independently at a later stage.

Trypanosomatidae are incapable of de novo purine biosynthesis, however they can salvage purines. They possess transporters for the uptake of purine bases from the host (Landfear, 2011) as well as the necessary phosphoribosyltransferases and enzymes involved in the interconversion of the purine nucleotides. Several of these enzymes have been located inside the glycosomes and/or identified as glycosomal enzymes by the possession of a PTS (reviewed by: Colasante et al., 2006; Opperdoes and Szikora, 2006; Vertommen et al., 2008). In contrast to the incapability for de novo purine synthesis, trypanosomatids do possess all enzymes for the synthesis of pyrimidine from glutamine, bicarbonate and aspartate. As described above, the fact that these enzymes produce PPi, a substrate for PPDK and initially maybe also PPi-PFK, dependent on the time of acquisition of these enzymes and the change in PFK's specificity for its phosphodonor (Fig. 7), may have been the selective force for the sequestration of these enzymes within the organelles.

The successive enzymes of isoprenoid synthesis by the mevalonate pathway, for producing squalene and sterols, are in most eukaryotes found in the cytosol and ER. In trypanosomatids, mitochondrion and glycosomes seem to be involved as well, with the key enzyme 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR) as an apparent glycosome-associated protein, despite the lack of a consensus PTS (Concepción et al., 1998; Heise and Opperdoes, 2000; Opperdoes and Szikora, 2006; Ginger et al., 2010). Solanesyl-diphosphate synthase, another enzyme involved in isoprenoid biosynthesis, was also located in glycosomes of *T. cruzi* (Ferella et al., 2006). The NADPH dependence of isoprenoid synthesis may have been a reason why part of this pathway became associated with the organelles (Fig. 7). However, it should be mentioned that a mitochondrial localisation has also been reported for HMGR (Peña-Diaz et al., 2004). Further studies will be required to definitely determine the role of glycosomes in isoprenoid biosynthesis.

8.7. Reactive oxygen metabolism

The evolution of glycosomes not only resulted in the acquisition of new enzymes and pathways but also in losses (Fig. 7 and Supplementary Fig. S1). For example, most of the enzymes of H_2O_2 metabolism – the hallmark of peroxisomes – were lost from glycosomes, even altogether from trypanosomatids. Glycosomes of extant trypanosomatids do not contain any H_2O_2-producing oxidases nor a catalase in many of the species. Catalase is present in the organelles of the bodonid *Trypanoplasma* (Opperdoes et al., 1988), but not in those of any of the parasitic trypanosomatids studied, suggesting that it was lost from them shortly after the Kinetoplastea split into the Trypanosomatida and Bodonina. Catalase is also present in *Crithidia* where, however, it is soluble rather than organelle-associated. Indeed the *Crithidia* genome has two closely-related catalase genes but interestingly their predicted amino-acid sequences show a high identity (75%) with catalases of spirochaete bacteria and lack a PTS, in agreement with its subcellular localisation (F. Opperdoes, personal communication). This strongly suggests a recent re-acquisition of catalase by this trypanosomatid through lateral gene transfer from a bacterium.

In the catalase-lacking trypanosomatids, H_2O_2 detoxification inside glycosomes still seems to occur, but by other enzymes. A trypanothione-dependent detoxification mechanism is found dually distributed in these trypanosomatids, in both their cytosol and glycosomes (Colasante et al., 2006; Opperdoes and Szikora, 2006). Furthermore, significant levels of ascorbate (vitamin C) are present in trypanosomatids (Clark et al., 1994) and play an important role in H_2O_2 detoxification. A plant-like ascorbate-dependent hemoperoxidase has been detected in the genomes of *T. cruzi*, *Leishmania* spp. and *C. fasciculata* but not in *T. brucei*. In *T. cruzi* the enzyme was located in the ER (Wilkinson et al., 2002), whereas in plants

multiple isoforms are present in various compartments including peroxisomes. However, none of the trypanosomatid sequences possesses a PTS. Sequences for the iron-ascorbate-oxidoreductases have also been identified in all trypanosomatids studied. Interestingly, eight different sequences for this enzyme, all having a PTS1, are present in *T. brucei* and indeed two isoforms have been detected in glycosomes of this parasite (Colasante et al., 2006). PTS1-containing homologues can also be detected in other *Trypanosoma* spp. but not in *Leishmania* or *Crithidia*. Iron-ascorbate-oxidoreductases function in the important ascorbate–glutathione cycle of plant peroxisomes to produce reduced ascorbate for detoxification of H_2O_2 (Noctor and Foyer, 1998). Nonetheless, trypanosomes are also capable of efficient non-enzymatic reduction of monodehydroascorbate to ascorbate by trypanothione (Krauth-Siegel and Lüdemann, 1996). All trypanosomatids studied, like many other eukaryotes but not humans, have the capacity for ascorbate synthesis (Wilkinson et al., 2005; Opperdoes and Coombs, 2007). The biosynthesis route is different in mammals, plants and yeasts, but in all organisms the last step of the pathway involves the conversion of an aldonolactone to ascorbate by a FAD-dependent oxidoreductase. Yet, this enzyme differs in substrate specificity, electron acceptor and subcellular location in each of these taxonomic groups. The oxidoreductase of trypanosomatids is most similar to the plant enzyme galactonolactone dehydrogenase which functions within mitochondria with cytochrome c as the electron acceptor. Indeed, the *T. brucei* enzyme can also use this substrate, as well as arabinonolactone to synthesise ascorbate, however it was not located in the mitochondrion but in glycosomes (Wilkinson et al., 2005). The *T. brucei* and *T. cruzi* enzymes contain a PTS1, however the enzymes of *Leishmania* and *Crithidia* lack such a targeting motif. The finding of both a galactonolactone dehydrogenase and an iron-ascorbate oxidoreductase only in glycosomes of *Trypanosoma* and not in other trypanosomatids suggests that a prominent role of ascorbate within these organelles was acquired only late in evolution, after the diversification of the Trypanosomatidae family.

Trypanosomatids also have several superoxide dismutase isoforms that produce H_2O_2 from superoxide radicals; one of them is present inside glycosomes (Dufernez et al., 2006; Wilkinson et al., 2006). There is also sequence evidence for a thiol metabolism to deal with oxidative stress in the cells, also in glycosomes (Opperdoes and Szikora, 2006). Together, the data suggest that glycosomal H_2O_2 metabolism – production and detoxification – has undergone considerable modification during evolution. All currently available data have been obtained for parasitic trypanosomatids. Future studies on free-living kinetoplastids should reveal whether the loss of peroxide-producing oxidases is related to the development of parasitism. Another interesting question to address is whether modifications of oxidative stress defence mechanisms inside the organelles and a possible redistribution of such mechanisms between cytosol and glycosomes have occurred to better deal with the oxidative attack by the host.

9. Glycosomes may have facilitated the development of parasitism

Parasitism evolved many times within the Kinetoplastea (Fig. 9) (Simpson et al., 2006). Most likely, aquatic free-living kinetoplastids first parasitised insects and subsequently, via the insects, vertebrates and plants became infected. Both evolutionary transitions meant that the metabolism of the kinetoplastid organisms undergoing them should have been sufficiently flexible so that the organism could develop a cyclic process of adaptations to different environments (i.e. develop a life-cycle): a cycle between an aquatic environment and an insect and a cycle between an insect and a vertebrate host. Moreover, many parasites have subsequently evolved in a way that allowed them to adapt to different places in the bodies of their hosts, resulting in even more complicated

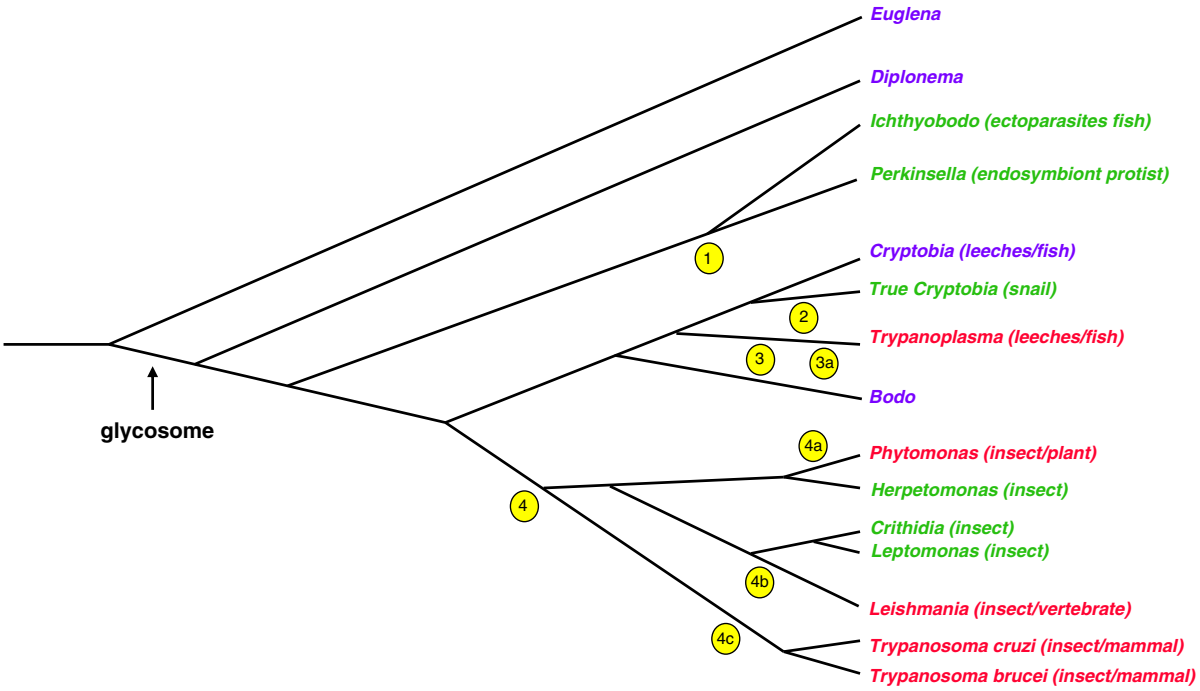

Fig. 9. Parasitism has arisen independently, multiple times in the Kinetoplastea. The phylogenetic relationships between extant organisms as published elsewhere (Simpson et al., 2006) are shown. The positions of the branch points of lineages are not according to an evolutionary time scale. Free-living organisms are indicated in mauve, monogenetic parasites (having only a single host in their life cycle) in green and digenetic parasites (life cycle involving two hosts) in red. Yellow circles with numbers 1–4 indicate the development of parasitism in a lineage, letters a–c behind the number refer to the establishment of parasitism in a second host resulting in a digenetic parasitic life cycle of the organisms of that lineage. The hosts are indicated to the right.

life-cycles: in the insect first on the mouth parts and subsequently often also within the digestive system and salivary glands, in the vertebrates extracellularly in the blood and subsequently often also intracellularly, in different cell types dependent on the host. The facts that parasitism evolved independently multiple times in the kinetoplastids and that in different lineages a complicated life-cycle could develop must have been made possible by a great metabolic adaptability of these organisms. We propose that this adaptability has to a large extent been made possible by the compartmentalisation into glycosomes of metabolic processes that had to undergo drastic and rapid changes in (some) transitions (Herman et al., 2008), similar to those observed for the adaptability of yeasts to different environments by compartmentalisation of their crucial pathways in peroxisomes (Bellu and Kiel, 2003; Manjithaya et al., 2010). Pathways, or parts of them, can be 'turned over' much more rapidly and efficiently upon induction, when they are compartmentalised than when present in the cytosol as individual enzymes. It therefore seems likely that the sequestering of core metabolic pathways into glycosomes may have greatly facilitated kinetoplastids to develop successfully at multiple times parasitism, both monogenetic and digenetic, – and to evolve this parasitism to great perfection – even though there is no reason to assume that metabolic compartmentalisation per se might be important for parasitism.

10. Conclusions

Glycosomes were first described 34 years ago, in 1977, as microbodies harbouring glycolytic enzymes in bloodstream-form *T. brucei*. Soon afterwards, they were also discovered in other trypanosomatid parasites, later in Bodonida and very recently in Diplonemida. In due course, these organelles were recognised as peroxisomes, when they were shown successively to share with peroxisomes from other organisms not only morphological aspects but also some enzymes and metabolic pathways and to have a similar biogenesis route involving orthologous proteins. Nonetheless, how the organelle originated in these protists and the reason why this organelle may have become established remained enigmatic for a long time. There are several reasons for this delay. First, the origin of peroxisomes themselves has been debated for years; only recently has an explanation been found – derived from the ER – that is now considered plausible by the majority of peroxisome researchers. Second, the initial identification of glycosomes was made for bloodstream-form *T. brucei* and throughout the last three decades by far most of the research has been performed on the organelles and their enzymes of these trypanosomes. These parasites can easily be grown and genetically manipulated, and research on their glycosomes is medically relevant (Verlinde et al., 2001; Galland and Michels, 2010; Brennand et al., 2011). However, these parasites are now considered in many respects not representative for the majority of the kinetoplastids. Their metabolic repertoire is very limited and the enzyme composition of their glycosomes, with up to 90% of their protein content made up of glycolytic enzymes, is much simpler than that of the corresponding organelles of all other members of this diverse group of protists. The studies on *T. brucei* glycosomes have provided a detailed insight into the properties of the glycolytic enzymes and the consequences of their compartmentalisation for the functioning of glycolysis in extant bloodstream-form trypanosomes. However, as realised over time and explained in this paper (Sections 2 and 4), this knowledge is probably of lesser relevance to understand the full physiological importance of glycosomes in other kinetoplastids and the reason why the organelles evolved in the first place. Such knowledge may now have started to develop by the availability of the genome sequences of a variety of trypanosomatids, permitting the lay out for these organisms of schemes of metabolic pathways that highlight the roles played by glycosomes in different pathways, not only in glycolysis (Section 8). Moreover, the finding that peroxisomes of notably methylotrophic yeasts, but also other eukaryotes, play a very important role in the rapid and efficient metabolic reprogramming during changes in nutrient availability, provided a stimulus to explore the role of glycosomes in trypanosomes undergoing changes under environmental conditions, notably during their life-cycle differentiation when such changes are encountered (Section 6).

In this paper, we proposed a scenario for the origin and evolution of glycosomes based on (i) the current knowledge of the evolutionary history of the Kinetoplastea, (ii) a comparison of the metabolism and role of glycosomes in it for different Trypanosomatina, as deduced from available genome sequences as well as from metabolic studies, and for some of these organisms at different life-cycle stages, and (iii) a comparison of the composition of the glycosomal proteome and function with those of the peroxisomes in non-kinetoplastid organisms. Key in addressing the question as to how peroxisomes acquired the major part of the glycolytic pathway to become glycosomes has been the realisation – as described by Martin (2010) – that, in a eukaryotic cell, minor mistargeting is a common feature and that the presence of even a small amount of an enzyme or of successive enzymes of a pathway targeted to a wrong compartment, could act as a metabolic unit on which natural selection can act to maintain and expand it when it provides an advantage to the cell (Section 7). Less information is available that allowed us to formulate a convincing answer on the question why glycolytic enzymes became established inside peroxisomes in the common ancestor of Kinetoplastea and Diplonemida. To answer this question, it would be important to have information about the growth conditions of this ancestral organism, information that is unlikely to be obtained. Yet, we hypothesised that this organism regularly encountered nutritional changes which necessitated considerable changes in its glycolytic or gluconeogenic activities and that the compartmentalisation of these enzymes inside peroxisomes facilitated its rapid and efficient adaptation to the new conditions.

Many uncertainties and questions remain. Our ideas about the origin and evolution of the organelle will, of course, always remain hypothetical. But when proteomic information, either predicted from genome sequences or determined by mass spectrometry, might become available about the glycosomes from non-trypanosomatid Kinetoplastea and Diplonemida, several of the questions about the evolution of the organelle may be answered in a plausible way. More uncertainty will probably always remain about the origin of the organelle. But perhaps additional information can be obtained to provide further support for our hypothesis as to how and why glycosomes originated, to refine our ideas or to allow the development of alternative hypotheses about the origin of the organelle.

In this paper, we argued that environmental changes of nutrients formed the driving force for the origin and the further evolution of glycosomes. One could then wonder whether the same compartmentalisation of glycolysis inside peroxisome-like organelles, or other subcellular compartments, has also occurred elsewhere in evolution, under similar selective forces. Indeed, we consider it feasible that this has happened, or may still occur, in other protists, but has not yet been detected. In this respect, it is important to realise that only a very limited number of protists have been biochemically characterised. In Kinetoplastea the sequestering of glycolytic enzymes was easily detected because several of the species in this order are parasites that cause important health problems to humans and livestock and therefore became topics of biomedical and veterinary research. Moreover, several of these parasites, most notably trypanosomes, could be adapted in such a way that they could be propagated under laboratory conditions; *T. brucei* can easily be grown to very high density in rodents and in in vitro culture. The fact that the blood-

stream forms of this species are entirely dependent on glycolysis for their ATP supply and that the compartmentalisation of their glycolytic enzymes was so extreme (the glycosomal matrix of bloodstream-form trypanosomes has a high protein density; 90% of it is made up by glycolytic enzymes) made it hard to overlook when studying these parasites. Since glycosomes were detected in *T. brucei*, specific searches have been performed for these organelles in related organisms – with success, although some of them being less easily grown in the laboratory and relying much less on glycolysis. The closest related organisms without detectable compartmentalisation of glycolytic enzymes are in the other branch of the Euglenozoa, the Euglenida. These latter organisms probably did not experience the driving force for the development of glycosomes, although it cannot be excluded that glycosomes originated in the common ancestor of Diplonemida, Kinetoplastea and Euglenida, but that ancestral organisms of the latter lineage lost the glycolytic enzymes again from their peroxisomes.

A last intriguing issue which remains is the question whether further support could be obtained for the notion, as postulated in this paper, that glycosomes played a facilitating role in the development of parasitism in the Kinetoplastea. Genetic studies with trypanosomatids have shown that mutations in autophagy affect the possibility of the parasites to differentiate and may diminish their virulence (reviewed in Brennand et al., 2011). It would be interesting to attempt to identify trypanosomatid mutants with deficiencies in glycosomes – their function, biogenesis or degradation by autophagy – that do not affect their functioning under steady-state growth conditions but drastically impair their ability to adapt to changing nutrient conditions or differentiation. The PEX14 procyclic mutant cell line prepared by Parsons and colleagues as described in Section 4 (Furuya et al., 2001), which is viable under glucose-depleted conditions, could be considered suggestive in this regard. Although such mutant cells do not prove a link between the presence of glycosomes and parasitism, they render it likely that glycosomes provide an increased versatility to the organisms necessary for differentiation and thus to develop a parasitic life-cycle.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ijpara.2011.10.007.

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Chapter 3

The function of glycosomes in the metabolism of trypanosomatid parasites and the promise of glycosomal proteins as drug targets

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Abstract

Trypanosomatids have the unique feature of compartmentalizing the major part of the glycolytic pathway inside peroxisome-related organelles called glycosomes. However, these organelles also contain enzymes of several other important pathways involved in both catabolic and anabolic processes. The enzyme content and the metabolic role of glycosomes differ between trypanosomatid species and between their life-cycle stages. Several of the glycosomal pathways have been shown to be important for the viability, pathogenicity and/or virulence of different trypanosomatid parasites. Additionally, the correct compartmentalization of glycosomal enzymes inside the organelles appeared to be vital for these pathogens. Therefore, many of these enzymes, as well as the proteins involved in the translocation of metabolites across the glycosomal membrane and peroxins, proteins responsible for the biogenesis of glycosomes, are candidate drug targets. Glycosomal enzymes and peroxins of *Trypanosoma brucei*, *Trypanosoma cruzi* and *Leishmania* spp. are being studied, and compounds which interfere with their functioning are being developed for their use as lead drugs against the diseases caused by these parasites. Potent, selective inhibitors of several enzymes have been obtained which exert trypanocidal activity on parasites cultured *in vitro* and have no or only little effect on growth of human cells. In addition, some compounds showed anti-parasite activity in experimentally infected animals.

1. Introduction

Pathogenic trypanosomatids share with all other representatives of the clade Kinetoplastea studied, as well as with representatives of the evolutionary related Diplonemida, the presence of organelles called glycosomes. Glycosomes are authentic peroxisomes, as revealed by their route of biogenesis. The biogenesis occurs in a similar way for all members of this quite diverse organelle family and involves homologous proteins. However, glycosomes distinguish themselves from other peroxisomes by the unique presence of the majority of the enzymes of the glycolytic pathway. This was first detected in *Trypanosoma brucei* [1] and later also in other kinetoplastids and a diplonemid species [2]. Insight into the full metabolic repertoire sequestered inside glycosomes has been obtained for the human pathogenic trypanosomatids by detailed biochemical studies as well as by the sequencing of the genomes of different species of African trypanosomes responsible for human sleeping sickness and the related disease 'nagana' in domestic animals, different strains of *Trypanosoma cruzi*, responsible for Chagas' disease in Latin America, and various species of *Leishmania* responsible for different manifestations of leishmaniasis [2]. The first six or seven (dependent on species and/or life cycle stage) enzymes of glycolysis, responsible for the conversion of glucose –or other hexoses– into 1,3-bisphosphoglycerate (1,3PGA) or 3-phosphoglycerate (3PGA), are found within the organelle, as well as other enzymes involved in carbohydrate metabolism, such as two enzymes of glycerol metabolism, and enzymes of the gluconeogenic, succinic fermentation and pentose-phosphate pathways (PPP) (Fig. 1). Most of these enzymes must have been acquired by peroxisomes in an ancestral kinetoplastid, leading to the formation of glycosomes. In subsequent evolution, the organelles also acquired enzymes of squalene and pyrimidine biosynthesis, purine salvage and ascorbate metabolism, while typical peroxisomal enzymes such as H_2O_2-producing oxidases and catalase were lost [2]. Other typical peroxisomal enzymes such as those involved in fatty-acid β-oxidation and ether-lipid biosynthesis were retained.

2. Glycosomes of *Trypanosoma brucei*

The human pathogenic stage of African trypanosomes, which lives in the host's bloodstream, is completely dependent on the fermentation of glucose taken up from the blood. In contrast to the other life-cycle stages, the repertoire of other metabolic enzymes is highly repressed in this bloodstream form; the mitochondrion does not have tricarboxylic acid (TCA) cycle enzymes, a functional respiratory chain and the capacity for oxidative phosphorylation. Furthermore, African trypanosomes don't have any carbohydrate or other material serving as energy storage. Under aerobic conditions, they synthesize all their ATP from the free energy obtained by the conversion of the sugar into pyruvate: two molecules of pyruvate and ATP from one molecule of glucose. Within the glycosomes, glucose is broken down into two 3PGA, while the investment of ATP in the hexokinase (HXK) and phosphofructokinase (PFK) reactions is balanced by the two ATP produced when the two 1,3PGAs are converted into 3PGAs by phosphoglycerate kinase (PGK) (Fig. 1). The two 3PGAs exit the glycosomes to the cytosol where the last three reactions, catalyzed by phosphoglycerate mutase (PGAM), enolase (ENO) and pyruvate kinase (PYK) occur. PYK is responsible for the net production of two ATP. The NADH formed during glycolysis inside the glycosomes, in the reaction catalyzed by glyceraldehyde-3-phosphate dehydrogenase (GAPDH), is reoxidized to NAD^+ by a system that shuttles the electrons to the mitochondrion where it reduces O_2 to H_2O without the formation of a proton-motive force and ATP. The shuttle involves a glycosomal NADH-dependent glycerol-3-phosphate dehydrogenase (G3PDH), a putative glycerol 3-phosphate/dihydroxyacetone phosphate (G3P/DHAP) exchange transporter in the glycosomal membrane and a glycerol-3-phosphate oxidase (GPO) system in the mitochondrial membrane. This GPO comprises a FAD-dependent G3PDH, ubiquinone-10 and an alternative oxidase (AO). Under anaerobic conditions, when the AO cannot function, NADH inside the glycosomes is still oxidized by the G3PDH, leading to G3P accumulation within the organelle. Glycosomes also contain a glycerol kinase (GK), whose normal function in cells is the ATP-dependent phosphorylation of glycerol to G3P. The equilibrium of the GK reaction favors strongly the phosphorylation; under physiological conditions the enzyme cannot convert G3P into glycerol with

concomitant ATP formation. However, the important G3P accumulation within the glycosomes under anaerobic conditions allows the reversal by mass action. Consequently, glucose is then converted into one molecule of pyruvate and one molecule of glycerol. The intraglycosomal ATP/ADP balance is thus maintained, with, per molecule of glucose consumed, one ATP formed by the PGK reaction and another one by GK, but the net ATP production by PYK is then only one (Fig. 1). This appears sufficient to keep the cells alive for some time, but not for growth. Consequently, the trypanosomes, which cannot sustain metabolic arrest, will die within hours under anaerobic conditions or when the GPO activity is inhibited by salicylhydroxamic acid (SHAM) [3].

Since bloodstream-form *T. brucei* is entirely dependent on sugar uptake and glycolysis for its free energy supply, both the uptake and glycolytic enzymes

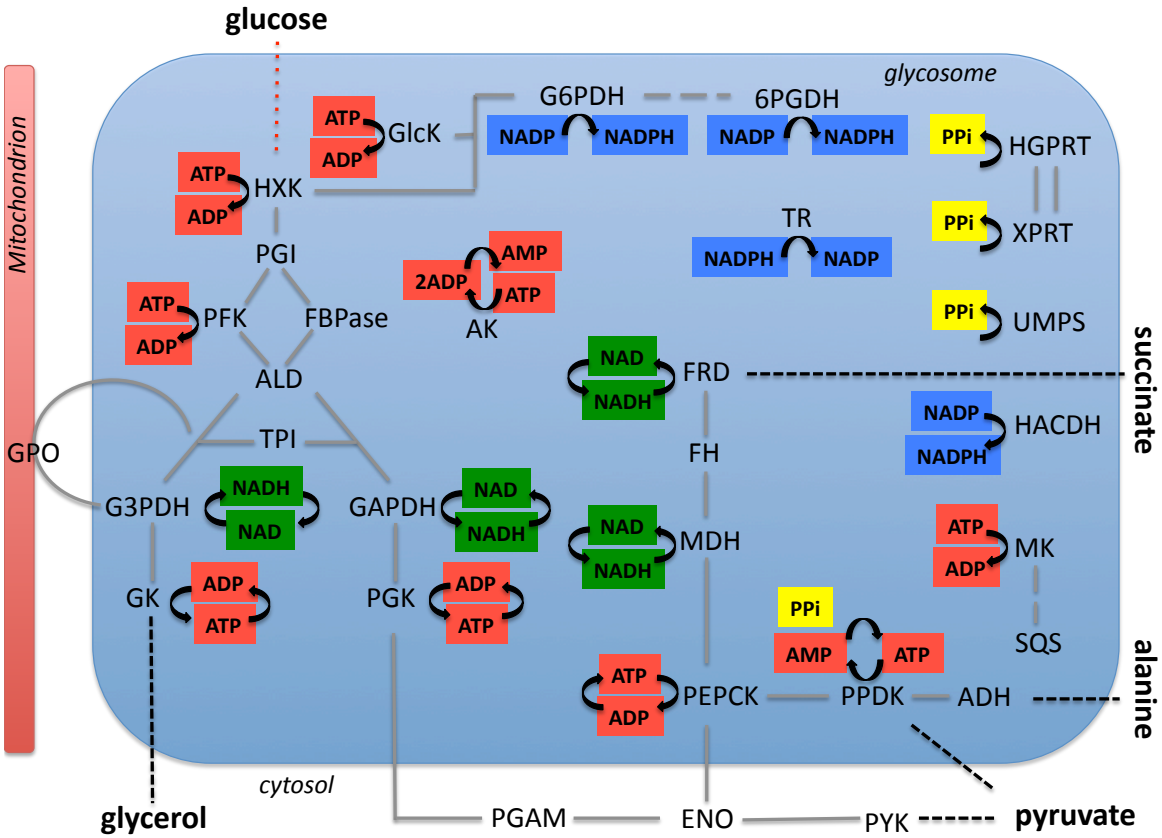

Figure 1. Diagram presenting enzymes of major metabolic pathways or parts of pathways found in glycosomes of different trypanosomatids and the crosstalk between these pathways by shared cofactors [NAD(H), NADP(H), ATP, ADP, AMP] and PPi. The substrate glucose and the end-products of its catabolism are given in bold. For complete schemes of all reactions occurring or postulated to occur inside glycosomes, see [2,37]. Abbreviations of enzyme names: (glycolysis/gluconeogenic/succinate production pathways) HXK, hexokinase; GlcK, glucokinase; PGI, 6-phosphoglucose isomerase (or glucose-6-phosphate isomerase); PFK,

phosphofructokinase; FBPase, fructose-1,6-bisphosphatase; ALD, aldolase; TPI, triosephosphate isomerase; G3PDH, glycerol-3-phosphate dehydrogenase; GK, glycerol kinase; GPO, glycerol-3-phosphate oxidase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PGK, phosphoglycerate kinase; PGAM, phosphoglycerate mutase; ENO, enolase; PYK, pyruvate kinase; PEPCK, phosphoenolpyruvate carboxykinase; MDH, malate dehydrogenase; FH, fumarate hydratase (or fumarase); FRD, fumarate reductase; PPDK, pyruvate phosphate dikinase; ADH, alanine dehydrogenase; AK, adenylate kinase; (pentosephosphate pathway) G6PDH, glucose-6-phosphate dehydrogenase; 6PGDH, 6-phosphogluconate dehydrogenase; (purine salvage) HGPRT, hypoxanthine-guanine phosphoribosyltransferase; XPRT, xanthine phosphoribosyltransferase; (pyrimidine synthesis) UMPS, UMP synthase; (sterol synthesis) MK, mevalonate kinase; SQS, squalene synthase; (fatty-acid β-oxidation) HACDH, 3-hydroxyacyl-CoA dehydrogenase; (pyrimidine synthesis); (antioxidant defense) TR, trypanothione reductase. Other abbreviation: PPi, inorganic pyrophosphate.

have already since several decades been considered as potential drug targets (reviewed in [4,5]). Indeed deprivation of these trypanosomes from glucose leads to death within minutes [6]. The essentiality, and thus the genetic validation as drug targets, has been obtained by RNA interference (RNAi) and conditional knockout experiments for the following glycosomal enzymes: HXK, PFK, aldolase (ALD), triosephosphate isomerase (TPI) and GAPDH, as well as the other enzymes PGAM, ENO, PYK and AO which are also involved in aerobic glycolysis in trypanosomes but not present in the organelles [3,7,8]. The plasma-membrane glucose transporter, as well as the pyruvate transporter which is responsible for the efflux of the trypanosome's glycolytic end product into the blood, have been chemically validated as drug targets [6,9,10].

A control analysis study of the glycolytic flux in bloodstream-form *T. brucei* has been performed to determine the distribution of control exerted by the different enzymes and to identify which steps of the pathway need the least inhibition to block the flux and, from that perspective, are the most promising drug targets. This involved both an *in silico* analysis, using a validated computer model developed on the basis of the kinetic data determined for all enzymes involved [11], and an experimental approach in which the activities of enzymes and transporters were varied *in situ* by genetic methods and/or inhibitors. The results showed that, under physiological conditions, by far most control resides in the glucose transporter, whereas the remaining control is shared by various enzymes: ALD, GAPDH, G3PDH, PGK, PGAM and ENO. Surprisingly, HXK, PFK and PYK are in excess and consequently exert no control, and ATP demand appeared

to have no control either unless PYK is inhibited [7-9,12,13]. Therefore, it was concluded that, from a network perspective, the glucose transporter is the most promising drug target, followed by ALD, GAPDH, G3PDH, PGK, PGAM and ENO. These results were experimentally confirmed by titrating the activities of enzymes and the glucose transporter by RNAi and inhibitors, with the observation that the transporter has approximately 40% control at 5 mM glucose [7-9,12,13]. Partial inhibition of each of the enzymes appeared sufficient to inhibit the glycolytic flux and kill the cells, even in the case of the three kinases which are in excess. Importantly, a compilation of all data showed that a reduction of the glycolytic flux by 30 – 50% is already sufficient to arrest growth [14].

Interestingly, a control analysis performed for glycolysis in human erythrocytes, which reside in the same environment as the trypanosomes, *i.e.* the blood, showed that, in contrast to trypanosomes, the flux is controlled by the ATP demand, not by the ATP supply and that the glucose transporter has no control at all [15]. In consequence, a differential control analysis indicated that the transporter is an even more promising drug target [16].

Glycosomes of bloodstream-form *T. brucei* are nearly completely devoted to glycolysis; over 90% of their protein content is comprised of glycolytic enzymes, in contrast to the procyclic-form trypanosomes living in the tsetse fly's midgut where this number is estimated to be only 40-50% [17]. Little is known about the importance of the other glycosomal enzymes of the bloodstream form and the possibility to use them as drug targets. Only PPP enzymes such as glucose-6-phosphate dehydrogenase (G6PDH) and 6-phosphogluconate dehydrogenase (6PGDH) have been chemically and genetically validated as targets [18-20]. The enzymes of both the oxidative and non-oxidative branches of the PPP have a dual distribution in trypanosomatids, in glycosomes and cytosol, with the ratio's varying dependent on enzyme, species and life-cycle stage. For *T. brucei* G6PDH approximately 40% was located inside glycosomes. The characterization of G6PDH, its validation as a drug target and its specific *in situ* inhibition by human steroids resulting in death of bloodstream-form cells have been reviewed elsewhere [21], and the PPP enzymes of trypanosomatids as drug targets will be described in a different chapter of this volume [22].

Sugar nucleotides are needed for the majority of glycosylation reactions. For *T. brucei* they are mostly synthesized by *de novo* pathways, *i.e.* by the bioconversion of an existing sugar or sugar nucleotide. Whereas in most organisms these pathways are present in the cytosol, work by Ferguson and coworkers has shown that in trypanosomes several steps of the synthesis of GDP-fucose, UDP-glucose, UDP-galactose and UDP-*N*-acetylglucosamine occur inside glycosomes (Fig. 2) [23-28]. These enzymes, as well GDP-mannose pyrophosphorylase (of which the subcellular localization has not yet been reported; and it has no recognizable PTS motif), which makes GDP-mannose [29], are essential for growth of bloodstream-form parasites, and thus may be considered as validated drug-target candidates. No detailed studies of these pathways have been performed as yet for other trypanosomatids.

3. Glycosomes of *Trypanosoma cruzi*

The life-cycle of *T cruzi* involves four developmental stages: in the insect vector the epimastigotes and metacyclic trypomastigotes and in the mammalian host the trypomastigotes living in the blood and amastigotes replicating intracellularly in different tissues and organs. The latter two are the clinically relevant forms for which drugs are required. *T. cruzi* has to adapt its metabolism to the wide range of environments encountered during its life cycle. The nutritional conditions vary from amino-acid rich with transiently available glucose for epimastigotes living in the insect's gut, to glucose-rich for blood trypomastigotes and to very low in sugars, but rich in a plethora of metabolites such as intermediates of carbohydrate metabolism, fatty acids, and amino acids as present in the mammalian cytosol for amastigotes. All developmental stages of *T. cruzi*, in contrast to *T. brucei,* possess a well-developed

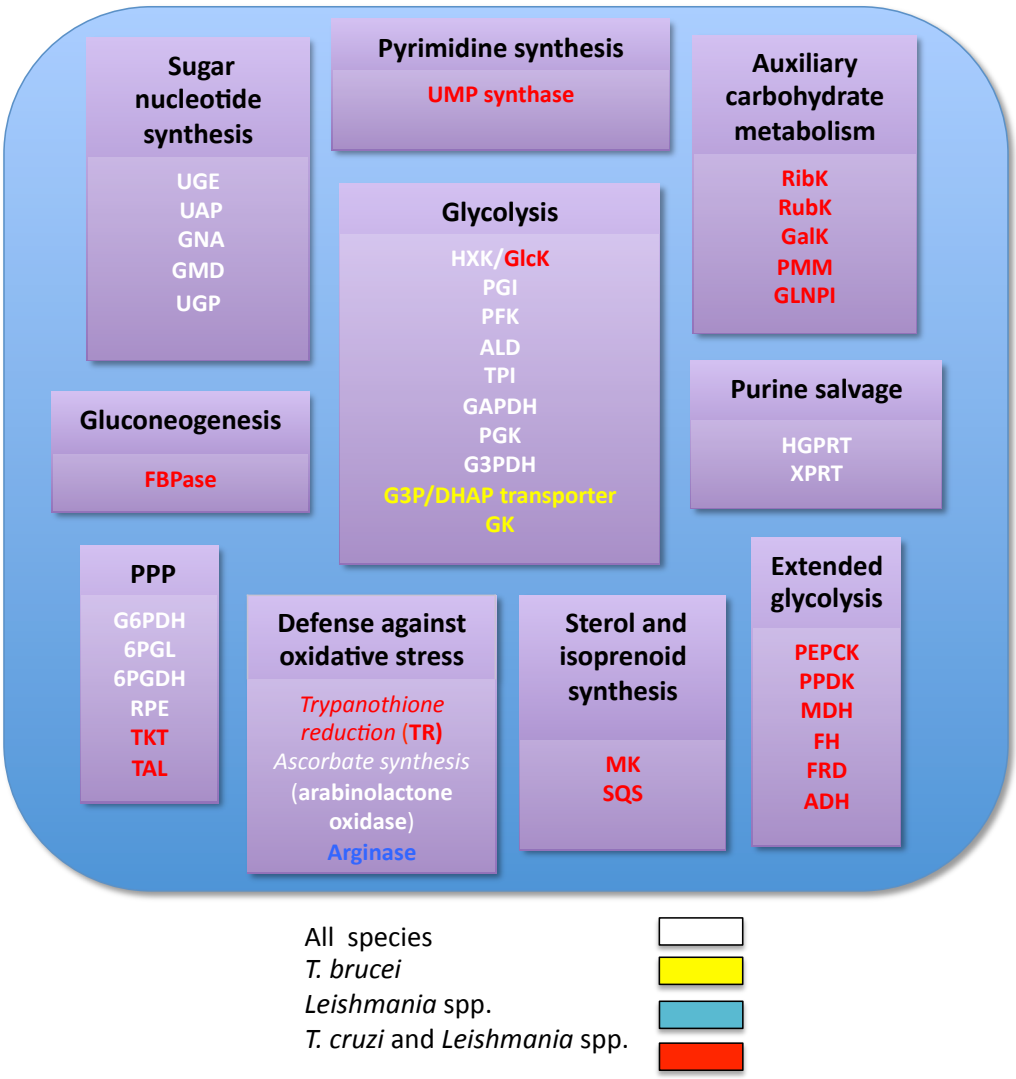

Figure 2. Inventory of the different metabolic processes and enzymes present inside glycosomes of the human pathogenic stages of the different trypanosomatids. The names of processes are given in black. The experimentally determined presence or inferred functionally importance of enzymes within glycosomes of the different trypanosomatid species (see main text and [2]) is indicated with different colors. Abbreviations: (glycolysis) HXK, hexokinase; GlcK, glucokinase; PGI, 6-phosphoglucose isomerase (or glucose-6-phosphate isomerase); PFK, phosphofructokinase; ALD, aldolase; TPI, triosephosphate isomerase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PGK, phosphoglycerate kinase; G3PDH, glycerol-3-phosphate dehydrogenase; G3P, glycerol 3-phosphate; DHAP, dihydroxyacetone phosphate; GK, glycerol kinase; (gluconeogenesis) FBPase, fructose-1,6-bisphosphatase; (extended glycolysis) PEPCK, phosphoenolpyruvate carboxykinase; PPDK, pyruvate phosphate dikinase; MDH, malate dehydrogenase; FH, fumarate hydratase (or fumarase); FRD, fumarate reductase; ADH, alanine dehydrogenase; pentosephosphate pathway (PPP) G6PDH, glucose-6-phosphate dehydrogenase; 6PGL, 6-phosphogluconolactonase; 6PGDH, 6-phosphogluconate dehydrogenase; RPE, ribulose-5-phosphate-3-epimerase; TAL, transaldolase; TKT, transketolase; (auxiliary carbohydrate metabolism) RibK, ribosekinase; RubK, ribulosekinase; GalK, galactosekinase; PMM, phosphomannomutase; GLNPI, glucosamine-6-phosphate isomerase; (sugar nucleotide synthesis) UGE, UDP-glucose 4'-epimerase; UAP, UDP-N-acetylglucosamine pyrophosphorylase; GNA, glucosamine 6-phosphate N-acetyltransferase; GMD, GDP-mannose dehydratase; UGP, UDP-glucose pyrophosphorylase; (purine salvage) HGPRT, hypoxanthine-guanine phosphoribosyltransferase; XPRT, xanthine phosphoribosyltransferase; (pyrimidine synthesis)

UMPS, UMP synthase; (sterol and isoprenoid synthesis) MK, mevalonate kinase; SQS, squalene synthase; (defense against oxidative stress) TR, trypanothione reductase.

mitochondrion with a TCA cycle and a complete, functional electron-transfer chain coupled to oxidative phosphorylation [30]. Nonetheless, when glucose is available, *T. cruzi* will oxidize it, but even under aerobic conditions only incompletely, to succinate, acetate, L-alanine and CO_2 [31,32].

T. cruzi glycosomes contain, similarly to those of *T. brucei* (section 2) and other trypanosomatids, the glycolytic enzymes responsible for the conversion of glucose into 3PGA [33]. The three final steps, converting 3PGA into pyruvate, occur in the cytosol. The kinetic properties of the glycosomal enzymes of the different trypanosomatid species are also very similar, including the apparent lack of activity regulation of HXK and PFK. However, the glucose fermentation pathway in each of the *T. cruzi* life-cycle stages and also in *Leishmania* spp. is extended to a succinate production pathway –also observed in procyclic *T. brucei*– in which the phosphoenolpyruvate (PEP), produced in the cytosol by ENO, enters the glycosomes, to be converted into succinate by the activities of a PEP carboxykinase (PEPCK), malate dehydrogenase (MDH), fumarate hydratase (FH) and a soluble, NAD-dependent fumarate reductase (FRD) [34,35] (Figs. 1 and 2). Alternatively, PEP can be dephosphorylated inside the glycosomes to pyruvate by a pyruvate phosphate dikinase (PPDK) with the concomitant formation of ATP and inorganic phosphate (Pi) at the expense of AMP and inorganic pyrophosphate (PPi). This PPi is an energy-rich by-product from glycosomal biosynthetic reactions; no pyrophosphatase is present to hydrolyze it. The pyruvate may be further reduced using NADH by a glycosomal alanine dehydrogenase (ADH). The presence of the additional NADH-dependent oxidoreductases (MDH, FRD, ADH) and kinases (PEPCK, PPDK) contribute to the maintenance of the intraglycosomal NAD/NADH and ATP/ADP balances. Contrary to the situation in bloodstream-form *T. brucei*, G3PDH and GK, also present in glycosomes [36,37], seem not to play an important role in balancing these cofactors in *T. cruzi* and *Leishmania* spp., but may rather act to form G3P for lipid metabolism from either DHAP or glycerol. Also different from

bloodstream-form *T. brucei* is the fact that biosynthetic processes seem to play a quantitatively more important role in glycosomes of these other parasites (and in procyclic *T. brucei*) and are coupled to the catabolic processes by producing PPi, to be used by PPDK, and their requirement of NAD(P)H and ATP (see Fig. 1 and below).

PEP is also a substrate of the cytosolic PEP mutase that catalyzes the conversion of PEP to phosphonopyruvate, the initial step in the formation of many naturally occurring phosphonate compounds in *T. cruzi* [38]. The amount of PEP that enters the glycosome would depend on the activity regulation of the allosteric enzyme PYK.

Glycolysis plays a less important role in *T. cruzi* than in bloodstream-form *T. brucei*. Epimastigotes live in an amino-rich environment but nonetheless will preferentially use glucose as an energy substrate during the time when it is available after the insect's blood meal [39], similar to insect-stage procyclic *T. brucei*. In contrast, trypomastigotes, living in the blood, may rely considerably on glycolysis, although little data are available. Indeed, most enzymes of the extended glucose breakdown to succinate and pyruvate (via both PYK and PPDK) have been detected in cells of this life-cycle stage (reviewed in [40]). Contradictory data have been reported about the energy metabolism of amastigotes. Early studies with an axenic culture model suggested an essentially glycolytic metabolism for this life-cycle form, fermenting glucose to succinate and acetate for their ATP production [41]. Indeed, most enzymes are detectable in amastigotes (reviewed in [40]). However, recent studies showed no indications for the expression of the plasma-membrane hexose transporter in amastigotes, contrary to epimastigotes and trypomastigotes where it was found to be active [42,43]. This would tally with the notion that free sugar levels are low in the cytosol of mammalian cells. It is feasible, however, that glycolytic intermediates such as phosphorylated hexoses or trioses are taken up from the cytosol of the host cell to be metabolized by the glycolytic pathway and, in addition to fatty acid and amino acid catabolism, contribute to the ATP supply. Indeed, the *T. cruzi* genome, but not those of *T. brucei* and *Leishmania* spp., codes for a hexose-phosphate transporter similar to that present in the membranes of bacteria, plastids and the mammalian ER [44]. Nonetheless, proteomic studies

indicated that the transition of trypomastigotes to amastigotes is accompanied by a dramatic shift from carbohydrate- to lipid-dependent energy metabolism [42]. Therefore, the glycolytic enzymes detected in glycosomes of amastigotes are probably necessary for gluconeogenesis to synthesize glucoconjugates.

T. cruzi and *Leishmania* contain also other enzymes in carbohydrate metabolism, but not found *T. brucei*, that are probably all present inside glycosomes as inferred from the presence of a peroxisome-targeting signal (PTS): sugar kinases other than a hexokinase, such as a glucokinase (GlcK) to be discussed below, ribosekinase, ribulosekinase and galactokinase, as well as phosphomannomutase and glucosamine-6-phosphate isomerase (Fig. 2) [2,37]. These enzymes may be important for the use of alternative sugars as energy source, but also for the formation of important or essential glucoconjugates.

Other pathways or parts of them are also present in *T. cruzi* glycosomes, such as the PPP, responsible for about 10% of the glucose-consumption flux in *T. cruzi* epimastigotes, considerably higher than in bloodstream form *T. brucei* [45]. All PPP enzymes were found in each of the four major life-cycle stages and for each of the enzymes a predominantly cytosolic localization was reported, but also a presence in glycosomes. Also found inside glycosomes are purine salvage, pyrimidine biosynthesis, biosynthesis of ether lipids, β-oxidation of fatty acids, some isoprenoid biosynthesis enzymes [46] and enzymes involved in protection against oxidative stress (reviewed in [40]) (Figs. 1 and 2).

All parasitic trypanosomatids are auxotrophic for purines. Purine bases are taken up from the host and interconverted into purine nucleotides by enzymes of the purine salvage pathway of which several have been located inside glycosomes and/or identified as glycosomal enzymes by the presence of a PTS [37]. Hypoxanthine is the predominant salvageable purine in the host milieu, rendering the enzyme hypoxanthine-guanine phosphoribosyltransferase (HGPRT) a crucial enzyme in the metabolism of the parasites and thus a potential drug target. This enzyme is present in the glycosomes. The *T. cruzi* HGPRT [47], as well as its homolog from other trypanosomatids, have been studied in detail.

Isoprenoids are important for trypanosomatids, for production of squalene and sterols. Isoprenoid synthesis in these parasites occurs via the mevalonate pathway. The ergosterol synthesis of *T. cruzi* has been studied in

detail, and several enzymes involved in it have been chemically validated in *in vitro* cultures (reviewed in [48,49]). About 45% of one of the enzymes, squalene synthase (SQS), has been located in the glycosomes of *T. cruzi* [46]. In addition, the likely synthesis of isoprenoids inside the glycosomes is supported by the presence of several intermediates such as squalene in the organelle's matrix and membrane [50], in agreement with similar observations made for mammalian peroxisomes [51]. More details about sterol synthesis in trypanosomatids will be presented in another chapter [52].

The organization of the metabolic network in glycosomes of *T. cruzi* suggests that the role of these organelles in this organism is fundamentally different from that in bloodstream form *T. brucei*. Whereas the organellar metabolism of the latter comprises almost only the catabolic process of glycolysis, that of *T. cruzi*, and also procyclic *T. brucei* and different *Leishmania* spp. life-cycle stages, has the catabolism of glucose via glycolysis to succinate and alanine and via the PPP connected to several biosynthetic pathways by the production and consumption of ATP and NAD(P)H and PPi (see Fig. 1).

Interestingly, PPi seems not only to form a metabolic link by being a product and substrate in glycosomal anabolism and catabolism, respectively, but it may also regulate the distribution of fluxes through these pathways by modulating the activity of some *T. cruzi* enzymes, such as PEPCK and HXK [35,53]. An increase of intraglycosomal PPi by a high biosynthetic activity will inhibit PEPCK and thus steer the PEP away from the succinate production towards PPDK and ADH, consequently using the PPi for ATP production and so increasing the free-energy yield from glucose. Moreover, PPDK and ADH will in this situation become crucial for maintaining the energy and redox balance within the glycosomes and thus sustaining the glycolytic flux [35]. Simultaneous HXK inhibition by PPi may promote glucose phosphorylation by the PPi-insensitive glucokinase (GlcK), also present in glycosomes of *T. cruzi* and *Leishmania* spp., but not *T. brucei* [54]. This GlcK produces preferentially the β-anomer of glucose 6-phosphate (G6P) [55], the substrate of G6PDH. Since the substrate of the second enzyme of the glycolytic pathway, glucose-6-phosphate isomerase, is α-G6P, the preferential product of HXK, the increase in PPi will thus

lead to a rerouting of the glucose flux from glycolysis to the PPP, and consequently stimulate the NADPH production for biosynthetic processes.

4. Glycosomes of *Leishmania* spp.

During their life cycle, *Leishmania* parasites alternate between their motile promastigote stage and non-motile amastigote stage, respectively, in their invertebrate vector and mammalian host. In mammals, the parasites thrive inside the phagolysosomes of macrophages or other cells such as neutrophils and dendritic cells [56]. *Leishmania* are capable of performing multiple metabolic adaptations to cope with the distinct environments in both hosts. The adaptations involve several metabolic pathways in different cell compartments including the glycosomes. Although the medically relevant form is the amastigote, glycosomes and their enzymes have been more intensively studied in promastigotes because these are more easily cultured. These studies have allowed the identification of important features of the metabolism inside glycosomes. *In silico* studies with the genomes of different *Leishmania* species have also provided invaluable information about the metabolic capacity of the parasite's glycosomes [37]. These studies, together with transcriptomic and proteomic analyses of the amastigote form and the analysis of virulence phenotypes of *Leishmania* mutants [57], have been important to identify aspects of the glycosome that are essential for the physiology of the amastigote form and may thus be selected as candidate drug targets.

Leishmania amastigotes have a reduced metabolism and a slow growth rate in comparison with promastigotes [58]. However, amastigotes seem to have a comparable glycosome density as promastigotes [59] and, similar to the promastigote form, glycosomes are involved in several metabolic processes that appear to be crucial for the amastigote form. Among these processes are carbohydrate, polyamine and lipid metabolism as well as purine salvage, pyrimidine synthesis and protection against oxidant stress (Fig. 2).

As the glycosomes in other trypanosomatids, those of *Leishmania*, in both its promastigote and amastigote stage, contain all first seven enzymes of the glycolytic pathway [60-62], with the glycosomal glycolytic enzymes slightly

upregulated, but the cytosolic enzymes of the final steps of glycolysis significantly down-regulated in the amastigote stage [63]. Hexose uptake is essential for growth and viability of the amastigote form, despite the low level of hexoses in the phagolysosome [64]. A hexose transporter null mutant cannot survive as amastigote in macrophages [65,66], highlighting the importance of sugar as a carbon source. This importance of sugar metabolism and the involvement of glycosomes therein in amastigotes are also underscored by the fact that gluconeogenesis is increased in the amastigote form. The gluconeogenic enzymes PEPCK and fructose-1,6-bisphosphatase (FBPase), both localized in the glycosomes, are up-regulated in this stage [63,67]. Indeed this latter enzyme, FBPase, is important for virulence in *L. major*. Parasites lacking this enzyme were unable to replicate in the macrophage's phagolysosome and failed to generate normal lesions [68], indicating that gluconeogenesis is essential for amastigotes. In addition, *Leishmania* amastigotes accumulate the reserve oligosaccharide mannogen (formerly known as mannan) in the cytosol [69]. This 4 to 40 mannose residues long oligosaccharide is required for a normal establishment of infection and survival in macrophages [70,71]. Mannose-α-1-phosphate, which is used for mannogen synthesis, is derived from hexose-phosphates formed in the glycosomes. *Leishmania* amastigotes are also dependent on hexosamines [72] scavenged from the phagolysosome which may contain glucosamine and N-acetylglucosamine. These hexosamines can be products of matrix proteoglycans and glycosaminoglycans internalized and degraded by the macrophages or by chitinase secreted by the amastigotes [73]. These exogenous hexosamines are phosphorylated and can be converted into fructose 6-phosphate within the glycosomes. Catabolism of the amino sugars inside the glycosomes is important for growth in macrophages and establishment of infection [74]. However, the sugars and amino sugars are almost certainly not only essential for N-glycosylation and oligosaccharide biosynthesis, but also for sustaining the flux through the PPP. The NADPH produced by this process is needed for oxidative stress protection, which is very important in the hostile environment of the macrophage's phagolysosome, and for nucleotide synthesis.

Whereas sugar catabolism plays only a minor role in the energy supply of *Leishmania* amastigotes, β-oxidation of fatty acids appears to be the main source of energy in this intracellular stage, as is the case in *T. cruzi* amastigotes. *In silico* analysis of the *Leishmania* genome led to the prediction that several enzymes of this pathway are associated with glycosomes [37], in agreement with results from earlier cell fractionation studies [75]. Proteomic and enzymatic analyses have shown that levels of enzymes involved in this pathway are increased in amastigotes [63,67]. However, the up-regulated enzymes of β-oxidation could not be clearly identified as glycosomal proteins [63,67,76]. Moreover, a correlated expression pattern was observed for β-oxidation of fatty acids with mitochondrial functions in differentiating *L. donovani* [63], suggesting that at least a major part of β-oxidation occurs in the mitochondrion. The fatty acids can be obtained from phospholipids through the up-regulated phospholipase A2 and monoglyceride lipase. These enzymes would also provide glycerol required for gluconeogenesis in the glycosome [63]. Glycosomes are also involved in the synthesis of isoprenoids for sterols and other products, for which the enzymes are distributed in different subcellular compartments [77,78]. The mevalonate kinase (MK) has been found associated with the glycosomal matrix in *L. major* and *T. brucei* [79].

Leishmania amastigotes are also able to use amino acids as carbon source, but the glycosome is not directly involved in this metabolism. Nonetheless, glycosomes contain the enzyme arginase that catalyzes the conversion of arginine into ornithine and urea as the first step in the biosynthesis of polyamines [80], necessary for the synthesis of trypanothione. This latter molecule is essential for the defense against oxidative stress [81]. The function of arginase is also associated with decreasing arginine levels in the phagolysosome to interfere with the host's nitric oxide production. Mutant parasites of different species of *Leishmania* lacking this enzyme have a reduced virulence; the pathology emerges less rapidly than in infection with wild-type parasites [80,82-85]. *Trypanosoma* species lack the arginase [85].

The glycosome also contains arabinolactone oxidase [86,87], the last enzyme of the synthesis of ascorbate. In conjunction with ascorbate peroxidase, this could play a role in the antioxidant defense in *Leishmania* when residing

within the phagolysosome. Indeed, *Leishmania* spp. contain an asorbate peroxidase, but its subcellular localization remains to be determined. In an *in vitro* infection model, a *L. donovani* strain overexpressing arabinonolactone oxidase showed a higher infection index than wild-type cells [87].

Like the other trypanosomatids, *Leishmania* is not able to synthesize purines *de novo* and scavenge them from the host. Four enzymes are required and two of them, HGPRT and xanthine phosphoribosyltransferase (XPRT), which have been located inside the glycosome [88,89], are critical for the infection of macrophages and mice [90]. Moreover, studies performed with *L. donovani* promastigotes have shown that the parasite responds rapidly to purine starvation by increasing the expression of the purine transporter and purine salvage enzymes, including HGPRT and XPRT [91]. This adaptation to purine starvation might occur in amastigotes as a response to changes in the phagolysosome. In contrast to purines, trypanosomatid parasites are able to synthesize pyrimidine nucleotides *de novo*. The last two enzymes of this pathway (orotate phosphoribosyltransferase and orotidine 5'-monophosphate decarboxylase) are fused into a bifunctional enzyme [uridine 5-monophosphate (UMP) synthase] that is also present inside the glycosome [85,92]. Promastigotes of *L. donovani* lacking UMP synthase were unable to proliferate in the absence of pyrimidine supplement [92]. However, whether this bifunctional enzyme is essential for amastigote physiology remains to be elucidated.

5. Essentiality of controlled communication across the glycosomal membrane

Like peroxisomes in mammalian and plant cells and in yeasts, glycosomes seem to be distinct entities with a well-defined metabolic repertoire for specific environmental conditions of the cells. Moreover, these organelles are also synthesized and degraded as separate entities (see section 6). Nonetheless, the metabolic network in the organelles should be well integrated in the overall network of the cell in order to function properly. Moreover, when the cell needs to change its metabolic repertoire to adapt to altered environmental conditions, such as occur during the life-cycle-related differentiation of trypanosomatid

parasites, the organellar network should change as well. Therefore, the cytosolic and glycosomal networks should be linked by devices for exchange of substrates, intermediates and products, and these links should allow different transmembrane solute fluxes when the networks in both compartments change. How is this solute translocation across the glycosomal membrane achieved and would the translocation devices involved be suitable drug targets? Studies on these questions have so far been mostly restricted to bloodstream-form *T. brucei*.

Like peroxisomal membranes, the glycosomal membrane is an apparent permeability barrier. This notion seems firmly established for bulky compounds such as ATP, NADH and fatty acids. The ATP consumption and synthesis are balanced inside the glycosome and an ATP/ADP ratio different from that in the cytosol is maintained (see section 2) [11]. For bloodstream form *T. brucei*, reoxidation of intraglycosomal NADH involves a mechanism by which the electrons are transferred from the cofactor to the mitochondrion by a shuttle mechanism. Three ABC transporters are present in the glycosomal membrane [93], one of them has been shown to import long-chain fatty acids such as oleoyl-CoA into the organelles [94]. Also smaller compounds such as glycolytic intermediates seem capable of crossing the glycosomal membrane, but only relatively slowly, suggestive of a low permeability. Upon addition of a pulse of radioactively labeled glucose to bloodstream trypanosomes, the label appears very rapidly in a small (20-30%) pool of intermediates, interpreted as those present inside the glycosomes, and in pyruvate, but only 60 times slower in a large (70-80%) pool of intermediates, the cytosol [95]. Also the possibility to reverse the GK reaction under anaerobic conditions, due to the increase in the glycosomal G3P concentration, is indicative of a low permeability of the membrane for this metabolite [1,96,97]. Furthermore, simulation of glycolysis using the kinetic model indicated the essentiality of a defined pool of phosphorylated glycolytic intermediates within the glycosome, separate from the cytosol, to enable a functional glycolytic flux [11,98]. Nonetheless, like for the membranes of peroxisomes from plant and mammalian cells and yeast, channel-forming activities have been found in preparations from purified glycosomal membranes [99]. The data available from this study, together with inference from data obtained for peroxisomes, suggest the presence of various,

distinct channels in the glycosomal membrane that allow the permeation of most low molecular-mass metabolites, including charged compounds such as the glycolytic phosphorylated intermediates. The nonetheless observed low equilibration of many of such intermediates between glycosomes and cytosol may be attributed to the existence of a multienzyme complex and channeling of metabolites directly between the active sites of successive enzymes without their release from the complex, as has similarly been observed for plant peroxisomes (reviewed in [100]). Additionally or alternatively, control of the fluxes through the channels by regulatory proteins or association of the channels with the enzymes and/or the presence of the Donnan potential due to the high intraglycosomal density of many proteins with a high pI may also be responsible for the low transmembrane exchange of many metabolites [99,101].

Despite the presence of channels, it cannot be excluded yet that the glycosomal membrane contains some transporter molecules for specific low molecular substrates, that have not been recognized because of a low similarity with known transporters and/or difficult identification by biochemical approaches.

Any protein involved in metabolite translocation across the glycosomal membrane is a potential drug target for several reasons: (1) the unique compartmentalization of glycolysis and some other processes inside glycosomes; (2) the proved essentiality of this compartmentalization, at least for glycolysis in bloodstream form trypanosomes (reviewed in [102,103]) and probably also for other processes in the other parasites as well; (3) the very low similarity between the glycosomal solute transporters identified so far and their human counterparts [93]; (4) the apparent absence of homologs of the so far only known peroxisomal channel protein, Pxmp2 [104] in the genomes of the trypanosomatids.

6. Essentiality of correct integration of glycosomal metabolism in the overall metabolism - relation to life-cycle differentiation

As described in the previous sections, the metabolic networks within glycosomes, like in peroxisomes, are well integrated within the cells' overall

metabolic network, although these organelles are synthesized and degraded as separate entities. The biosynthesis of the organelles should be well coordinated with the growth of the cells for the proper functioning of the metabolism. Biosynthesis of peroxisomes/glycosomes occurs by growth and division of existing organelles, in which proteins are posttranslationally imported from the cytosol into the matrix or inserted into the membrane, and lipids are obtained from the endoplasmic reticulum (ER) by non-vesicular transport. Moreover, both lipids and specific membrane proteins can also be recruited via membrane vesicles from distinct regions of the ER. These processes involve a number of specific proteins called peroxins (acronym PEX) which perform their functions through cascades of interactions and conformational changes (reviewed in [102,103]). Degradation of the organelles is achieved by a specific form of autophagy called pexophagy. Under normal growth conditions, the levels of autophagy and pexophagy in a cell, including trypanosomatid parasites, are relatively low. However, under conditions of stress or when there is a need for remodeling the structure or metabolic repertoire of a cell, these processes are upregulated. Indeed, autophagy is importantly increased during differentiation of trypanosomatids [105,106] and, in the case of *T. brucei*, also an increased turnover of glycosomes by pexophagy is observed [107]. Autophagy involves a considerable number of specific autophagy-related proteins or ATGs as well as several proteins shared with other processes.

About 10 peroxins of *T. brucei* have been identified, most of them involved in import of matrix proteins, some others in insertion of proteins into the glycosomal membrane or in glycosome proliferation. Knockdown of the expression of each of these PEX proteins leads to arrest of glycosome biogenesis as shown by the presence of all or part of the newly synthesized matrix proteins in the cytosol, and growth retardation followed by death. The effect is particularly dramatic in bloodstream form trypanosomes. An explanation why this is so detrimental for the cell was offered by the finding that the glycosomal enzymes involved in glucose metabolism, and probably also other enzymes, cannot function properly outside the organelles because of a lack of activity regulation mechanisms [97,98]. This, together with the low similarity of the trypanosome PEX amino acid sequences compared to that of their human

counterparts (varying from about 35% until less than 15% or barely detectable [102]), render these proteins promising drug targets. Also some ATGs and other proteins involved in autophagy have been shown to be essential for trypanosomatid parasites, particularly during their differentiation [105,106]. This has not yet been demonstrated for proteins specifically involved in glycosome degradation, but it is likely the case. Therefore, also these ATGs, of which the sequences are not very well conserved [108], may be interesting drug targets.

7. What has been achieved so far in target characterization?

The most prominent aspects of glycosomes are glycolysis and its reverse process of gluconeogenesis. As discussed in the previous sections, glycolysis is essential and thus a drug target in the pathogenic form of *T. brucei*. This is probably also the case for trypomastigotes of *T. cruzi*. Furthermore, the production of G6P for glucoconjugates and mannogen from sugars and fatty acids by HXK and gluconeogenic enzymes is vital for *Leishmania* amastigotes. The same is probably the case for *T. cruzi* amastigotes. Each of the enzymes shared by the glycolytic and gluconeogenic pathways is thus a validated potential drug target in the three parasites, and PFK and PYK also in *T. brucei*, while FBPase, PEPCK and possibly also PPDK are targets in the intracellular trypanosomatids. Progress has been made in the characterization of many of these enzymes such as development of protein expression systems, detailed kinetic characterization, protein crystallization and structure determination. Results are summarized in Table 1. Many proteins, particularly the glycolytic enzymes, are quite well conserved between the different trypanosomatids, while they are much more different from the corresponding proteins from other groups such as mammals. In some cases similar reactions are even catalyzed in the parasites and human by non-homologous enzymes, such as PGAM and –only in *T. brucei*– the enzyme eventually responsible for the reoxidation of glycolytically produced NADH, AO. This often offers the possibility to use information about unique structural and/or functional features from an enzyme of only one of the trypanosomatids to develop potent inhibitors that are effective

on the corresponding enzyme of each of the parasites with no or little effect on the human counterpart. Nonetheless, for optimization of such inhibitors for a specific enzyme of each parasite by structure-activity

Table 1. Potential drug targets in glycosomes of trypanosomatid parasites and the stage of drug development

Enzyme	Target				Crystal structure available	Selective inhibitor available	Inhibitor with *in vitro* anti-parasite activity available	Inhibitors with *in vivo* anti-parasite activity available
	Tb	*Tc*		*L*				
	BF	T	A	A				
Glycolysis Gluconeogenesis/ Succinate production								
HXK	V	L	N	V		*Tb* / *Tc*	*Tb* / *Tc*	*Tc*
GlcK	–	N	N	L	*Tc*			
PGI	V	L	L	L	*L*			
PFK	V	P	U	U	*Tb*	*Tb*	*Tb*	
ALD	V	L	L	L	*Tb* / *L*	*Tb*		
TPI	V	L	L	L	*Tb* / *Tc* / *L*			
GAPDH	V	L	L	L	*Tb* / *Tc* / *L*	*Tb* /*Tc* / *L*	*Tb* / *Tc*	
PGK	V	L	L	L	*Tb*			
G3PDH	V	P	P	P	*L*			
GK	U	U	U	U				
(PGAM)	V	L	L	L	*Tb* / *L*			
(ENO)	V	L	L	L	*Tb*			
(PYK)	V	L	U	U	*Tb* / *L*	*Tb*	*Tb*	
PEPCK	–	P	U	P	*Tc*			
MDH	–	P	U	P				
FH	–	L	U	P				
FRD	–	P	U	P				
PPDK	–	P	U	p	*Tb*			
ADH	–	P	U	P				
FBPase	–	U	L	V				
(Glucose transporter)	V	L	U	V				
PPP								
G6PDH	V	L	L	L	*Tc*	*Tb* / *Tc*	*Tb* / *Tc*	
6PGDH	V	L	L	L	*L*	*Tb*	*Tb*	
sterol synthesis								
SQS	U	V	V	V		*Tc*	*Tc*	*Tc*
purine salvage								
HGPRT	?	V	V	V	*Tc*	*Tc* / *L*	*Tc* / *L*	*Tc*
sugar nucleotide synthesis								
UGE	V	L	L	L	*Tb*			
UAP	V	L	L	L				
GNA	V	L	L	L	*Tb*			
GMD	V	L	L	L				
UGP	L	L	L	L	*Tb*			

Abbreviations: *Tb*, *T. brucei*; *Tc*, *T. cruzi*; *L*, *Leishmania* spp.; BF, bloodstream form; T, trypomastigote; A, amastigote; V = validated; L = likely; P = possibly; U = unlikely (but likely or possibly in combination with another enzyme); N = not known; – = not present. For abbreviations of proteins, see main text and/or legends to Figures 1 and 2. Enzymes given between parentheses are glycolytic enzymes present exclusively in the cytosol but important for the glycolytic flux through the glycosome; similarly for the plasmamembrane glucose transporter. The likelihood about enzymes as drug targets for *T. cruzi* trypomastigotes is in part inferred from knockdown and knockout studies on glucose-grown procyclic *T. brucei* that is considered a good model system for this *T. cruzi* life-cycle stage (published data and personal communication by F. Bringaud, Université Bordeaux Segalen, France).

relationship (SAR) analysis, structural information for each enzyme in some cases appeared advantageous to exploit its subtle, unique features.

HGPRT from both *T. cruzi* and *L. tarentolae* has been characterized in detail and crystal structures are available for drug design [109,110].

The PPP is very important for each of the trypanosomatids, notably for the production of NADPH to deal with the host's oxidative defense by which the

parasites are attacked. In *T. brucei*, G6PDH and 6PGDH, the NADPH-producing enzymes of the oxidative branch, have been validated as drug targets by RNAi, but not those of the non-oxidative branch; transketolase and transaldolase were even undetectable in bloodstream forms. G6PDH and 6PGDH have been characterized in detail (reviewed in [20,21]) and crystal structures are available for the *T. cruzi* and *T. brucei* enzymes, respectively. For further discussion of the PPP as target, see elsewhere in this volume [22].

Four glycosomal enzymes involved in the biosynthesis of sugar nucleotides which are necessary for glycosylation reactions, have been genetically validated as drug targets in *T. brucei* [UDP-glucose 4'-epimerase (UGE), UDP-N-acetylglucosamine pyrophosphorylase (UAP), glucosamine 6-phosphate N-acetyltransferase (GNA) and GDP-mannose dehydratase (GMD), see section 2]. These enzymes have been biochemically characterized and the crystal structures of UDP-glucose 4'-epimerase and glucosamine 6-phosphate N-acetyltransferase, as well as UDP-glucose pyrophosphorylase (UGP), have been determined at high resolution [27,28,111]. Of these, UGP catalyzes the condensation of glucose 1-phosphate with UTP, UGE interconverts UDP-glucose and UDP-galactose and GNA acetylates glucosamine 6-phosphate using acetyl-CoA [27,28]. These structures will provide a framework for the design of inhibitors against the parasite enzymes.

8. What has been achieved so far in the development of inhibitors of glycosomal enzymes or processes

Some drugs used against trypanosomatid-borne diseases appear to exert inhibitory actions on glycosomal enzymes *in vitro*. The most prominent example is the sleeping sickness drug suramin. It inhibits all seven glycolytic enzymes isolated from *T. brucei* glycosomes as well as the cytosolic PYK with IC_{50} values between 3 and 100 μM [112,113]. That does not imply that this drug kills the parasite by inhibition of glycolysis; it is doubtful that such a large molecule (molecular mass 1297 Da) easily crosses the glycosomal membrane. Moreover, suramin has an effect on many more enzymes, also mammalian glycolytic and

other enzymes, and it inhibits growth of cultured trypanosomes with an ED_{50} value as low as 50 nM.

New inhibitors for glycolytic enzymes from trypanosomatid glycosomes and cytosol are being obtained by different approaches which involve synthesis after structure-based design and catalytic-mechanism based design, structure-based database mining, medium- or high-throughput screening (HTS) of compound libraries and combinations of these approaches. As mentioned in section 7, crystal structures are available for several of these enzymes from *T. brucei*, *T. cruzi* and/or *L. mexicana* (Table 1). Based on these structures, fructose 6-phosphate analogs –furanose sugar amino amides– have been developed as inhibitors of *T. brucei* PFK that have IC_{50} values of about 25 μM and trypanocidal activity on cultured parasites in the low micromolar range with much less effect on cultured human fibroblasts. These fructose 6-phosphate analogs were also tested on trypanosomatid PYKs, that have a binding site for the allosteric activator fructose 2,6-bisphosphate with a quite different architecture than the fructose 1,6-bisphosphate binding site of the human PYK isoenzymes. Indeed, some of these compounds also inhibited this enzyme and killed the parasites at similar IC_{50} and ED_{50} values, respectively [114], although the binding of the compounds in the effector-binding site has not been ascertained.

Crystal structures have been determined for both *T. brucei* and *L. mexicana* aldolase. Although their active sites are quite similar to that of mammalian aldolases, the minimal differences that exists and knowledge of the catalytic mechanism has allowed the development of a quasi-irreversibly binding inhibitor, 5-formyl-6-hydroxy-2-naphthyl di-sodium phosphate, that inactivated the trypanosomatid enzymes at low micromolar concentrations without effect, or only very weakly on mammalian aldolases, even at 1 mM [115]. Pro-drug forms of this and similar charged compounds were able to kill cultured trypanosomes with ED_{50} values in the low micromolar range with no effect on mammalian cells [116].

High-resolution crystal structures have been determined for the glycosomal GAPDH of each of the three trypanosomatids. These GAPDHs are characterized by a unique binding site for the adenosine moiety of the cofactor NAD^+. Most striking among the various differences is a hydrophobic cleft

adjacent to the 2'OH group of the adenosine ribose. The combined differences have been exploited for the synthesis of adenosine analogs with ribosyl C2' and adenosyl N^6 substitutions that act as selective inhibitors of the parasite enzyme with IC_{50} values as low as 0.06 μM [117-119]. These inhibitors block pyruvate production of *T. brucei*, kill cultured bloodstream-form trypanosomes but not human fibroblasts and are also active on *Leishmania* axenic amastigotes and intracellular *T. cruzi*. N^6,N^2 substituted adenosine analogs also inhibited *T. brucei* PGK, with IC_{50} values of approximately 30 μM, and inhibited growth of *T. brucei* and intracellular *T. cruzi* at ED_{50}'s of 20 μM. However, the selectivity compared to murine fibroblasts was only 2-fold [120].

For several trypanosomatid enzymes, assays have been developed for HTS of large libraries of small compounds. For *T. brucei* HXK this has resulted in the identification of 10 compounds with IC_{50} values between 0.05 and 41 μM, 20 – 17,000-fold and 2 – 1720-fold, respectively, more potent than the previously identified *T. brucei* HXK inhibitors londiamine and quercetin [121]. Seven of the compounds inhibited growth of bloodstream-form *T. brucei* with $0.03 \leq ED_{50} \leq 3$ μM, while the corresponding values for human cell lines were > 12.5 μM, suggesting at least a 400-fold greater toxicity toward parasites for the most potent inhibitor. For PFK and PYK, submicromolar inhibitors have been obtained with trypanocidal activity in the low micromolar range (unpublished results). The potency of these inhibitors is currently being optimized by SAR analysis.

Trypanosomatid HXKs are inhibited by PPi, although at quite different concentrations and by different mechanisms: for *T. cruzi* (Ki = 0.5 mM, mixed type), and *Leishmania* spp. (Ki = 0.035 mM, competitive with ATP) [53,62]. *T. brucei* possesses two almost identical HXK isoforms, HK1 and HK2. Oligomers of HK1 are active, whereas oligomers of HK2 are not. But hetero-oligomers have even an increased activity, up to 3-fold. HK1 is not inhibited by PPi, but HK2 endows the oligomers sensitivity towards PPi [122]. These specific properties of trypanosomatid HXKs were used to develop bisphosphonates as inhibitors. Bisphosphonates are metabolically inert inorganic PPi analogs in which the oxygen bridge between the two phosphorus atoms has been replaced by a carbon substituted with various side chains [123]. Aromatic amino-methylene

bisphosphonate derivatives have been identified as potent and selective inhibitors of *T. cruzi* hexokinase. These compounds blocked glycolysis and growth of both extracellular epimastigotes and intracellular amastigotes, with a selectivity ratio of more than three orders of magnitude [124,125] and therefore constitute a promising new class of anti-*T. cruzi* agents. Bisphosphonates have been shown already to be active both *in vitro* and *in vivo* against *T. cruzi* without apparent toxicity to the host cells [49]. Since millions of people have already been treated with bisphosphonates and since they have proved anti-Chagasic activity, such compounds as inhibitors of PPi-susceptible glycosomal enzymes appear attractive for further development as chemotherapeutic agents using structure-based drug design. A crystal structure of a trypanosomatid HXK that would allow, by SAR analysis, further optimization of any of the available inhibitors is not yet available. In contrast, a crystal structure has been determined for the *T. cruzi* GlcK [55], but this enzyme is not susceptible to PPi [54], thus unlikely inhibited by the bisphosphonate compounds developed for the HXK. No GlcK inhibitors have been developed to date.

SQS, also involved in sterol synthesis and with a dual glycosomal and mitochondrial/microsomal localization in both *T. cruzi* and *L. mexicana* [46], has been chemically validated as a chemotherapeutic target in both parasites [48]. *T. cruzi* SQS has been produced as a soluble, fully active, recombinant enzyme and used to identify quinuclidine derivatives acting as parasite-enzyme specific inhibitors [126,127]. Two of such compounds, E5700 and ER-119884, display very potent anti-*T. cruzi* activity *in vitro* and one of them (E5700) was able to provide full protection against death and completely arrested development of parasitemia in a murine model of acute disease when given orally [128]. This was the first report of an orally active SQS inhibitor as an anti-infection agent [49]. A new class of anti-*T. cruzi* agents, aryloxyethylthiocyanates, comprises 4-phenoxyphenoxyethylthiocyanate (WC-9); it acts also by selectively inhibiting the parasite's SQS [49,129]. Sterol synthesis of *T. cruzi* as drug target is discussed in much greater detail in a different chapter [52].

Several enzymes involved in the trypanosomatids' unique peroxide detoxification cascade involving trypanothione have PTS motifs, indicating a potentially glycosomal localization, although most or almost all activity seems to

be present in the cytosol (reviewed in [37]). For *T. cruzi,* but not *T. brucei* the activity was partially associated with glycosomes whereas the situation for *Leishmania* spp. is unknown [130,131]. The design and tests of specific trypanothione reductase (TR) inhibitors has received much attention from several research groups in the last 15 years and many families of compounds have been identified as TR inhibitors and trypanocidal agents *in vitro* (reviewed in [49]). Thioridazine, a known inhibitor of TR *in vitro* [132], is able to reduce the parasitemia, increase survival and prevent cardiac damage in murine models of acute Chagas' disease [133,134]. For a more detailed discussion of the trypanothione system as a drug target in trypanosomatids, the reader is referred to chapters elsewhere in this book [135-137].

Inhibitors with trypanocidal activity at micro- and submicromolar concentrations, respectively, are also available for the partially in glycosomes located PPP enzymes G6PDH and 6PGDH [21,138] and are discussed in more detail elsewhere in this volume [22].

The glycosomal enzyme HGPRT, involved in purine salvage, has been proposed as a target for antiparasitic chemotherapy. The enzyme has already been used in development of trypanocidals, by exploiting the fact that it can phosphoribosylate the hypoxanthine analog allopurinol (4-hydroxypyrazolo(3,4-*d*)pyrimidine); the ribonucleotide analog produced is incorporated into RNA during transcription resulting in its degradation and consequently a decreased protein synthesis in the trypanosomatids [139]. Although the allopurinol is also metabolized by the human enzyme, its selectivity against trypanosomatids is attributed to the higher specificity activity of the enzyme in the parasites as well as the fact that humans can synthesize purines *de novo* [140]. In leishmaniasis, the efficacy of this molecule alone is not sufficient for routine clinical use, but combined with meglumine antimoniate it is applied as a parasitostatic in infected dogs [141,142]. Allopurinol was shown to be active in murine models of acute Chagas' disease [143], and has also been used to treat patients with the chronic form of the disease [144].

The available *T. cruzi* and *L. tarantolae* HGPRT crystal structures [109,110] may allow to exploit differences between the active site of the parasite and human enzymes for the design of parasite-enzyme selective inhibitors or

improved subversive substrates as better trypanocidals. Purine and pyrimidine analogs have been tested with IC_{50} values in the micromolar range, including allopurinol [110]. Allopurinol appeared to be more efficient for inhibition of the *Leishmania* than the *T. cruzi* enzyme [109,110]. A docking program was used to screen the Available Chemicals Directory for ligands of the active site of the *T. cruzi* enzyme [145]. This yielded inhibitors with *K*i's between 0.5 and 17 μM, some of them being effective in inhibiting growth of parasites in infected mammalian cells at low micromolar concentration. Further optimization of HGPRT ligands as trypanocidals seems warranted.

9. Discussion and Conclusions

In each of the trypanosomatid parasites, glycosomes are very important. In the pathogenic stages of all of them, several glycosomal processes or functions have been shown to be essential for viability or pathogenic virulence. The organelles offer potential drug targets at several levels: (1) in the enzymes they harbor, (2) in metabolic communication between their matrix and the cytosol via solute transporters and channels, (3) and in their biogenesis and degradation.

Although glycolysis remains the most remarkable process inside glycosomes, research during the last 30 years has demonstrated that additionally a considerable number of other metabolic processes, involving many enzymes, are present inside these organelles (reviewed in [2]). An increasingly number of these processes and enzymes also appeared to be essential for the viability or virulence of the pathogenic stages of one or all of the trypanosomatid parasites. In this chapter, we described the processes and enzymes that have been shown to be essential or at least important, the progress made in the characterization of a considerable number of these candidate drug targets and the state of the art in development of compounds that, in a selective manner, inhibit such target enzymes of the parasites, growth of parasites and decrease or cure infections in animal models.

An aspect of glycosomes that has not yet been a topic of drug-discovery related research concerns the connection between the metabolism processes in the glycosomal matrix and the cytosol. Until very recently nothing was known

about how metabolic substrates, intermediates and products cross the glycosomal membrane. Nonetheless, such solute translocation is essential for the proper functioning of the parasites' metabolism and thus their viability. Recently obtained data discussed in this chapter show that almost certainly, bulky solutes are translocated by specific transporters, while the majority of the metabolites that have to cross the membrane and have a molecular mass smaller than 400 Da do so via some channels with low substrate selectivity [93,94,99,101]. Furthermore, the solute transporters have a very low sequence similarity with their human counterparts and no homologs can be found of the known human peroxisomal solute channel in the trypanosomatid genome databases. Therefore, the glycosomal solute transporters and channels offer also interesting perspectives for future drug discovery. A corollary of the existence of channels in the glycosomal membrane is that drugs targeted to glycosomal matrix proteins should preferably have a mass that permits their translocation through the aqueous pore formed by the channel-forming proteins, thus not larger than approximately 400 Da.

For *T. brucei,* the proper compartmentalization of glycolysis has been shown to be essential. Due to their peculiar properties of some glycolytic enzymes, the process can only function inside glycosomes; even partial mislocalization is detrimental to the cell. This will certainly also be the case under glycolytic growth conditions of the other parasites, such as is probably the case for *T. cruzi* trypomastigotes, but glycolysis seems not very important for the intracellular *T. cruzi* and *Leishmania* spp. amastigotes. However, it is conceivable that also other glycosomal processes that are essential for the parasites need complete compartmentalization for proper functioning but this remains to be established. The essentiality of proper sequestering of enzymes inside glycosomes implies that the organelle's biogenesis and thus the peroxins mediating this process are good drug targets. This approach of drug discovery is currently actively being pursued, because peroxins are not well conserved between human and trypanosomatid. Indeed preliminary results are promising. Similarly, the degradation of glycosomes by autophagy, and thus autophagy-related proteins involved in it are probably promising drug targets in those pathogenic stages where reprogramming of glycosomal metabolism during life-

cycle differentiation plays a role for viability or virulence. This notion has been extensively discussed in [106]), but needs further research to substantiate it.

Glycosomes have been first described 35 years ago, as microbodies containing glycolytic enzymes in *T. brucei* [1]. Since then much research has been devoted to the demonstration of the organelles in other Kinetoplastea, the identification of other metabolic processes inside the organelles and the characterization of the glycolytic and some other glycosomal enzymes of the human-parasitic trypanosomatid species. In the last 10-15 years our knowledge of the organelle has increased enormously thanks to the systems biology and reverse genetics approaches being used in the understanding of its functioning, the studies of its biogenesis and all the information that became available through the sequence of the trypanosomatid genomes and functional genomics projects. Together with the advances made in the drug discovery process by improvement of structure-based drug design and mining methods, introduction of high-throughput screening of large compound libraries, this has led to a situation that the research on drug discovery for glycosomal enzymes has accelerated significantly in recent years. It is hoped that this research will soon lead to compounds for clinical trials and eventually to therapies for people suffering from trypanosomatid infections.

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Chapter 4

Study of the molecular mechanism involved in recycling of matrix protein receptor, PEX5, during glycosome biogenesis in *Trypanosoma brucei*.

General methods

1. Trypanosomes *in vitro* and *in vivo* cultures

***In vitro* cultures**: Monomorphic bloodstream and procyclic-form *T. brucei* strain Lister 427 (hereafter called *T. brucei* 427), cell lines 449 (Biebinger *et al.,* 1997) that were used in this study constitutively express the *Escherichia coli* tetracycline (Tet) repressor gene from the chromosomally integrated plasmid pHD449, also endowing phleomycin resistance. This cell line is metabolically indistinguishable from the wild type. Bloodstream forms were cultured in HMI-9 medium containing 20% heat-inactivated foetal calf serum (Gibco) and 0.18 µg/ml phleomycin (Cayla) at 37°C under water-saturated air with 5% CO_2. Procyclic trypanosomes were grown in SDM-79 medium (Brun and Schönenberger, 1979) supplemented with 12% foetal calf serum and 0.5 µg/ml phleomycin at 28°C under water-saturated air with 5% CO_2.
Cultures were always harvested in the exponential growth phase, *i.e*., at densities lower than $2x10^6$ cells/ml for bloodstream forms and $2x10^7$ cells/ml for procyclic cells, by centrifugation at 700 x *g* for 10 min.

2. Transfections

Bloodstream form: The method used for transfection of bloodstream-form trypanosomes has previously been described by Krazy and Michels (2006). Briefly, a total of $1x10^7$ exponentially growing trypanosomes ($2x10^6$ cells/ml) were used per transfection. Cells were harvested by centrifugation at 700 x *g* in a HS-4 Sorvall rotor, during 10 min and washed once with 10 ml of pre-warmed (37°C) Cytomix [(2 mM EGTA, 120 mM KCl, 0.15 mM $CaCl_2$, 10 mM K_2HPO_4, pH 7.6, 25 mM HEPES, 5 mM $MgCl_2$, 0.5% glucose, 1 mM hypoxanthine, 100 µg/ml fat free bovine serum albumin (BSA)] (Van den Hoff *et al.,* 1992). Cells were then resuspended in 500 µl of cytomix. A volume of 400 µl of the cell suspension was

transferred and mixed with 10 µg of linearized DNA in a sterile electroporation cuvette. Electroporation of trypanosomes was done with a pulse of 1250 volts, 25 Ω and 50 mF applied during 240-250 µsec and transfected cells were immediately transferred to 12 ml of pre-warmed (37°C) HMI-9 medium without antibiotics and cultured at 37°C. 20 h later, the cells were diluted twice in HMI-9 medium and appropriate antibiotics were added (hygromycin 5 µg/ml, blasticidin 5 µg/ml, puromycin 0.1 µg/ml). Cells were plated in 24 well plates and incubated during 1 week before clone selection.

Procyclic form: The method used has previously been described by Krazy and Michels. (2006). A total of $2x10^7$ of exponential growing trypanosomes ($5x10^6$–$1x10^7$ cells/ml) were used per transfections. Cells were harvested by centrifugation at 700 x *g* in a HS-4 Sorvall rotor, during 10 min and washed once with ice-cold Zimmerman Post-Fusion Medium (ZPFM) (132 mM NaCl, 8 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 1.5 mM $Mg(CH_3COO)_2$, 90 µM $Ca(CH_3COO)_2$) (McCulloch *et al*., 2004). Cells were then resuspended in 500 µl ice-cold ZPFM and transferred to a sterile cuvette. 10 µg of linearized DNA was mixed with cells and incubated on ice for 10 min. A pulse of 1800 Volts, 25 Ω and 50 mF during 700 µs was applied. After electroporation cells were immediately transferred to 4.5 ml of prewarmed (28°C) SDM-79 without antibiotics and cultured at 28°C during 20 h. Cells were diluted twofold in fresh medium and corresponding antibiotics were added (hygromycin 50 µg/ml, blasticidin 10 µg/ml, puromycin 1 µg/ml). 1 ml of cells were placed in the top wells of 24 wells plates and serially threefold diluted in the bottom direction of the plate in conditioned SDM-79 medium supplemented with antibiotics at their proper concentration. Positive clones were selected after 10 days of incubation at 28°C from the bottom wells of the plates.

3. Growth measurements

Cultures of 1 ml were started at $2x10^5$ cells/ml (bloodstream form) and $2x10^6$ cells/ml (procyclic form) at time 0. Motile cells were daily counted in a Bürker-Türk (Merck) cell counting grid (0.1 mm in depth). After dilution up to the initial

cell density, cells were recounted similarly. Accumulative growth curves were constructed by plotting the $\log_{10}$ of the product of cell density (cells/ml) and total dilution as a function of time.

4. Molecular biological techniques

Standard methodologies in molecular biology were carried out essentially according to Sambrook *et al.* (1989). Chemicals and enzymes were obtained from Fermentas, Roche and Promega.

4.1. Bacterial strains:

XL1-blue (Stratagene)	*endA1 gyrA96(nal^R) thi-1 recA1 relA1 lac glnV44 F'[::Tn10 proAB$^+$ lacI^q Δ(lacZ)M15 Amy Cm^R] hsdR17(r_K^- m_K^+)*
JM107	*endA1 glnV44 thi-1 relA1 gyrA96 Δ(lac-proAB) [F' traD36 proAB$^+$ lacI^q lacZΔM15] hsdR17(R_K^- m_K^+) λ$^-$*
Bl21(DE3) (Novagen)	*F$^-$ ompT gal dcm lon hsdS$_B$(r_B^- m_B^-) λ(DE3 [lacI lacUV5-T7 gene 1 ind1 sam7 nin5])*
TOP10 (Invitrogen)	*F- mcrA Δ(mrr-hsdRMS-mcrBC) φ80lacZΔM15 ΔlacX74 nupG recA1 araD139 Δ(ara-leu)7697 galE15 galK16 rpsL(Str^R) endA1 λ$^-$*
DB3.1	*F- gyrA462 endA1 glnV44 Δ(sr1-recA) mcrB mrr hsdS20(r_B^-, m_B^-) ara14 galK2 lacY1 proA2 rpsL20(Sm^r) xyl5 Δleu mtl1*

Bacteria were grown in LB-Luria Bertani medium for all molecular cloning procedures.

4.2. Yeast strains:

BY4741 (wild type)	Euroscarf Acc. No. Y00000	*(MATa; his3Δ 1; leu2Δ 0; met15Δ 0; ura3Δ 0)*
BY4742 (wild type)	Euroscarf Acc. No. Y10000	*(MATα ; his3Δ 1; leu2Δ 0; lys2Δ 0; ura3Δ 0)*
PEX22 Orf: YAL055w	Euroscarf Acc. No. Y00352	*(BY4741; Mat a; his3D1; leu2D0; met15D0; ura3D0; YAL055w::kanMX4)*
PEX22 Orf: YAL055w	Euroscarf Acc. No. Y20352	*(*BY4743*; Mat a/a; his3D1/his3D1; leu2D0/leu2D0; lys2D0/LYS2; MET15/met15D0; ura3D0/ura3D0; YAL055w::kanMX4/YAL055w*
PEX4 haploid: Orf: YGR133w	Euroscarf Acc. No. Y14763	BY4742; *Mat a; his3D1; leu2D0; lys2D0; ura3D0; YGR133w::kanMX4*
SFY526 (Clontech)		*MATa, ura3–52, his3–200, ade2–101, lys2–801, trp1–901, leu2–3, 112, canr, gal4–542, gal80–538,URA3::GAL1-lacZ*

All yeast strains were maintained in YPD medium. After transformation, positive clones were selected in minimal medium lacking the corresponding auxotrophy markers.

4.3. Gene cloning procedures

Bacterial recombinant proteins:

The full ORF of *TbPEX4*, *TbPEX22* and the Tbmonoubiquitin gene were amplified by PCR from genomic DNA of *T. brucei* 427 using the following sets of primers: TbPEX4 forward 5'-CGACATATGATGTGTAGAGTTTGTAGGAG-3' and TbPEX4 reverse 5'- TAGGATCCCTATCTCCCATCATTGAAAG-3'; TbPEX22 forward 5'-CGACATATGATGCATCGCAAACCTCTGTTTG-3' and TbPEX22 reverse 5'-TA GGATCCTCAGAACAACAGTTCGGATAAAC-3'; Tbmonoubiquitin forward 5'-CGACATATGATTGATAAAATGCAGATCTTC-3' and Tbmonoubiquitin reverse 5'-TTAGGATCCCTAACCACCTCGAAGACG. TbPEX4FL and TbPEX22FL were cloned using cloning vector pJET1.2 (Thermo Scientific), while the Tbmonoubiquitin gene was cloned using vector pTZ57R (Thermo Scientific). Subsequently all constructs were sequenced. The three genes were then transferred to the bacterial expression vector pET28a (Novagen) between the restriction sites *Nde*I-*Bam*HI. This vector directs the expression of N-terminally $(His)_6$-tagged recombinant proteins under the control of the Lac promoter.

RNAi constructs:

Fragments of the genes *TbPEX4* and *TbPEX22* [*PEX4* sense fragment between nucleotides at the positions 20-510 (491 bp) of the gene and antisense fragment between positions 20-460 (441 bp)] and [*PEX22* sense fragment from position 20 to 576 (559 bp) and antisense fragment from position 20 to 520 (500 bp)] were amplified by PCR from *T. brucei* 427 genomic DNA using the following sets of primers: TbPEX4 sense forward 5'-GTAAAGCTTGTTTAATAACTGTTGTTATGC-3' and TbPEX4 sense reverse 5'-TTACTCGAGATCATTGAAAGGTGGTGC-3', TbPEX4 antisense forward 5'-TTACTCGAGGTGAGTTATAACCCATTAC-3' and TbPEX4 antisense reverse 5'-GTAGGATCCGTTTAATAACTGTTGTTATGC-3'; TbPEX22 sense forward 5'-GTAAAGCTTTGAAACTATATGGAACGAAATGC-3' and TbPEX22 sense reverse 5'-TTACTCGAGTTCGGATAA AGA AGAGATGCTC-3'; TbPEX22 antisense forward 5'-TTACTCGAGCAACGTTGGGTAAGACAAGAC-3' and TbPEX22 antisense reverse 5'-GTAGGATCCTGA AACTATATGGAACGAAATGC-3'. Sense and antisense fragments were first individually cloned using the cloning vector pTZ57R and sequenced. After digestion with the corresponding restriction enzymes (*Hind*III-*Xho*I sense fragments and *Xho*I-*Bam*HI antisense

fragments) the purified inserts were simultaneously ligated in the trypanosome specific tetracycline-inducible expression vector pHD677 between the restriction sites *Hind*III/*Bam*HI vector by a triple ligation reaction. Constructs from positive clones were verified by sequencing.

Yeast two-hybrid constructs:

The full-length genes *TbPEX4, TbPEX22, TbPEX2, TbPEX10* and *TbPEX12* and truncated versions (*TbPEX4IR* from nucleotide position 387 to 519, *TbPEX22IR* from 255 to 530, *TbPEX2RD* from 631 to 999, *TbPEX10RD* from 532 to 897 and *TbPEX12RD* from 889 to 1185) were amplified by PCR from *T. brucei* 427 genomic DNA using the following sets of primers: TbPEX4FL forward 5'-CGGAATTCATGTGTAGAGTTTGTAGGAGT-3' and TbPEX4FL reverse 5'-CGGGATCCCTACTCCCCATCATTGAAAG-3', TbPEX4IR forward 5'-CGGAATTCGACGCCGAAGCCCCTTC-3' and TbPEX4IR reverse 5'-CGGGATCCCTACTCCCCATCATTGAAAGG-3', TbPEX22FL forward 5'-CGGAATTCATGCATCGCAAACCTCTGTTTG-3' and TbPEX22FL reverse 5'-CGGGATCCTCAGAACAA CAGTTCGGATAAAC-3', TbPEX22IR forward 5'-CGGAATTCGAATGGAGGGATTGCGC-3' and TbPEX22IR reverse 5'-CGGGATCCACCCACCAGAACAACGTT, TbPEX2FL forward 5' CGGAATTCATGGCTTGGATAACTGATGCTG-3' and TbPEX2FL reverse 5'-CGGGATCCTCACTGTGCCCATGGGGTG-3', TbPEX2RD forward 5' CGGAATTCATGCTTTCACTTCGGCTAGTAT-3' and TbPEX2FL reverse (idem for TbPEX2FL reverse), TbPEX10FL forward 5'-TCCCCCGGGTATGCAACCGGCCACTGCAC-3' and TbPEX10FL reverse 5'-ACGCGTCGACCTACGACGCACCGCTGTCG-3', TbPEX10RD forward 5'-TCCCCCGGGTATGCTCGAGCTGTTGATTCGG-3' and TbPEX10RD reverse (idem for TbPEX10FL reverse), TbPEX12FL forward 5'-TCCCCCGGGTATGATAGAGAGTAATTTGCTCTC-3' and TbPEX12FL reverse ACGCGTCGACGCACTCATAAATGCGTCGAAC-3', TbPEX12RD forward 5'-TCCCCCGGGTATGGAATTCAGTGGCAACACG-3' and TbPEX12RD reverse 5'-ACGCGTCGACTCAGCACTCATAAATGCGTCG-3'. Digested fragments were cloned after ligating them directly in the yeast expression vectors pGAD424 and pGBT9 (Clontech) between the restriction sites *Eco*RI/*Bam*HI (*TbPEX4* and *TbPEX22*)

and *Sma*I/*Sal*I (*TbPEX10* and *TbPEX12*). These vectors direct the synthesis of fusion proteins $GAL4_{BD}$ and $GAL4_{AD}$, respectively. Constructs from positive clones were analyzed by gene sequencing.

GFP-tag fusion proteins:

The *TbPEX4* and *TbPEX22* full-length genes were amplified by PCR from *T. brucei* 427 genomic DNA using the following sets of primers: TbPEX4FL-GFP forward 5'-CGGAATTCATGTGTAGAGTTTGTAGGAGT-3' and TbPEX4FL-GFP reverse 5'-CGGGATTCTCCCCATCATTGAAAGGTG-3', GFP-TbPEX4FL forward 5'-ACGCGTCGACATGTGTAGA GTTTGTAGGAGTTT-3' and GFP-TbPEX4FL reverse 5'-CGGGATCCCTACTCCCCATCATTGAAAGG-3', TbPEX22FL-GFP forward 5'-GTAAAGCTTATGCATCGCAAACCTCTGTTTG-3' and TbPEX22FL-GFP reverse 5'-GTAGGATTCAAGAACAACAGTTCGGATAAAGAAG-3', GFP-TbPEX22FL forward 5'-GTAAAGCTTGGATGCATCGCAAACCTCTG-3' and GFP-TbPEX22FL reverse 5'-GTAGGATTCTCAGAACAACAGTTCGGATAAAG-3'. PCR amplified fragments were digested with *Hind*III and *Bam*HI and ligated in the trypanosome-specific vectors pGN1 and pGC1 containing already the Green-Fluorescent Protein (GFP) gene (previously prepared by the insertion of the EGFP gene into the trypanosome-specific tetracycline-inducible expression vector pHD1336 (Yernaux *et al.*, 2006)). After cloning, constructs were analyzed by sequencing.

Myc-tag fusion proteins.

The full-length genes for monoubiquitin and TbPEX22 were amplified by PCR from *T. brucei* 427 genomic with the following set of primers: MycUbi forward 5'-GTAAAGCCTATTGATAAAATGCAGATCTTCG-3' and MycUbi reverse (idem for Tbmonoubiquitin reverse), MycTbPEX22 forward (idem to GFP-TbPEX22FL forward) and MycTbPEX22 reverse (idem for TbPEX22FL-GFP reverse). PCR-amplified fragments digested with *Hind*III-*Bam*HI were cloned using the trypanosome-specific tetracycline-inducible expression vector pHD1701 (gift from Dr. Frank Voncken), which directs the synthesis of a N-terminal 3x-Myc-tagged fusion protein. Constructs from positive clones were verified by gene sequencing.

TAP-tag fusion protein.

The full-length *TbPEX22* gene was amplified by PCR from *T. brucei* 427 genomic DNA using the following set of primers: TAP-tagTbPEX22 forward 5'-GGGGCCCATGCATCGCAAACCTCTGTTG-3' and TAP-tagTbPEX22 reverse 5'-ACTCTAGAGAACAACAGTTCGGATAAAGAAG-3'. The PCR fragment, digested with the restriction enzymes *Apa*I/*Xba*I, was cloned directly using the trypanosome-specific tetracycline-inducible expression vector pHD918 which directs the synthesis of a C-terminally TAP-tagged fusion protein.

PEX4 knock-out construct

A segment of the 5'UTR region of the *TbPEX4* locus from position -581 to -16 and a segment of the 3'UTR region comprising the last 22 bp of the ORF of *TbPEX4* until position +592 was amplified by PCR from *T. brucei* 427 genomic DNA using the following sets of primers: 5'UTRKOTbPEX4 forward 5'-ATCGCCATGG*ATTAAA*GTTAACTAGAGTTGTAATGTTATATACTTTG-3' and 5'UTRKOTbPEX4 reverse 5'-TTTAAGAATTCAAATGTTCTAGACTCAATTACGTACAAACAGGAAC-3', 3'UTRKOTbPEX4 forward 5'-ACCTTTCTAGAATGGGCTCGAGAGACGAGTGGAATTG-3' and 3'UTRKOTbPEX4 reverse 5'-GCTCTGAATTCTTCTTCGTTAACAATGCAGAAAAAAGAC-3'.

Each fragment was individually cloned using the pGEMT easy vector (Promega) to create the plasmids pGEMT-5'UTRTbPEX4 and pGETMT-3'UTRTbPEX4, respectively. Subsequently, the 3'UTRTbPEX4 fragment was liberated from the vector and subcloned after ligating it in the pGEMT-5'UTRKOTbPEX4 vector between the restriction enzymes *Eco*RI and *Xba*I to generate the pGEMT5'-3'UTRKOTbPEX4. The puromycin resistance gene (*Streptomyces alboniger* puromycin N-acetyltransferase gene) and blasticidin resistance gene (*Aspergillus terreus* blasticidin S deaminase gene) were excised from plasmid pGEMT-puro and pGEMT-bla (kind gift from Dr. Frédéric Bringaud) using the restriction enzymes *Xba*I and *Xho*I and each individually ligated in pGEMT5'-3'UTRKOtbPEX4 to generate the pGEMT5'-PURO-3'UTRKOtbPEX4 and pGEMT5'-BLA-3'UTRKOtbPEX4. These two plasmids were then digested with the restriction enzyme *Hpa*I to liberate the fragment TbPEX4-5'-PURO-3'UTR and

TbPEX4-5'-BLA-3'UTR containing the corresponding selection markers flanked by the 500 bp of the UTR region of *TbPEX4* locus. These DNA fragments were sequentially used to transfect *T. brucei* procyclic and bloodstream forms and replace each allele of the *PEX4* gene by homologous recombination for the respective antibiotic resistance markers.

GFP-PTS1 expression vector

The gene coding for GFP with at its C-terminus fused a PTS-1 targeting signal (SKL) was amplified by PCR from the yeast expression vector pEW88 (kind gift from Dr. Ben Distel) using the following set of primers: GFP-SKL forward 5'-GTAAAGCTTATGAGTAAAGGAGAAGAACTTTTC-3' and GFP-SKL reverse 5'-CGGGATCCCTAGAGTTTTGAGTGCAGTGG-3'. The amplified PCR fragment was digested with *Hind*II and *Bam*HI and ligated to the trypanosome-specific tetracycline-inducible expression vectors pHD1336 and pHD677.

Yeast mutants complementation constructs

The full-length genes *TbPEX4* and *TbPEX22* were amplified by PCR from *T. brucei* 427 genomic DNA using the following sets of primers: TbPEX4 forward 5'-ACGCGTCGACATGTGTAGAGTTTGTAGGAG-3' and TbPEX4 reverse 5'-TAAGAATGCGGCCGCCTACTCCCCATCATTGAAAG-3', TbPEX22 forward 5'-ACGCGTCGACATGCATCGCAAACCTCTGTTTG-3' and TbPEX22 reverse 5'-TAAGAATGCGGCCGCTCAGAACAACAGTTCGGATAAAG-3'. The amplified fragments were digested with the restriction enzymes *Sal*I and *Not*I and individually cloned using the Gateway entry vector pENTR1A (Invitrogen). Constructs from positive clones were checked by gene sequencing. The pENTR1A-TbPEX4 and pENTR1A-TbPEX22 constructs were used in a LR reaction (Invitrogen) where the genes were transferred by homologous recombination to the Destination vectors pAG413GDP and pAG415GDP (Addgene) to generate the pAG413GDP-TbPEX4 and pAG415GDP-TbPEX22 constructs. These plasmids were used to direct the constitutive expression of respectively the *TbPEX4* and *TbPEX22* genes in *S. cerevisiae* under control of the GAPDH promoter. Constructs of positive clones were analyzed by gene sequencing.

4.4. Genome integration analysis

Total genomic DNA was extracted from $5x10^7$ cells using the Magnesyl KF genomic system (Promega) and the Kingfisher Labsystem (Thermo Scientific). Briefly, after selection of positive clones (resistant to the respective antibiotic markers) of *T. brucei* procyclic and bloodstream form transfected with the relevant plasmids containing RNAi and knock-out constructs, cells were further analyzed to confirm the correct integration of the corresponding gene(s) by PCR from genomic DNA. The criteria to analyze the integration of a RNAi construct in the genome were based on a PCR reaction performed using a forward primer that hybridized in the loop region of the hairpin sequence of the RNAi construct (*TbPEX4* loop forward 5'-ACTGAAGCTGGTGCACCACCT-3', *TbPEX22* loop forward 5'-TTCTGGTGGGTTCCAGTGCAA-3') and a reverse primer that annealed to an internal sequence on the resistance marker (hygromycin reverse 5'-CCTTTGCCCTCGGACGAGTGC-3'). The criteria to analyze the integration of *TbPEX4* knock-out constructs were based on a PCR reaction performed with the following sets of primers: TbPEX4-RTPCR forward 5'-AAGGTGGTAGTTTTTGTCTTCG-3' and TbPEX4-RTPCR reverse 5'-TCCTGCGTCGCAGTTGAAG-3', Blast forward 5'-ATGGCCAAGCCTTTGTCTCAAG-3' and Blast reverse 5'-ACCAGAGGGCAGCAATTCACG-3', Puro forward 5'-ATGACCGAGTACAAGCCCACG-3' and Puro reverse 5'-CAGGAGGCTTTCCATCTGTTG-3', Tubulin forward 5'-GCGCGAAATCGTCTGCGTTCAGG-3' Tubulin reverse 5'-CGACGTACGGAGTCCATTGTACC-3'. The negative result of a non-amplification of the *TbPEX4* gene and the simultaneous positive results in amplification of the blasticidin and puromycin resistance marker genes were used as evidence for successful double gene knock-out.

4.5. Semiquantitative RT-PCR and qPCR

Total RNA was isolated from $5x10^7$ cells using the SV Total RNA Isolation System (Promega). After quantification, 2 µg of total RNA were used to synthesize cDNA using the RevertAid™ H Minus First Strand cDNA Synthesis Kit (Thermo Scientific) following the manufacturer's instructions. Total cDNA was diluted 4-fold and used as template for PCR amplification using the following internal

primers (TbPEX4 RTPCR forward and TbPEX4RTPCR reverse, TbPEX22 RTPCR forward 5'-AGCCTCTTTCCTTAGAAGGA-3' and TbPEX22 RTPCR reverse 5'-CAGAAAAGAATATTATGTCGTG-3', Tubulin forward and Tubulin reverse. Fixed volumes were loaded into slots of 2% agarose gels which, after electrophoresis, were stained with Syber green (Invitrogen). The intensity of the bands was quantified in a Kodak station and normalized to the intensity of the loading control tubulin. For qPCR analysis of the ΔPEX4 cell line, cDNA was not diluted after synthesis and used for a qPCR reaction with 10 µl of KAPA SYBR® FAST Bio-Rad iCycler™ qPCR Master Mix (Sopachem, Kapa Biosystem, Eke, Belgium) and 10 pmol of each primer. A single denaturing step at 94°C for 300 s was followed by 40 cycles: 94°C for 30 s and 60°C for 3 s. For quantification, the copy number of each mRNA was normalized to the tubulin mRNA copy number. The following sets of primers were used: TbPEX4 RTPCR forward and TbPEX4RTPCR reverse, Tb927.5.1000 forward 5'-CTCTTCGTCGCATTCAAAAG-3' and Tb927.5.1000 reverse 5'-CGTCCACTCCATTGCAGTT-3', Tb11.01.5510 forward 5'-TGGCGTATGGTACATCCAC-3' and Tb11.01.5510 reverse 5'-CAACATGCGGAGTTGTGATT-3', Tb927.7.2540 forward 5'-TGCCAGGGTTGCTATAGAC-3' and Tb927.7.2540 reverse 5'-CTGCTTTGGACATACATAC-3', Tb927.8.6090 forward 5'-CTTCCCTGTGAGTGATAATC-3' and Tb927.8.6090 reverse 5'-CTGTACGCAAGCACTTCTG-3', Tb927.2.3720 forward 5'-GCCATGCACCTCATAATGCG-3' and Tb927.2.3720 reverse 5'-CGACTCCATTAAAAGGCCG-3', Tb09.211.0050 forward 5'-GTCATTTCCATGTGACAATTC-3' and Tb09.211.0050 reverse 5'-CTTCATGGCTTCAGCTTCAT-3', Tb927.4.3460 forward 5'-GAAGGAGTTACGCGACATC-3' and Tb927.4.3460 reverse 5'-CTGGTACTCGCTGTTGAAC-3', Tb11.01.5790 forward 5'-CCTCAGGAAACTCCTTACGC-3' and Tb11.01.5790 reverse 5'-CATCAATGCCTCCATTAGAG-3', Tb927.4.2710 forward 5'-CATTAGTCGTAGTGCTCCG-3 and Tb927.4.2710 reverse 5'-CTCATTGAGGACACGCATAA-3', Tb927.4.3190 forward 5'-GAAGGAGTTACGCGACATC-3' and Tb927.4.3190 reverse 5'-CCACAGTTCTTGCGCAATG-3', Tubulin forward and Tubulin reverse.

5. Immunological techniques

5.1. Antibodies production. Purified full-length recombinant proteins (TbPEX4, TbPEX22, TbMonoUbiquitin) were used as antigens to raise antisera in rabbits. A rabbit polyclonal antiserum, based on a synthetic peptide corresponding to the C-terminal residues of TbPEX4 (RMYAITEAGAPPFNDGE), was customized and affinity purified by Perbio Science - Thermo Scientific.

5.2. Western blot analysis

Immunoblotting was performed according to a standard procedure (Towbin *et al.,* 1979) with slight modifications. Briefly, proteins were size separated by electrophoresis on SDS-polyacrylamide gels (SDS/PAGE) and then transferred to a nitrocellulose membrane (Amersham Biosciences) using a wet transfer tank (Hoefer Instruments). Non-specific binding of antibodies to the membrane was prevented by blocking in PBS supplemented with 5% dried low-fat milk for 1 h. Incubation with primary antibodies was done in PBS supplemented with 1% of dried low-fat milk overnight at room temperature. Membranes were subsequently washed by consecutive 10 min incubations in PBS (2X), PBS supplemented with 0.05% of NP-40 (1X) and PBS (2X). Incubation with the secondary antibody was done in PBS supplemented with 1% of dried low-fat milk for 1 h at room temperature. For detection of the following proteins, polyclonal antisera raised in rabbits were used at the indicated dilutions: TbPEX5 (1:20,000) and affinity purified TbPEX5 (1:5,000), TbPEX4 (1:4,000), TbPEX4 peptide based (affinity purified) (1:300), TbUbiquitin (1:10,000), Human-Ubiquitin (Santa-Cruz) (1:300), TbPEX11 (1:2,500), TbPEX14 (1:20,000), TbEnolase (1:150,000), TbAldolase (1:200,000), cytosolic glyceraldehyde-3-phosphate dehydrogenase (TbcGAPDH) (1:10,000), hexokinase (TbHXK) (1:100,000) and pyruvate-phosphate dikinase (TbPPdK) (1:25,000). Furthermore, a mouse monoclonal antibody against TbHSP60 (1/100) was used (courtesy of Dr. Frédéric Bringaud) and a monoclonal anti-Myc (Invitrogen) (1:3,000). The secondary antibodies, anti-rabbit IgG and anti-mouse IgG conjugated to horseradish peroxidase (Rockland Immunochemicals) were diluted 1:10,000 and visualised with the ECL Western Blotting System, a

luminol-based system (Amersham Biosciences).

5.3. Immunoprecipitation

5.3.1.1. *Native immunoprecipitation*: The procedure employed has previously been described by Leung *et al*, (2011). Briefly, cells ($1x10^8$) were harvested by centrifugation at 800 x *g* in a HS-4 Sorvall rotor for 10 min at 4°C and then washed twice in PBS. Cells were lysed in 1 ml of TEN buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA) containing 1% Triton X-100 and Protein Block cocktail (Fermentas) for 1 h at 4°C in a rotation device. Lysates were then centrifuged at 16,000 x *g* in a T15A61 VWR rotor for 15 min at 4°C to remove nuclei and cell debris, and the supernatant was transferred to a fresh tube. One microliter of monoclonal anti-Myc antibody was added to each sample, and the mixture was incubated overnight at 4°C. Immune complexes were isolated by the addition of 50 µl of protein A-Sepharose (Amersham Biosciences) and incubation for 2 h at 4°C while rotating. Sepharose beads were then washed with PBS, followed by centrifugation at 16,000 x *g* in the same rotor for 15 s. The supernatant was removed and the washes were repeated twice. Subsequently, the beads were resuspended in 1X SDS Laemmli buffer and incubated at 95°C for 10 min. Samples were subjected to protein electrophoresis and western blot analysis.

5.3.1.2. *Denaturing immunoprecipitation*: The following procedure has previously been described by Chung *et al.* (2008). Briefly, samples of $1x10^8$ cells were pelleted at 700 x *g* in a HS-4 Sorvall rotor for 10 min at 4°C, washed in PBS and lysed by the addition of 100 ml of radioimmunoprecipitation assay (RIPA) buffer [25 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS and Protein Block Cocktail (Fermentas)] for 15 min on ice. Lysates were centrifuged at 16,000 x *g* in a T15A61 VWR rotor for 30 s to remove nuclei and cell debris, and the supernatants transferred to fresh tubes. 13 µl of 10% SDS was added to each supernatant and the suspensions were then incubated at 95°C for 5 min, after which they were diluted with 750 µl of dilution buffer (50 mM Tris–HCl, pH 7.5, 1.25% Triton-X-100, 190 mM NaCl, 6 mM EDTA) and protease inhibitor cocktail (Fermentas). 50 µl of protein A-Sepharose

(Amersham Biosciences) (prewashed and resuspended in dilution buffer) was added to the supernatants and incubated at 4°C for 2 h. Samples were then centrifuged at 16,000 x *g* in the same rotor for 5 min at 4°C and the supernatants transferred to fresh tubes. One µl of anti-PEX5 antibody was added to each sample which were then incubated at 4°C overnight on a rotating device. Immune complexes were then isolated by the addition of 50 µl of protein A–Sepharose on a rotating device for 2 h at 4°C. Subsequently, Sepharose beads were washed twice with wash buffer I (50 mM Tris-HCl, pH 7.5, 0.1% Triton-X-100, 0.02% SDS, 150 mM NaCl and 5 mM EDTA) and twice with wash buffer II (50 mM Tris-HCl, pH 7.5, 0.02% Triton X-100 and 1 M NaCl). A further centrifugation step was performed for 15 s at 16,000 *x g*, and the remaining supernatant was removed by pipetting. Samples were resuspended in 1 x Laemmli buffer, denatured at 95°C for 5 min and subjected to SDS–PAGE followed by western blot analysis.

5.4. Immunofluorescence

The procedure used for immunofluorescence has previously been described by Galland *et al.* (2007). Briefly, procyclic and bloodstream-form trypanosomes ($1x10^7$ cells) were centrifuged at 918 x *g* in a T15A61 VWR rotor for 5 min at 4°C and washed twice in ice-cold PBS. Cell fixation was done with 4% formaldehyde in PBS for 10 min at room temperature in a rotating device followed by a permeabilization step involving treatment with 1% Triton X-100 for 15 min. Finally cells were incubated for 1 h with PBS containing 0.5 M glycine and washed once in ice-cold PBS. After applying of trypanosomes to poly-L-lysine coated microscope slides during 1 h at room temperature, unspecific binding was blocked by incubation in PBS containing 5% BSA for 45 min at 37°C, followed by incubation with primary antiserum in PBS containing 2% BSA at the following dilutions: mouse monoclonal anti-*Tb*TPI (1:5,000), rabbit polyclonal *Tb*Aldolase (1:1,500) and anti-*Tb*gGAPDH (1:5,000). After extensive washes with PBS, cells were incubated for 45 min at 37°C with fluorescein-conjugated secondary antibodies (Alexa Fluor 488 anti-mouse IgG, Alexa Fluor 488 anti-rabbit, Alexa 568 anti-mouse IgG and Alexa 568 anti-rabbit IgG (Molecular Probes, Invitrogen) in PBS containing 2% of BSA at a 1:1,000 dilution. After

extensive washes with PBS, cells were incubated with 4',6-diamidino-2-phenylindole (DAPI) (1:500) in PBS for 30 min at room temperature followed by extensive washes in PBS. Cells were finally mounted in DAKO (DAKO North America, Inc.) and visualized using a using a Zeiss Axiovert 200 inverted fluorescence microscope coupled to an MRC-1024 confocal scanning laser imaging system (BioRad). Digital images were captured using the software AxioVision 4.8.2 (Zeiss).

6. Biochemical Techniques

6.1. Differential centrifugation

A total of $8x10^9$ exponentially growing wild-type bloodstream ($5x10^5$ cells/ml) and procyclic ($5x10^6$ cells/ml) trypanosomes were collected, centrifuged at 680 x *g* in a SLA-3000 Sorvall rotor for 15 min at 4°C and washed twice in isotonic STE buffer (250 mM sucrose, 25 mM Tris-HCl, pH 7.4, 1 mM EDTA). Cells were homogenized (microscopically checked) in a pre-chilled mortar with carborundum powder as described previously by Opperdoes and Borst (1977). Differential centrifugation was performed as follows: the suspension was taken up in 5 ml of STE buffer supplemented with a cocktail of protease inhibitors (Fermentas) and centrifuged at 30 x *g* in a SM-24 Sorvall rotor for 3 min at 4°C. The pellet was washed twice with 0.5 ml of STE buffer and then discarded. The pooled supernatants (5 ml) representing the cell homogenate were centrifuged at 1,500 x *g* in the same rotor for 10 min giving the nuclear fraction as pellet. The nuclear fraction was resuspended in 5 ml of STE buffer. The post-nuclear supernatant was then centrifuged at 5,000 x *g* in the same rotor for 10 min giving the large-granular (mitochondria-enriched) fraction as pellet. The large granular fraction was resuspended in 5 ml of STE buffer. In order to obtain the small-granular (glycosomes-enriched) fraction, the supernatant was centrifuged at 15,000 x *g* in the same rotor for 30 min and the pellet resuspended in 5 ml of STE. A further centrifugation step (140,000 x *g* in a 50Ti Beckman rotor for 1 h) gave the microsomal fraction (pellet) and the cytosol devoid of all organelles (supernatant). Equal amounts of protein of each fraction were analyzed by SDS-PAGE followed by western blotting.

6.2. Isopycnic centrifugation

Exponentially growing procyclic-form trypanosomes (1.5 litre; $5x10^6$ cells/ml) were centrifuged at 680 x *g* in a SLA-3000 Sorvall rotor for 15 min at 4°C, washed 1X with 150 ml of TEDS buffer (25 mM Tris-HCl, pH 7.8, 1 mM EDTA, 1 mM DTT, 250 mM sucrose) followed by a second wash with 50 ml of the same buffer. Cells were homogenized (microscopically checked) in a pre-chilled mortar with carborundum powder as described previously by Opperdoes and Borst (1977). Differential centrifugation was done as described above to prepare a large granular fraction enriched in mitochondria and glycosomes. 2.5 ml of this large granular fraction was loaded on the top of a preformed linear 20-40% (v/v) Optiprep density gradient (33 ml) mounted on top of a 3.5 ml 50% (v/v) Optiprep cushion according to the manufacturer's instruction (Optiprep Application sheet S09, Axis-shield). The gradients were centrifuged at 4°C in a VTi-50 vertical rotor (Beckman) at 100,000 x *g* overnight, using the slow acceleration and deceleration mode. Fractions of 1 ml were collected from the bottom of the tube using an automatic fraction collector. Protein concentrations were determined by Bradford method. Proteins present in 300 µl of each fraction were precipitated with TCA and cold acetone according standard procedures. Western blot analysis of each fraction was performed as previously explained. The diffraction index was measured for each fraction and the density calculated.

6.3. Selective permeabilization with digitonin

Exponentially growing procyclic ($5x10^6$ cells/ml) and bloodstream ($5x10^5$ cells/ml) form *T. brucei* were centrifuged at 700 x *g* in a HS-4 Sorvall rotor for 10 min at 4°C, washed twice in ice-cold STE buffer (25 mM Tris-HCl, pH 7.4, 250 mM sucrose and 1 mM EDTA), and then resuspended in 0.5 ml of the same buffer. The protein content of the cell suspension was determined and aliquots of cells containing 100 µg of protein mixed with HBSS-IP buffer (1.3 mM $CaCl_2$, 5 mM KCl, 0.3 mM KH_2PO_4, 0.5 mM $MgCl_2$, 0.4 mM $MgSO_4$, 138 mM NaCl, 4 mM $NaHCO_3$, 0.3 mM Na_2HPO_4, 15 mM Hepes, pH 7.6) supplemented with protease inhibitors (Proteoblock, Fermentas) to adjust each volume to 100 µl. Different concentrations of digitonin (Fluka) (from 0.01 to 1 mg digitonin/mg of protein)

were added to each suspension followed by incubation for 4 min at room temperature and immediate centrifugation at 20,000 x *g* in a T15A61 VWR rotor for 2 min at 4°C. As controls (no release of enzymes or total release) were taken cells that were not treated with digitonin and cells to which 0.5% Triton X-100 (final concentration) was added. After centrifugation of the suspensions, 80 µl of the supernatant were taken for further analysis and the organellar pellet was washed once in HSSB-IP buffer, centrifuged at 20,000 x *g* in the same rotor for 2 min at 4°C and finally resuspended in 80 µl of HSSB-IP buffer. Supernatant and pellet samples corresponding to 50 µg of protein were resolved by SDS-PAGE and analyzed by western blotting.

6.4. Bacterial recombinant protein expression and purification

Conditions used for induction of the expression of recombinant proteins were as follows: (1) *E. coli* BL21(pET28TbMonoUbi), growing in 100 ml LB supplemented with 30 µg/ml kanamycin was induced to express TbMonoubiquitin at OD_{600nm} = 0.4, by addition of 1 mM of isopropyl β-D-1-thiogalactopyranoside (IPTG), followed by continued culturing at 28°C for 4 h. (2) *E. coli* BL21 (pET24TbPEX5) and *E. coli* BL21 (pPROEXTbPEX5), growing in 100 ml LB supplemented with 30 µg/ml kanamycin and 100 µg/ml, respectively, were induced at OD_{600nm} = 0.4, by using 1 mM of IPTG and further growth at 37°C for 4 h. (3) *E. coli* BL21 (pET28PGK) grown in LB plus 1 M sorbitol (50 ml) supplemented with 30 µg/ml of kanamycin, was induced at OD_{600nm} = 0.5 by the use of 1 mM of IPTG and incubation at room temperature overnight. (4) *E. coli* BL21(pET28TbPEX4) and BL21(pET28TbPEX22) (100 ml each) were grown in the ZYM-5052 autoinduction medium (0.5% yeast extract, 25 mM Na_2HPO_4, 25 mM KH_2PO_4, 50 mM NH_4Cl, 5 mM Na_2SO_4, 2 mM $MgSO_4$, 0.5% glycerol, 0.05% glucose and 0.2% lactose), supplemented with 30 µg/ml kanamycin at 16°C for 72 h and 30° for 24 h (TbPEX4 and TbPEX22, respectively).

6.4.1.1. *Native Purification*. N-terminally $(His)_6$-tagged TbMonoUbiquitin was purified using affinity chromatography with immobilized nickel using an ÅKTA system (GE Healthcare). Briefly, cells were harvested by centrifugation at 2,000 × *g* in a HS-4 Sorvall rotor for 15 min at 4 °C and the pelleted cells were

resuspended in 10 ml of Binding buffer (50 mM Tris-HCl, pH 7.5, 500 mM NaCl) supplemented with a cocktail of protease inhibitors (Roche) and broken by two passages through a French pressure cell at 6.9 MPa. Subsequently nucleic acids were degraded by incubation with 1 U of benzonase (Merck) at 37 °C for 15 min. The lysate was then centrifuged at 20,000 × *g* in a SS-34 Sorvall rotor for 15 min at 4 °C and the supernatant used to load a pre-equilibrated His-TRAP column (Amersham Biosciences). The resin was washed with 10 volumes of Binding buffer containing 10 mM imidazole followed by 10 volumes of Binding Buffer with 20 mM imidazole. TbMonoUbiquitin was eluted with a linear gradient of imidazole (20 mM - 500 mM). 13 fractions of 500 µl were collected. The purity of the protein was assessed by SDS-PAGE and its concentration determined by the Bradford method using the Bio-Rad protein assay with BSA as standard in the presence of 10 µl of elution buffer. TbPGK was purified as described (Zomer *et al*., 1998).

6.4.1.2. *Inclusion bodies purification*: N-terminally $(His)_6$-tagged TbPEX4, TbPEX22 and TbPEX5, and C-terminally tagged TbPEX5, all full-length proteins, were purified under denaturing conditions using affinity chromatography with immobilized nickel (TbPEX4 and TbPEX22) or immobilized cobalt (TbPEX5). Briefly, cells were harvested by centrifugation at 2,000 × *g* in a HS-4 Sorvall rotor for 15 min at 4 °C and the pelleted cells were resuspended in 20 ml of buffer A (20 mM Tris-HCl, pH 8, 200 mM NaCl) supplemented with a cocktail of protease inhibitors (Roche) and broken by two passages through a French pressure cell at 6.9 MPa. Nucleic acids were subsequently degraded by incubation with 1 U of Benzonase (Merck) at 37 °C for 15 min. The lysate was then centrifuged at 20,000 × *g* in a SS-34 Sorvall rotor for 15 min at 4 °C and the pelleted inclusion bodies were resuspended in 12.5 ml of Buffer B (20 mM Tris-HCl, pH 8, 200 mM NaCl, 1% sodium deoxycholate and 1% CHAPS) and incubated for 30 min at 4°C on a rotating device. The centrifugation step was repeated as previously and the inclusion bodies resuspended in Buffer C (20 mM Tris-HCl, pH 8, 200 mM NaCl, 0.25% sodium deoxycholate) and incubated similarly. This step was repeated three more times and finally the inclusion bodies were washed in 12.5 ml of Buffer A to remove the detergent. The pelleted inclusion bodies were

resuspended in 4 ml of Buffer D (20 mM Tris-HCl, pH 8, 100 mM NaCl, 8 M urea) and incubated overnight at 4°C with agitation. Protein suspensions were diluted to a urea concentration of 6 M before purification. The solubilized proteins were then purified by affinity chromatography with immobilized nickel in a His-Link Resin (Promega) (TbPEX4 and TbPEX22) or TALON resin (Clontech) (TbPEX5) under denaturing conditions following the instruction of manufacturers. Proteins were eluted with 5 ml of Buffer E (20 mM Tris-HCl, pH 8, 100 mM NaCl, 6 M urea, 500 mM imidazole). The purity of the protein was assessed by SDS-PAGE and its concentration determined by the Bradford method using the Bio-Rad protein assay with BSA as standard in the presence of 10 µl of elution buffer.

6.5. TbPEX5 antibodies purification

Purified full-length recombinant TbPEX5 was covalently linked to a Sepharose 4B CNBr (Amersham Biosciences) resin following the manufacturer's instructions. Briefly, the resin was equilibrated with 10 bed volumes of Binding buffer (0.1 M $NaHCO_3$, pH 8.3, 0.5 M NaCl). Purified TbPEX5TbPEX5 (0.63 mg) diluted 4 times in Binding buffer was incubated with the resin overnight at 4°C on a rotating device. Unbound protein was collected in the flow-through. Four extensive washes were carried out with Binding buffer and a blocking step was performed by incubation with Blocking buffer (0.2 M glycine, pH 8) for 2 h at 4°C on a rotating device. Two washing steps were performed with 10 bed volumes of Buffer A (0.1 M CH_3COONa, 0.5 M NaCl, pH 4) followed by 10 bed volumes of Binding buffer. These sequential washes were repeated three times. For the antibodies purification the TbPEX5 activated resin was washed with 10 bed volumes of PBS followed by 10 bed volumes of Buffer B (0.1 M glycine, pH 2.5) and finally with 10 bed volumes of PBS. TbPEX5 antisera (200 µl was diluted 2-fold in PBS) and passed by gravity three times through the resin. Extensive washes were performed with PBS until the A_{280nm} was constant. The purified antibodies were eluted with 6.4 ml of Buffer B and collected in 8 fractions of 800 µl and instantly equilibrated to neutral pH by the addition of 10 µl of 2 M Tris-HCl, pH 11. The absorbance of the fractions was measured and the fractions with the highest protein content were used to test the reactivity with the antigen TbPEX5 from trypanosome lysates by western blotting.

6.6. TAP-tag purification of TbPEX22

One litre of exponentially growing wild-type procyclic form *T. brucei* ($5x10^6$ cells/ml) and procyclic form trypanosomes expressing TbPEX22-TAPtagged were induced with 0.5 µg/ml of tetracycline for 12 h. Differential centrifugation was performed to produce a small granular fraction enriched in glycosomes, which were used as starting material for the TbPEX22-TAP-tagged purification. Briefly, small granular pellets were solubilized in 2 ml of Buffer IPP150 (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% NP40 and 2% of digitonin) for 2 h at 4°C on a rotating device. The protein solution was centrifuged at 100,000 *x g* in a 50Ti Beckman rotor for 1 h at 4°C and the supernatant (solubilized membrane proteins) was recovered for further TAP-tag purification. This protein solution enriched in glycosomal proteins was incubated in 1 ml wet IgG resin (Amersham Biosciences) pre-equilibrated in Buffer IPP150 overnight at 4°C. The resin was washed with 10 ml of IPP150 buffer followed by a single wash with TEV protease buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% NP40, 0.5 mM EDTA, 1 mM DTT). Purified recombinant TEV protease (70 U) was diluted in 1 ml of TEV protease buffer, added to the resin and an incubation was performed for 2 h at 16°C for 2 h. Protein complexes were eluted by gravity (1 ml) and precipitated by chloroform/methanol (1:3 v/v) (Wessel and Flugge, 1984). Pelleted proteins were dissolved in 100 µl of 1X Laemmli Buffer and boiled at 100°C for 5 min. Proteins in samples taken during the successive purification steps were size-fractionated by SDS-PAGE followed by western blot analysis.

6.7. Protease protection assay

Protein protection assays were performed using a method previously described by Igoillo-Esteve *et al.* (2011). Briefly, *T. brucei* 427 wild-type bloodstream and procyclic form cells were processed as for differential centrifugation. The small granular fraction was used as a source of intact glycosomes. Glycosomes were resuspended in iso-osmotic buffer (250 mM sucrose, 10 mM $MgCl_2$ and 25 mM Tris-HCl, pH 7.8), and the glycosomal integrity was determined by hexokinase activity measurement according to Visser and Opperdoes (1980). Samples containing 100 µg of total glycosomal protein, with or without 0.3% Triton X-100, were incubated in the presence or absence of 60 µg/ml of Proteinase K

(Roche) at 0 °C for 1 h. The proteolytic activity was stopped by the addition of 10 mM phenylmethylsulfonyl fluoride (PMSF). Proteins were then precipitated with chloroform/methanol (1:3 v/v), dissolved and size-fractionated by SDS–PAGE followed by western blot analysis.

6.8. PTS-1 *in vitro* binding assay

Exponentially growing *T. brucei* 427 bloodstream (400 ml) and procyclic cells (200 ml) were centrifuged at 700 x *g* in a HS-4 Sorvall rotor for 10 min at 4°C. Cells were lysed in Binding buffer (0.1 M triethanolamine (TEA)-HCl, pH 7.6, 1 mM $KHPO_4$, 200 mM KCl, 0.1% Triton-X-100) in the presence of a cocktail of protease inhibitors (Fermentas) for 1 h on ice. DNA was degraded using 1 U of Benzonase (Merck) for 15 min at 37°C. Two cycles of sonication of 15 s at 4°C were performed prior to centrifugation at 13,800 x *g* in a T15A61 VWR rotor for 10 min at 4°C. Protein concentration was determined by the Bradford method and lysates normalized to 1 mg/ml. Lysates (2 mg total protein) were incubated in a pre-equilibrated Talon resin (Clontech) overnight at 4°C as a clearing step to eliminate proteins that bind unspecifically to the resin. The flow-through was collected, proteins were quantified and the cleared lysate used as starting material for the *in vitro* binding experiment. The imidazole in the purified TbPGK sample was diluted to 10 mM by several cycles of concentration and dilution in Centricon devices (30 kDa) (Millipore). Subsequently, 1 mg of the recombinant protein was bound to a pre-equilibrated Talon resin overnight at 4°C. After two extensive washes (with 10 bed volumes), the resin was activated for the *in vitro* binding experiment. Cleared trypanosome lysates (0.5 mg total protein) were incubated with the TbPGK-Talon resin overnight at 4°C. The flow-through was collected and washes were done with 10 bed volumes of binding buffer. Elution of TbPGK and trypanosome proteins that had interacted with it was done in two fractions of 500 µl with 50 mM and 100 mM of imidazole, respectively. The total protein in the elution fractions were precipitated with chloroform/methanol and dissolved in 1X Laemmli buffer. Interacting proteins were analyzed by SDS-PAGE followed by western blotting.

6.9. Protease susceptibility studies

Exponentially growing *T. brucei* 427 bloodstream (400 ml) and procyclic (200 ml) cells were centrifuged at 700 x *g* in a HS-4 Sorvall rotor for 10 min at 4°C. Cells were washed once in PBS, resuspended in 1 ml of lysis buffer (0.1 M TEA-HCl, pH 7.6, 1 mM $KHPO_4$, 200 mM KCl, 0.1% of Triton X-100) and immediately transferred to 4 eppendorf tubes to each of which protease inhibitors from different commercial sources (ProteoBlock Protease Inhibitor Cocktail from Fermentas; Complete Mini, EDTA-free Protease Inhibitor Cocktail tablets from Roche; home-made cocktail: 1 µM Pepstatin, 1 µM Leupeptin, 10 µM AEBSF, E-64 0.25 µM, PMSF 0.75 mM) were added. Cells were incubated on ice for 20 min and treated with 1 U of Benzonase for 15 min at 37°C. Lysates were centrifuged at 13,800 x *g* in a T15A61 VWR rotor for 15 min at 4°C and the supernatants recovered. Protein suspensions were kept at 4°C overnight before being analyzed by SD-PAGE followed by western blotting.

6.10. Protein turnover analysis

Cultures of 50 ml of exponentially growing procyclic form ($1x10^6$ cells/ml) and 100 ml of bloodstream form ($5x10^5$ cells/ml) *T. brucei* 427 were treated with 50 µg/ml of the ribosome inhibitor cycloheximide (Sigma) during 6 h. Samples of 12.5 ml were collected after 0, 3, 4.5 and 6 h and cell lysates prepared using the ProteoJET Membrane Protein Extraction Kit (Fermentas) following the instructions by the manufacturer. Briefly, cells were centrifuged at 700 x *g* in a HS-4 Sorvall rotor for 10 min at 4°C and washed once in 1 ml of Wash buffer containing a protease inhibitor cocktail (ProteoBlock from Fermentas). The cell pellet was then resuspended in 50 µl of Permeabilization buffer supplemented with the same protease inhibitor cocktail and incubated on a rotating device at 4°C for 30 min. The cell lysate was centrifuged at 16,000 x *g* in a T15A61 VWR rotor for 15 min at 4°C and the supernatant (cytosolic fraction) was collected in a fresh tube. The pellet was then treated with 50 µl of Membrane Extraction Buffer supplemented with protease inhibitors and incubated at 4°C in a thermomixer at 1,400 rpm for 30 min. The suspension was then centrifuged at 16,000 x *g* in the same conditions and the supernatant (Organellar Fraction) recovered in a fresh tube. All samples were frozen at –80°C until their analysis by SDS-PAGE followed by western blotting.

6.11. *In vitro* import and export of TbPEX5

Assays for *in vitro* import and export reactions of TbPEX5 were done according to Okumoto *et al.* (2011) with slight modifications. ^{35}S-Labelled TbPEX5 was synthesized *in vitro* using the construct pET24TbPEX5 as template in the TNT Quick Coupled transcription/translation system (Promega) with ^{35}S-methionine from Perkin-Elmer, following the manufacturer's instructions. Proper *in vitro* synthesis was confirmed by SDS-PAGE followed by autoscanning of the gels. Differential centrifugation of procyclic wild-type and procyclic ΔPEX4 *T. brucei* cells (800 ml of exponentially growing trypanosomes at 8x10^6 cells/ml) was performed in the presence of Import buffer (0.25 M sucrose, 5 mM Hepes-KOH, pH 7.4, 0.1% ethanol, 5 mM methionine and 50 mM KCl) to obtain a post-mitochondrial supernatant as source of intact glycosomes and cytosol. The post-mitochondrial supernatant was split in two equal parts: one part was treated by ultracentrifugation at 100,000 x *g* in a 50Ti Beckman rotor for 1 h at 4°C to obtain a cytosolic fraction from each cell line and the second part was used for the *in vitro* import and export assays. Proteins from cytosolic and post-mitochondrial fractions were quantified and normalized to a concentration of 1 mg/ml. The post-mitochondrial fraction from wild-type and ΔPEX4 cells (400 µg) were aliquoted in eppendorf tubes (in duplicate) and incubated with 10 µl of ^{35}S-TbPEX5 in the presence of an ATP regenerating system (3 mM $MgCl_2$, 1 mM ATP, 10 mM creatine phosphate and 50 µg/ml creatine kinase) at 26 °C for 30 min. *In vitro* import of ^{35}S-TbPEX5 was verified in one of the duplicate reaction mixtures by a protease protection assay performed as described above. After stopping the reaction by the addition of PMSF, a sample of this proteolytically treated material, corresponding to 10% of the total suspension, was taken and centrifuged at 20,000 x *g* in a T15A61 VWR rotor for 30 min at 4°C. The supernatant was collected and the organellar pellet was washed with 1 ml of import buffer before finally resuspending it in 100 µl of import buffer. The second duplicate of the *in vitro* import reaction, after its incubation period, was centrifuged at 20,000 x *g* in the same rotor during 30 min at 4°C and used for the *in vitro* export reaction. The supernatant was recovered and the organellar pellet resuspended in an equivalent volume of the corresponding cytosolic fraction in the presence of the ATP regenerating system and then split in two

equal parts. The export reaction was performed at 26°C for 30 min and stopped on ice (a negative control had been kept on ice). Reaction mixtures were then separated in a cytosolic and organellar fraction by centrifugation at 20,000 x *g* in the same rotor for 30 min at 4°C. Fractions of the *in vitro* import and *in vitro* export were analyzed by SDS-PAGE followed by autoscanning of the gels in a Typhus scanner (GE Healthcare) and by western blotting.

6.12. Pulse-chase experiments

Pulse-chase experiments were done as Coppy *et al.* (2005). A culture of 120 ml exponentially growing procyclic-form trypanosomes ($5x10^6$ cells/ml) was centrifuged at 700 x *g* in a HS-4 Sorvall rotor and resuspended in 40 ml of SDM79 medium without methionine. Cells were metabolically labelled with 250 µCi/ml ^{35}S-methionine during 1 h at 28°C and then chased by addition of an excess of non-labelled methionine (5 mM). Samples of 12.5 ml were taken at 0, 1.5 and 3 h and processed for TbPEX5 immunoprecipitation as described above. Elution fractions were analyzed by SDS-PAGE followed by autoscanning of the gels as well as by western blotting.

6.13. Proteasome inhibition and cellular fractionation.

Exponentially growing *T. brucei* wild type bloodstream ($5x10^5$ cells/ml) (100 ml) and procyclic ($5x10^6$ cells/ml) forms, the procyclic RNAi cell lines RNAiPEX6 and RNAiPEX12, as well as procyclic ΔPEX4 cells (50 ml) were treated for 6 h with 1 µM of the proteasome inhibitor lactacystin (Sigma). Cells were centrifuged at 700 x *g* in a HS-4 Sorvall rotor and washed once in STE buffer. Cell pellets were resuspended in a minimal volume of STE buffer (300 µl) and the protein content in each cell suspension quantified by the Bradford method. Aliquots of cells containing 300 µg of protein were mixed with HBSS-IP-NEM buffer (HSSP-IP buffer supplemented with 20 mM of N-ethylmaleimide (NEM) to adjust each volume to 300 µl. Cells were treated with 0.1 mg digitonin/mg of protein) for 4 min at room temperature and immediately centrifuged at 20,000 x *g* in a T15A61 VWR rotor for 2 min at 4°C. 280 µl of the supernatant (cytosolic fraction) were taken. The organellar pellet was washed once in HSSB-IP-NEM buffer, centrifuged at 20,000 x *g* for 2 min at 4°C and finally resuspended in 150 µl of

HSSB-IP-NEM buffer. Supernatant (135 µl) and pellet samples (67.5 µl) were diluted for SDS-PAGE in 4X Laemmli buffer containing or not DTT (25 mM). Samples containing DTT were boiled at 100°C for 5 min, while samples without DTT were heated at 56°C for 10 min. The proteins in 60 µl of the preparations were resolved by SDS-PAGE and analyzed by western blotting.

6.14. RNAi experiments

T. brucei bloodstream and procyclic RNAi cell lines for TbPEX5 (described previously by Galland *et al.,* 2007), TbPEX4 and TbPEX22 were induced with 1 µg/ml and 5 µg/ml of tetracycline in exponentially growing cells and the cell density was daily determined. Samples were taken after 24 (BfTbPEX5 and PFTbPEX5) and after 24, 48, and 72 h for TbPEX4 and TbPEX22, while diluting cultures up to the initial cellular density after sample collection. Cells were collected and processed for total RNA extraction (as described) and for cytosolic and organellar protein extraction. TbPEX5RNAi protein extraction was done by lysis of the cells in PBS-CHAPS (0.6%) supplemented with a protease inhibitor cocktail (Roche) during 30 min at 4°C. Two cycles of sonication (15 s each) at 4°C were performed before centrifugation at 16,000 x *g* in a T15A61 VWR rotor. The soluble fraction was collected for western blot analysis. TbPEX4RNAi and TbPEX22RNAi protein extraction was done by using the commercial ProteoJET Membrane protein extraction kit (Fermentas) following the manufacturer's instructions. Cytosolic and organellar fractions were obtained as described and further analyzed by SDS-PAGE followed by western blotting.

7. Live cell imaging

Live cell imaging was performed as described by Price *et al.* (2010). Briefly, $1x10^7$ procyclic-form *T. brucei* cells (wild-type and ΔPEX4 expressing GFP-PTS1) were centrifuged at 980 *x g* in a T15A61 VWR rotor for 10 min at 4°C. Cell pellets were gently washed in ice-cold PBS and finally resuspended in 50 µl of ice-cold PBS. 10 µl of each cell suspension was gently mixed by flickering with 200 µl of ice-cold PBS-Cygel (Alexis-Biochemicals) and kept on ice. A drop of 30-40 µl of the mixtures were spotted onto 50x22 mm glass coverslips and

immediately covered with a similar coverslip allowing the cells to spread. Preparations were allowed to solidify and were immediately analyzed in a Zeiss Axiovert 200 inverted fluorescence microscope coupled to an MRC-1024 confocal scanning laser imaging system (BioRad). Digital images were captured using the software AxioVision 4.8.2 (Zeiss).

8. Transmission electron-microscopy

Trypanosomes were collected by centrifugation at 700 x *g* in a HS-4 Sorvall rotor for 10 min at 4°C, washed in PBS-Ca/Mg (PBS, pH 7.0, with 3.6 mM $CaCl_2$ and 3.0 mM $MgCl_2$) and fixed by resuspension of the pellets in 2% glutaraldehyde in phosphate buffer, pH 7.0. After incubation for 30 min at 4°C the samples were washed by centrifugation in veronal buffer, post-fixed in 1% OsO_4 in veronal buffer for 1 h at 4°C, washed by centrifugation in veronal and finally pelleted in 2% agar. Small blocks of the pellet were stained "in bloc" in 0.5% uranyl acetate in veronal buffer overnight in the dark at 4°C. The next day, after extensive washing, the blocks were dehydrated in a graded series of ethanol and embedded in Spurr. Ultrathin sections of 70 nm were made with a Reichert Ultramicrotome and sections on grids were stained with uranyl acetate and lead citrate, 10 min each. Samples were examined by transmission electron microscopy (Philips, CM12) at 80 kV.

9. Scanning electron-microscopy

Trypanosomes were allowed to sediment on poly-L-lysine coated coverslips for 30 min. The samples were subsequently rinsed three times in PBS-Ca/Mg (PBS, pH 7.0, with 3.6 mM $CaCl_2$ and 3.0 mM $MgCl_2$) at room temperature and fixed in successively 0.1 M cacodylate buffer with 0.1 % glutaraldehyde for 30 min, 1% glutaraldehyde for 30 min and finally 2% glutaraldehyde overnight. After three wash steps in 0.1 M cacodylate buffer, post-fixation was performed in 1% OsO_4 in cacodylate buffer for 4 h. Subsequently, the samples were washed extensively in this buffer for 30 min, then 6 x 10 min in water and finally dehydrated by overnight incubation in 70% ethanol. The next day samples were further

dehydrated in a graded series of ethanol, critical point dried, mounted and sputter coated with a 10 nm gold layer. Samples were then examined in a CM12 electron microscope (Philips) at 60 kV by use of the secondary electron detector (scanning mode).

Analysis: Images were imported in AxioVision (4.8.2; Zeiss, Germany)
Statistics: Statistical evaluation was performed with SYSTAT (version 10, SPSS, Chicago, USA)

10. Mass spectrometry

TbPEX5 from bloodstream and procyclic form trypanosomes was immunoprecipitated as described above. Elution samples were analyzed by SDS-PAGE followed by western blotting. Duplicate gels were run and protein staining was done with Krypton (BioRad) following the manufacturer's instructions. Gels were scanned in an Odyssey station (LI-COR) and protein bands corresponding to TbPEX5, at 100 kDa and 70 kDa, were excised for mass spectrometric analysis.

11. Yeast two-hybrid experiments

Yeast two-hybrid analyses in strains SFY526 and HF7c were performed as described by Fransen *et al.* (2001). Double transformants were selected on SD synthetic medium without tryptophan and leucine. Interactions were tested using the β-galactosidase filter assay.

Chapter 5

Study of the molecular mechanism involved in the recycling of matrix protein receptor, PEX5, during glycosome biogenesis in *Trypanosoma brucei*

Results and discussion

This chapter presents our investigation of glycosome biogenesis in *T. brucei*. The main objective of this research project has been the elucidation of the recycling mechanism of TbPEX5, the glycosomal matrix protein receptor responsible for the transport of PTS-1 proteins to the glycosomes. Equally relevant has been the identification of new factors involved in TbPEX5 recycling: the ubiquitin-conjugating enzyme TbPEX4 and its membrane-anchor protein TbPEX22.

1. Determination of the nature of different TbPEX5 forms in wild-type *T. brucei*.

The *T. brucei* glycosomal matrix protein receptor TbPEX5 has been previously identified and functionally characterized by de Walque *et al*. (1999) and Galland *et al.* (2007). This work established that TbPEX5 is essential for bloodstream and procyclic trypanosomes, interacts with TbPEX7 and is required for import of PTS-1, PTS-2 as well as I-PTS proteins. Additionally, Krazy and Michels (2006) identified and functionally characterized members of the PEX5 recycling complex, TbPEX6, TbPEX10 and TbPEX12, which were also shown to be essential peroxins for glycosomal protein import. Their homologues have been previously demonstrated to be directly involved in the recycling of PEX5 in yeast, mammalian and plant cells. In *T. brucei*, however, insight into the function of these peroxins in the molecular mechanism of TbPEX5 recycling has not been yet obtained. Interestingly, non-published data obtained by Krazy and Michels, indicate the intriguing existence of multiple forms of TbPEX5 with different molecular weight in wild-type cells as well as in the RNAi cell lines of these peroxins (RNAiTbPEX6, RNAiTbPEX10 and RNAiTbPEX12).

TbPEX5 is encoded by a single copy gene (Tb927.5.1100) with an ORF that predicts the synthesis of a protein with a subunit molecular weight of 72 kDa.

However, western blot analysis performed with a polyclonal antiserum raised against a quasi full-length recombinant protein revealed two different molecular weight bands: a band that migrates at 72 kDa as predicted and a slower migrating band of 80-100 kDa.

As an initial step in our research on the elucidation of the molecular mechanism involved in the recycling of the matrix protein receptor TbPEX5, we have employed several techniques that aimed to identify the nature of the different molecular weight forms of TbPEX5 previously observed.

1.1. PEX5 antibody purification and western blot analysis.

In order to establish the specificity of the TbPEX5 antiserum used in previous western blot analyses, we have performed an antibody purification based on affinity chromatography. First, we have expressed insoluble C-terminally His-tagged TbPEX5 in *E. coli* (Figure 17A). We managed to obtain a nearly pure recombinant protein after washing the inclusion bodies with detergents, followed by a denaturing step with a high concentration of urea. To further improve the purity, the His-tagged-TbPEX5-urea fraction was loaded on a column of Talon resin with immobilized cobalt under denaturing conditions. Intriguingly, the majority of the recombinant TbPEX5 was recovered in the flow-through and washes (Fig. 17B) with a very minor amount present in the elution fractions (data not shown). Subsequently, the TbPEX5-urea fraction was covalently bound to a CNBr-Sepharose column and used as bait to purify antibodies from the polyclonal antiserum raised against TbPEX5. When tested on a total extract from *T. brucei* procyclic and bloodstream-form cells, the purified antibodies showed, similarly as the non-purified TbPEX5 antiserum, that TbPEX5 exists as at least two forms with different molecular weights: the form with the predicted weight of 72 kDa and another slower migrating form of 100 kDa (Fig. 17C). This result confirmed the specific recognition of the TbPEX5 forms in trypanosome lysates by the antiserum. This purified antibody was used in all subsequent experiments related to the identification of TbPEX5 forms.

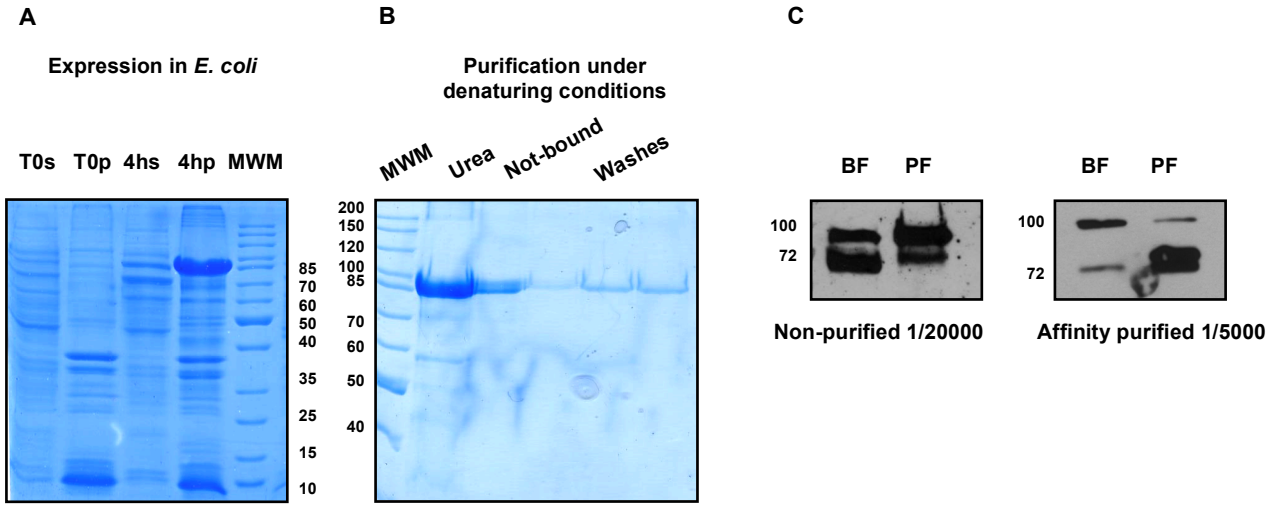


Figure 17. **A**. ***Expression of recombinant TbPEX5 in E. coli.*** . MWM: molecular weight markers. T0s: lysate non-induced soluble fraction. T0p: lysate non-induced pellet fraction. 4hs: lysate 4 hours of IPTG induction soluble fraction. 4hp: lysate 4 hours of IPTG induction pellet fraction. **B**. ***Purification of recombinant TbPEX5 under denaturing conditions.*** Inclusion bodies containing insoluble C–terminally His-tagged TbPEX5 were washed with detergents and finally denatured in the presence of 8 M urea. TbPEX5-urea was used as a starting material for the TbPEX5 purification by affinity chromatography using immobilized cobalt (Talon resin) under denaturing conditions. TbPEX5 was mainly recovered in the non-bound fractions. The TbPEX5-urea fraction was then used as antigen for TbPEX5 antibodies purification. **C.** ***Western blot analysis of different TbPEX5 forms***. A similar profile (recognition of two bands) was obtained using non-purified and affinity purified anti-PEX5 antibodies in bloodstream (BF) and procyclic (PF) form *T. brucei*. (Note that proteins in the blotted gels were separated at different resolutions).

1.2. TbPEX5 expression silencing by RNA interference.

After having tested the purified TbPEX5 antibodies, which show high specificity, we analyzed the available TbPEX5 RNAi cell line in order to see if any changes in the abundance of the different TbPEX5 molecular weight bands after RNAi induction would be coordinated. Protein samples were collected 24 h after induction by tetracycline and immunoblotting was performed. As shown in Figure 18, the two most intense bands (70 and 100 kDa) present in bloodstream and procyclic wild-type cells, disappeared completely after RNAi induction. This indicates that indeed TbPEX5 exists as at least two different molecular weight forms in *T. brucei* wild-type cells.

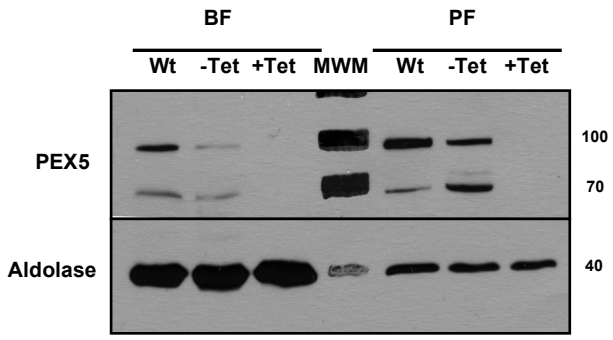


Figure 18. ***RNAi silencing of TbPEX5***. TbPEX5 RNAi cell lines were grown in the presence (+Tet) and absence of the inducer tetracycline (-Tet). Samples of wild type (Wt), induced and non-induced cells were taken after 24 h after induction of the (+Tet)-cells and processed for immunoblotting using affinity purified TbPEX5 antibodies.

1.3. Mass spectrometry analysis of immunoprecipitated TbPEX5.

As a first attempt to isolate native TbPEX5 to perform a further analysis of the specific nature of the distinct forms, we have immunoprecipitated them under denaturing conditions from *T. brucei* wild-type procyclic and bloodstream forms. Initially, different amounts of the polyclonal non-purified TbPEX5 antibodies were tested to standardize the proper conditions. As shown in Figure 19,, the two bands of 72 kDa and 100 kDa detected after western blotting in lysates of bloodstream and procyclic form trypanosomes, using the purified anti-TbPEX5, were successfully immunoprecipitated with 1 µl of TbPEX5 antiserum as seen in the elution fractions (Fig. 19A), strongly suggesting once more that indeed the two bands correspond to TbPEX5. The total protein mixture of the immunoprecipitated material, containing PEX5 forms as confirmed by western blotting, was separated by SDS-PAGE and the proteins were subsequently stained using the Krypton Infrared Protein Stain reagent. The gel was scanned by infrared light and the bands corresponding to the known sizes of immunoprecipitated PEX5 forms were excised from the gel. The corresponding proteins were subjected to overnight trypsin digestion and then the tryptic peptides analyzed by mass spectrometry (in collaboration with Dr Didier Vertommen of the PHOS-Unit, de Duve Institute). The masses of the detected peptides were "BLASTed" against the *T. brucei* trypsin peptide database. The resulting peptide profiles indicate that in both bloodstream and procyclic forms the two immunoprecipitated proteins (72 kDa and 100 kDa) recognized by the

purified anti-PEX5 antibodies, correspond indeed to the PTS-1 receptor TbPEX5 (Table 2). It should be mentioned that the peptide profile obtained was not sufficient to reconstruct the entire TbPEX5 protein sequence. Therefore, it was impossible to determine whether the 72 or 100 kDa form has either a N-terminal/C-terminal deletion or extension, respectively, or posttranslational modifications that could explain the difference in molecular weight.

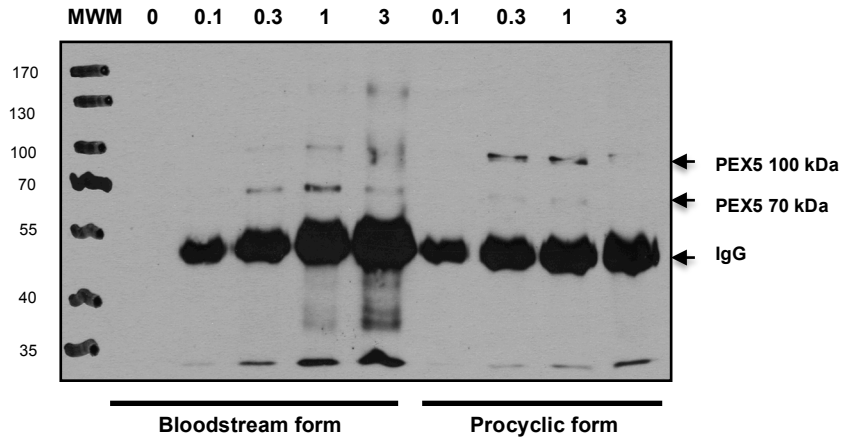


Figure 19. ***Two different molecular weight forms of TbPEX5 are immunoprecipitated***. TbPEX5 immunoprecipitation was performed using different volumes (µl) of the non-purified TbPEX5 antibodies in detergent-treated total lysates of bloodstream and procyclic trypanosomes. The eluted proteins were used for immunoblotting with affinity purified TbPEX5 antibodies. The best condition for immunoprecipitation was proven to involve the use of 1 µl of TbPEX5 antibodies.

Table 2. ***Mass spectrometry analysis of immunoprecipitated TbPEX5*.**

Bands	Acc.	Cove	Pept	#Aas	MW (Da)	Score	Description
BF1 (100 kDa)	TbPEX5	19.08	7	655	72049	63.48	|Peroxisome targeting signal 1 receptor|Trypanosoma brucei|chr 5|TIGR||Manual
BF2 (70 kDa)	TbPEX5	4.73	2	655	72049	17.20	|Peroxisome targeting signal 1 receptor|Trypanosoma brucei|chr 5|TIGR||Manual
PF1 (100 kDa)	TbPEX5	12.37	4	655	72049	57.48	|Peroxisome targeting signal 1 receptor|Trypanosoma brucei|chr 5|TIGR||Manual
PF2 (70 kDa)	TbPEX5	7.18	4	655	72049	33.91	|Peroxisome targeting signal 1 receptor|Trypanosoma brucei|chr 5|TIGR||Manual

Cove: Sequence coverage in percentage
Pep: Number of unique peptides identified
Aas: Length of polypeptide
Score: as calculated by algorithm sequest

1.4. Behaviour of recombinant TbPEX5 in SDS-PAGE.

In initial studies it has been observed that PEX5 from plants, mammals and *P. pastoris* presented aberrant migrations pattern in SDS-PAGE (Wiemer *et al.,* 1998; Wiemer *et al.,* 1995; McCollum *et al.,* 1993) with higher apparent molecular weights than predicted from their amino-acid sequences. Indeed this was also reported for recombinant and native TbPEX5 (de Walque *et al.,* 2001). In order to explore more profoundly if TbPEX5 behaves in a similar way, we have expressed the recombinant full-length TbPEX5 in *E. coli* and analyzed the migration pattern in SDS-PAGE followed by western blotting. As shown in Figure 20, the N-terminally and C-terminally His-tagged TbPEX5 migrate both with an apparent molecular weight of 100 kDa. This molecular weight is about 25 kDa higher than the predicted one of the His-tagged full-length recombinant protein (74 kDa). This result suggests that the native full-length TbPEX5 may behave in a similar way in SDS-PAGE, which explains the observation made in all previous experiments where TbPEX5 seemed to exist in a higher molecular weight form than expected from its amino-acid sequence. This result indicates indirectly that, under the conditions tested, the faster migrating form of 72 kDa is a modified form, somehow derived by proteolytic degradation or processing from the 100 kDa TbPEX5 or by an alternative splicing process.

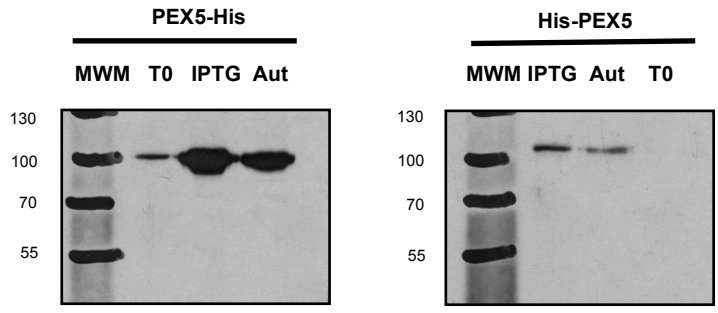


Figure 20. ***Behaviour of recombinant TbPEX5 in SDS-PAGE***. Total proteins (10 µg) from *E. coli* strain BL21 which expressed the full-length TbPEX5 with either a N-terminal or C-terminal His-tag, were analyzed by SDS-PAGE followed by western blotting using an anti-His antiserum. Two conditions for expression were tested: 1 mM of IPTG (IPTG) and use of Autoinduction medium (Aut). (MWM: molecular weight markers; T0: Non-induced).

1.5. TbPEX5 susceptibility to parasite endogenous proteases.

In order to explore the possibility that the native TbPEX5 (100 kDa) can be modified by endogenous proteases we have analyzed the presence of TbPEX5 in *T. brucei* bloodstream and procyclic lysates prepared in the presence of protease inhibitors cocktails obtained from different commercial sources. As shown in Figure 21A, in *T. brucei* bloodstream and procyclic total lysates, made in the absence of protease inhibitors, the abundance of the slower migrating form of TbPEX5 was importantly decreased in both developmental forms, whereas the shorter form remained not affected (Notice that in the case of the bloodstream form lysate, an exceptional strong proteolysis of the shorter form occurred in this experiment, that was not observed in any of the subsequent experiments). By contrast, whenever a protease inhibitor cocktail was included in the lysate (no matter which commercial source), the higher molecular weight form was more stabilized and the shorter one remained equally stable. This result may suggest that the full-length TbPEX5 is susceptible to (an) unknown parasite protease(s) present in the lysate, which is (are) only partially inhibited by common protease inhibitors. It should be mentioned that all biochemical experiments performed so far in this research project, involved the disruption of trypanosomes in a classical lysis (detergent containing) buffer, which was always supplemented with a protease inhibitor cocktail. Under these conditions, we observed systematically two molecular weight forms of TbPEX5 and, in procyclic cells, occasionally three (72, 80, 100 kDa).

Since PEX5 is known as a cytosolic PTS-1 receptor that docks at the peroxisomal/glycosomal membrane and –as shown for yeast peroxisomes– forms a transient pore in the membrane (Meinecke *et al*., 2010), we intended to identify which pool of TbPEX5 –the main one (cytosolic TbPEX5) or the glycosome associated one (membrane bound TbPEX5)– was affected by the proteolytic modification. As a strategy, we have produced lysates of the *T. brucei* procyclic form in the presence and absence of protease inhibitor cocktails from different commercial sources. We have used a commercial protein extraction system (ProteoJet Membrane Extraction Kit, Fermentas) that we have tested for use in the production of a cytosolic fraction by disruption of the plasma

membrane without affecting the integrity of organellar (glycosomal) membranes.

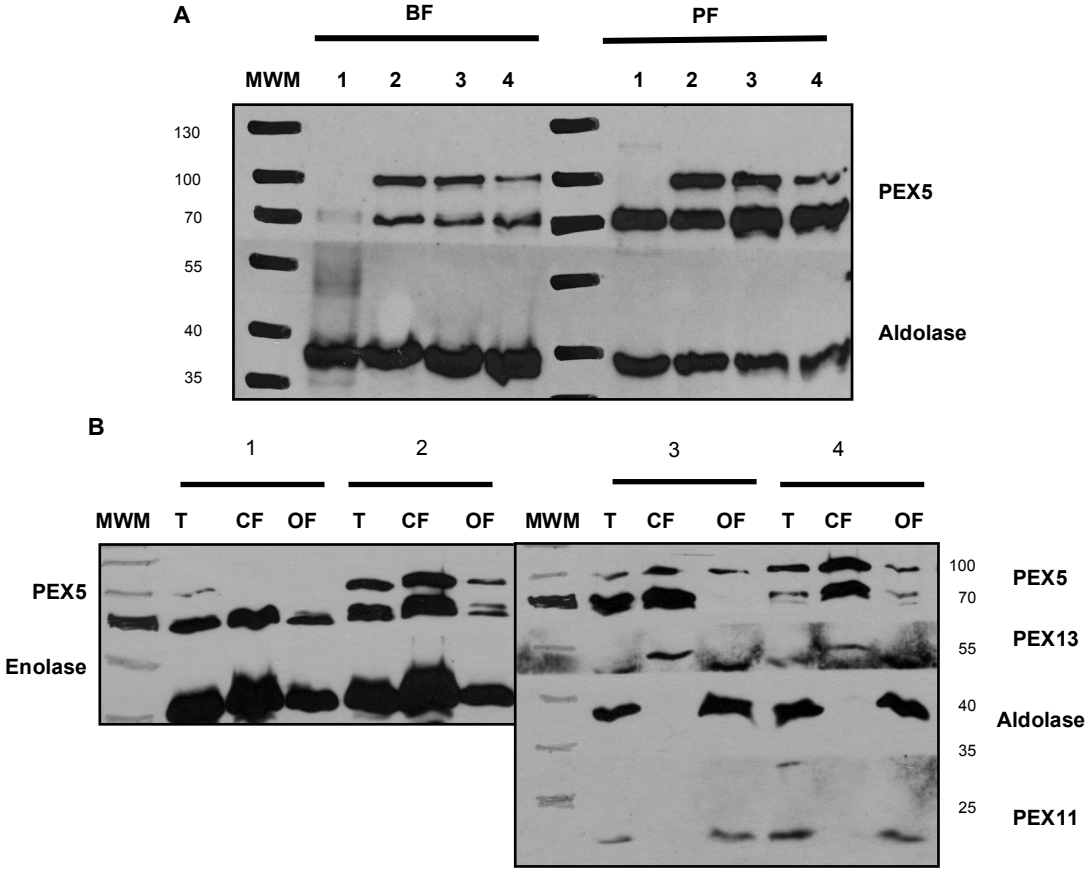


Figure 21. ***Susceptibility of TbPEX5 to endogenous proteases***. **A**. Total lysates of bloodstream and procyclic form *T. brucei* were incubated overnight in the presence or absence of different cocktails of protease inhibitors. Samples were analyzed by western blotting using anti-PEX5 purified antibodies. 1: No protease inhibitors, 2: Home-made protease inhibitors cocktail, 3: Fermentas® Proteoblock, 4: Roche ®. **B**. *T. brucei* procyclic cells were lysed in the presence or absence of different cocktails of protease inhibitors. 1: No protease inhibitors, 2: Home-made protease inhibitors cocktail, 3: Fermentas® Proteoblock, 4: Roche ®. After centrifugation at 16,000 x *g* the total lysate was separated in a cytosolic fraction (CF), which contains the majority of TbEnolase and an organellar fraction (OF) in which several glycosomal proteins were shown to be present: Aldolase, PEX13 and PEX11.

After separation of the cytosolic proteins from the organellar fraction by centrifugation at 16,000 *x g*, we have evaluated the abundance of different forms of TbPEX5 by western blot analysis. Figure 21B shows once more that the TbPEX5 slower migrating form (100 kDa) is susceptible to proteases in the absence of protease inhibitors. This processing was still observed in the presence of protease inhibitors from different sources, as observed in the previous experiment. The two TbPEX5 forms were more abundant in the cytosolic fraction (rich in enolase) than in the organellar fraction that contains

the glycosomal marker proteins TbPEX11 and aldolase, thus confirming its expected subcellular location. We also observed that the two forms of TbPEX5 were detected at much lesser extent in the organellar fraction, which was expected since this peroxin cycles between the cytosol and glycosomal membrane. However, we cannot exclude that the detection of this protein could (also) have been due to a cross-contamination of the organellar fraction with cytosol, since the cytosolic marker enolase was also well detectable in the organellar fraction. Assuming that the TbPEX5 forms detected in the organellar fraction were indeed due to their real, physiological presence in the glycosomal membrane, this result could suggest that the proteolytic processing of TbPEX5 occurs in the cytosol leading to a faster migrating form that could also interact with the glycosomal membrane while importing PTS-1 proteins. An alternative explanation could be that the unknown protease is also present in the glycosomal membrane where it would be able to process the TbPEX5 molecules that reach the organelle in their act of performing a round of import.

1.6. Subcellular distribution of TbPEX5 and its association with glycosomes.

In the previous experiment we could observe that when *T. brucei* bloodstream and procyclic forms were treated with a commercial protein extraction system (ProteoJet Membrane Extraction Kit, Fermentas) containing mild detergents, cytosolic proteins (including the majority of the two forms of TbPEX5) were released in the supernatant. Notably, a small amount of TbPEX5 was detected in the organellar fraction where the major part of each of the glycosomal markers such as aldolase and TbPEX11 (Fig. 21B) and other glycosomal membrane peroxins such as PEX13 and PEX14 (data no shown) remained associated.

In order to determine with more accuracy the capacity of the different forms of TbPEX5 to interact with the glycosomal membrane, we have analyzed the presence of TbPEX5 in glycosomes-enriched fractions obtained by isopycnic centrifugation of a large granular fraction of procyclic cells using a commercial medium (Optiprep) (Fig. 37B). Interestingly, we detected the two main forms of TbPEX5 (72 and 100 kDa) (data not shown) in the fractions 13-16, rich in the glycosomal markers aldolase and PEX11. This result indicates that also the

processed form of TbPEX5 might be able to interact with the docking complex (TbPEX14 and TbPEX13) present in the glycosomal membrane. This would imply that some part of the unstructured N-terminal region of TbPEX5, known to be responsible for interaction with TbPEX14 and TbPEX13, must have been preserved from the proteolytic processing. Previously, it has been shown that, of the three pentapeptide motifs present in the N-terminal half of TbPEX5, no. 1 and 3 can bind to TbPEX14 (Choe *et al*., 2003) and no 3 also to TbPEX13 (Verplaetse *et al*., 2009), indicating that if an interaction between the processed receptor and the docking complex occurs, it must happen exclusively through the third pentapeptide motif of TbPEX5.

1.7. Functional study of the different molecular weight forms of TbPEX5 by PTS-1 in vitro binding assays.

In order to study if the two main forms of TbPEX5 are equally functional for PTS-1 import, we have used an *in vitro* binding assay in which recombinant purified His-tagged PGK (a PTS-1 containing protein) was immobilized in an cobalt-based affinity resin (Talon) and used as a ligand for native TbPEX5. Since the interaction between PEX5 and the PTS-1 peptide involves PEX5's C-terminal TPR containing domain, we hoped to gain knowledge if this domain would have been affected in the fast migrating form of TbPEX5 (72 kDa). Lysates were produced from *T. brucei* bloodstream and procyclic forms in the presence of proteases inhibitors and were first cleared from non-specifically interacting proteins in a PGK-free Talon resin. The cleared lysate was then incubated with the Talon-PGK resin. The protein content of the original lysates, as well as the solutions collected after the wash steps of the column and fractions obtained with imidazole concentrations that, in prior experiments, had been shown to cause elution of PGK from the column were analyzed by western blot using anti-His and affinity purified anti-PEX5 antibodies. As shown in Figure 22, in both bloodstream (A) and procyclic (B) cells, we could detect the 100 and 72 kDa protein in the initial cell lysate. After clearing the lysate from the non-interacting proteins we could only detect the faster migrating form of TbPEX5 (72 kDa) indicating that 1) the overnight incubation at 4°C was sufficiently long to allow the unknown protease to process all molecules of the 100 kDa form (as a result

of the time-dependent loss of activity of the protease inhibitors cocktail) or 2) that the slower migrating form of TbPEX5 has affinity for the cobalt ions of the non-PGK containing resin. We could, however, exclude this latter possibility since western blot analysis of the fraction eluted from such an empty resin for the presence of TbPEX5 was negative (data not shown). Taking into account that only the TbPEX5 form of 72 kDa and not the native one of 100 kDa was present in the cleared lysate, we restricted our *in vitro* binding study to this species of TbPEX5. Western blot analysis showed that TbPEX5 (72 kDa) was recovered in the TbPGK eluted fraction indicating the capability of this TbPEX5 form to interact with the PTS-1 motif present in TbPGK. This is an indication that the C-terminal TPR domain of TbPEX5 is functional for PTS-1 binding. This capacity implies that the structure of the domain is intact and therefore this part of the protein is not shortened.

One can then speculate that this faster migrating form is produced by a proteolytic degradation at the N-terminal side of the full-length TbPEX5 (100 kDa). This would be in agreement with the results of the TbPEX5 interaction with the glycosomal membrane, since the region responsible for the binding of TbPEX5 to the docking complex (TbPEX13-TbPEX14) includes the third pentapeptide motif (Fig. 22B), which is positioned between amino acids 318-322, leaving an area of more than 30 kDa for N-terminal proteolytic processing of the 100 kDa native form TbPEX5 to produce a TbPEX5 of 72 kDa that is still capable of interacting with glycosomes.

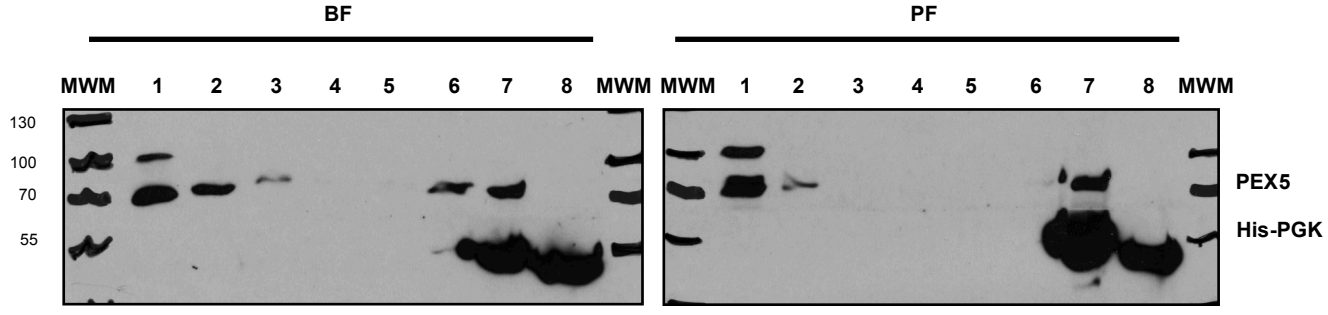


Figure 22. ***The short form of TbPEX5 interacts with the PTS-1 motif containing TbPGK***. Total lysates of bloodstream and procyclic form *T. brucei* were analyzed by affinity chromatography under native conditions using a Talon resin with N-terminally His-tagged TbPGK bound. The TbPGK interacting proteins were eluted with imidazole and further analyzed by western blotting using affinity purified anti-PEX5 and anti-His antibodies for PGK detection. 1: Total lysate, 2: Total lysate after free-Talon incubation, 3: Elution from free-Talon resin (Non-specific interacting proteins), 4: Flow-through from PGK resin, 5: Wash 1, 6: Wash 2, 7: Elution with 50 mM imidazole, 8: Elution with 100 mM imidazole.

1.8. Turnover analysis of different molecular weight bands of TbPEX5. Intending to study the stability of the different forms of TbPEX5, we have performed turnover studies where the synthesis of new proteins was impaired by the addition of cycloheximide, a drug that interferes with translational elongation in eukaryotes. The turnover of TbPEX5 as well as other control proteins was analyzed by western blot. It should be mentioned that, due to the effect on the protein translation caused by this drug, cell growth is compromised during the treatment. For this reason we decided to study the turnover of TbPEX5 for a period of 6 h to avoid the cells entering in cellular arrest. Figure 23 shows that in bloodstream and procyclic forms the levels of the slower migrating form of TbPEX5, enolase and aldolase remain stable during 6 h of cycloheximide treatment, indicating a slow turnover. However, the shorter form of TbPEX5 seems to be less abundant after 6 h of the treatment, which suggests that this form has a degradation rate slightly faster than the 100 kDa TbPEX5 form. The impossibility to study in this experiment a control protein with a half-life shorter than the glycolytic enzymes, because of lack of an antiserum for a rapidly turned-over protein, makes it difficult to conclude if the two forms of TbPEX5 differ significantly in turnover rate, and hence if they are equally recycled.

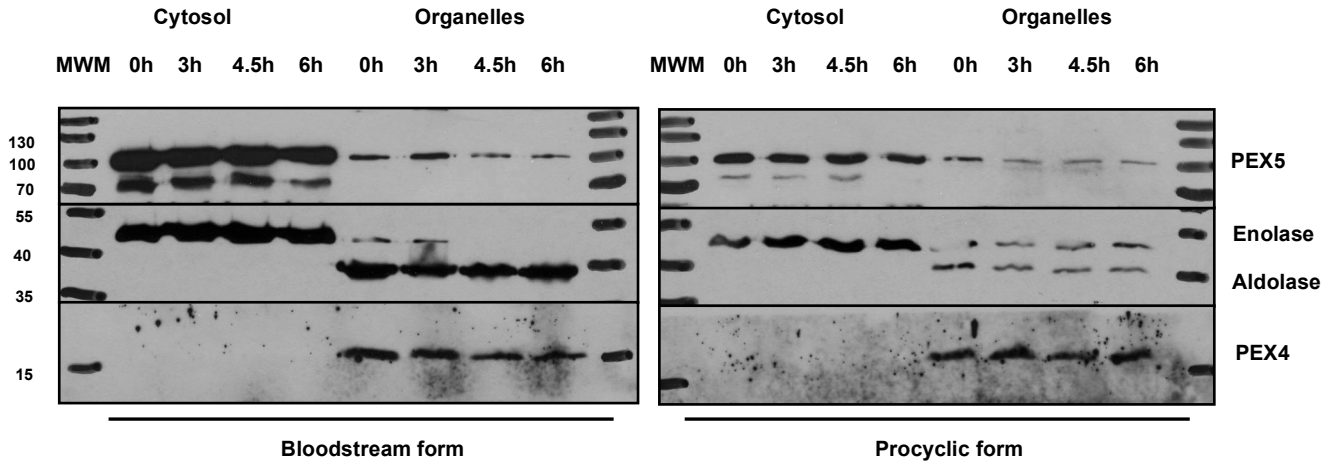


Figure 23. ***Analysis of TbPEX5 turnover.*** *T. brucei* bloodstream and procyclic forms were grown in HMI-9 and SDM79, respectively, in the exponential phase. Then, cultures were supplemented with cycloheximide. Samples were collected 0, 3, 4.5 and 6 h after the drug had been added. Cells were lysed in the presence of a cocktail of protease inhibitors and separated in cytosolic and organellar fractions after centrifugation at 16,000 x *g*. Western blot analyses were done using 40 µg of proteins (bloodstream cytosol and organellar fractions), 10 µg (procyclic cytosol) and 30 µg (procyclic organellar). Antibodies against TbPEX5 (affinity-purified), TbPEX4 and control proteins like Enolase and Aldolase were used.

***1.9. Metabolic labelling of TbPEX5*:** Several lines of evidence suggested to us that the native TbPEX5 species is object of a form of proteolytic processing that cannot be totally inhibited by protease inhibitors cocktails. We have observed this consistently, after disruption of the cells using whatever lysis buffer: Commercial ProteoJet Membrane Extraction Kit (Fermentas), Home-made PBS-CHAPS 0.6%, Home-made RIPA Buffer, Home-made PGK-binding buffer, or by direct resuspension of the cellular pellet in 1X Laemmli buffer) and always in the presence of standard protease inhibitor cocktails from different commercial sources as well as home-made cocktails. One important question about this processing was whether it occurs *in vitro* when proteases are released from lysosomes and/or other cellular compartments during cell lysis by detergents, or if it occurs also *in vivo* inside the cell. To address this question we performed pulse-chase metabolic labelling of TbPEX5 in wild-type procyclic form *T. brucei*. Our working hypothesis was based on the following, alternative assumptions: *(1) TbPEX5 processing is a physiological process*: In that case it occurs in live cells, probably by a specific protease. The expectation would be that the process is retarded or arrested upon lysis of the cells, when TbPEX5 and the protease are largely diluted. The ratio TbPEX5(100 kDa)/PEX5(72 kDa) will be a function of the rate of synthesis of TbPEX5 and its processing. Logically, these should together be in a steady state during growth, implying that the ratio remains constant. *(2) PEX5 processing is not physiological*, but is expected to occur upon lysis of the cell when proteases come in contact with the peroxin. Inside intact cells there would be virtually only TbPEX5(100 kDa), but upon breaking of the cells the ratio TbPEX5(100 kDa)/TbPEX5(72 kDa) decreases in time, with a (possibly first-order) rate and to an extent that depends on the conditions (that affect the activity of the proteases and their accessibility to the PEX5L).

The design of the experiment involved that a short pulse of radiolabelled ^{35}S-methionine, at a low concentration and with a high specific activity, would be given to the trypanosomes followed by a chase of non-labelled methionine at the usual, high concentration. This causes the synthesis of new TbPEX5 with a high ^{35}S-specific activity during the short pulse time, followed by synthesis of non-labelled TbPEX5. Consequently, the concentration of radiolabelled ^{35}S-TbPEX5

will decrease in time with a rate that is a function of the (regular) rate of TbPEX5 synthesis and TbPEX5 degradation (by proteasomes and/or autophagy). Assuming that TbPEX5 turnover in normally growing trypanosomes is low, which has been proved in the previous experiment, the dilution rate of labelled TbPEX5 is probably a function of the trypanosomes' growth rate.

If TbPEX5 processing is physiological, the expected result would be that the radiolabelled TbPEX5 is synthesized as the TbPEX5 100 kDa. This TbPEX5 is intracellularly converted into the TbPEX5 72 kDa in the regular way and with the regular speed. Therefore, the level of ^{35}S-TbPEX5 100 kDa decreases in time while that of ^{35}S-PEX5 72 kDa increases in time. However, ^{35}S-PEX5 100 kDa and ^{35}S-PEX5 72 kDa are both diluted with similar rates by non-labelled TbPEX5 forms. The combined result of these two processes is that the concentration of both ^{35}S-PEX5 100 kDa and ^{35}S-PEX5 72 kDa decreases in time, but also the ratio ^{35}S-PEX5 100 kDa / ^{35}S-PEX5 72 kDa in the successive samples. If TbPEX5 processing is not physiological, the expected results would be that ^{35}S-TbPEX5 is synthesized as TbPEX5 100 kDa. It remains intracellularly as ^{35}S-TbPEX5 100 kDa, but after the chase this radiolabelled form is diluted by non-labelled TbPEX5 100 kDa. Upon lysis of the cells, the ^{35}S-TbPEX5 100 kDa is (as the non-labelled TbPEX5 100 kDa) cleaved into ^{35}S-TbPEX5 72 kDa, until the proteolytic action is stopped (by inhibitors and or low temperature). If all samples taken in time are treated similarly, the ratio ^{35}S-TbPEX5 100 kDa / ^{35}S-PEX5 72 kDa is the same in each sample, but the total radioactivity of ^{35}S-TbPEX5 100 kDa + ^{35}S-PEX5 72 kDa decreases in the successive samples as a result of the chase.

We consider that the results may become more complicated if: TbPEX5 processing is physiological but the proteolytic process is not immediately stopped after lysis of the cells. Or if in both cases, physiological or not physiological processing, proteolysis is not stopped during storage of the samples on ice (in the presence of protease inhibitors) before their further processing for immunoprecipitation of TbPEX5 as seems to be the case from our previous experiments.

In the practical execution of the experiment, we chased with cold methionine during 0, 1.5 and 3 h and the collected fractions were used to immunoprecipitate TbPEX5. Figure 25 shows that non-labelled native TbPEX5 (100 kDa) was successfully immunoprecipitated while non-labelled TbPEX5 72 kDa was detectable only at a low level. This could suggest that poor proteolytic processing occurred or that the TbPEX5 antiserum did not recognize with high affinity this form of the protein. Figure 24 shows the autoscanning of the eluted fractions in which only a very faint band of the ^{35}S-TbPEX5 (100 kDa) was observed. Unfortunately, these results were not informative regarding the nature of the processing of TbPEX5, since we could not detect any ^{35}S-TbPEX5 (72 kDa) besides the non-labelled TbPEX5 (72 kDa) that was shown to be present by western blotting. This precluded to compare the ratio of the two forms as a function of the chase-time and hence to draw any conclusion about whether the proteolytic processing is physiological or not.

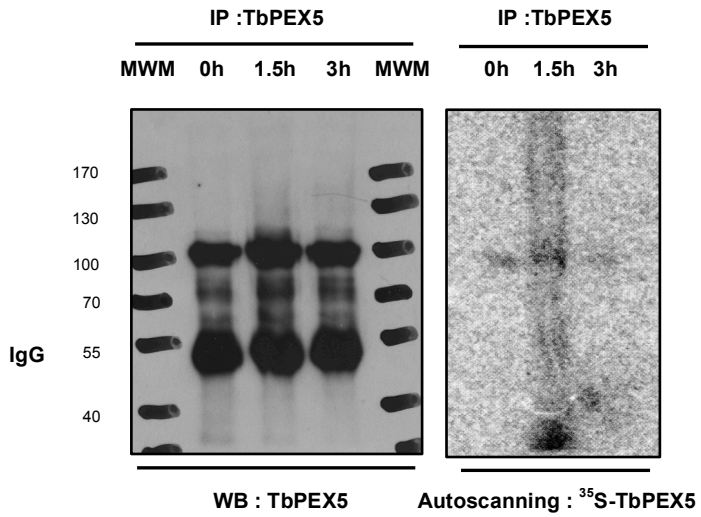


Figure 24. ***Metabolic labelling of TbPEX5***. Wild-type procyclic form *T. brucei* cells were metabolically labelled in SDM79 (-methionine) during 1 h with ^{35}S-methionine. A chase was done with an excess of cold methionine and samples were successively collected after 0, 1.5 and 3 h and kept on ice. Immunoprecipitation of TbPEX5 was performed and the elution fractions were analyzed by western blotting and autoscanning.

2. TbPEX5 Ubiquitination and its role in the receptor recycling

As has been described in Chapter 1, the current model of peroxisomal matrix protein import implies that the cytosolic receptor PEX5 cycles between the cytosol, where it recognizes the PTS-1 containing proteins, and the peroxisomal membrane, where its delivers its cargo into the peroxisomal matrix (Dammai *et al.,* 2001; Platta and Erdmann, 2007). It has been well established that in yeasts and mammalian cells, the receptor PEX5 is ubiquitinated at the peroxisomal membrane (Platta *et al*., 2004; Kiel *et al*., 2005a; Carvalho *et al*., 2007). Initially it was found that PEX5 was ubiquitinated in some yeast mutants where peroxins involved in the recycling of PEX5 were affected, notably the PEX4-PEX22 complex and the AAA^{+}-ATPases PEX1 and PEX6, and the latter's membrane anchor protein PEX15 (Platta *et al.,* 2004; Kiel *et al.,* 2005a). However, later it was found that the receptor PEX5 is also monoubiquitinated in wild-type cells and that this represents one of the recycling stages of PEX5 (Kragt *et al*., 2005). Moreover, recent research has identified that the monoubiquitination of PEX5 occurs at a conserved cysteine residue present in the N-terminal part of the protein (Grou *et al*., 2009). In *T. brucei* several homologues of peroxins involved in PEX5 docking and recycling have been identified and functionally characterized (Moyersoen *et al*., 2004; Galland *et al*., 2007; Verplaetse *et al*., 2009; Krazy and Michels, 2006; Saveria *et al*., 2006). Although an important function in glycosomal protein import has been assigned to these peroxins, based on the phenotypic analysis performed in the respective RNAi mutants, no studies about their specific function in the recycling of TbPEX5 have been performed so far. The studies of PEX5 recycling in yeasts and mammalian cells which support the notion that the molecular mechanism based on differential mono- or polyubiquitination of the matrix protein receptor PEX5 at the peroxisomal membrane regulates its recycling, raised the question whether TbPEX5 is also ubiquitinated and, if so, where in the cell this modification occurs and which proteins are involved.

2.1. Ubiquitination studies in wild-type bloodstream and procyclic form T. brucei.

After having determined the nature of the different molecular weight forms of TbPEX5 detected by western blot, and identified the 100 kDa species as the native form and the 72 kDa as a proteolytically processed form which is probably generated *in vitro* by specific parasite protease(s), we concentrated on the study of possible slower migrating forms of TbPEX5 (not yet observed) that could correspond to the migration pattern of TbPEX5 modified by the addition of 1, 2, 3 or 4 ubiquitin moieties.

Initially we intended to study TbPEX5 ubiquitination by western blot analysis using purified TbPEX5 antisera as well as TbUbiquitin antisera. For that purpose we have cloned, expressed and purified recombinant *T. brucei* Monoubiquitin (Fig. 25A) and used to raise a polyclonal antiserum (kindly produced by Dr. Juan Luis Concepción) that could provide better quality results (higher specificity and affinity) in the recognition of ubiquitinated proteins in *T. brucei* lysates than the already tested commercial human-ubiquitin antisera (Santa Cruz). The rationale for raising a TbUbiquitin specific antiserum was that it had been shown that, despite the high degree of identity in the primary structure of human and *T. cruzi* ubiquitin, the substitution of only a few amino acids was sufficient to distinguish parasite versus host proteins in immunological tests such as ELISA and western blot analysis (Telles *et al.,* 1999). In Figure 25B is shown the high affinity of the produced antiserum for the purified recombinant monoubiquitin protein. However, when comparing the anti-human and anti-*T. brucei* ubiquitin antisera, no improvement could be observed in the recognition of the ubiquitinated proteins in trypanosome lysates (data not shown). Indeed, both antisera generated a “smear”-like pattern probably due to the abundance of ubiquitinated proteins over a whole range of molecular weights present in *T. brucei* lysates.

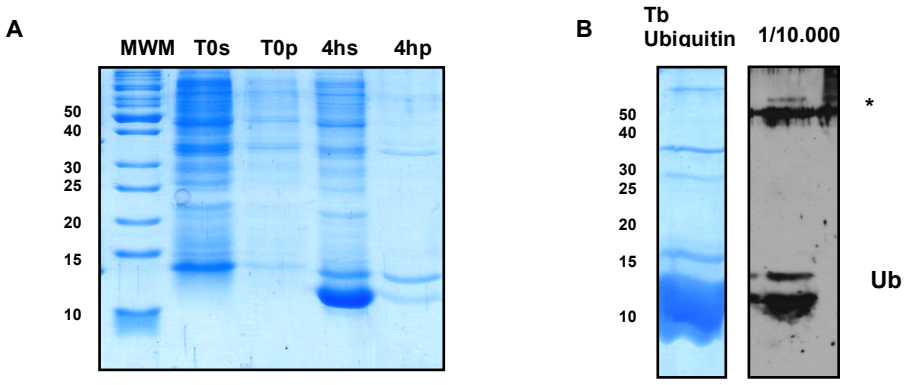


Figure 25. ***Expression of recombinant T. brucei Ubiquitin*** **in** ***E. coli*** **and purification**. **A**. MWM: molecular weight markers. T0s: lysate non-induced soluble fraction. T0p: lysate non-induced pellet fraction. 4hs: lysate 4 hours of IPTG induction soluble fraction. 4hp: lysate 4 hours of IPTG induction pellet fraction. **B.** Soluble His-tagged TbUbiquitin was affinity purified and used to raise polyclonal antiserum. The antiserum, at a 1/10,000 dilution, detected the recombinant protein. * Correspond to unspecific recognition by the antiserum.

When western blot analyses were performed using affinity purified TbPEX5 antibodies on total lysates (Fig. 26), as well as on an immunoprecipitated fraction of TbPEX5 (Fig. 19), no slower migrating TbPEX5 bands that could correspond to ubiquitin-modified TbPEX5 were detected. Attempts were done by studying the receptor in trypanosomes in their exponential and stationary growth phases, both bloodstream and procyclic cells, by cell fractionation (cytosolic and organelles separation by the commercial system ProteoJET, Fermentas) in the presence of the unspecific inhibitor of de-ubiquitinating enzymes (NEM) which has been proved to increase the detection of ubiquitinated species of PEX5 in other systems (Kragt, *et al.,* 2004). In neither of these cases higher molecular-weight PEX5 bands could be detected (Fig. 26).

We decided then to explore the possibility that proteasome inhibitors such as the commercially available lactacystin, could increase the likelihood of detecting polyubiquitinated forms of TbPEX5, since these species are known to be degraded by the proteasome in yeast and hence have been successfully stabilized in the presence of similar inhibitors such as MG132 (Debelyy *et al.,* 2011). For that purpose *T. brucei* wild-type bloodstream and procyclic cultures were treated with 1 µM of lactacystin during 6 h. A cell fractionation was performed in the presence of 20 mM of NEM by using a low concentration of digitonin (0.1 mg digitonin/mg of protein).

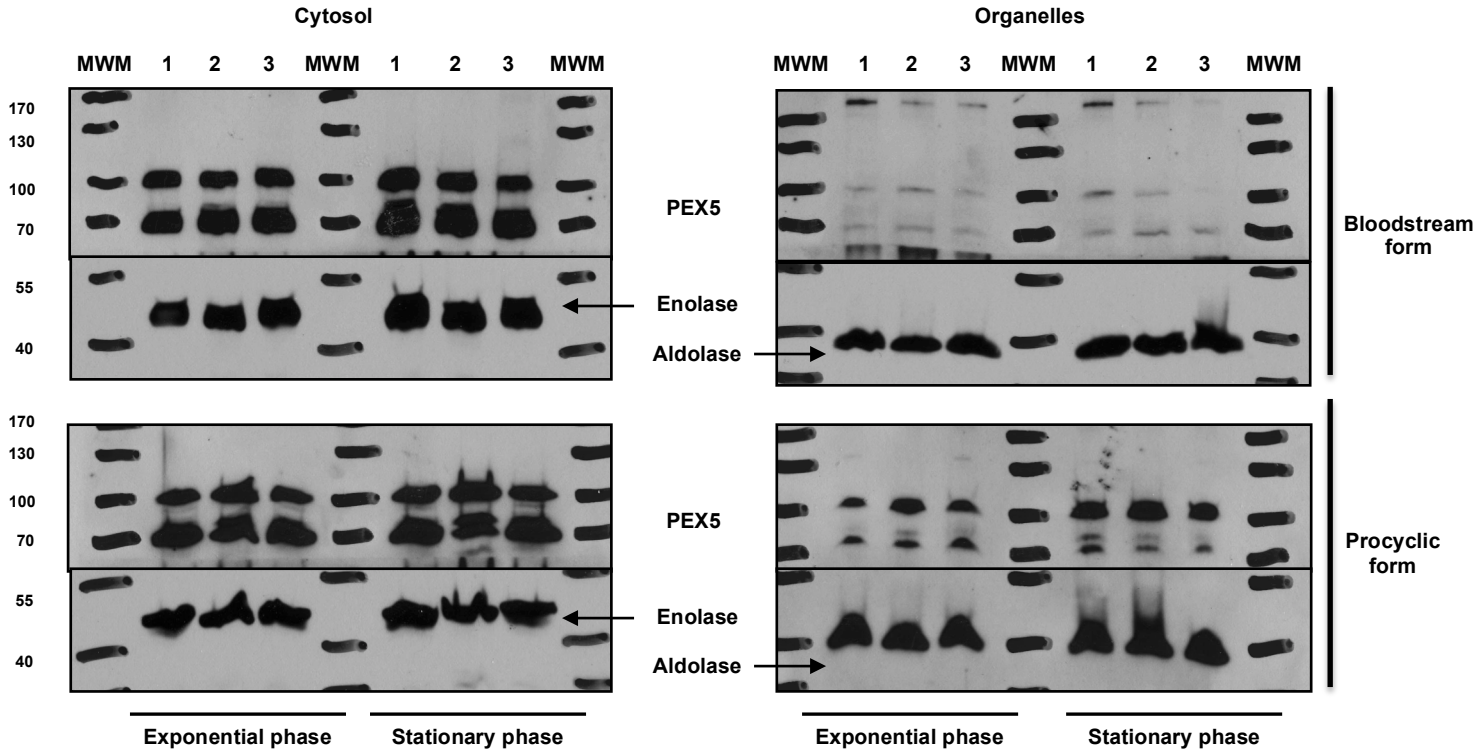


Figure 26. ***TbPEX5 susceptibility to NEM.*** *T. brucei* bloodstream and procyclic cells in the exponential and stationary growth phase were lysed in the presence of 20 mM of N-ethylmaleimide (NEM) and a cocktail of protease inhibitors (PI) (Fermentas®). After lysis, the cellular extract was centrifuged at 16,000 x *g* to separate cytosolic components from the organellar fraction. 30 µg of protein from each fraction were analyzed by western blot using affinity purified TbPEX5 antibodies as well as antisera against control proteins (Enolase and Aldolase). **1**: +NEM/+PI; **2**: -NEM/+PI; **3**: +NEM/-PI.

This concentration had previously been proved to selectively permeabilize the plasma membrane, releasing the cytosolic protein content and leaving the organelles (including glycosomes) intact. After centrifugation at high speed these two fractions were efficiently separated and protein samples were prepared in Laemmli buffer with or without DTT. The treatment in the presence and absence of DTT has been efficiently used in studies with yeast and mammalian cells to distinguish cysteine-linked monoubiquitin in PEX5 from lysine-linked polyubiquitin PEX5 (Miyata *et al.,* 2012; Okumoto *et al.,* 2011). Western blot analysis done with the non-purified TbPEX5 antiserum (Fig. 27) shows that the native TbPEX5 100 kDa form was highly abundant in the cytosolic fraction (as the cytosolic marker enolase), while a minor part was associated with the organellar fraction where the glycosomal marker aldolase was highly abundant.

Interestingly, it was found that, in the conditions tested, a slower migrating form (about 110 kDa) detected with the non-purified TbPEX5 antiserum was present exclusively in the cytosolic fraction of both bloodstream and procyclic cells. This band was absent from the organellar fraction where by contrast the TbPEX5 antiserum recognized a much slower migrating band of about 130 kDa. The presence or absence of the proteasome inhibitor lactacystin did not affect the abundance of the additional TbPEX5 species, indicating no role of the proteasome inhibition in the stabilization of these modified forms of TbPEX5. No changes were observed either by the presence or absence of DTT, suggesting that a possible modification of a cysteine residue, as have been previously found in mammalian and yeast PEX5, seems unlikely. Nevertheless, we cannot exclude that the insensitivity to DTT it is due to the concentration of DTT (25 mM) used in our experiments. This concentration might not have been sufficient to reduce the thioester bond that is formed between PEX5 and the ubiquitin. This aspect of

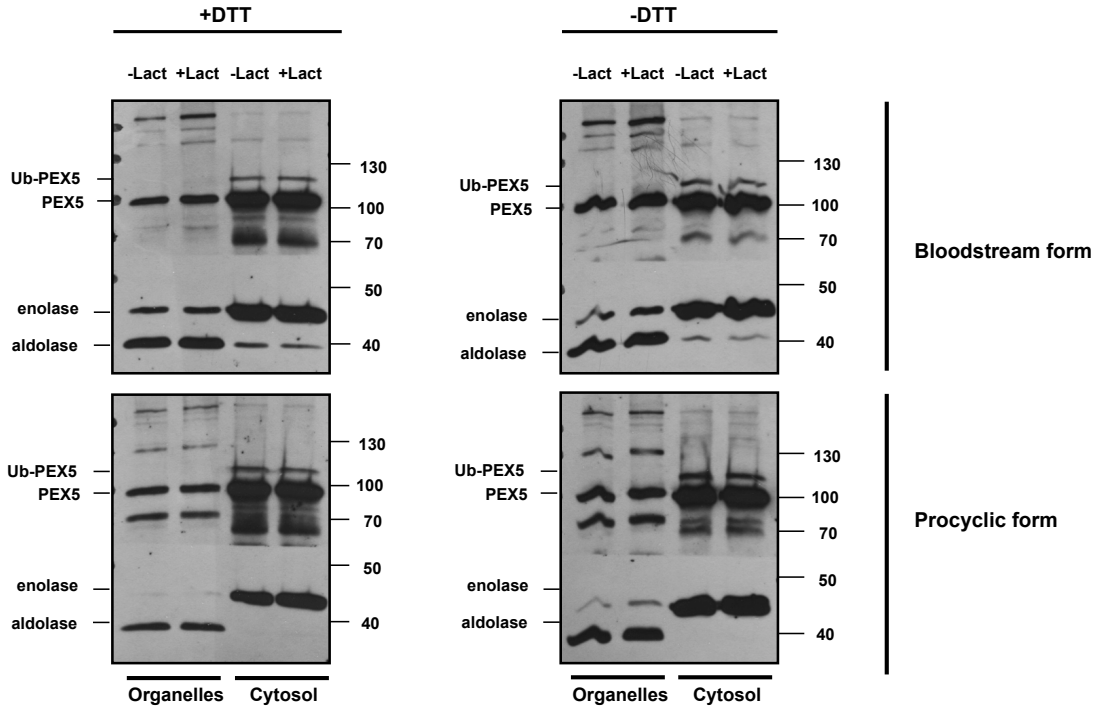


Figure 27. ***A slower migrating TbPEX5 form is present in T. brucei wild-type cells.*** Wild-type BF and PF cells were cultured in the presence of the proteasome inhibitor lactacystin for 6 h. Cytosolic and organellar fractions were prepared after treatment of cells with 0.1 mg of digitonin/mg of protein in the presence of 20 mM of NEM followed by centrifugation. Protein samples were prepared in Laemmli buffer containing DTT or not. A slower migrating form of TbPEX5 (Ub-PEX5) was exclusively detected in cytosolic fractions, irrespective of the presence of DTT.

TbPEX5 ubiquitination it is currently under investigation. Based on the obtained result, we speculate that the form of 110 kDa represents monoubiquitinated TbPEX5 (the product of the addition of a 8.5 kDa ubiquitin moiety), a modification occurring most probably at a lysine residue, and we don't exclude that the 130 kDa form could represent a polyubiquitinated form.

As a second strategy, we have created a wild-type procyclic *T. brucei* cell line that expresses an N-terminally 2x-Myc-tagged-Ubiquitin protein (with a predicted molecular weight of 12.5 kDa) under the control of a tetracycline-inducible promoter. After standardization of the best expression conditions, we have treated the wild-type procyclic and Myc-tagged Ubiquitin cell line with lactacystin for 6 h and performed a cell fractionation similarly as described above, but in the presence or absence of NEM. In this manner, we aimed to get insight into the question if inhibition of deubiquitinating enzymes affects the presence of the additionally detected TbPEX5 forms. Western blot analysis was done as shown in Figure 28.

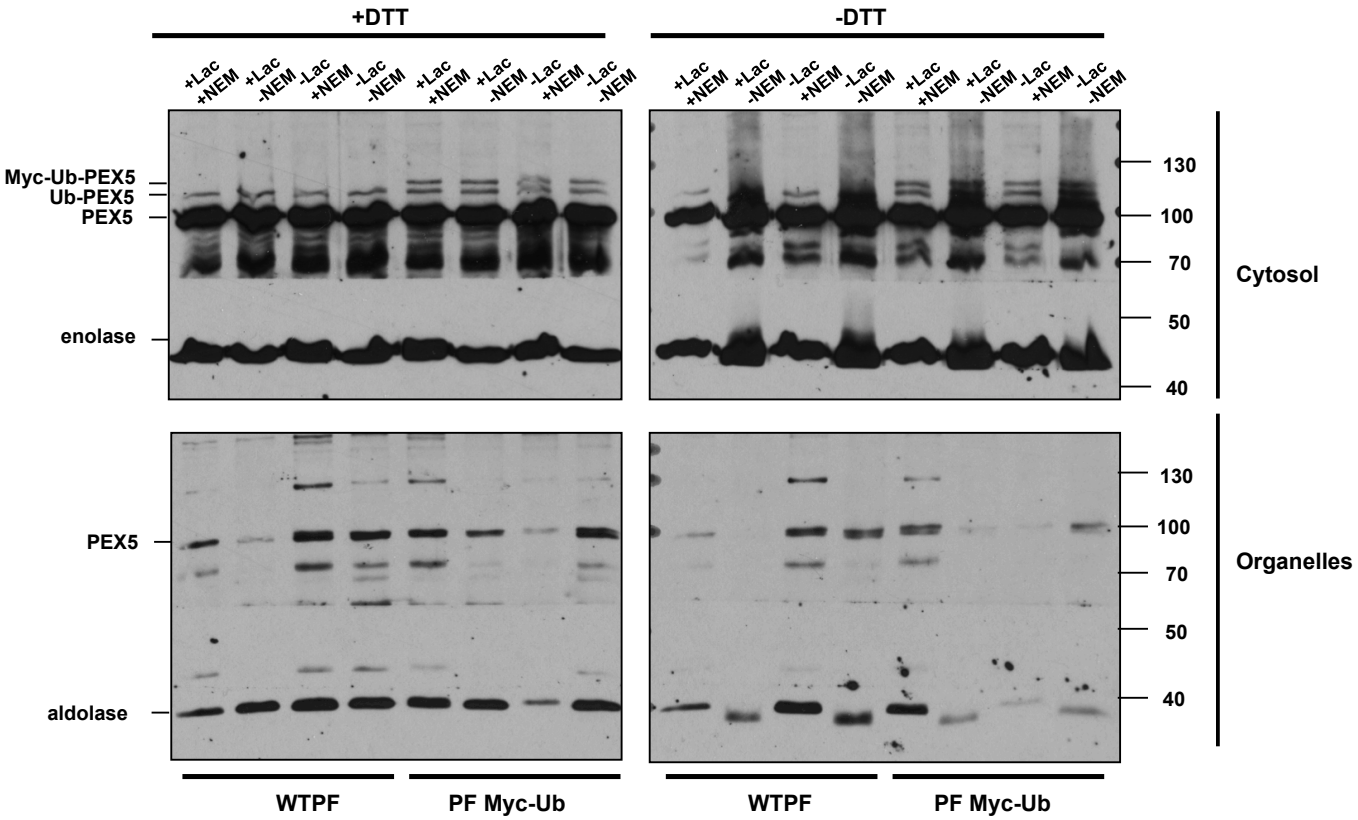


Figure 28. ***A second slower migrating TbPEX5 form is detected upon expression of Myc-Ubiquitin.*** Trypanosomes of wild-type PF and a PF Myc-Ub cell line expressing 2X-Myc-Ub were treated as in Fig. 27, but in the presence or absence of NEM. A slower migrating band was detected by the PEX5 antiserum; it corresponds to Myc-Ub-TbPEX5 and is exclusively found in cytosolic fractions. Both slower migrating forms of TbPEX5 were lactacystin/NEM independent.

We found that upon Myc-ubiquitin expression in procyclic cells, a new slower migrating form of about 115 kDa was detected by the TbPEX5 antiserum in the cytosolic fractions, in addition to the 110 kDa form detected also in the wild-type cells and that is probably due to the modification by the endogenous ubiquitin. Upon expression of Myc-ubiquitin no changes in the molecular weight were observed in the band of 130 kDa that is detected exclusively in organellar fractions. Therefore, we discard the possibility that this band corresponds to a polyubiquitinated form of TbPEX5 and rather assume that is should be attributed to cross reactivity of the antiserum with another protein. Regarding the effect of the proteasome inhibitor lactacystin, we confirmed once more that the addition of this drug does not lead to stabilization of the slower migrating forms of TbPEX5 in the wild-type and Myc-ubiquitin cell line, suggesting that these forms correspond to stable species of TbPEX5 occurring in the cell. We could neither observe a difference in the abundance of the slower migrating forms of TbPEX5 when cells were fractionated in the absence or presence of NEM, showing that the inhibition of deubiquitinating enzymes is not required for the detection of this modified TbPEX5 form. We could notice that protein migration was somewhat disturbed when samples were analyzed in the absence of both NEM and DTT (Fig. 28). This makes it difficult to draw any conclusions about the relative abundance of all species of TbPEX5 between the different treatments. The data collected in this experiment strongly suggest that part of the cytosolic TbPEX5 is monoubiquitinated in wild-type cells and that the N-terminal Myc-tagged-Ubiquitin can be efficiently conjugated to the receptor (probably conjugated to a lysine residue in TbPEX5) forming species of Myc-tagged-Ubiquitin-TbPEX5 which are also resistant to proteasome degradation and to the activity of deubiquitinating enzymes.

As a final proof of ubiquitination of TbPEX5 we have analyzed the immunoprecipitated TbPEX5 fraction from a previous experiment (Metabolic labelling of TbPEX5 in wild-type procyclic cells) using the commercial antibodies raised against human ubiquitin. Figure 29 shows that the eluted fraction of TbPEX5's immunoprecipitation contains the native form (100 kDa) as well as the slower migrating form of 110 kDa (faintly detected). When this sample was analyzed with human-ubiquitin antibodies, a band of 110 kDa was detected. It should be noticed that also a band at approximately the same molecular weight as the native TbPEX5 was detected with the human-ubiquitin antiserum, however we attribute this to a cross-reaction of the antiserum due to the high amount of the TbPEX5 protein present in the elution fraction.

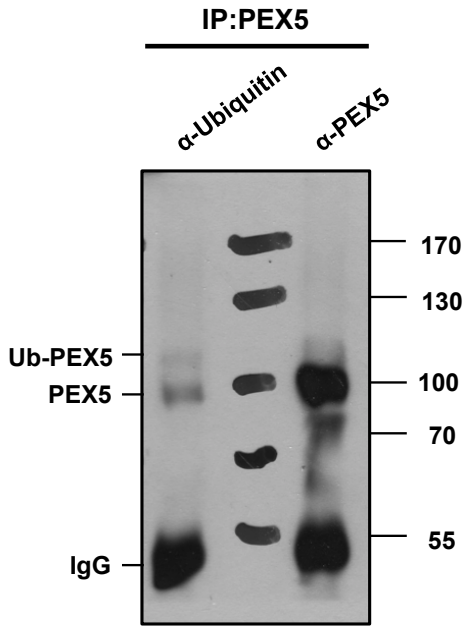


Figure 29. ***The slower migrating form of TbPEX5 detected in wild-type cells corresponds to Ubiquitin-TbPEX5.*** TbPEX5 was immunoprecipitated from *T. brucei* wild-type PF cells. Western blot analysis was performed with the elution fraction using antisera against human ubiquitin and TbPEX5. The slower migrating form of TbPEX5 was detected by the anti-ubiquitin antiserum proving that TbPEX5 is ubiquitinated in wild-type cells.

All evidence collected so far demonstrates that cytosolic TbPEX5 can be ubiquitinated in wild-type bloodstream and procyclic forms. The ubiquitinated form of TbPEX5 seems to represent a very small fraction of the total receptor present in the cytosol (as inferred from the low intensity of the signal in western blots) and seems to be stable even in the presence of active deubiquitinating enzymes as well as an active proteasome. Intriguingly, we did not find in any of the conditions tested, ubiquitinated species of TbPEX5 associated with the organellar fraction containing glycosomes. However, we do not exclude that indeed ubiquitinated TbPEX5 molecules can be present in the organellar membrane, since the putative ubiquitinating machinery is associated with the glycosomes. A probable explanation of the apparent absence is that in normal conditions in wild-type trypanosomes the steady-state level of these molecules may be very low, making their detection difficult with the limited sensitivity of western blot analysis.

3. Identification and characterization of additional components involved in PEX5 recycling.

During the last decade several peroxins involved in glycosome biogenesis of *T. brucei* have been identified and their role in the different steps of the process has been elucidated. These include members of the matrix protein import pathway such as TbPEX2, TbPEX5, TbPEX6, TbPEX7, TbPEX10, TbPEX12, TbPEX13, TbPEX14, as well as TbPEX11 which plays a role in glycosome division and TbPEX19, the peroxisomal membrane protein chaperonin/receptor. However, other peroxins have not been yet identified. Among them are: (1) PEX3, involved in peroxisome membrane formation; (2) PEX8, an assembly factor which connects the docking and the RING-finger complexes to form a competent importomer; (3) PEX15/PEX26 which is the membrane anchor protein of the AAA^{+}-ATPases PEX1 and PEX6 complex in yeast and mammalian cells, respectively; and (4) the complex comprised of the ubiquitin-conjugating enzyme PEX4 and its membrane anchor PEX22.

3.1. Sequence homology searching: TbPEX4 and TbPEX22.

We aimed to identify other peroxins involved in the late steps of matrix-protein import. More precisely we started looking for TbPEX4. This ubiquitin-conjugating enzyme has been shown to be essential for peroxisome biogenesis and plays a role in the PEX5 retrieval from peroxisomes in different yeasts and mammalian cells through the cysteine-specific monoubiquitination of the PTS-1 receptor PEX5. By reciprocal BLAST analysis using as a query sequences of proteins with confirmed PEX4 activity (ScPEX4, PpPEX4, PaPEX4 and HpPEX4) (Fig. 30A), we could identify a sequence for a typical ubiquitin-conjugating enzyme with putative PEX4 features in the *T. brucei* genome database (*T. brucei* strain TREU927; GeneDB, accession code Tb927.8.920). This gene was amplified from the DNA of our laboratory strain *T. brucei* Lister 427 by PCR, cloned and sequenced. It is predicted to code for a protein of 171 amino acids with a molecular weight of 19 kDa and an isoelectric point of 5. The critical Cys residue in the ubiquitin-conjugating domain is also conserved in the C-terminal half of the putative TbPEX4. We made a multiple alignment of this sequence and those

predicted for other trypanosomatids and the respective yeast PEX4s (Fig. 30A). The identity between the protein of *T. brucei* and other trypanosomatids is between 60-70% and 26-36% with regard to different yeast species.

At a later stage, we also identified a candidate for PEX22 in the *T. brucei* genome database (previous accession code: Tb10.6k15.3180; current code: Tb927.10.7480) by sequence homology searching using as a query the *Arabidopsis thaliana* PEX22 sequence in a reciprocal BLAST analysis (Fig. 30B). This peroxisomal membrane protein has been described in different species of yeasts and in *A. thaliana* as the anchor that binds PEX4 to the membrane. It shows a very low overall sequence similarity with its homologues, however it shares with them a characteristic single transmembrane segment present in the N-terminal part. It is noteworthy to mention that initially no result was obtained when using the *S. cerevisiae* PEX22 sequence as a query in the BLAST search. The putative *T. brucei* PEX22 gene was amplified by PCR, cloned and sequenced. It codes for a 22.1 kDa protein with an isoelectric point of 7.8 and a predicted transmembrane segment formed by amino acids 15 to 37. The putative *T. brucei* PEX22 protein has orthologues in *T. cruzi* (Tc00.1047053509715.26) and *L. major* (LmjF36.3050), which also contain the conserved predicted transmembrane segment in the N-terminal half and show similarities with the other PEX22s in their sequence and calculated molecular weight (Fig. 30B). The percentages of identity between the putative *T. brucei* PEX22 and its *T. cruzi* and *L. major* orthologues are 50% and 33%, respectively.

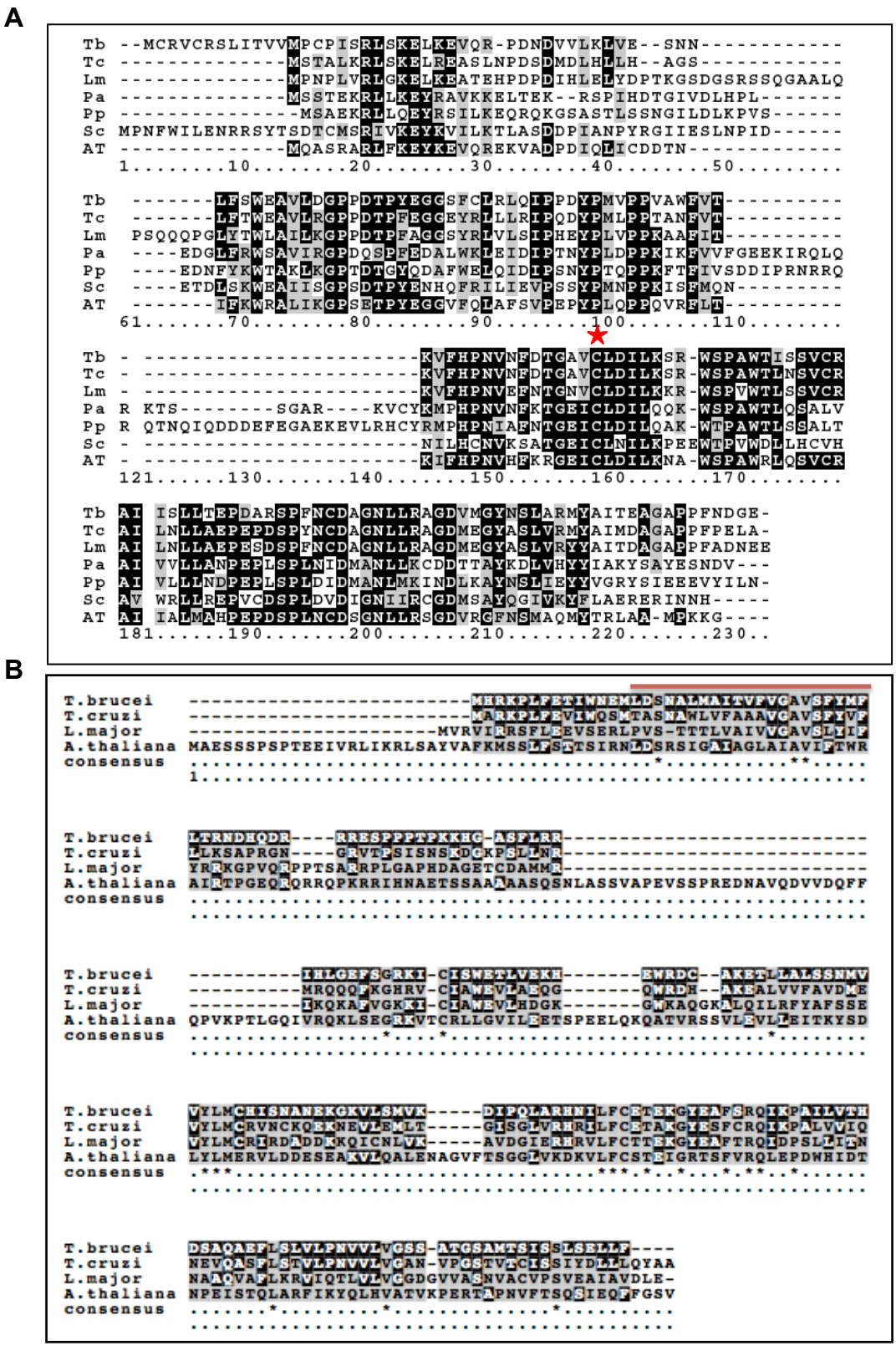


Figure 30. ***Alignment of TbPEX4 and TbPEX22 with homologous amino-acid sequences***. **A**. Alignment of PEX4. Tb: *T. brucei*. Tc: *T. cruzi*. Lm: *L. major*. Pa: *Hansenula polymorpha*. Pp: *Pichia pastoris*. Sc: *Saccharomyces cerevisiae*. AT: *Arabidopsis thaliana.* ★ *indicates* the conserved cysteine residue involved in ubiquitin conjugation. **B.** Alignment of PEX22. The characteristic N-terminal transmembrane segment is indicated with a red bar above the alignment.

3.2. In silico interaction studies of TbPEX4-TbPEX22.

Very recently, Williams *et al.* (2011), published the crystal structure of the *S. cerevisiae* PEX4-PEX22 complex (Fig. 15). They identified the region responsible for the interaction between the two proteins, which indeed does not overlap with any other interaction interface in PEX4. Moreover, they demonstrated that the binding of PEX22 enhances the *in vitro* ubiquitination capacity of PEX4 and the *in vivo* PEX5 ubiquitination, thus revealing a novel mechanism of Ubiquitin-conjugating regulation.

We have performed an *in silico* interaction analysis of the TbPEX4 and TbPEX22 candidates using the published data about the interaction surface in the yeast PEX4-PEX22 complex. This analysis was done in collaboration with the authors of the cited paper. We have first aligned the protein sequences of the pairs TbPEX4-ScPEX4 (alignment starting at position 1) as well as TbPEX22-ScPEX22 (starting at position 50). Figure 31 shows that the identified interacting residues in ScPEX4 and ScPEX22 (shaded boxes) are relatively well conserved in the TbPEX4 and TbPEX22 amino-acid sequences.

Additionally, we have analyzed the intrinsic protein disorder and globularity profile (GlobPlot 2.3) of TbPEX22 and compared it with the profile of ScPEX22. As is shown in Figure 32, both proteins posses a predicted transmembrane segment in the N-terminal region (ScPEX22 amino acids 15-52 and TbPEX22 amino acids 15-37), followed by a disordered region and a large globular domain.

A

```
Alignment starting at position 1

ScPex4p MPNFWILENRRSYTSDTCMSRIVKEYKVILKTLASDDPIANPYRGIIESLNPIDETDLSK

TbPex4p MCR--VCRSLITVVMPCPISRLSKELKEVQRDPDND---------VV--LKLAESNNLFS

        * .  : ..  : .    :**: ** * : :   .*         ::  *:  :..:* .

ScPex4p WEAIISGPSDTPYENHQFRILIEVPSSYPMNPPKISFMQNNILHCNVKSATGEICLNILK

TbPex4p WEAVLDGPPDTPYEGGSFCLRLQIPPDYPMVPPVA-WFVTKVFHPNVNFDTGAVCLDILK

        ***::.**.*****. .* : :::*..*** **   :: .:::* **:  ** :**:***

ScPex4p PEEWTPVWDLLHCVHAVWRLLREPVCDSPLDVDIGNIIRCGDMSAYQGIVKYFL-AERE-

TbPex4p S-RWSPAWTISSVCRAIISLLTEPDARSPFNCDAGNLLRAGDVMGYNSLARMYAITEAGA

        . .*:*.* :    :*:  ** ** . **:: * **::*.**: .*:.:.: :  :*

ScPex4p --RINNH

TbPex4p PPFNDGE
```

B

```
Alignment starting at position 50

ScPex22p DSVKENKKARKSKCIIMS-KSIQGLPIKWEEYA--------ADEVVLL------------

TbPex22p PPTPKKHGASFLRRIHLGEFSGRTICISWETLVEKHEWRDCAKETLLALSSNMIVYLMCH

          .. ::: *   : * :.  * : : *.**  .        *.*.:*

ScPex22p VPTSHTDGSMKQAIGDAFRKTKNEHKIIYCDSMDGLWSCVRRLGKFQCILNSRDFTSSGG

TbPex22p ISNANEKGKVLSMVKDIPQ--LARHNILFCETEKGYEAFSRQIKP--AILVTHDSAQA-E

         :..:: .*.: . : *  :    .*:*::*:: .*  :  *::    .** ::* :.:

ScPex22p SDAAVVPEDIGRFVKFVVDSDVE-DVLIDTLCN

TbPex22p FLSLVLPNVV-LVGSSATGSAMTSISSLSELLF

           : *:*: :  . . ...* :     :. *
```

Figure 31. ***Interacting residues of ScPEX4-ScPEX22 are conserved in the putative TbPEX4-TbPEX22 sequences***. A. Alignment of ScPEX4 and TbPEX4 (from residue 1) (A) and ScPEX22 and TbPEX22 (from residue 50) (B) are shown. Residues responsible for the interaction in the yeast complex are highlighted in shaded boxes. Conserved residues in the trypanosome proteins are indicated (*, :, .).

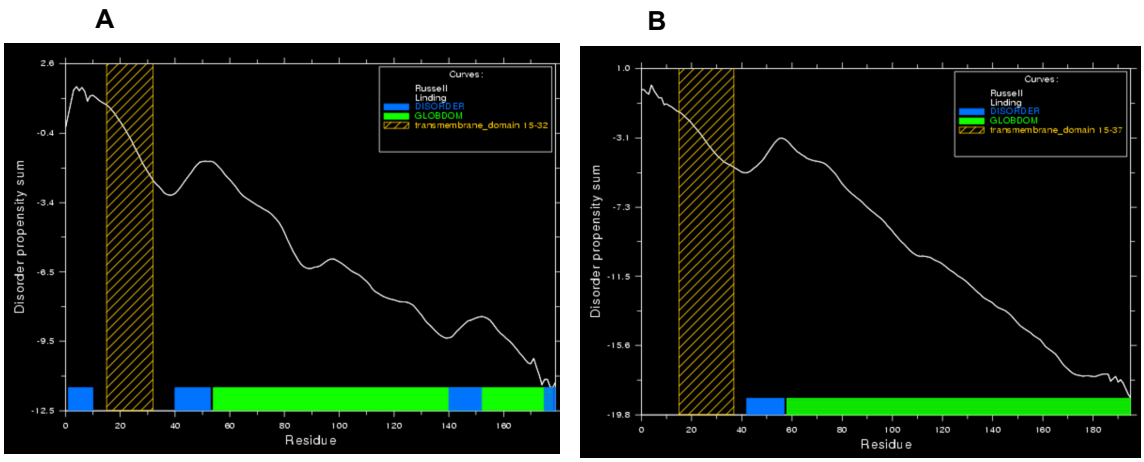


Figure 32. ***Comparison of the globular profile of ScPEX22 (A) and TbPEX22 (B)***. Plots show the disorder propensity along the protein sequence. Several structural predictions show strong similarities: a transmembrane segment at the N-terminal region (dashed bar) followed by a disordered segment (blue box) and a large globular domain (green bar).

Furthermore, we have constructed a structure model of the two *T. brucei* candidate peroxins in complex, based on the structure of its homologues in *S. cerevisiae* (Fig. 33). The *in silico* model shows that the amino acids predicted to be involved in the interaction are oriented in such a way that molecular contacts in the model occur, indicating that in principle the two proteins would be able to interact. The majority of the hydrophobic interacting residues are conserved and additionally some compensating mutations seem to have occurred (Ex: ScPex22 Arg130 interacts electrostatically with ScPex4 Asp151) (Fig. 33C). In the *T. brucei* model these are both replaced, with Gln and Asn, respectively, a pair that could make side chain H-bonding interactions. The most relevant residues (ScPEX4 Tyr 172 and ScPEX22 Lys 133), that stabilize the interaction in the ScPEX4/ScPEX22 complex, are not conserved in the *T. brucei* peroxins. These residues have been replaced by Met in TbPEX4 and Pro in TbPEX22, two residues that can interact by Van de Waals forces due their hydrophobic character.

In summary, this bioinformatics analysis strongly suggests that the two candidates, TbPEX4 and TbPEX22, posses structural features very similar to the authentic ScPEX4-ScPEX22 complex.

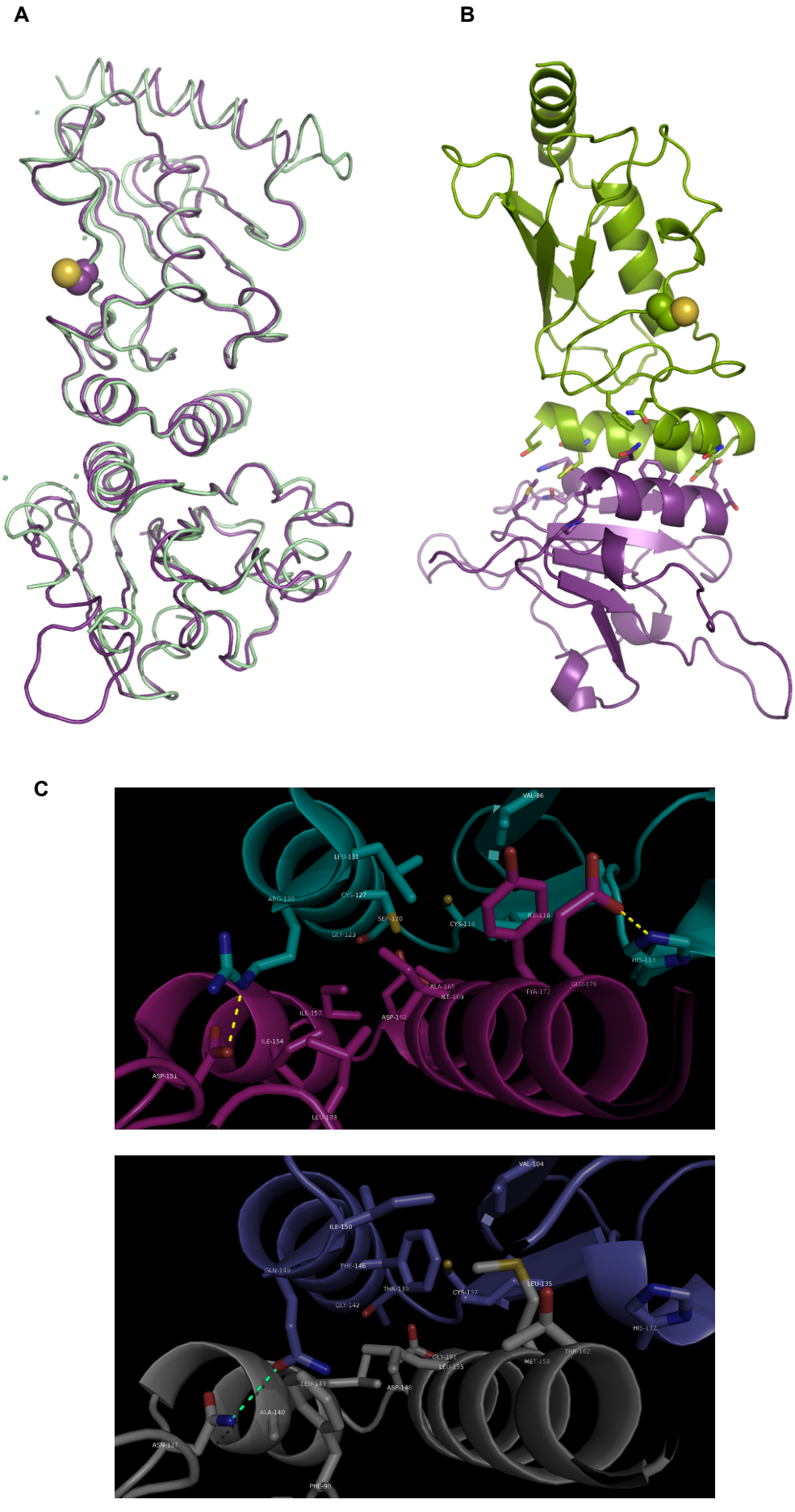

A
B
C
VAL-86
LEU-131
ARG-130
CYS-127
SER-120
GLY-123
CYS-118
ILE-116
HIS-113
ALA-165
ILE-169
TYR-172
GLU-176
ILE-157
ASP-162
ILE-154
ASP-151
LEU-103
VAL-104
ILE-150
GLN-149
PHE-146
THR-139
CYS-137
LEU-135
GLY-142
HIS-132
GLY-151
LEU-155
MET-158
THR-162
LEU-143
ASP-148
ALA-140
ASN-127
PHE-90

Figure 33. ***Structural model of the TbPEX4/TbPEX22 complex.*** **A.** ScPEX4-ScPEX22 crystal structure (grey) with a TbPEX4-TbPEX22 (purple) overlapping structural model**. B**. Model of TbPEX4 (green)-TbPEX22 (purple) in complex. Amino acids possibly involved in the interaction are displayed. **C.** Structural detail of the interacting region of ScPEX4-ScPEX22 complex (top) and the same region of TbPEX4-TbPEX22 model (Bottom). ScPEX4 is displayed in pink and ScPEX22 in blue. Side-chains of the residues involved in the interaction are shown and salt-bridges are highlighted in yellow. TbPEX4 is presented in grey and TbPEX22 in purple. Side-chains of the amino acids predicted to be involved in the interaction are shown. A compensating hydrogen bond is coloured in green.

3.3. Protein-protein interaction studies

Several of the interactions that occur between peroxins involved in the peroxisomal and glycosomal matrix protein import have been elucidated through studies with the yeast two-hybrid system (Williams *et al.,* 2011; Verplaetse *et al.,* 2009). In our lab this system has been successfully used to characterize TbPEX5-TbPEX14, TbPEX5-TbPEX13 and TbPEX13-TbPEX14 interactions, in most of these cases allowing the identification of specific regions that participate in the interface of the interacting peroxins (Verplaetse *et al.,* 2009).

We have decided to use the same approach to gain insight into the possible interactions between TbPEX4-TbPEX22 but also interactions involving the RING-finger ubiquitin ligases TbPEX2/TbPEX10/TbPEX12 as well as the receptor TbPEX5. To that end, we have cloned the full-length *TbPEX4, TbPEX22, TbPEX2, TbPEX10, TbPEX12* genes in the yeast expression vectors pGBT9 and pGAD424, which allow the constitutive expression of fusion proteins that contain either the $GAL4_{BD}$ or the $GAL4_{AD}$ fused to the respective peroxin. After having transformed *S. cerevisiae* strain SFY526 with a combination of these plasmids, or with empty vectors (as negative controls), we performed yeast two-hybrid analyses by using the β-galactosidase filter assay, in order to study the protein-protein interaction between the two peroxins. Results were compared to a positive control (plasmids containing the full-length TbPEX5 and N-terminal TbPEX14). Unfortunately, no interaction was detected in any of the pairs of peroxins tested (Table 4). We decided then to restrict our analysis to those regions of the proteins predicted to be responsible for interactions (the RING-finger domains in the case of TbPEX2, TbPEX10 and TbPEX12). In the case of the

TbPEX4/TbPEX22 pair we therefore analyzed the capacity of interaction based on the already identified interacting region in the ScPEX4/ScPEX22 complex (Williams *et al.,* 2011) and predicted to exist also in the TbPEX4 and TbPEX22 candidates. Interactions were analyzed similarly by using the β-galactosidase filter assay. Our experiments did not reveal interactions between the RING-finger domains of the peroxins TbPEX2/TbPEX10/TbPEX12. We equally obtained negative results when the interactions of individual RING domains and the full-length TbPEX4 were assessed (Table 3). Remarkably, we found a positive interaction between the C-terminal region of TbPEX4 (residues 149-192) and TbPEX22 C-terminal half (residues 86-177) (Table 3). This result is in agreement with the yeast peroxin data by Williams *et al.* (2011); the minimal interacting region found by the yeast two-hybrid system corresponds to a similar interface in the model of the *T. brucei* complex. The fact that this interaction was not detected with the full-length proteins could be due to incorrect folding of one or both proteins and/or an inefficient nuclear transport in the yeast cells, especially in the case of TbPEX22, which possesses a predicted transmembrane region in its N-terminal part.

Our attempts to validate the *in vivo* protein-protein interaction between the TbPEX4 and TbPEX22 candidates by alternative techniques such as co-immunoprecipitation with Myc-tagged TbPEX22 and copurification with TAP-tagged TbPEX22 were unfortunately unsuccessful (data not shown). Nevertheless, we are tempted to argue that it is likely that we have identified the real TbPEX22 homologue for the following reasons: 1) we detected a positive interaction in the yeast two-hybrid system, 2) the *in silico* analysis of TbPEX4/TbPEX22 sequences and structure model show a remarkable similarity in the theoretical conformation of the two proteins in complex, and 3) the fact that the reciprocal BLAST analysis only yielded a single candidate in the *T. brucei* genome, with similar features as those present in PEX22 proteins previously identified in other organisms.

Table 3. Summary of the results from protein-protein interaction studies by the yeast-two hybrid system. (FL= Full length, RING=RING domain, IR= Interacting region). Truncated versions are indicated with the positions of the amino acids retained. (These results are based on the blue/white filter assay of the β-galactosidase activity, of which the pictures are not shown).

pGAD424	pGBT	Interaction results
TbPEX4 FL	**TbPEX10 FL**	*Negative*
TbPEX4 FL	**TbPEX12 FL**	*Negative*
TbPEX4 FL	**TbPEX2 FL**	*Negative*
TbPEX4 FL	**TbPEX10 RING (177-298)**	*Negative*
TbPEX4 FL	**TbPEX12 RING (296-395)**	*autoactivating*
TbPEX4 FL	**TbPEX2 RING (210-347)**	*Negative*
TbPEX4 FL	**TbPEX22 FL**	*Negative*
TbPEX4 IR (149-192)	**TbPEX22 IR (86-177)**	*Negative*
TbPEX2 FL	**TbPEX4 FL**	*Negative*
TbPEX2 RING (210-347)	**TbPEX4 FL**	*Negative*
TbPEX2 FL	**TbPEX10 FL**	*Negative*
TbPEX2 RING (210-347)	**TbPEX10 FL**	*Negative*
TbPEX2 FL	**TbPEX10 RING (177-298)**	*Negative*
TbPEX2 RING (210-347)	**TbPEX10 RING (177-298)**	*Negative*
TbPEX2 FL	**TbPEX12 FL**	*Negative*
TbPEX2 RING (210-347)	**TbPEX12 FL**	*Negative*
TbPEX2 FL	**TbPEX12 RING (296-395)**	*autoactivating*
TbPEX2 RING (210-347)	**TbPEX12 RING (296-395)**	*autoactivating*
TbPEX10 FL	**TbPEX4 FL**	*Negative*
TbPEX10 RING (177-298)	**TbPEX4 FL**	*Negative*
TbPEX10 FL	**TbPEX2 FL**	*Negative*
TbPEX10 RING (177-298)	**TbPEX2 FL**	*Negative*
TbPEX10 FL	**TbPEX2 RING (210-347)**	*Negative*
TbPEX10 RING (177-298)	**TbPEX2 RING (210-347)**	*Negative*
TbPEX10 FL	**TbPEX12 FL**	*Negative*
TbPEX10 RING (177-298)	**TbPEX12 FL**	*Negative*
TbPEX10 FL	**TbPEX12 RING (296-395)**	*autoactivating*
TbPEX10 RING (177-298)	**TbPEX12 RING (296-395)**	*autoactivating*
TbPEX12 FL	**TbPEX4 FL**	*Negative*
TbPEX12 RING (296-395)	**TbPEX4 FL**	*Negative*
TbPEX12 FL	**TbPEX2 FL**	*Negative*
TbPEX12 RING (296-395)	**TbPEX2 FL**	*Negative*
TbPEX12 FL	**TbPEX2 RING (210-347)**	*Negative*
TbPEX12 RING (296-395)	**TbPEX2 RING (210-347)**	*Negative*
TbPEX12 FL	**TbPEX10 FL**	*Negative*
TbPEX12 RING (296-395)	**TbPEX10 FL**	*Negative*
TbPEX12 FL	**TbPEX10 RING (177-298)**	*Negative*
TbPEX12 RING (296-395)	**TbPEX10 RING (177-298)**	*Negative*
TbPEX5 FL	**TbPEX4 FL**	*Negative*
TbPEX5 FL	**TbPEX10FL**	*Negative*
TbPEX5 FL	**TbPEX10 RING (177-298)**	*Negative*
TbPEX5 FL	**TbPEX2 FL**	*Negative*
TbPEX5 FL	**TbPEX2 RING (210-347)**	*Negative*
TbPEX5 FL	**TbPEX12 FL**	*Negative*
TbPEX5 FL	**TbPEX12 RING (296-395)**	*autoactivating*
TbPEX22 FL	**TbPEX4 FL**	*Negative*
TbPEX22 IR (86-177)	**TbPEX4 IR (149-192)**	*Positive*
TbPEX5 FL	**TbPEX14 N-Terminal**	*Positive*

3.4. Expression and purification of recombinant peroxins TbPEX4 and TbPEX22. Production of antisera

We have ligated the *T. brucei* PEX4 and PEX22 genes in the expression vector pET28 and expressed separately in *E. coli* the corresponding proteins each with an N-terminal His-tag. The expressed proteins were insoluble under all conditions used (different media, temperatures, induction times). The inclusion bodies were washed with detergents followed by a denaturation step in 8 M of urea. Denatured TbPEX4 and TbPEX22 were then purified by affinity chromatography using immobilized nickel under denaturing conditions (Fig. 34A and 34B). The purified proteins were used to raise polyclonal antisera in rabbits. Although the antisera recognized easily the respective recombinant antigens on western blots (Fig. 34C and 34D), the PEX22 antiserum did not recognize the natural protein in trypanosome lysates, which may be an indication that it is expressed in low amount in the parasites and/or that the most immunogenic epitopes of the denatured recombinant protein are not well exposed on blots prepared of proteins from trypanosome lysates. In contrast, the PEX4 antibodies allowed us to confirm the expression of a protein with the expected molecular weight, mainly associated with a membrane fraction from both procyclic- and bloodstream-form trypanosomes (Fig. 34C).

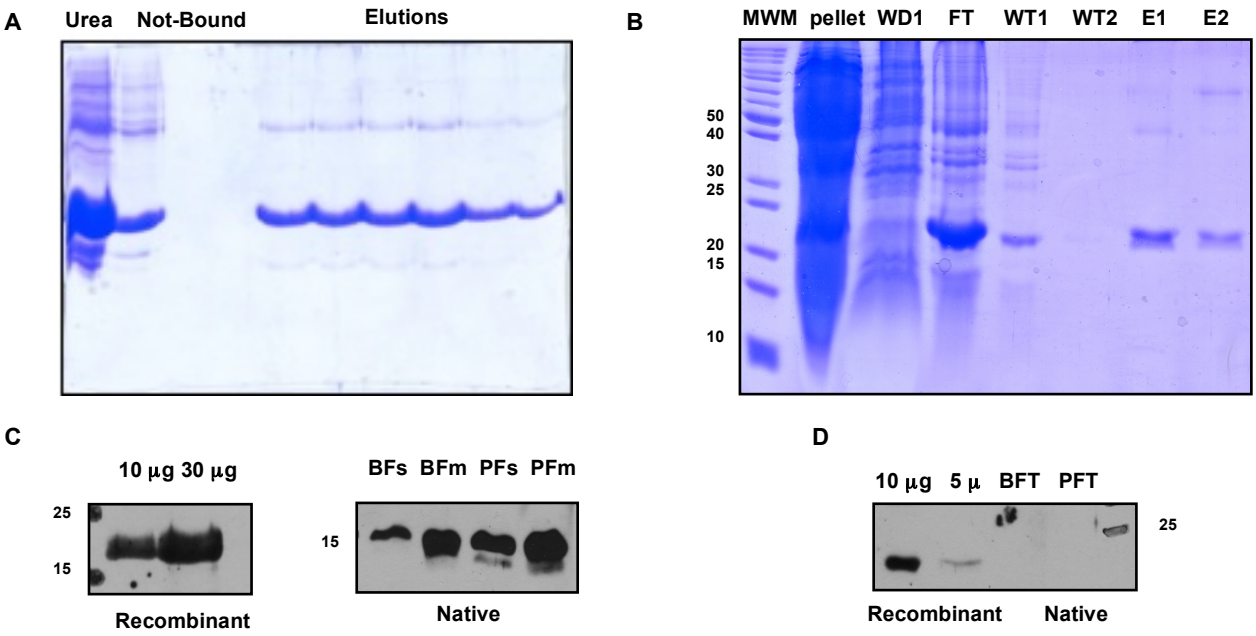


Figure 34. ***Expression and purification of recombinant TbPEX4 (A) and TbPEX22 (B***). Recombinant His-tagged TbPEX4 and TbPEX22 were expressed as insoluble proteins and purified from inclusion bodies using affinity chromatography with immobilized nickel (**A**) and cobalt TALON (**B**) resins. MWM: molecular weight markers. WD1: wash detergent 1. FT Talon: flow through TALON resin. WT1: wash TALON 1. WT2: Wash TALON 2. E1 and E2: elution with 200 and 300 mM of imidazole, respectively. **C** and **D**. Purified proteins were used to raise polyclonal antisera. The TbPEX4 antiserum recognizes the purified recombinant and the native protein in soluble and membrane fractions from bloodstream and procyclic form *T. brucei* (**C**). The TbPEX22 antiserum recognizes the recombinant protein but not the native protein in lysates from bloodstream or procyclic form trypanosomes (D).

3.5. Subcellular localization: Differential and isopycnic centrifugation and confocal fluorescence microscopy.

Initially we analyzed the presence of the putative TbPEX4 in subcellular fractions of wild-type bloodstream and procyclic form cells prepared by differential centrifugation. We used the matrix protein aldolase and membrane protein TbPEX11 as glycosomal marker proteins. A representative example of a fractionation experiment performed with bloodstream form cells is shown in Figure 36. The putative TbPEX4 protein appeared to be present, as expected, in the homogenate and post-nuclear fractions, while after fractionation it seemed to be most abundant in the large and small granular fractions (Fig. 36A). The distribution of aldolase and TbPEX11 indicates that the fractionation was well performed and that the distribution profile of putative TbPEX4 corresponds with a glycosomal localization.

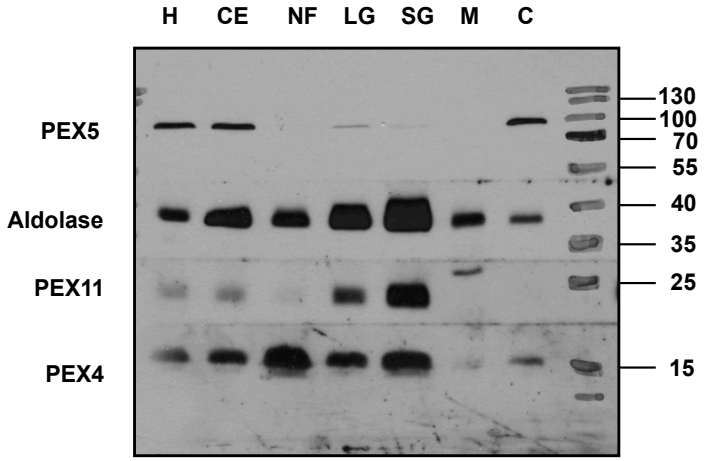


Figure 36. ***TbPEX4 is associated with glycosomes at their cytosolic face.*** **A.** *T. brucei BF* cells were fractionated by differential centrifugation and the fractions analysed by western blotting. H: Homogenate, CE: cellular extract, NF: Nuclear Fraction, LG: large granular fraction, SG: small granular fraction, M: microsomes, C: cytosol. TbPEX4 was identified in the small granular fraction enriched in the glycosomal matrix and membrane markers Aldolase and TbPEX11.

As additional evidence, we have detected the putative TbPEX4 in glycosomes purified from linear density gradients made with lysates of procyclic cells (Fig. 37B) as well as bloodstream form trypanosomes (data no shown) using the commercial Optiprep medium. It should be mentioned that isopycnic centrifugation performed under this condition leads unavoidable also to the formation of so-called "glycosome ghosts" which are formed as a result of osmotic effects causing breakage of glycosomes followed by resealing or leakage of matrix content during the cell fractionation, generating less dense glycosomal particles which co-migrate with lighter fractions in the gradient. This phenomenon was evidenced by the presence of a minor amount of Aldolase and TbPEX11, the glycosomal matrix and membrane marker, respectively, together with TbPEX4 in fractions 23-28 of the gradient (Fig. 37B).

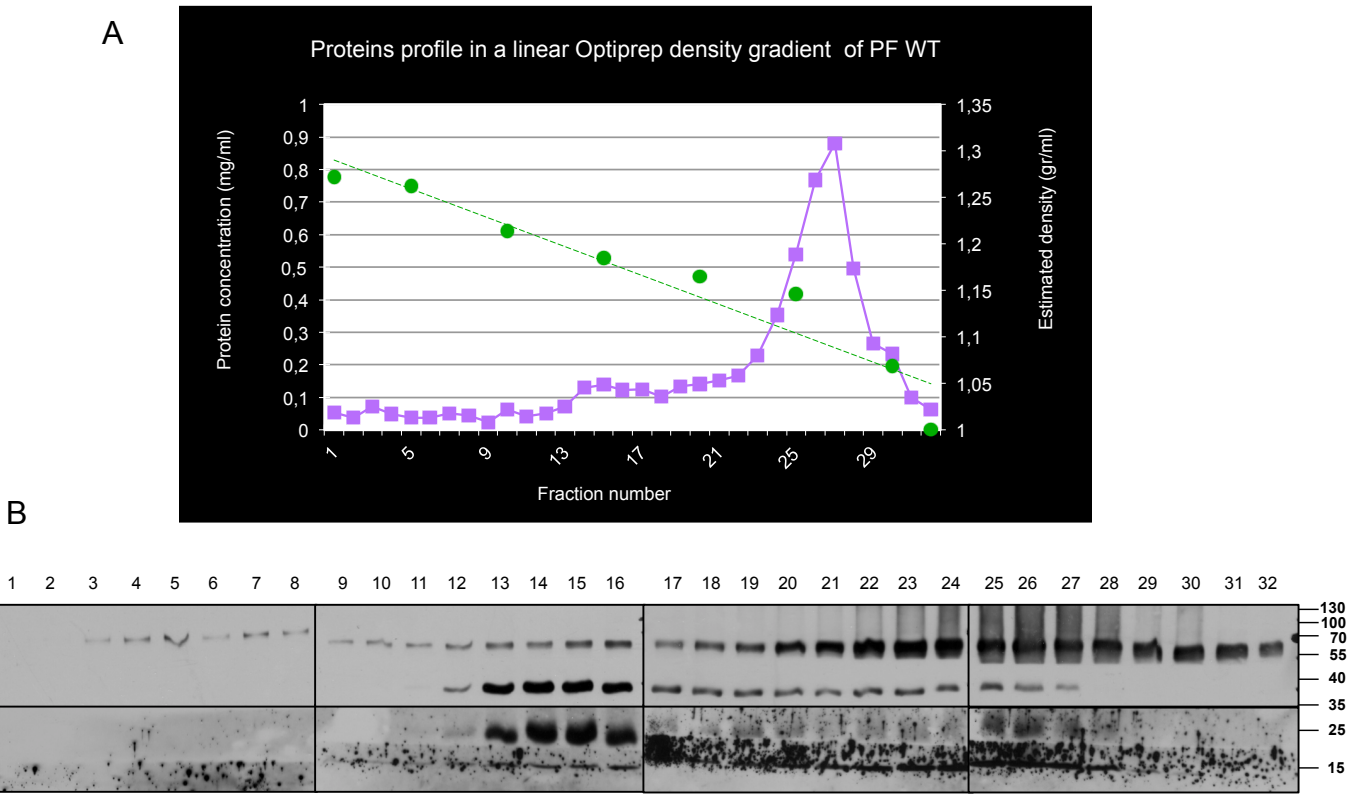


Figure 37. ***TbPEX4 is present in glycosome-rich fractions of a isopycnic density gradient.*** Glycosomes from *T. brucei* procyclic forms were purified by isopycnic centrifugation using commercial Optiprep medium. **A**. Protein profile in the linear density gradient. **B**. Western blot analysis was performed for the collected fractions. Antisera directed against the glycosomal matrix marker aldolase, glycosomal membrane marker TbPEX11, as well as TbPEX4 were used. Additional organelles like the mitochondria were examined with antibodies against TbHSP60.

Furthermore, we performed selective permeabilization of wild-type bloodstream and procyclic cells with digitonin, which allowed us to observe consistently by western blot that TbPEX4 is predominantly associated with organellar fractions. The profile obtained corresponds with the localization observed for the glycosomal membrane marker PEX11 (data not shown).

We have also used an alternative approach in which we have studied by confocal immunomicroscopy the localization of a GFP-TbPEX4 fusion protein in bloodstream and procyclic wild-type cells. For this purpose we inserted the *TbPEX4* gene in two GFP gene-containing expression vectors (pGC1, pGN1), which allowed the construction of stable cell lines for the tetracycline-inducible expression of N- and C-terminal fusion proteins of GFP and TbPEX4 in *T. brucei*. After transfection of *T. brucei* bloodstream and procyclic cells, we induced the N- and C-terminal GFP-TbPEX4 and TbPEX4-GFP fusion proteins and subsequently analyzed the subcellular localization by confocal fluorescence microscopy. In the case of the TbPEX4-GFP expressing clones, we were unable to see any fluorescence after induction with tetracycline. By contrast, in the clones that expressed the TbPEX4 protein GFP-tagged at the N-terminal part (Fig. 38) we could observe colocalization with the glycosomal marker aldolase, made detectable by immunofluorescence.

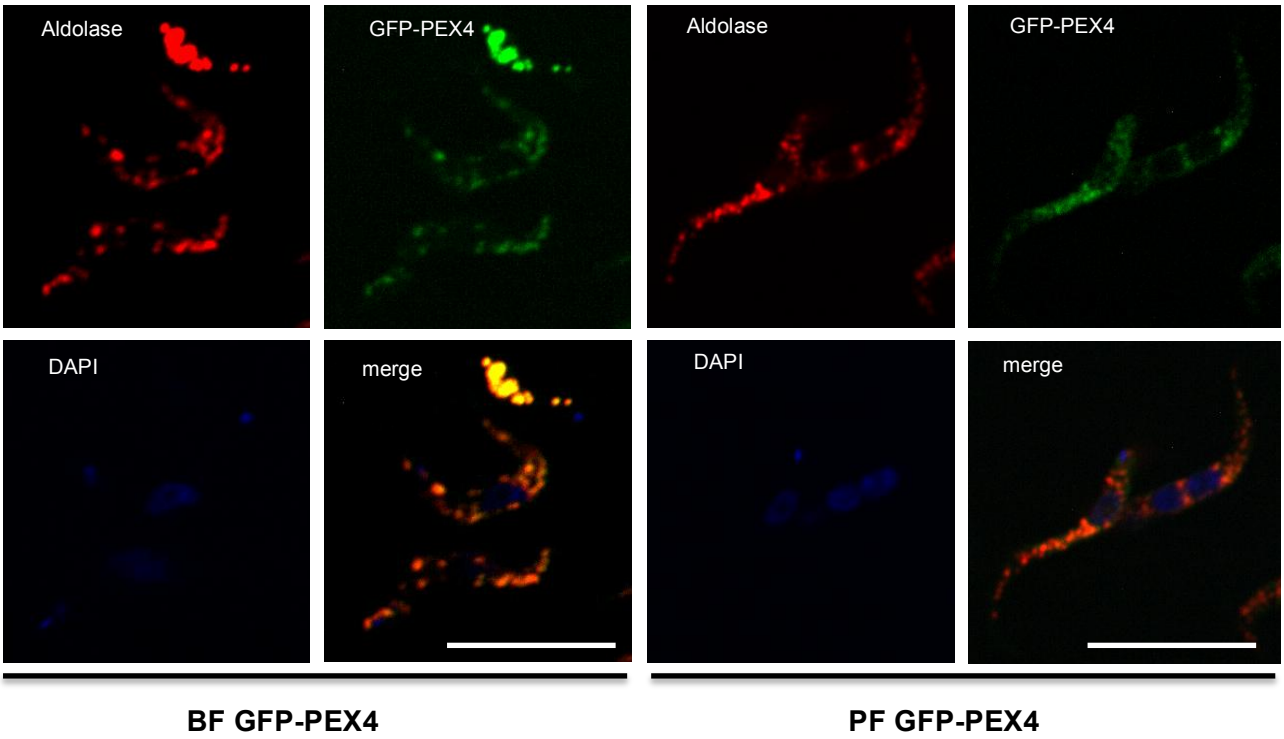


Figure 38. ***GFP-TbPEX4 is present in T. brucei glycosomes***. An N-terminal GFP-TbPEX4 fusion protein was expressed in *T. brucei* BF and PF cells. Confocal immunomicroscopy was performed using Aldolase as glycosomal marker. GFP-TbPEX4 was shown to colocalize with Aldolase suggesting its association with glycosomes. Scale bar: 10 µm.

After having confirmed the localization of TbPEX4 as a glycosomal enzyme, we wanted to know whether the protein is located in the matrix or associated with the cytosolic face of the glycosomal membrane. To investigate its topology we have performed protease protection assays on purified glycosomes obtained by differential centrifugation. In Figure 36B are shown the results obtained with glycosomes from bloodstream form *T. brucei*, however we have done the same analysis with glycosomes from procyclic cells and obtained similar results. Intact purified glycosomes were incubated with proteinase K in the presence or absence of Triton X-100. After having stopped the proteolytic reaction, proteins were precipitated and analyzed by western blotting using several glycosomal markers. TbPEX4 was efficiently degraded by the proteinase K irrespective of whether the glycosomal membrane was permeabilized with Triton X-100 or not. This behaviour indicates that TbPEX4 is associated to the glycosomal membrane side facing the cytosol, as was also observed for TbPEX6 and the C-terminal part (containing the SH3 domain) of TbPEX13 that are known to be exposed to the cytosol. TbPEX11, an integral membrane protein, was in all cases protected

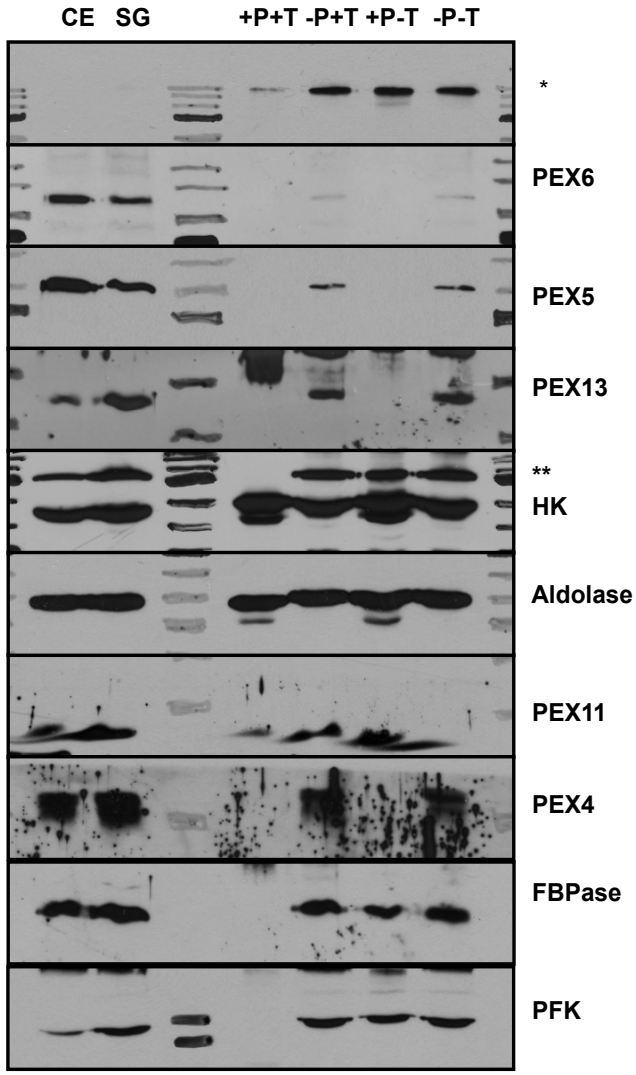


Figure 39. ***TbPEX4 is associated with glycosomes at their cytosolic face***. Intact glycosomes (latency of 95%) present in a small granular fraction obtained by differential centrifugation were used to perform a protease protection assay. CE: Cellular extract, SG: small granular, +P/+T: Proteinase K/Triton X-100, -P/+T: non-Proteinase K/Triton X-100, +P/-T: Proteinase K/non-Triton X-100, -P/-T: non-Proteinase K/non-Triton X-100. TbPEX4 was shown to be not-protected against Proteinase K proteolysis when glycosomes were not permeabilized with Triton X-100, similarly as the AAA^{+}-ATPase TbPEX6, TbPEX13 (SH3 domain), but in contrast to the glycosomal matrix proteins Fructose-1,6-bisphosphatase (FBPase), Phosphofructokinase (PFK) and the glycosomal membrane marker TbPEX11, which were protected under that condition. Intriguingly, the glycosomal matrix proteins Hexokinase (HXK) and Aldolase were susceptible to the proteinase K in the absence of Triton. * cross reacting band (aldolase), ** Cross-reacting band (HXK).

against proteolysis by proteinase K. The analysis of glycosomal matrix proteins such as FBPase and PFK shows that glycosomes were intact during the proteolytic treatment. Other glycosomal matrix markers such as Aldolase and Hexokinase showed a more intriguing behaviour. Both proteins seem to be only

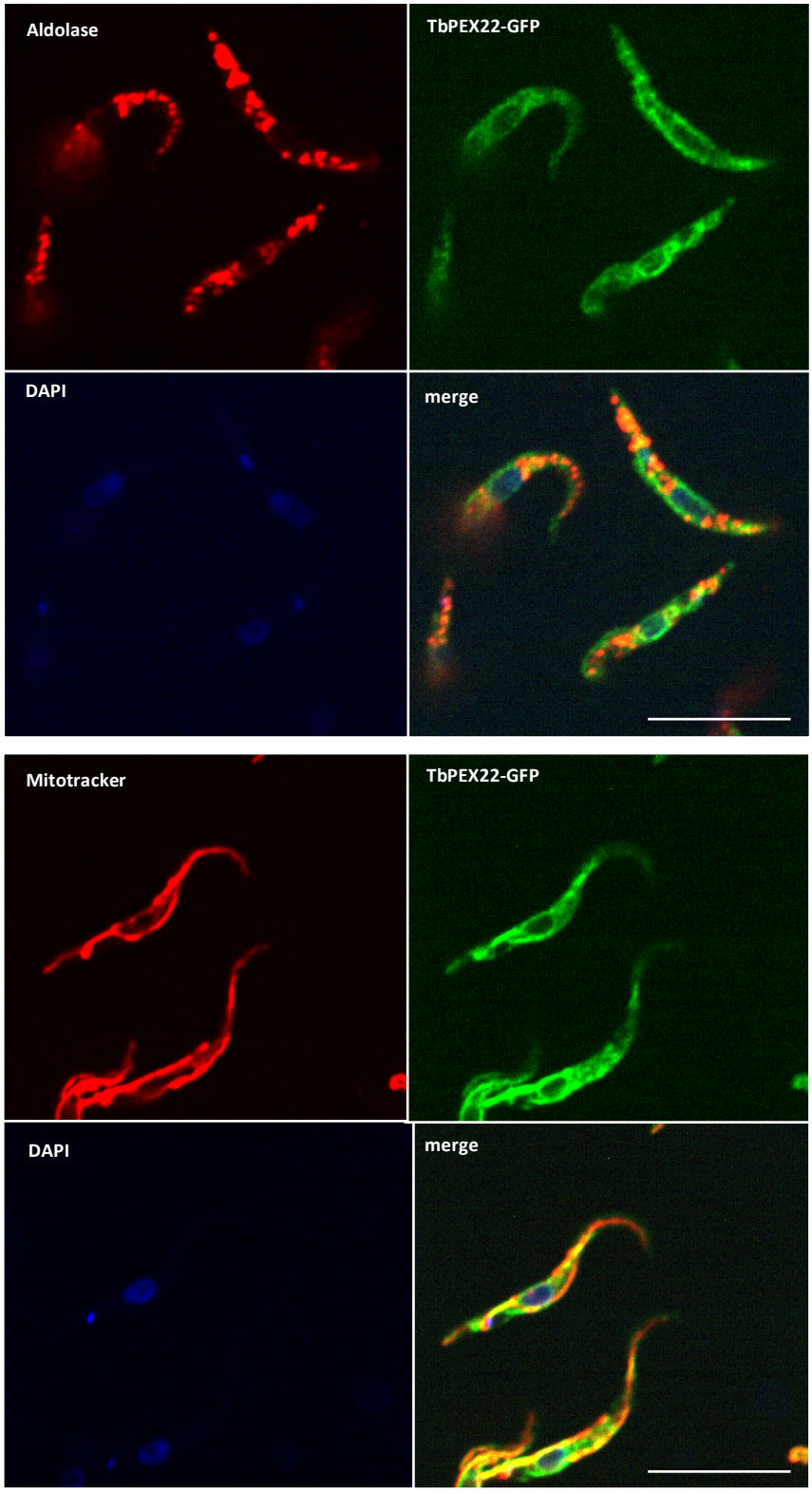
slightly sensitive to proteinase K. In addition, it was observed that such minor proteolysis occurred also when the glycosomal membrane was intact. Since the glycosome-rich fraction had a high latency (based on hexokinase activity measurement) and the fact that FBPase and PFK were efficiently protected against the proteinase K degradation in the absence of Triton X-100, we feel confident about the integrity of the organelles during the assay. Based on this intriguing observation and similar non-published data obtained previously in our laboratory where aldolase and hexokinase showed abnormal behaviour in topology assays, we are tempted to speculate about the occurrence of the different glycolytic enzymes within a multienzyme complex inside the glycosomes (see also the General Discussion in this thesis), with Aldolase and Hexokinase (both PTS-2 containing proteins) associated also with the membrane exposing some of their (probably N-terminal) residues to the external face of the glycosomal membrane. Nevertheless, further experiments would be necessary to analyze this issue in detail.

With regard to our analysis of the subcellular localization of the putative TbPEX22, we were hampered, because the standard immunological approaches were not possible due to the non-reactivity of the available antiserum with the natural PEX22 in whole cells and lysates. We have then opted for studying it by determining the subcellular localization of GFP and Myc-tagged fusion proteins. Our first approach was the expression of N- and C-terminal TbPEX22–GFP fusion proteins and their subsequent analysis by confocal immunomicroscopy. We have inserted the *TbPEX22* gene in the plasmids pGN1 and pGC1, described earlier in this chapter for the tetracycline-inducible expression of TbPEX4-GFP constructs. Procyclic positive clones were obtained for the expression of the fusion protein with the GFP at either the N- or C-terminal end. However, the fluorescence intensity of the N-terminally GFP-tagged TbPEX22 was very low, making difficult its detection by fluorescence microscopy. By contrast we managed to obtain procyclic cells that strongly expressed C-terminally tagged TbPEX22-GFP, which were used for the subcellular localization studies. As shown in Figure 40A, the pattern of the TbPEX22-GFP protein shows localization mostly spread through the cytosol with some intense labelling in some regions of

the cell, but notably not at all colocalizing with the glycosomal marker aldolase. Since the pattern observed seemed reminiscent to a mitochondrial localization (Price *et al.,* 2010), we decided to compare the pattern of TbPEX22-GFP with the localization of mitotracker which was used to label cells in culture. As demonstrated in Figure 40, the fluorescence pattern of TbPEX22-GFP corresponds nicely with that of the mitotracker staining. This is a strong suggestion of mitochondrial localization for this putative peroxin. Nevertheless, we do not exclude the possibility that, due to the very high expression of the TbPEX22-GFP protein [as evident from the strong intensity of the fluorescence even at very low concentrations of the inducer tetracycline (0.5 µg/ml)] (data not shown), the protein could have been driven to wrong cellular compartments or the GFP fused at its C-terminal end has interfered with its glycosome targeting.

Alternatively, we have expressed a N-terminally Myc-tagged TbPEX22 in procyclic cells and analyzed the pattern by confocal immunofluorescence microscopy using a monoclonal Myc antibody. Figure 40B shows a punctate pattern of the Myc antibody that colocalizes only partially with the glycosomal marker Aldolase, suggesting the presence of the Myc-TbPEX22 in these organelles. No mitochondrion-like pattern was detected in these cells as can be seen by the no-colocalization with mitotracker (Fig. 40B). The analysis of control conditions where no primary antibodies were added proved a good specificity of the secondary antibodies (data not shown). The two strategies thus suggest contradictory localizations. At this stage, we don't dare to claim a mitochondrial or glycosomal localization for the putative TbPEX22. We expect that more research using appropriate, more sensitive TbPEX22 antibodies will certainly lead to solving this issue in the future.

A



(see legend on the next page: Fig. 40A)

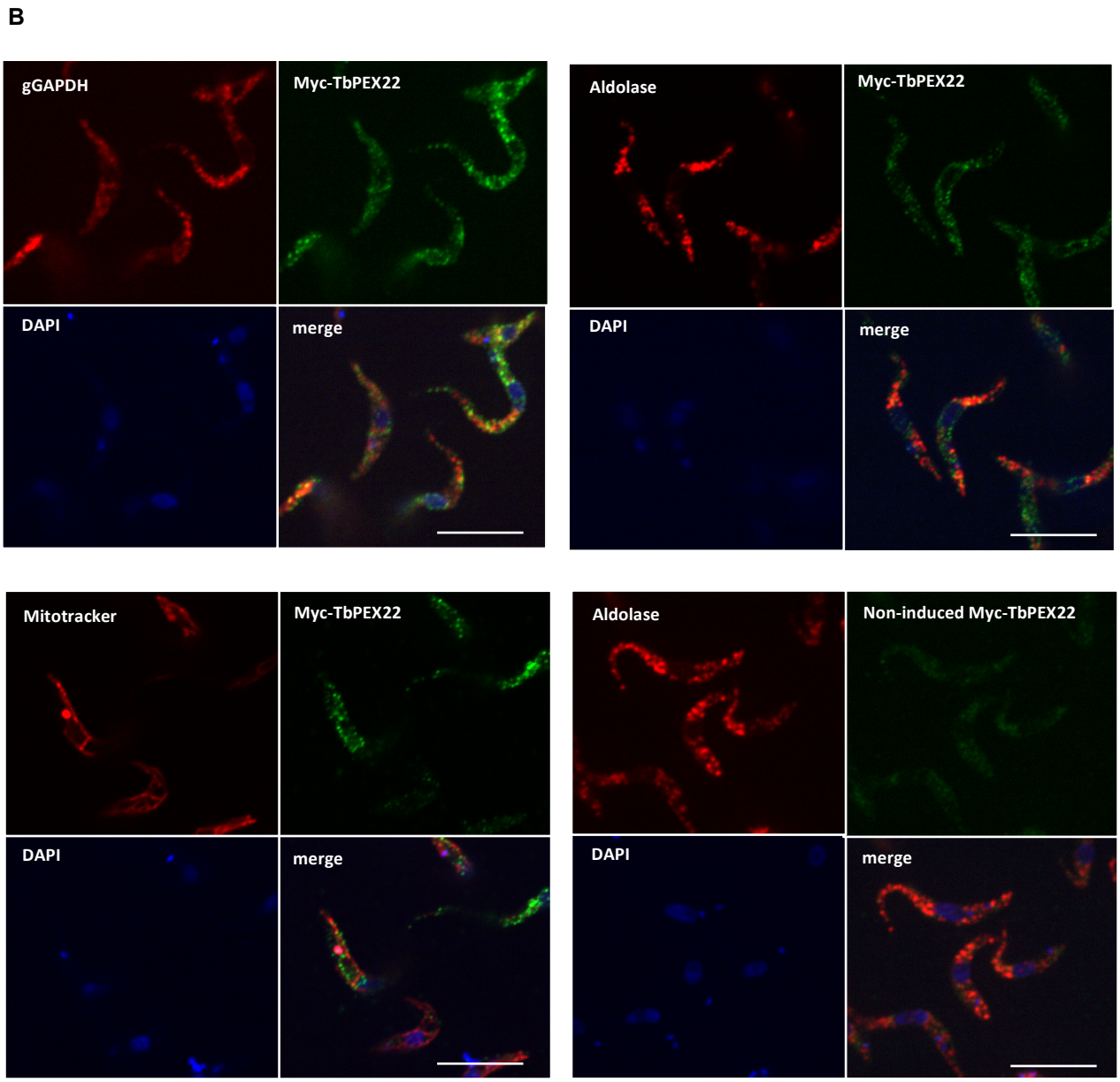


Figure 40. ***The subcellular localization of TbPEX22 is intriguing.*** **A.** A TbPEX22-GFP fusion protein was expressed in procyclic cells. Confocal fluorescence microscopy was performed and GFP fluorescence was compared with that of the glycosomal marker Aldolase and mitotracker. Strong colocalization was observed with the mitotracker indicating an association ofTbPEX22-GFP with the mitochondrion. **B.** A Myc-TbPEX22 was expressed in procyclic cells and confocal fluorescence microscopy was performed afterimmunostaining with monoclonal Myc-antibody. Localization of Myc immunofluorescence was compared with that of the glycosomal markers Aldolase and gGAPDH as well as with that of the fluorescence by the mitotracker. Myc-TbPEX22 staining shows a punctuate pattern that partially colocalized with glycosomes. No colocalization with mitochondrion was observed. Cells not induced for expression of Myc-TbPEX22 show a faint signal indicating a minor leakage of the promoter. Scale bar: 10 µm.

3.6. Functional analysis

In order to characterize the function of putative *T. brucei* peroxins we started by using RNAi, which has proved to be an appropriate technique for the functional study of several glycolytic enzymes (Moyersoen *et al.,* 2004; Albert *et al*., 2005; Cáceres *et al*., 2010) as well as different peroxins in *T. brucei* (Krazy and Michels, 2006; Galland *et al.,* 2007; Verplaetse *et al.,* 2009).

3.6.1.1. Construction of cell lines for RNAi against PEX4 and PEX22 and phenotypic analysis.

First we ligated, in a tandem array, a sense and antisense fragment each corresponding to the nearly full-length TbPEX4 or TbPEX22 gene in the trypanosome-specific pHD677 vector. This expression vector makes it (in principle) possible to create stable RNAi cell lines for the tetracycline-inducible expression of double-stranded RNA molecules. The recombinant DNA constructs were used to transfect bloodstream and procyclic forms in the case of TbPEX4 and only the procyclic form in the case of TbPEX22. It should be noted that induction of RNAi for TbPEX4 and TbPEX22 and the corresponding phenotypic analysis of the cells have been performed several times and that the selected results presented here are representative of all those experiments. After RNAi induction of the TbPEX4 RNAi cell line, we have confirmed a small decrease of TbPEX4 mRNA by RT-PCR (Fig. 41A). Importantly, we consistently observed that the strongest RNAi effect was achieved 24 h after induction, after which the mRNA levels increased again to nearly those observed under non-induced conditions. At the protein level we did not observe a prominent RNAi effect at any other time point after induction, including 24 h (Fig. 41B). The very limited sensitivity of the RNAi cell line to the RNAi induction has complicated our attempts to use this knock-down approach as a tool for functional studies of TbPEX4. Obviously, the phenotypic analysis of cells induced for RNAi against TbPEX4, which involved determination of the effect on cell growth (Fig. 41C), a study of the different molecular-weight forms of TbPEX5 (Fig. 42A), and an analysis of alterations in glycosomal matrix protein import [(studied by selective permeabilization with digitonin (Fig. 42B) and fluorescense immunomicroscopy

(Fig. 42C)], for more than 48 h was unlikely to reveal the real function of TbPEX4 with such cell lines. Indeed, no effect of RNAi induction was observed on any of the mentioned features. It should be mentioned that, after having encountered negative results with the expression vector pHD677, we have transferred the TbPEX4 RNAi construct to vector pHD1336, which in our hands is a better expression system than the pHD677. These two expression plasmids contain the *T. brucei* PARP promoter under the control of the *E. coli* tetracycline operator but differ in the 5' and 3' UTR region added to the inserted gene: while pHD677 has a VSG gene 3'-UTR (low inducible expression in procyclics), pHD1336 has an actin gene 3'-UTR. Nevertheless, a similar low response to tetracycline induction was obtained for the RNAi cell line created with the pHD1336-based PEX4 plasmid as with the pHD677-based one.

Regarding the RNAi performed against TbPEX22, we could observe a dramatic decrease of the mRNA by RT-PCR upon RNAi induction (Fig. 41A). Nevertheless, due to the lack of a suitable antiserum to study the decrease of TbPEX22 at the level of the protein by western blot analysis, and the absence of a growth phenotype (Fig. 41C), we could not establish the essentiality of TbPEX22 with this RNAi cell line. However, the TbPEX22 RNAi cell line was used for an attempt to analyze the predicted function of this protein in anchoring TbPEX4 to the glycosomal membrane. This was done by western blot analysis of TbPEX4 in the cell and its compartments. Total levels of TbPEX4 were not affected in the TbPEX22 induced cells (Fig. 41B). Neither could we observe a mislocalization of TbPEX4 from the organellar membrane to the cytosol when induced cells were analyzed by cell fractionation with digitonin (data not shown).

Taken all these data together, we conclude that the RNAi cell lines that we have created did not respond properly to achieve a strong depletion of the cellular levels of TbPEX4 and most probably also of TbPEX22, making a full functional characterization of these peroxins in *T. brucei* difficult. Other approaches like yeast mutants complementation and the generation of knockout cell lines were subsequently considered.

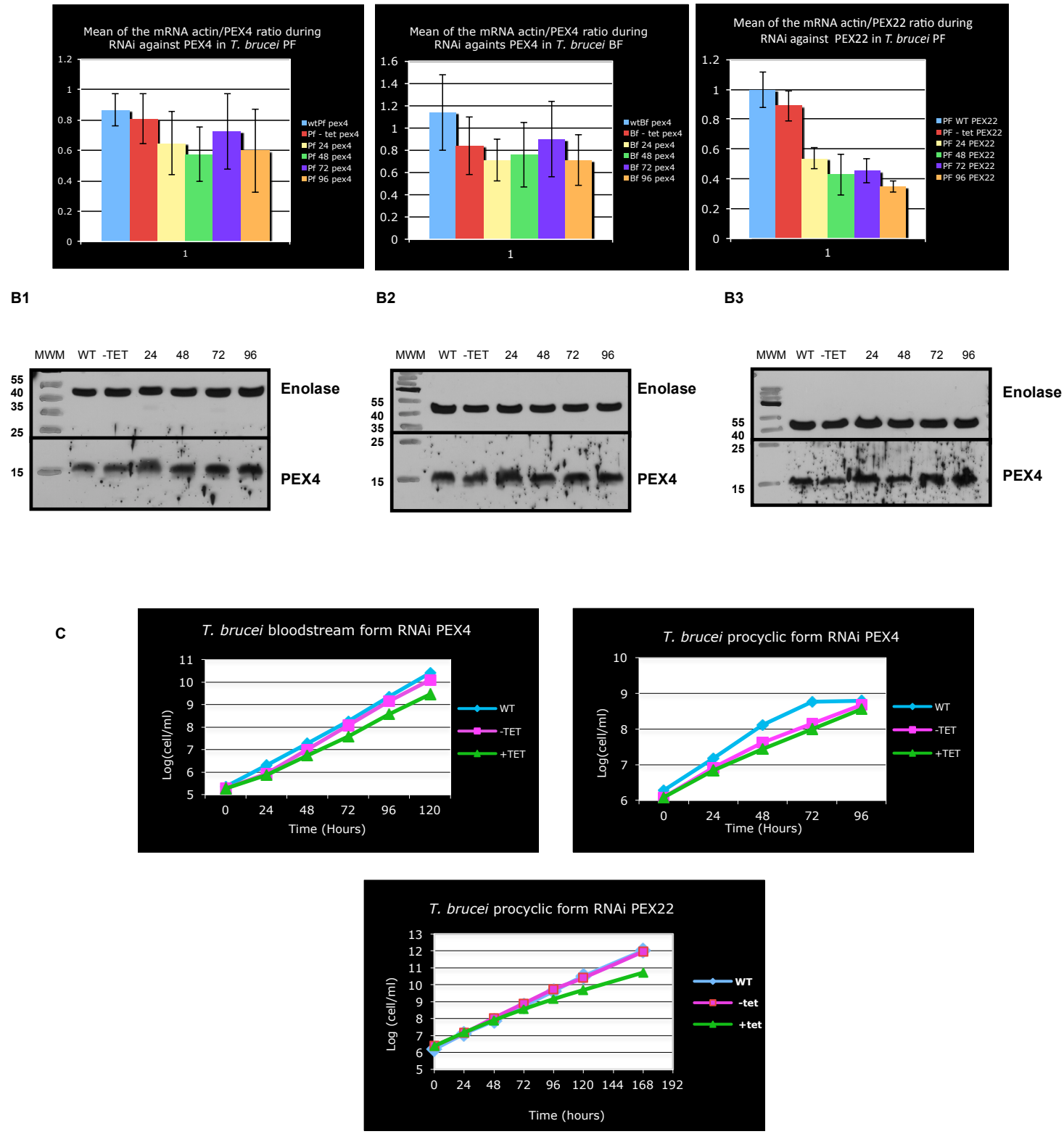


Figure 41. ***Silencing of TbPEX4 and TbPEX22 by RNA interference***. **A**. RT-PCR analysis of TbPEX4 RNA levels in PEX4-RNAi cell lines of bloodstream and procyclic form trypanosomes and TbPEX22 RNA levels in a PEX22-RNAi cell line of the procyclic form. Results are shown as the mean of four independent semi-quantitative RT-PCR analyses performed during RNAi induction. **B**. Western blot analysis of TbPEX4 during RNAi induction in TbPEX4 bloodstream (**B1**) and procyclic (**B2**) RNAi cell lines and a TbPEX22 procyclic (**B3**) RNAi cell line. **C**. Growth rate of induced TbPEX4 bloodstream and procyclic RNAi cell lines and a TbPEX22 procyclic RNAi cell line.

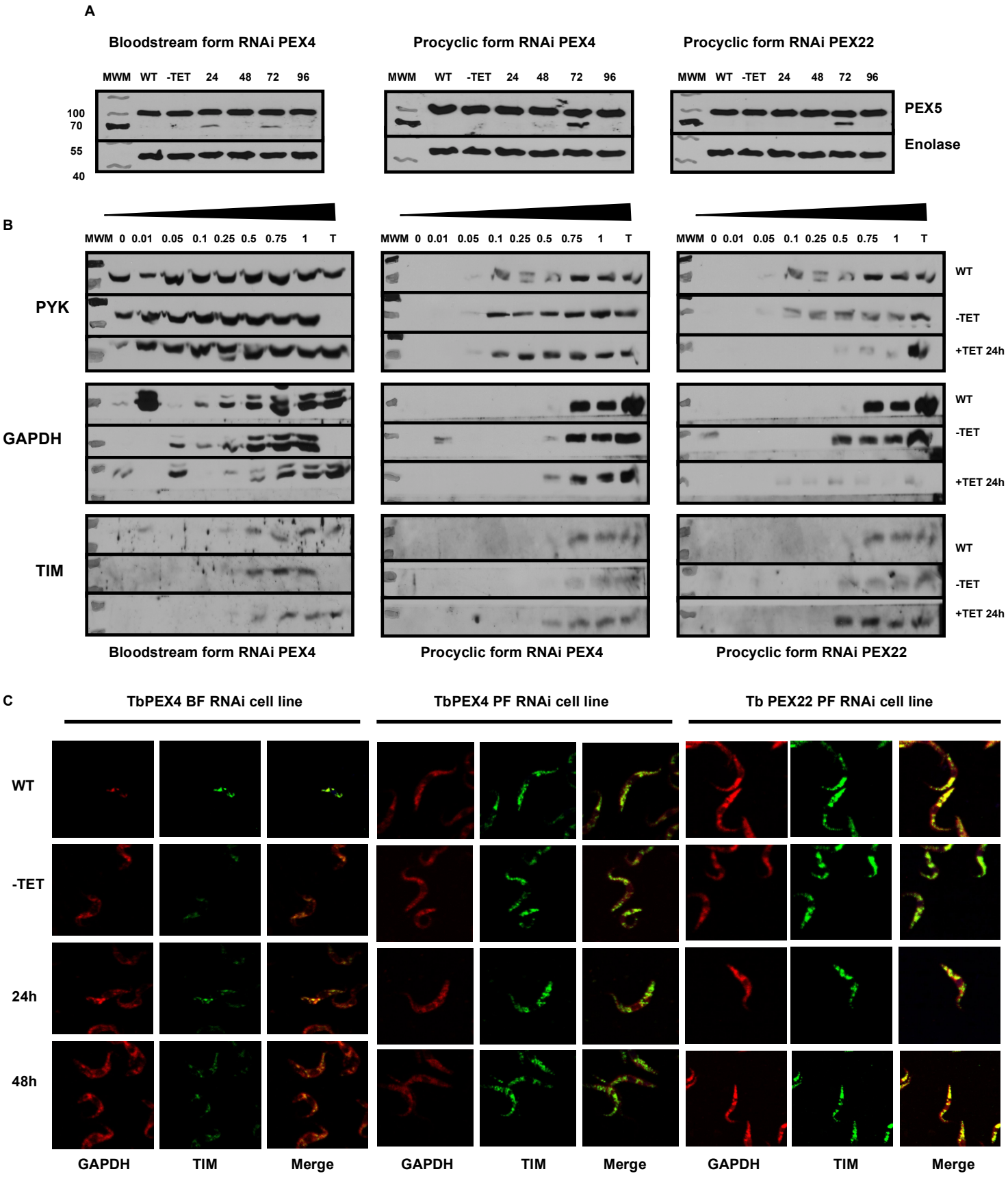

A
Bloodstream form RNAi PEX4
Procyclic form RNAi PEX4
Procyclic form RNAi PEX22
MWM WT -TET 24 48 72 96
100
70
55
40
PEX5
Enolase
B
MWM 0 0.01 0.05 0.1 0.25 0.5 0.75 1 T
PYK
GAPDH
TIM
WT
-TET
+TET 24h
Bloodstream form RNAi PEX4
Procyclic form RNAi PEX4
Procyclic form RNAi PEX22
C
TbPEX4 BF RNAi cell line
TbPEX4 PF RNAi cell line
Tb PEX22 PF RNAi cell line
WT
-TET
24h
48h
GAPDH
TIM
Merge

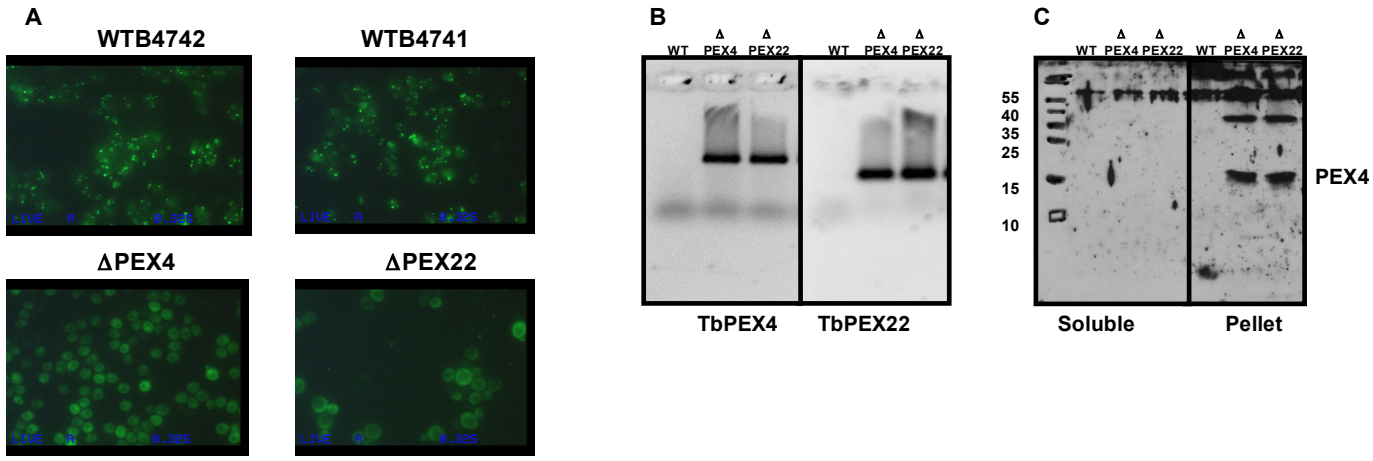
Figure 42. ***Phenotypic analysis of cells in which the expression of TbPEX4 and TbPEX22 was not silenced by RNA interference***. **A**. Abundance and distribution of TbPEX5 during RNAi of TbPEX4 and TbPEX22. **B**. Subcellular fractionation by digitonin titration of TbPEX4 bloodstream and procyclic RNAi cell lines and a TbPEX22 procyclic RNAi cell line during the RNAi induction. Immunoblots were prepared of the different fractions and assayed for the presence of the cytosolic marker pyruvate kinase (PYK), the glycosomal PTS-1 marker glyceraldehyde-3-phospate dehydrogenase (GAPDH) and the I-PTS glycosomal marker triosephosphate isomerase (TIM). **C**. Confocal immunomicroscopy of TbPEX4 bloodstream and procyclic RNAi cell lines and a TbPEX22 procyclic RNAi cell line during RNAi induction showing the localization of the glycosomal matrix enzymes GAPDH and TIM.

3.6.1.2. Yeast mutant complementation.

As an alternative strategy to study the function of TbPEX4 and TbPEX22, we used yeast mutant complementation, an approach already used by others to prove the function of the PEX4 and PEX22 homologues of *A. thaliana (*Zolman *et al.,* 2005). The ΔPEX4 and ΔPEX22 *S. cerevisiae* mutants are characterized by the incapacity to transport PTS-1 proteins into peroxisomes. This phenotypic characteristic can be visualized by fluorescence microscopy when a GFP-PTS1 protein is expressed in the respective mutants. We have introduced in ΔPEX4 and ΔPEX22 mutants as well as in wild-type cells, an inducible expression plasmid (pEW88), which expresses a GFP-PTS1 protein under control of the catalase promoter. The matrix protein import phenotype was observed by fluorescence microscopy after the induction of peroxisome proliferation in a minimal medium containing oleic acid as the only carbon source. As expected, the punctate green fluorescence pattern was only visible in wild-type cells while in the ΔPEX4 and ΔPEX22 mutants a diffuse green fluorescence was observed dispersed in the cytosol (Fig. 43A). After having confirmed the phenotype of ΔPEX4 and ΔPEX22 mutants, we expressed the corresponding TbPEX4 and TbPEX22 proteins in the *S. cerevisiae* mutants using the constitutive yeast expression vectors pAG415-ccd and pAG413-ccd. Initially we confirmed the expression by RT-PCR in the case of TbPEX4 and TbPEX22 and by western blot analysis in the case of TbPEX4 (Fig. 43B and 43C). We have assessed complementation by fluorescence microscopy and by growing the transfected mutants, as well as positive and negative controls, on oleic acid containing liquid and solid media. The results indicate that there was no phenotype complementation when the single TbPEX4 and TbPEX22 proteins were

expressed in the respective ΔPEX4 and ΔPEX22 *S. cerevisiae* mutants (Fig. 43D1 and 44A). We also tried complementation by coexpressing the two proteins, TbPEX4 and TbPEX22, in each single mutant in order to preserve the species-specific protein-protein interaction that should make possible the proper function of these peroxins. However, we did not observe any complementation either (Fig. 43D2, 44B and 44C). To exclude a possible partial complementation of the PTS-1 import phenotype, we have performed a more detailed microscopic analysis of the singly and doubly complemented *S. cerevisiae* ΔPEX4 mutant in collaboration with experts in the yeast field (experiment performed by Vishal Kalel in the group of Prof. Ralf Erdmann at the Ruhr-University of Bochum, Germany), confirming our previous results of non-phenotype complementation. Together these results suggest that TbPEX4 and TbPEX22 are not able to restore the ScPEX5 monoubiquitination, which is a prerequisite for the proper peroxisomal matrix protein import in this organism.

Nevertheless, we have reasoned that a possible explanation for this result could come from the fact that the *TbPEX4* and *TbPEX22* genes were under control of a constitutive promoter. This could have caused an over-expression of the respective proteins, which may then have been incorrectly folded and deposited as inclusion bodies in the cell. Indeed, after having confirmed the expression of TbPEX4 in the ΔPEX4 and ΔPEX22 *S. cerevisiae* mutants by western blot analysis, we observed that essentially all expressed protein was present in the insoluble fraction (Fig. 43C). Because of this observation, we cannot exclude the possibility that, in principle, the putative TbPEX4 and TbPEX22 have the capacity to complement the defect in peroxisomal protein import in the ΔPEX4 and ΔPEX22 *S. cerevisiae* mutants when expressed in the proper form, at the proper level and in the correct compartment.


A
WTB4742
WTB4741
ΔPEX4
ΔPEX22
B
WT
ΔPEX4
ΔPEX22
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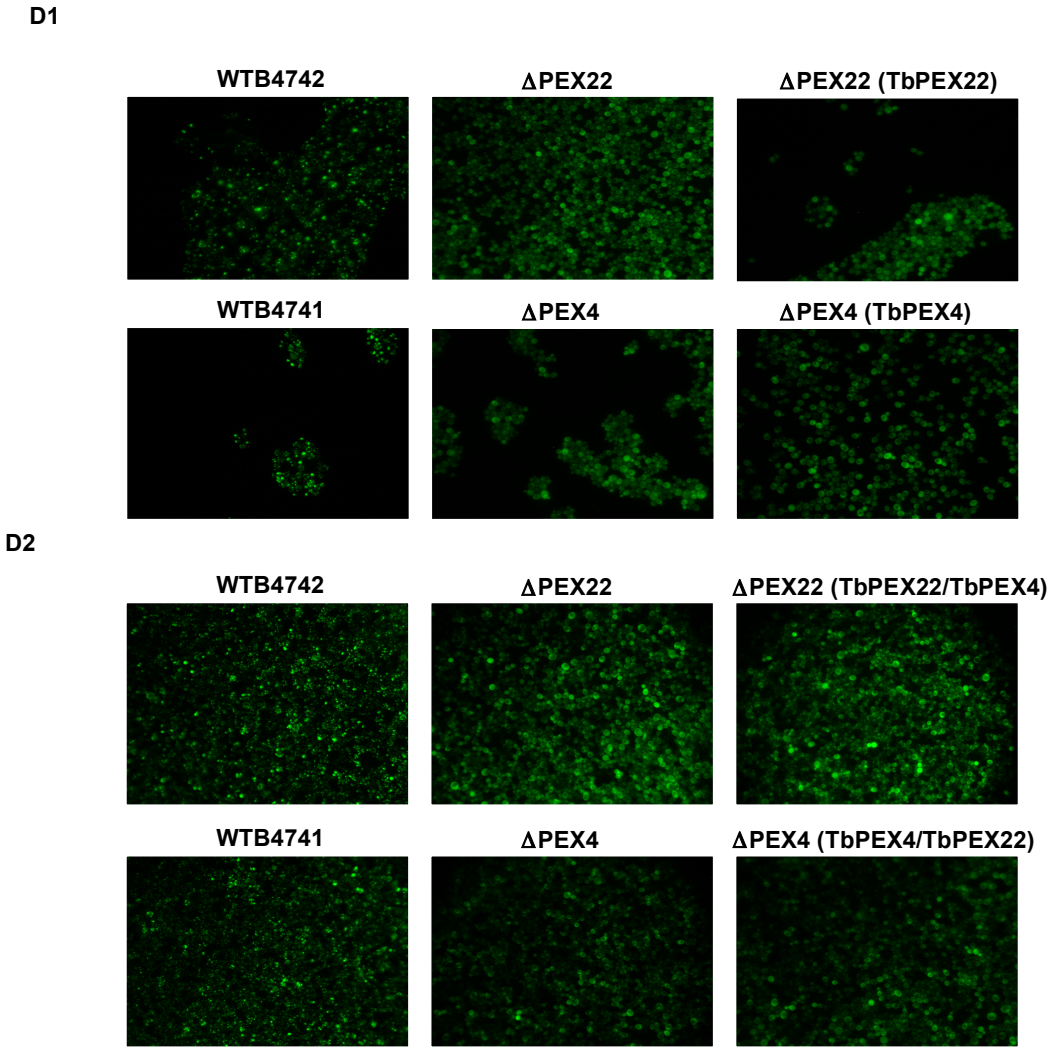
PEX4
Soluble
Pellet


D1
WTB4742
ΔPEX22
ΔPEX22 (TbPEX22)
WTB4741
ΔPEX4
ΔPEX4 (TbPEX4)
D2
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ΔPEX4
ΔPEX4 (TbPEX4/TbPEX22)

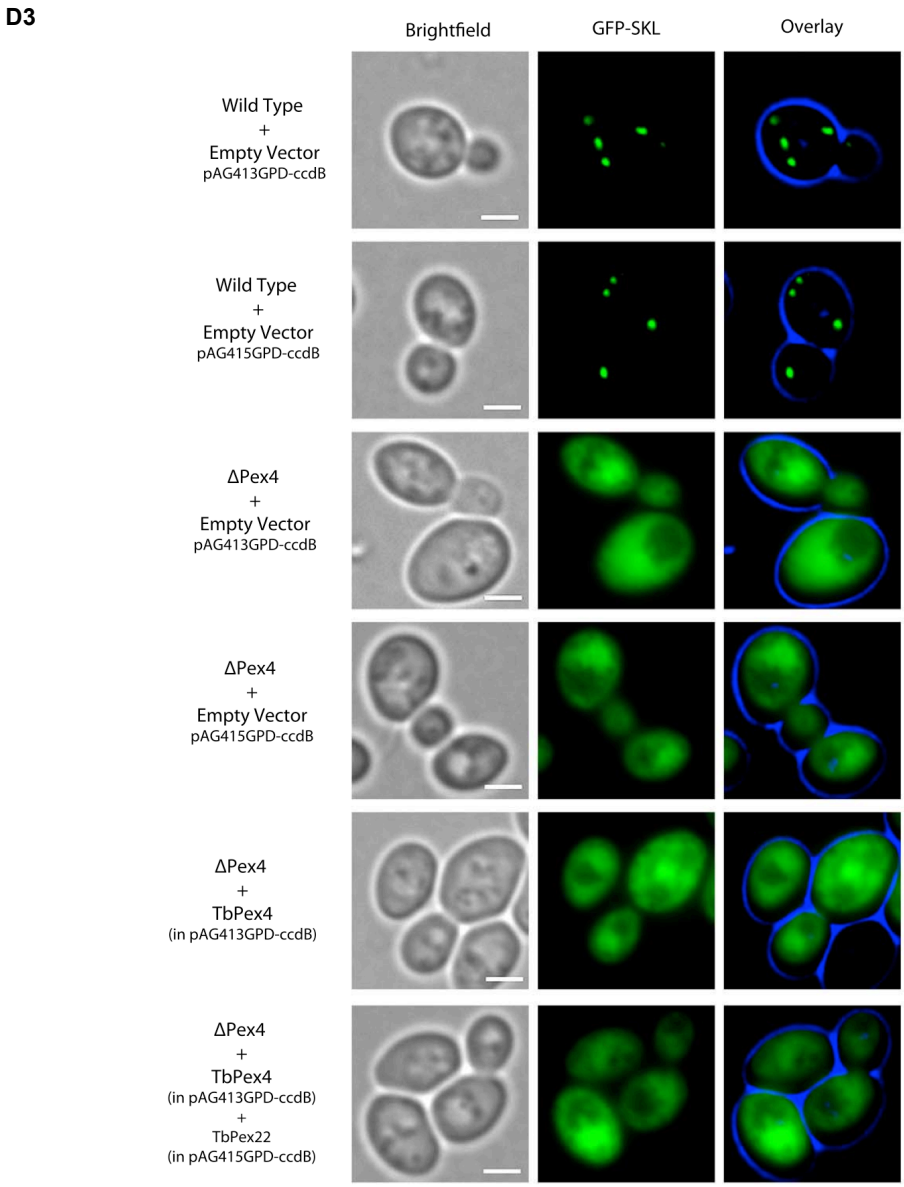


Figure 43. ***Complementation of yeast mutants by TbPEX4 and TbPEX22***. **A**. Analysis of matrix protein import using GFP-PTS1. Wild-type cells show a green fluorescent punctate pattern indicating functional peroxisomal import. ΔPEX4 and ΔPEX22 yeast mutants display diffuse cytosolic fluorescence indicative for a defect in peroxisomal protein import. **B** and **C**. TbPEX4 and TbPEX22 expressed in *S. cerevisiae* ΔPEX4 and ΔPEX22 mutants. (**B**) RT-PCR analyses of TbPEX4 and TbPEX22 were performed on wild-type and mutant cells after their transformation with empty vectors and the expression plasmids containing the *TbPEX4* and *TbPEX22* genes, respectively. (**C**) Western blot analysis using TbPEX4 antiserum was performed on *S. cerevisiae* wild-type and mutant cells after their transformation with empty vectors and the expression plasmids containing the *TbPEX4* and *TbPEX22* genes. The totality of TbPEX4 was found associated with the pellet fraction. **D.** Phenotypic analysis of complemented yeast mutants**. D1**. Single complementation. *S. cerevisiae* wild-type (negative control) and ΔPEX4 and ΔPEX22 mutant cells expressing TbPEX4 and TbPEX22, respectively, were analyzed microscopically for functional peroxisomal import. The diffuse cytosolic fluorescence pattern indicated the absence of functional complementation. **D2**. *S. cerevisiae* ΔPEX4 and ΔPEX22 mutants coexpressing both TbPEX4 and TbPEX22 show equally no phenotype restoration. **D3**. Detailed microscopic analysis of *S. cerevisiae* ΔPEX4 after single (TbPEX4) and double complementation (TbPEX4-TbPEX22) confirm the lack of phenotype restoration.

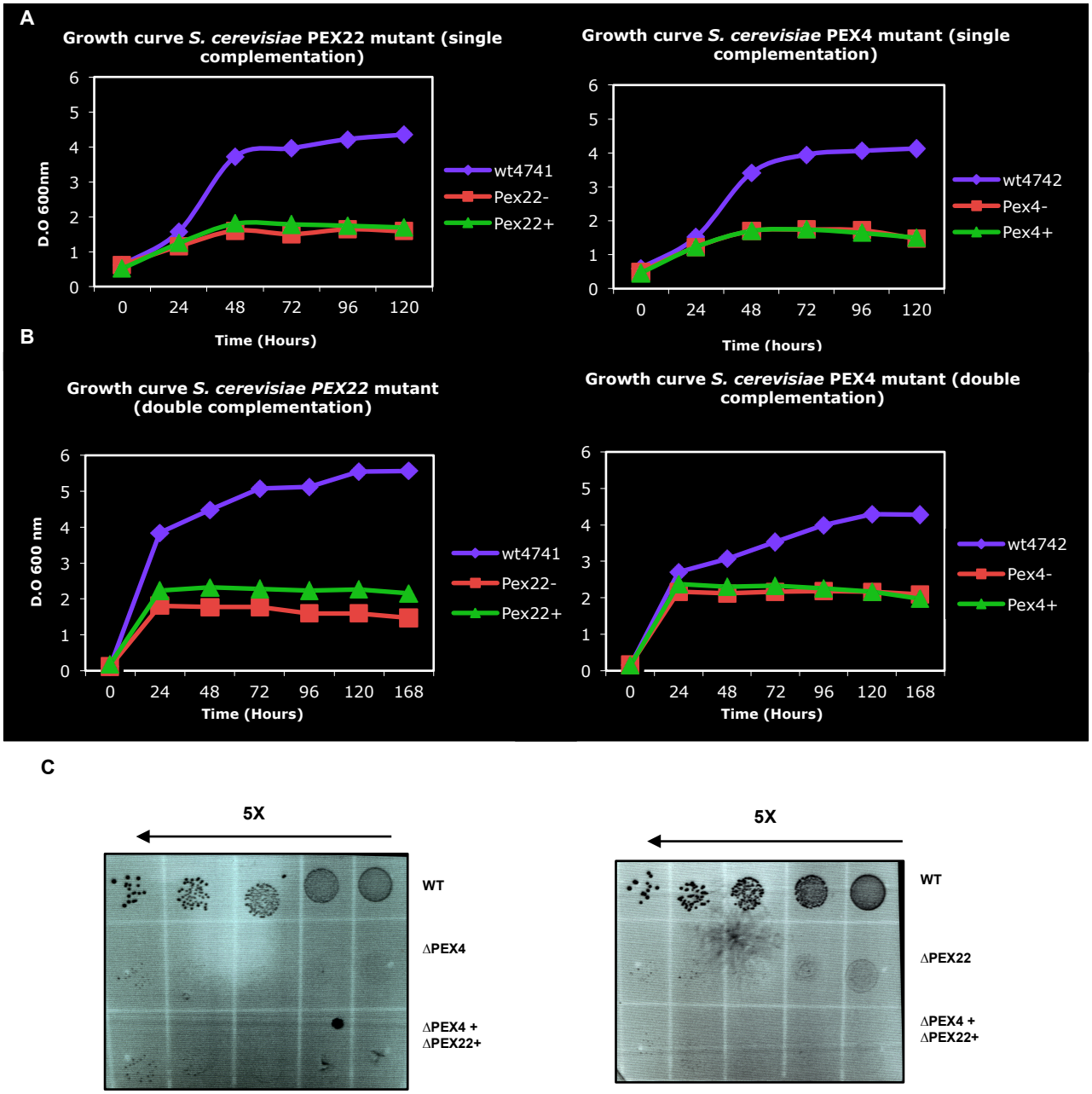


Figure 44. ***Growth behaviour in oleic acid-containing medium of yeast mutants complemented with TbPEX4 and TbPEX22.*** **A. Single complementation**. S. *cerevisiae* wild-type (negative control) and ΔPEX4 and ΔPEX22 mutant cells expressing TbPEX4 and TbPEX22, respectively, were grown in liquid (**A**) and on solid media (data not shown) containing oleic acid as the sole carbon source. The growth rate was determined by optical density at 600 nm in the case of the liquid cultures and by serial dilution of cell cultures on the solid media. **B** and **C**. **Double complementation**. *S. cerevisiae* ΔPEX4 and ΔPEX22 mutants coexpressing both TbPEX4 and TbPEX22 were grown in liquid (**B**) and on solid media (**C**). Phenotype restoration was observed neither by single nor by double TbPEX4 and TbPEX22 complementation.

3.6.1.3. Knock-out of the TbPEX4 gene

We have decided to study the molecular function of the TbPEX4 candidate by a knockout strategy. Two constructs were prepared that contain either the blastidicin or the puromycin resistance gene flanked by the 5'-UTR and 3'-UTR regions of the *TbPEX4* locus. We initially transfected *T. brucei* bloodstream and procyclic cells with the construct that contains the blasticidin resistance gene. Positive clones were selected for resistance to the antibiotic as well as by the confirmation of the genomic integration of the blasticidin resistance marker through PCR amplification. Selected clones of both life-cycle stage forms were then transfected with the puromycin resistance gene construct and positive clones were analyzed similarly. In the case of bloodstream form cells, we never succeeded in replacing both *TbPEX4* alleles since no cell growth was observed after the second round of selection with puromycin. A few times cloned populations of surviving cells were expanded and shown by genomic PCR to possess still an intact copy of the *TbPEX4* gene (data not shown). We interpreted this as the result of a gene duplication event, prior to the antibiotic resistance gene integration, that is a well-known phenomenon, particularly in bloodstream-form cells exposed to certain selective pressure to the gene to be knocked out (e.g. see Eid and Sollner-Webb, 1991). We also tried to create bloodstream-form *PEX4* conditional knockout cells by transfecting the blasticidin resistance clones created in the first round with a construct that expressed, under a tetracycline inducible promoter, a copy of the full-length *TbPEX4* gene in an hygromycin resistance vector (pHD677_TbPEX4_rescue). After selection in HMI-9 medium supplemented with blasticidin and hygromycin, positive clones were analyzed by genomic PCR for the integration of both antibiotic resistance genes. The selected clones were then transfected with the *TbPEX4* knockout construct harbouring the puromycin resistance gene followed by selection in HMI-9 medium supplemented with blasticidine, hygromycin, puromycin and tetracycline in order to induce expression of the *TbPEX4* rescue copy during selection. When growing cells were checked by PCR for genomic integration of all resistance gene markers and then analyzed in the absence or presence of tetracycline for a growth phenotype as well as by western blot analysis for the presence of

TbPEX4, it was noticed that, unfortunately, all clones still expressed the TbPEX4 protein even in the absence of tetracycline (data not shown). It was then evident for us that the second allele was not efficiently replaced, suggesting once more a gene duplication event. We can infer from this fact that TbPEX4 might be an important enzyme in the bloodstream form since cells seem reluctant to losing their gene.

In the case of the procyclic form, several clones were shown to have lost the *TbPEX4* gene and acquired the respective antibiotic resistance genes. This was proved by genomic PCR and by RT-PCR (Figure 45 shows the analysis performed with the clone used for the phenotypic characterization).

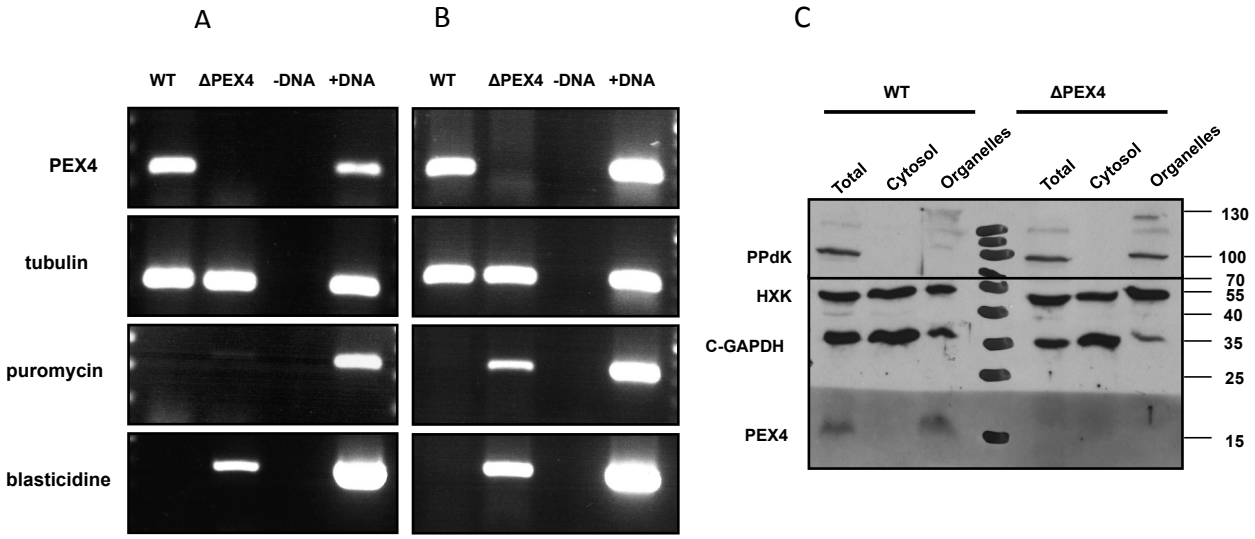


Figure 45. ***Validation of the ΔPEX4 procyclic T. brucei cell line.*** Both alleles of the TbPEX4 gene were successfully replaced by the blasticidin and puromycin resistance genes. **A**. PCR amplification of genes from genomic DNA of the wild-type (WT) and the ΔPEX4 (KOPEX4) cell line. **B**. RT-PCR analysis of cDNA prepared from transcripts of genes of the wild-type and the ΔPEX4 cell line. **C**. Western blot analysis of the TbPEX4 protein in cytosolic and organellar fractions of wild-type cells and the ΔPEX4 cell line. Cytosolic marker: Glyceraldehyde-3-phosphate dehydrogenase (C-GAPDH), dual cytosolic and glycosomal marker: Hexokinase (HXK), glycosomal marker: procyclic specific Pyruvate-phosphate dikinase (PPdK). The three approaches confirm the effective knockout of both alleles of the TbPEX4 gene and that the encoded protein is absent in the ΔPEX4 cell line.

Surprisingly, when we analyzed the ΔPEX4 cell line by western blotting we could still observe a protein band that migrated at approximately the same molecular weight as TbPEX4 (data not shown). This intriguing result made us perform a new analysis on the specificity of the TbPEX4 antiserum, which had been raised against the purified full-length denatured recombinant protein. This analysis

was based on western blots of samples obtained by cellular fractionation of *T. brucei* bloodstream and procyclic forms as well as on lysates of *E. coli* BL21, which express the full-length TbPEX4. In all cases the antiserum recognized a band of ≈18 kDa associated with the organellar fraction of both bloodstream and procyclic trypanosomes and a band corresponding to ≈20 kDa (the expected molecular weight for the full-length protein with fused to its N-terminus a His-tag) in the bacterial lysate (data not shown). We reasoned that a possible explanation for this result could be that, under the knockout conditions (absence of a PEX4-like protein which is essential for peroxisomal-matrix protein import in yeast cells and expected to be also the case in trypanosomes), another ubiquitin-conjugating enzyme (of similar molecular weight and similar structural features) is overexpressed to take over the function of TbPEX4. This would be possible if there is a potential redundancy in the function of UCB enzymes, allowing an enzyme whose principal function is in another process to function also, but possibly less efficiently, in the glycosomal protein import system. If that would be the case, our western blot result can be understood if the antiserum raised against the TbPEX4 protein could recognize conserved epitopes also present in the other, overexpressed UBC protein(s). The *T. brucei* genome apparently codes for 15 different putative ubiquitin-conjugating enzymes. In order to test the hypothesis of overexpression of a similar UBC protein in the ΔPEX4 cell line we have used three different strategies. First, we have performed 2D-electrophoresis on lysates of procyclic wild-type cells and the ΔPEX4 cell line with the aim of identifying by western blot, using the available TbPEX4 antiserum, a protein of similar molecular weight but with a different isoelectric point. Since we lacked the required equipment to perform this analysis in our home laboratory, experts of the platform for proteomic and protein analysis at the "Université catholique de Louvain" have assisted us. Despite several attempts in which proteins from the wild-type and ΔPEX4 cells were separated by 2D electrophoresis and analyzed on western blot, we were not able to detect a protein spot that might correspond to either TbPEX4 or other possible UBC proteins above the background signal, even in the wild-type cells (data not shown). When 2D Coomassie blue-stained gels from wild-type and ΔPEX4 cells were compared, with the aim of identifying proteins that could

reflect differences between the two cell lines, one protein spot migrating at ≈26 kDa and with an acidic isoelectric point was found to be evidently more abundant in the ΔPEX4 cells. We have tried to identify the protein corresponding to this spot by mass spectrometry, however without any success (data not shown).

As an alternative, we decided to customize antisera against a peptide specific for TbPEX4 but absent in the other putative *T. brucei* UBC proteins and with predicted good immunogenic properties according to bioinformatics analysis performed at Thermo Scientific. After immunization of two rabbits, the peptide-specific antisera were purified by affinity chromatography and the non-purified as well as the purified antisera were delivered for testing. Using the purified antisera we could definitively validate the PEX4 knockout status of our cell line (Fig. 45C). Remarkably, the new non-purified peptide-based TbPEX4 antisera still recognized a protein of similar molecular weight in the ΔPEX4 cell line. This result strongly suggests that another UBC protein of similar molecular weight was actually overexpressed in the ΔPEX4 cell line.

To investigate this possibility further, we analyzed by qPCR the mRNA levels of 10 putative UBC proteins, selected from the *T. brucei* genome database (Table 4) in wild-type and ΔPEX4 procyclic cells. The results obtained showed consistently that five different putative UBC mRNAs were overexpressed, in a statistically significant manner, in the ΔPEX4 cell line (Fig. 46). Highly interesting is the fact that three of these transcripts code for proteins with a very similar molecular weight as TbPEX4 (Table 4). In order to gain insight into the possible function of the ubiquitin-conjugating enzymes overexpressed in the ΔPEX4 cell line, we have performed a preliminary reciprocal BLAST analysis of the putative repertoire of *T. brucei* UBC enzymes, using as a query the characterized UBC enzymes from *S. cerevisiae* and human.

Table 4. ***Annotated Ubiquitin-conjugating enzymes in the T. brucei genome***. GeneDB accession numbers, predicted molecular mass, length (number of amino acids) and isoelectric point (PI) are displayed. The TbPEX4 candidate is highlighted in yellow. The expression of genes labelled with arrows was studied in the ΔPEX4 cell line. Blue arrows indicate genes whose expression did not change upon TbPEX4 knockout, while green arrows indicate genes of which overexpression was statistically proved.

		Mass (Da)	amino acids	pI
	Tb927.8.920	18,992 Da	172	4.6
→	Tb927.5.1000	16,766	148	6.52
→	Tb11.01.5510	24,056	216	4.44
	Tb927.2.2460	22,570	198	8.7
→	Tb927.7.2540	25,088	226	4.17
→	Tb927.8.6090	17,157	155	5.16
→	Tb927.2.3720	27,215	251	6.52
→	Tb09.211.0050	17,132	150	8.45
→	Tb927.4.3460	19,118	167	8.18
→	Tb11.01.5790	28,163	247	4.36
	Tb11.02.0815	15,654	138	6.93
→	Tb927.4.2710	18,140	158	6.27
→	Tb927.4.3190	24,366	220	7.43
	Tb927.7.6960	34,193	310	4.8
	Tb927.8.6510	22,937	198	6.95

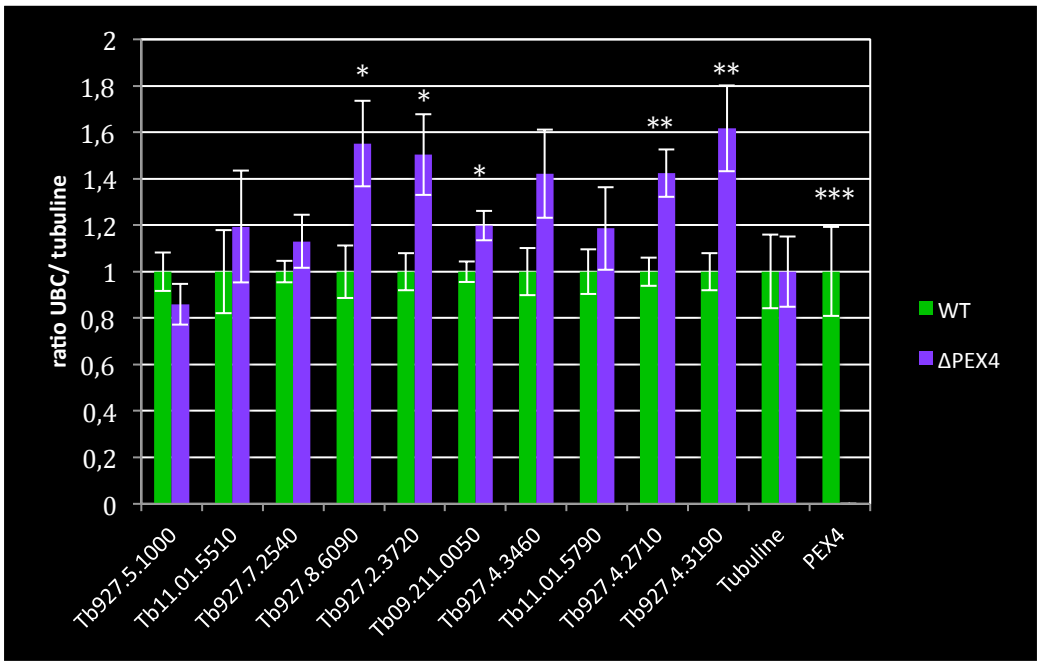


Figure 46. ***Expression levels of putative T. brucei Ubiquitin-conjugating enzymes (UBC) coding genes in the ΔPEX4 cell line.*** Total RNA from three different biological samples was extracted and used to synthesize cDNA. qPCR analysis of wild-type cells and the ΔPEX4 cell line was performed on three technical replicates and mRNA expression levels of 10 putative UBC proteins were compared. mRNA abundance was normalized to tubulin and data were analysed for statistical relevance via a student test (homoscedastic with 2 tail distribution). * $p<0.1$, ** $p<0.01$, ***$p<0,001$.

The results obtained are the following:

(Function of the UBC orthologues in yeast and human were retrieved from the Uniprot database).

Genes that did not change upon knockout of TbPEX4:

- Tb927.5.1000. This gene is the firm orthologue of the *ScUBC1/ScUBC4/ScUBC5*, genes coding for three redundant ubiquitin-conjugating enzymes involved in the selective degradation of regulated, short-lived, misfolded or abnormal proteins localized in the ER lumen or membrane via the ubiquitin-proteasome system. Remarkably, these three proteins have been shown to be redundant in the polyubiquitination of PEX5 in yeast mutants affected in peroxisomal matrix protein receptor recycling. This gene seems not be significantly overexpressed in the ΔPEX4 cell line.
- Tb11.01.5510. This gene is the firm orthologue of *ScUBC8*, coding for an enzyme mainly involved in the ubiquitination of histone H2A, but also in the negative regulation of gluconeogenesis though the proteasome-dependent degradation of fructose-1,6-bisphosphatase (FBPase).
- Tb927.2.2540. This gene is the firm orthologue of *ScUBC2*, encoding a protein which plays an important role in transcription regulation by giving a specific tag for epigenetic transcriptional activation, elongation by RNA polymerase II, telomeric silencing, and histone methylation. Is equally involved in post-replication repair of UV-damaged DNA and in N-end rule-dependent protein degradation.
- Tb927.4.3460 and Tb11.01.5790. These are likely orthologues of the genes *ScUBC6* and *ScUBC7*, respectively, coding for two enzymes with proved function also in the degradation of misfolded or regulated proteins localized in the ER lumen or membrane via the ubiquitin-proteasome system. Nevertheless, Tb11.01.5790 was also recognized as the most probably orthologue (with

identical similarity index) of *ScUBC3*, the gene of an enzyme that regulates several cellular processes including the ubiquitination of histone H2A, acting in the initiation of DNA replication (transition of the G1 to the S phase in the cell cycle), the ubiquitination and subsequent proteasomal degradation of target proteins including CDC6, CDC4 and CDC53 as well as the regulation of methionine biosynthesis genes.

Genes overexpressed upon knockout of TbPEX4:

- Tb09.211.0050. This gene is the firm orthologue of *S. cerevisiae UBC13* and human *UBE2N*. The encoded protein mediates the transcriptional activation of target genes. It plays a role in the control of progress through the cell cycle and differentiation. It is also involved in the error-free DNA repair pathway and contributes to the survival of cells after DNA damage. Importantly, the Tb09.211.0050 gene codes for a protein of similar molecular weight as TbPEX4 (17.37 kDa).
- Tb927.8.6090. This gene was recognized as the firm orthologue of *ScUBC11*, encoding an enzyme required for the destruction of mitotic cyclins and proteins that maintain sister chromatid cohesion in animal cells and in *Schizosaccharomyces pombe*, but proved not be essential in *S. cerevisiae.* In humans, UBE2C acts as an essential factor of the anaphase promoting complex/cyclosome (APC/C), a cell cycle-regulated ubiquitin ligase that controls progression through mitosis. It acts by initiating 'Lys-11'-linked polyubiquitin chains on APC/C substrates, leading to the degradation of APC/C substrates by the proteasome and promoting mitotic exit. The protein encoded by this gene has a very similar predicted molecular weight as TbPEX4 (17.15 kDa).
- Tb927.2.3720. This is a firm orthologue of human *UBE2S*, but not orthologous to any yeast gene by reciprocal BLAST. The protein acts together with UBE2C/UBCH10 in the anaphase promoting complex/cyclosome (APC/C), a cell cycle-regulated ubiquitin ligase that controls progression through mitosis by specifically elongating 'Lys-11'-linked polyubiquitin chains initiated by the E2 enzyme on APC/C substrates, enhancing the degradation of APC/C substrates by the proteasome and promoting mitotic exit. It also acts by elongating ubiquitin chains initiated by the E2 enzyme UBE2D1/UBCH5 *in vitro*; it is however unclear

whether UBE2D1/UBCH5 acts as an E2 enzyme for the APC/C *in vivo*.
Thus the proteins encoded by Tb927.8.6090 (the counterpart of human UBE2C) and Tb927.2.3720 (the counterpart of human UBE2S), both up-regulated at the transcript level, could act together to interfere with the cell cycle.

Interestingly, the two genes which showed to be the more significantly overexpressed in the ΔPEX4 cell line (Tb927.4.2710 and Tb927.4.3190) (Figure 46), did not yield a positive hit for being an evident orthologue of any of the ScUBC neither human UBC genes in our analysis. We cannot exclude, however, that Tb927.4.3190 could be a putative orthologous gene of *ScUBC6*, since it resulted in our analysis as the second hit with a high similarity index. More intriguing is the fact that the protein encoded by the gene Tb927.4.2710 seems not to be obviously related to any known UBC protein in *S. cerevisiae*, opening the possibility that it could be a trypanosome-specific gene product and plays an interesting role in the phenotype obtained in the ΔPEX4 cell line. Importantly, this gene codes for a protein with a predicted molecular weight of 18.14 kDa, the UBC protein with a molecular size most similar to that of TbPEX4.

Besides that this analysis represents only a rough approximation to the identification of possible orthologues of UBC proteins in *T. brucei*, for us it was an interesting and useful approach for trying to understand our previous data on the validation and characterization of the ΔPEX4 cell line.

Taking all these data together, we conclude that we successfully knocked out both alleles of the TbPEX4 gene in procyclic cells and we hypothesize that the conditions established for preparing the knockout in this cell line have created compensatory/adaptive mechanisms in which the expression of other UBC enzymes were upregulated above wild-type levels (three of these UBCs having a very similar molecular weight as TbPEX4). This could have had a direct effect on the phenotype associated with the absence of TbPEX4 for glycosome biogenesis, as well as in another cellular process such as the cell cycle regulation as will be discussed later.

Once we had confirmed the knockout of both alleles of the *TbPEX4* gene, we initiated the phenotypic characterization of the mutant cells, which involved, as previously with the RNAi cell lines, the study of growth behaviour, glycosomal matrix protein import and the ubiquitination of TbPEX5.

We observed that upon knockout of TbPEX4, the procyclic-form cells present a decreased growth rate when compared with wild-type cells (Figure 47). Although this decrease with a factor two seems not be very pronounced, taking into account the essentiality of glycosome biogenesis for the viability of the cells as shown in many previous studies (reviewed by Moyersoen *et al.*, 2004; Galland and Michels, 2010), it was consistently found in the phenotypic characterization. Very interestingly, we observed unexpected features with regard to the morphology and motility of the ΔPEX4 cells. These features involved a reduced cell size, a slower displacement of the cells in culture and an abundance of dividing cells, that remained two-by-two attached to each other because an apparently uncompleted longitudinal cleavage furrow. These seemingly glycosome-biogenesis unrelated defects will be discussed later.

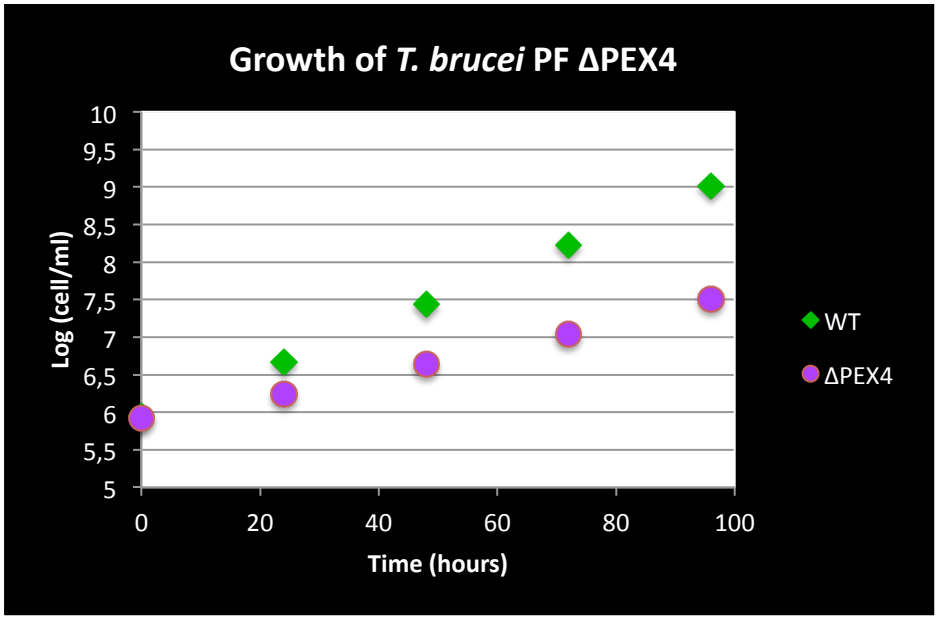


Figure 47. ***Growth behaviour of the ΔPEX4 cell line.*** Cellular growth of wild-type and ΔPEX4 procyclic cells was followed during 5 days in SDM79 medium. ΔPEX4 cells were shown to have a lower growth rate (by a factor 2) compared to wild-type cells.

To determine if glycosomal protein import is affected in the ΔPEX4 cells, we employed the following approach: first we created wild-type and ΔPEX4 cell lines that express GFP-PTS1 under control of a tetracycline-inducible promoter. To achieve that, we transfected wild-type cells with the pHD1336_GFP-PTS1 plasmid and the ΔPEX4 cells with the pHD677_GFP-PTS1. Positive clones were selected in the presence of the respective selectable markers and checked for the expression of GFP in the cells by fluorescence microscopy. Figure 48 shows a representative image of the fluorescence pattern obtained with the two cell lines when immobilized live cells where analyzed. Expression of GFP-PTS1 in both cases resulted in a typical glycosomal punctate pattern indicating the capacity of the wild-type as well as the ΔPEX4 cells of efficiently importing PTS-1-containing proteins into the organelle.

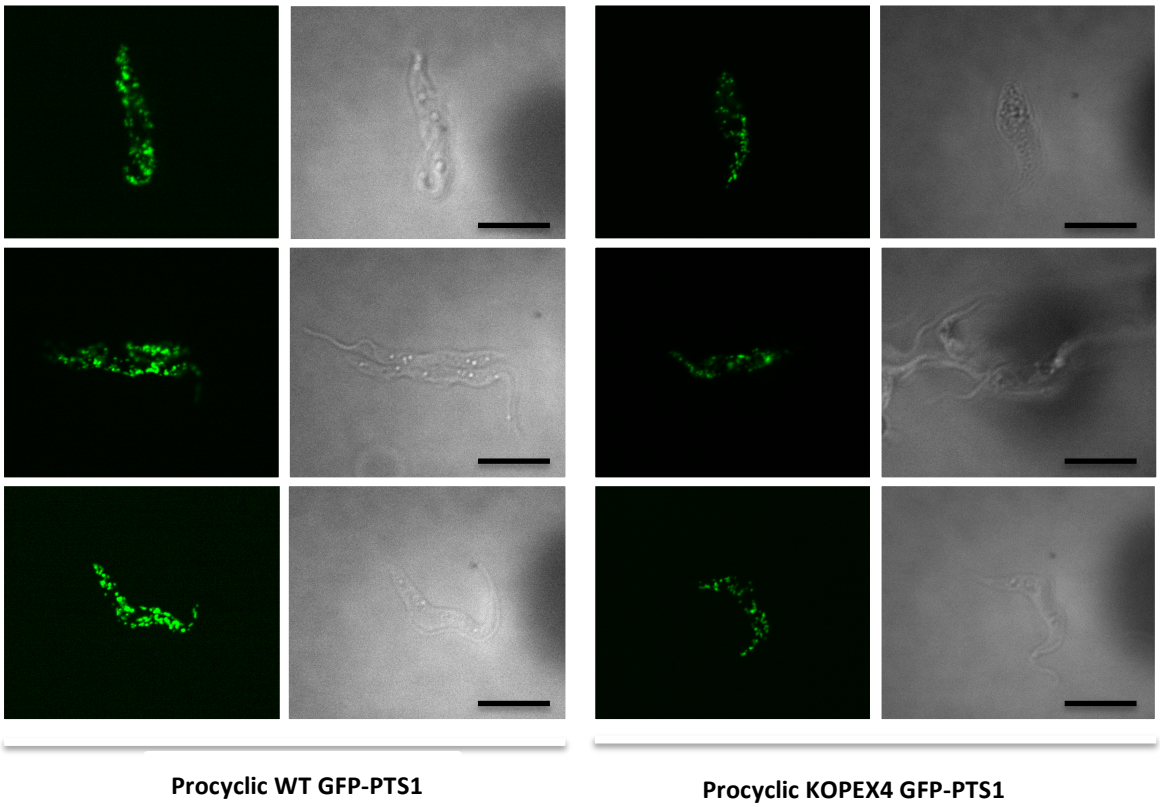


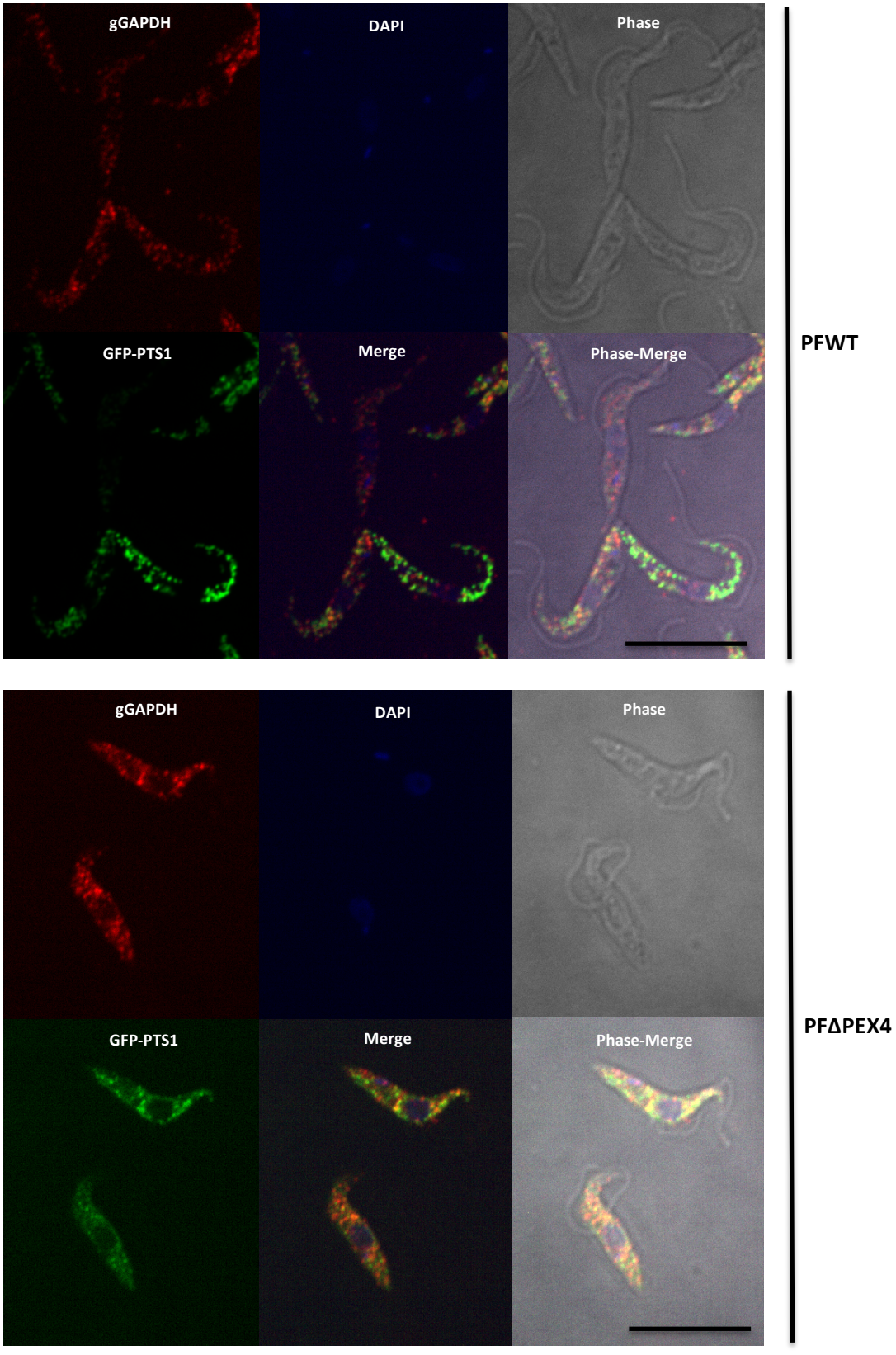
Figure 48. ***Glycosomal matrix protein import analysis in live cells***. Wild-type (WTPF) and ΔPEX4 live cells expressing GFP-PTS1 were analysed by confocal fluorescence microscopy. A punctuate pattern, typical of glycosomes, was observed in both cases. Scale bar: 10μm.

At this point we decided to validate the correct localization of the GFP-PTS1 protein by comparing it with well-known glycosomal enzymes as gGAPDH (a

PTS-1 containing protein) using confocal immunomicroscopy. Figure 49 shows a representative image of the results obtained. We could easily see in the wild-type as well as in the ΔPEX4 cells the colocalization of GFP positive signals with the immunostained gGAPDH, confirming its glycosomal localization and validating our system to study glycosomal protein import in live cells. In addition to a glycosomal staining for the GFP-PTS1 protein, also a fluorescent shadow-like signal throughout the cell body was observed in the ΔPEX4 cells, but which was absent in the wild type trypanosomes. Although this cytosol-like staining was not prominent, it might be an indication of partial mislocalization. In spite of the good colocalization of the two PTS-1 containing proteins, a very intriguing observation came from images of the wild-type cells; some cells presented exclusively the punctate red pattern coming from the immunostaining of gGAPDH and not the green fluorescence from the expressed GFP-PTS1 protein, while some other cells were exclusively positive for the GFP-PTS1 fluorescence but lacked the immunostaining of gGAPDH. More striking was the fact that some cells presented, besides glycosomes in which both signals merged, also puncta in which either one or the other fluorescent label was present. This observation suggests that different populations of glycosomes may exist in a cell. No scenario how such a heterogeneity may develop has been reported yet, but different explanations are possible. The notion of heterogeneity fits with the preliminary finding of apparently variable ratio of immature and mature glycosomes in a given cell population during growth from the stationary phase into the exponential phase during culturing of procyclic cells (Dr Meredith Morris, University of Clemson, USA, personal communication).

Taking these data together, we conclude that the absence of TbPEX4 does not lead to a prominent cytosolic accumulation of PTS-1 containing proteins as has been found for *S. cerevisiae* ΔPEX4 mutants. It should be mentioned however, that the deletion of PEX4 in *P. pastoris* did not cause an important mislocalization of peroxisomal matrix proteins, even though its role in peroxisome biogenesis has been validated and well described. We speculate that one of the *T. brucei* UBC enzymes overexpressed in the ΔPEX4 cell line (notably the candidate trypanosome-specific Tb927.4.2710), could have taken over, at least to a large extent, the function of TbPEX4 in the ubiquitination of TbPEX5, retaining the

possibility of a functional recycling of the receptor and thus glycosome biogenesis.



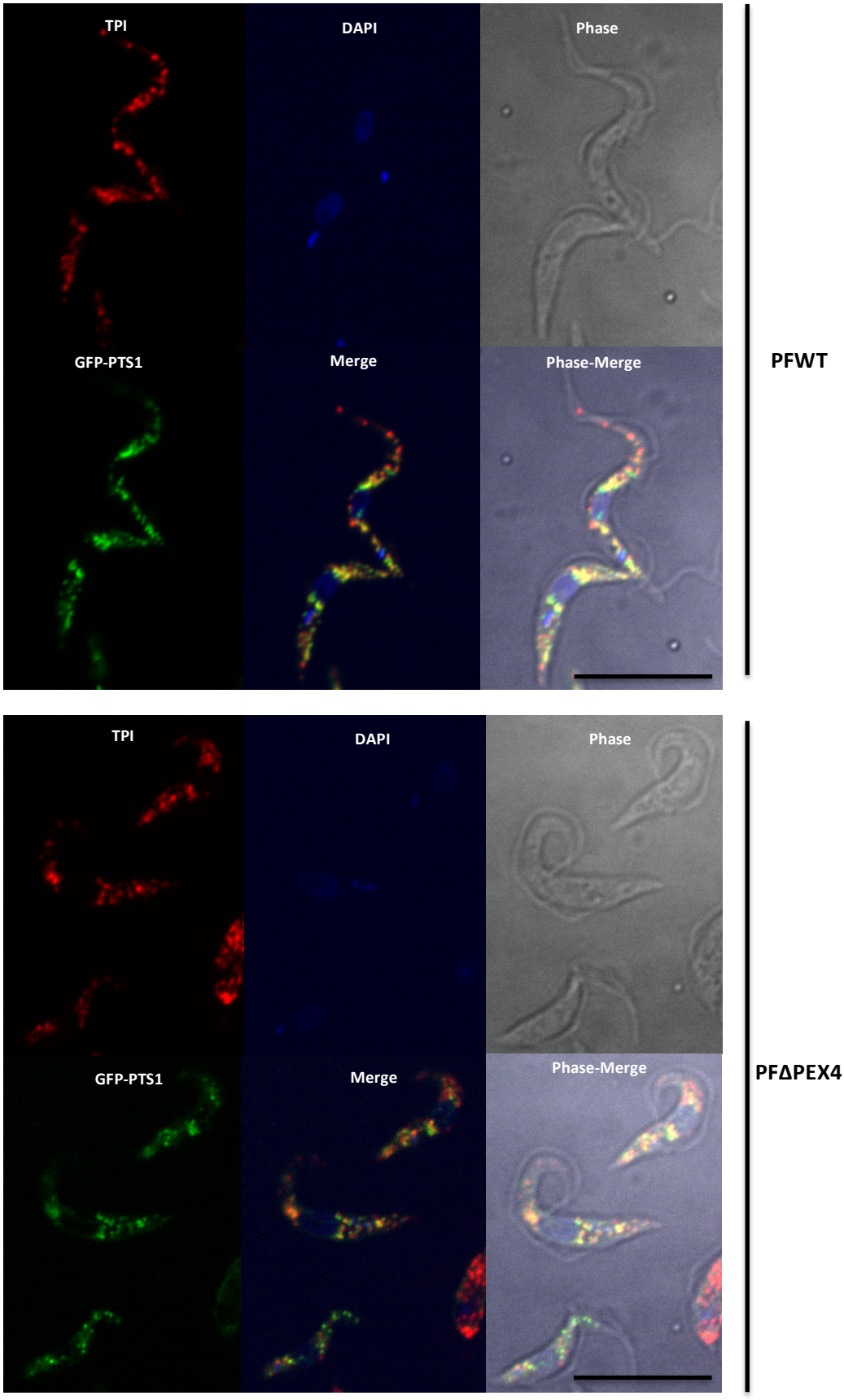

TPI
DAPI
Phase
GFP-PTS1
Merge
Phase-Merge
PFWT
TPI
DAPI
Phase
GFP-PTS1
Merge
Phase-Merge
PFΔPEX4

Figure 49. ***Validation of the glycosomal localization of the GFP-PTS1 protein in the procyclic wild-type cells and the ΔPEX4 cell line***. GFP-PTS1 expressing cells were fixed, permeabilized and immunolabelled with antisera for gGAPDH (PTS-1 containing protein) (A) and TPI (I-PTS-containing protein) (B). The colocalization of the GFP-PTS1 protein with glycosomal matrix markers was studied by confocal fluorescence microscopy. In both cases, GFP-PTS1 colocalized with the two glycosomal markers. A partial mislocalization of GFP-PTS1 and gGAPDH was found in the PEX4 cells (A). Scale bar 10 μm.

To determine, at the molecular level, if the ΔPEX4 cell line was affected in its TbPEX5 recycling, we performed an analysis of the receptor's ubiquitination using the same strategy that we established for the study of this modification in wild-type cells (see Result sections 2). Cellular fractionations of wild-type, ΔPEX4 and wild-type Myc-Ubiquitin cell lines were performed in the presence of NEM by selectively permeabilization of the plasma membrane with a low concentration of digitonin. Cytosolic and organellar fractions were prepared for SDS-PAGE in Laemmli buffer containing or not DTT and subsequently analyzed by western blotting. The blots were decorated with TbPEX5, Enolase and Aldolase antibodies. Figure 50 shows that TbPEX5 is in the ΔPEX4 cell line an abundant cytosolic protein as in wild-type cells, as well as in cells expressing Myc-Ubiquitin. No changes were observed in the steady-state levels of the protein in the cytosol neither in the organellar fraction of the mutant cells when compared to wild-type and Myc-Ubiquitin cells, indicating no important accumulation in the glycosomal membrane. Importantly, we observed that the slower migrating form of TbPEX5, which corresponds to the ubiquitinated form previously demonstrated to occur in bloodstream and procyclic cells, was importantly decreased in the ΔPEX4 cell line to nearly non-detectable levels. The abundance of the ubiquitinated form of TbPEX5 was shown once more not to be affected by the presence of DTT in the protein preparation, suggesting that this form of ubiquitination is lysine dependent. This result clearly shows that the protein identified as TbPEX4 plays a role in this form of ubiquitination of TbPEX5. This finding of a lysine-dependent monoubiquitination by PEX4 was somehow surprising, because the current model of peroxisome biogenesis in yeast cells assigned to PEX4 the function of cysteine-dependent monoubiquitination of PEX5, which occurs in wild-type cells to signal PEX5 retrieval from the membrane by the translocases PEX1/PEX6 during the receptor recycling. However, in the absence of PEX4, as well as in the absence of

its anchor protein PEX22, it has been repeatedly found that yeast PEX5 is polyubiquitinated at a lysine residue, not by PEX4 but by redundant UBC proteins (UBC4, UBC5, UBC1), to target the receptor for proteasomal degradation. This situation is not considered as a default process during receptor recycling, but instead as a quality control mechanism to dispose of excess PEX5 when it accumulates in the membrane as a consequence of the impaired recycling signal in the absence of PEX4. Our data seem to indicate that no accumulation of TbPEX5 occurs in the glycosomal membrane when the ubiquitination signal, given by TbPEX4, is affected. Since we cannot exclude the possibility of a functional redundancy in the ubiquitin-conjugating system involved in the receptor recycling (since a faint signal of ubiquitinated TbPEX5 is still detected in the ΔPEX4 mutant), we could not establish with certainty if the total abolishment of ubiquitination of the receptor would cause its accumulation on the glycosomal membrane. This result is also in agreement with the lack of a PTS-1 protein import defect in the ΔPEX4 cell line.

In order to study in more detail the molecular function of TbPEX4 in the export of TbPEX5 from the glycosomal membrane we performed a preliminary experiment of *in vitro* import and export of TbPEX5 using a methodology proved functional in experiments with mammalian peroxisomes (Okumoto *et al.*, 2011). First, we produced *in vitro* radioactively labelled ^{35}S-TbPEX5, using a commercial cell-free coupled transcription-translation system. Appropriate synthesis was verified by analysis of 10% of the reaction product by SDS-PAGE followed by autoradiography of the gel. Figure 51A1, shows an intense signal corresponding to the full-length TbPEX5. In order to obtain a source of intact glycosomes to perform the *in vitro* import/export experiment we performed differential centrifugation of procyclic-form wild-type and ΔPEX4 cells. A post-mitochondrial fraction (PMF) containing glycosomes, cytosol and microsomes was obtained after centrifugation at 5,000 *x g*. This fraction was used for the *in vitro* import experiment as well as an equivalent portion as a source of cytosol after its ultracentrifugation. This cytosolic fraction supplemented with an ATP regenerating system was used for the *in vitro* export experiment. After incubation of the PMFs from wild-type and ΔPEX4 cells with ^{35}S-TbPEX5, a

protease protection assay was performed followed by centrifugation to verify appropriate import of the radiolabelled TbPEX5 into the organelles. Samples were analyzed on SDS-PAGE followed by western blotting or radioactive autoscanning. In Figure 51B1, is shown that, in both wild-type and in ΔPEX4, radiolabelled TbPEX5 was highly abundant in the total fraction as well as in the supernatant of the samples not treated with proteinase K. A very reduced amount of the radiolabelled protein was associated with the organellar fraction of the untreated sample, indicating a very minor protein import. No signal of TbPEX5 was detected after immunoblotting in the proteinase K treated samples, suggesting that proteolysis has been effective. When these fractions where analyzed by western blot using antibodies against TbPEX5, as well as against the cytosolic marker Enolase and the glycosomal marker Aldolase, we could confirm that indeed the centrifugation step after the proteinase K treatment led to the separation of the majority of glycosomes from the cytosolic fraction (see abundance of Aldolase in the organellar pellet). However, a remaining amount of Aldolase was still detected in the cytosol. We could equally observe that the initial amount of protein in the ΔPEX4 sample was considerably higher than in that of the wild-type, making difficult the interpretation of the data (more TbPEX5 was associated with the organellar pellet). It was surprising that the proteinase K treatment seems to have been very efficient in the degradation of cytosolic TbPEX5 (radiolabelled and endogenous) but not of Enolase (a cytosolic protein) neither of Aldolase after solubilization of the glycosomal membrane by Triton X-100 treatment. Since the signal detected in the proteinase K+/Triton X-100- (designated as P+/T- O) in the autoscanned gel was extremely low, we can assume that the *in vitro* import of ^{35}S-TbPEX5 did not efficiently occur, neither into glycosomes from wild-type cells nor into those from ΔPEX4 trypanosomes. Taking in consideration that our system was not experimentally ideal, since analyzed samples contained the endogenous TbPEX5 proteins, which cycle between the cytosol and the glycosomal membrane, it is not surprising that the ^{35}S-TbPEX5 could not be associated with the glycosomes due to the likely physical occupancy by the endogenous protein in the organelle. Indeed, by a longer exposure of the *in vitro* import western blot films, we could detect a small

amount of TbPEX5 associated with the organellar fraction in the P+/T- O condition, thus supporting our hypothesis.

The analysis of the *in vitro* export experiment corroborates that actually no association of the ^{35}S-TbPEX5 occurred since no signal was observed in the organellar neither in the cytosolic fraction where the protein was supposed to be released (Fig. 51C).

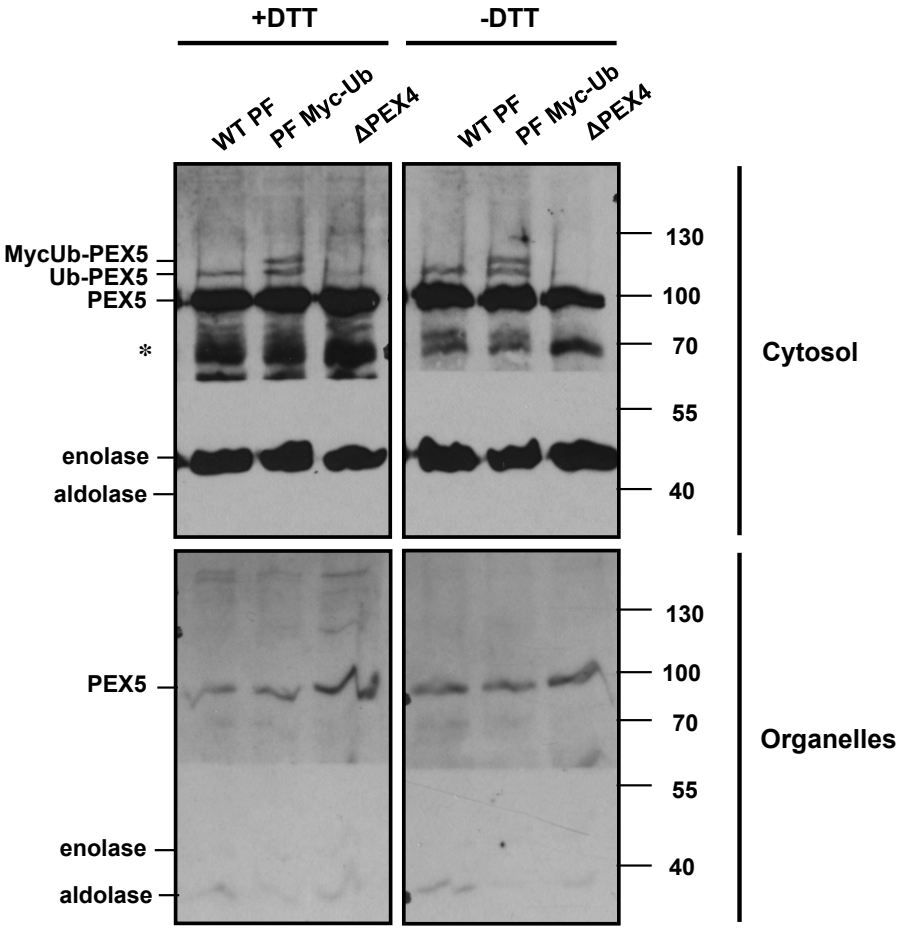


Figure 50. ***TbPEX5 ubiquitination is impaired in the ΔPEX4 cell line***. Cytosolic and organellar fractions were obtained by selective permeabilization of the plasma membrane with a low concentration of digitonin in the presence of 20 mM of NEM. Western blot analysis shows an important decrease in the level of the Ub-TbPEX5 form in the ΔPEX4 cell line, indicating that TbPEX4 functions as an ubiquitin-conjugating enzyme involved in TbPEX5 ubiquitination in wild-type cells. No important changes in the steady-state levels of TbPEX5 were observed in the cytosolic neither in the organellar fraction. The * indicates the *in vitro* proteolytically processed form of TbPEX5.

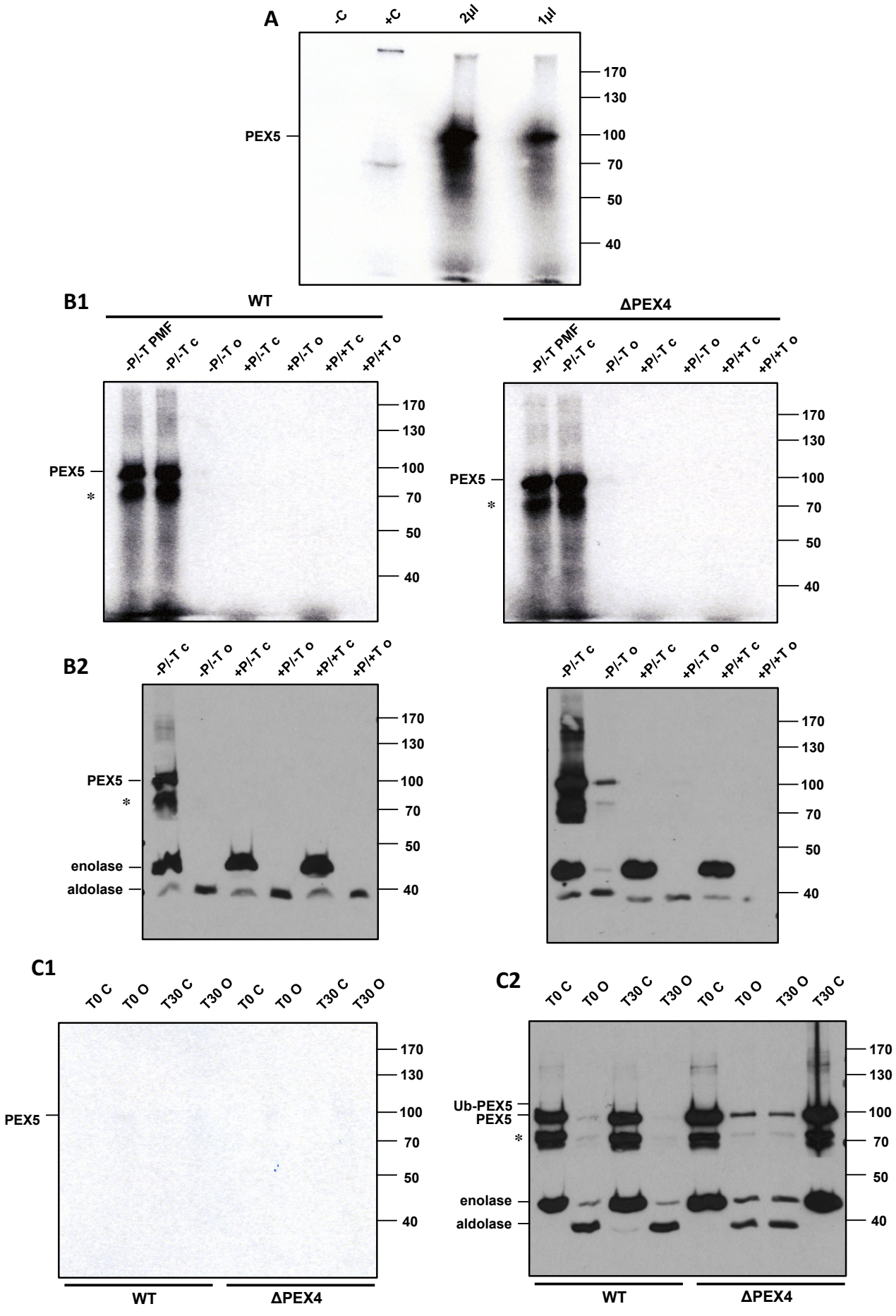

A
-C
+C
2µl
1µl
PEX5
170
130
100
70
50
40
B1
WT
ΔPEX4
-P/-T PMF
-P/-T c
-P/-T o
+P/-T c
+P/-T o
+P/+T c
+P/+T o
PEX5
*
B2
enolase
aldolase
C1
T0 C
T0 O
T30 C
T30 O
C2
Ub-PEX5
PEX5

Figure 51. ***In vitro import/ export of ^{35}S-TbPEX5 in glycosomes of wild-type and ΔPEX4 cells***. **A**. *In vitro* transcription/translation of TbPEX5 was performed in a cell-free commercial system. Different amounts of the DNA template (plasmid pET24-TbPEX5-His-tagged) were used (1 µl and 2 µl). –C: no DNA in the preparation; +C: Luciferase cDNA from the kit. **B**. *In vitro* import experiment. **B1** Autostaining of ^{35}S-TbPEX5 in wild-type and in ΔPEX4 cells shows an abundant amount of *in vitro* synthesized ^{35}S-TbPEX5 in the post-mitochondrial fraction (PMF) and the cytosolic fraction of proteinase K untreated samples (-P/-T), while a very low amount of ^{35}S-TbPEX5 was barely detectable in the organellar fraction of the same sample. Proteinase K treatment was efficient to degrade the ^{35}S-TbPEX5 in the cytosolic fraction whenever it was included in the assay (+P/-T and +P/+T). **B2**. Western blotting analysis of *in vitro* import samples shows endogenous TbPEX5 as well as ^{35}S-TbPEX5 (assuming that the signal obtained comes from the contribution of the signal provided by the recognition by the antiserum of both types of protein) abundantly present in the cytosolic fraction of cells not treated with proteinase K. A minor amount of TbPEX5 was detected in the organellar fraction of the ΔPEX4 sample. The western blot analysis also shows the efficacy of proteinase K in degrading endogenous TbPEX5. The localization of the cytosolic (Enolase) and glycosomal (Aldolase) markers shows that the cell fractionation resulted in proper separation of the glycosomes in the organellar pellet with only some small contamination to the cytosolic fraction. No cross-contamination of the organellar pellet with cytosolic proteins was observed. **C**. *In vitro* export of ^{35}S-TbPEX5. **C1** Autoscanning of ^{35}S-TbPEX5 shows no detectable signal in the organellar pellet (O), neither in the cytosol (C) before (T0) and after (T30) the *in vitro* export reaction in the wild-type and the ΔPEX4 samples. **C2**. Western blot analysis of the *in vitro* export samples shows that endogenous TbPEX5 was highly abundant in the cytosolic fraction used for the export of ^{35}S-TbPEX5 (T0 C, T30 C) in both wild-type and ΔPEX4 cells. Also noticeable is the monoubiquitinated form of TbPEX5 in the wild-type samples T0 C and T30 C. The signal obtained from the cytosolic marker Enolase suggests a small cross-contamination of the organellar pellet with cytosolic proteins (probably due to the lack of a washing step after the centrifugation), which can explain the presence of endogenous TbPEX5 strongly associated with the organellar pellet especially in the ΔPEX4 samples (T0 O, T30 O). Migration patterns of molecular-weight markers are reproduced at the right-hand side of each panel.

Irrespective of the negative results of this experiment, we would like to stress two observations: 1) We showed that ^{35}TbPEX5 added to the PMS was processed by the same mechanism of proteolysis that converts the native TbPEX5 (100 kDa) to the faster migrating form (70 kDa) (Fig. 51B1). This is in agreement with our previous data indicating an *in vitro* processing of TbPEX5 by (a) specific unknown protease(s). 2) We could detect once more the monoubiquitinated form of TbPEX5 in the cytosolic fraction of wild-type cells while no-signal was observed in the simple of ΔPEX4 cells (Fig. 51C2).

3.6.1.3.1.1. Glycosome-independent morphology phenotype.

As was mentioned above, we observed consistently by light microscopy that cultured, growing ΔPEX4 cells presented an apparent smaller cell size and somewhat irregular motility patterns. Moreover, there seemed a prevalence of dividing cells remaining attached to each other by an apparently incomplete separation of the cleavage furrow. However, these features were difficult to define more precisely and to quantify at the resolution of regular phase-contrast microscopy.

In order to gain insight into the effect at the ultrastructural level of the knocking-out of the *TbPEX4* gene and the simultaneous overexpression of some other UBC genes, we decided to perform scanning and transmission electron-microscopy of the ΔPEX4 cells. This was done in collaboration with Dr Patrick Van Der Smissen and Prof. Pierre Courtoy in the Cell Biology Unit of our home institute, with the available Platform for Imaging Cells and Tissues. Wild-type and ΔPEX4 procyclic cells were grown and harvested in the exponential phase ($5x10^6$ cells/ml) and then treated and fixed for scanning and transmission electron-microscopy as described in Chapter 4 (General methodologies).

Figure 52 and 53 show the results obtained. When observing the cell morphology by scanning electron-microscopy, a large part of the cell population presented an aberrant cell shape with a shorter and wider cell body compared to the characteristic elongated-thin shape of normal procyclic trypanosomes (Figure 52).

In an effort to quantify this morphology defect, we analyzed the projected area of the cell body. The results obtained by examination of 100 cells of each sample are presented in Table 5. The mean area of projections was 8773 nm^2 for wild-type cells and 5840 nm^2 for ΔPEX4 cells. A "Kolmogorov-Smirnov Two Sample Statistical Test" corroborated the extremely high significance of this cell size difference (Table 6). This unexpected phenotype raised the question whether this effect could be attributed to a defect in a glycosome-associated event or if it was related to an alteration in another cellular process, for example in the cell cycle.

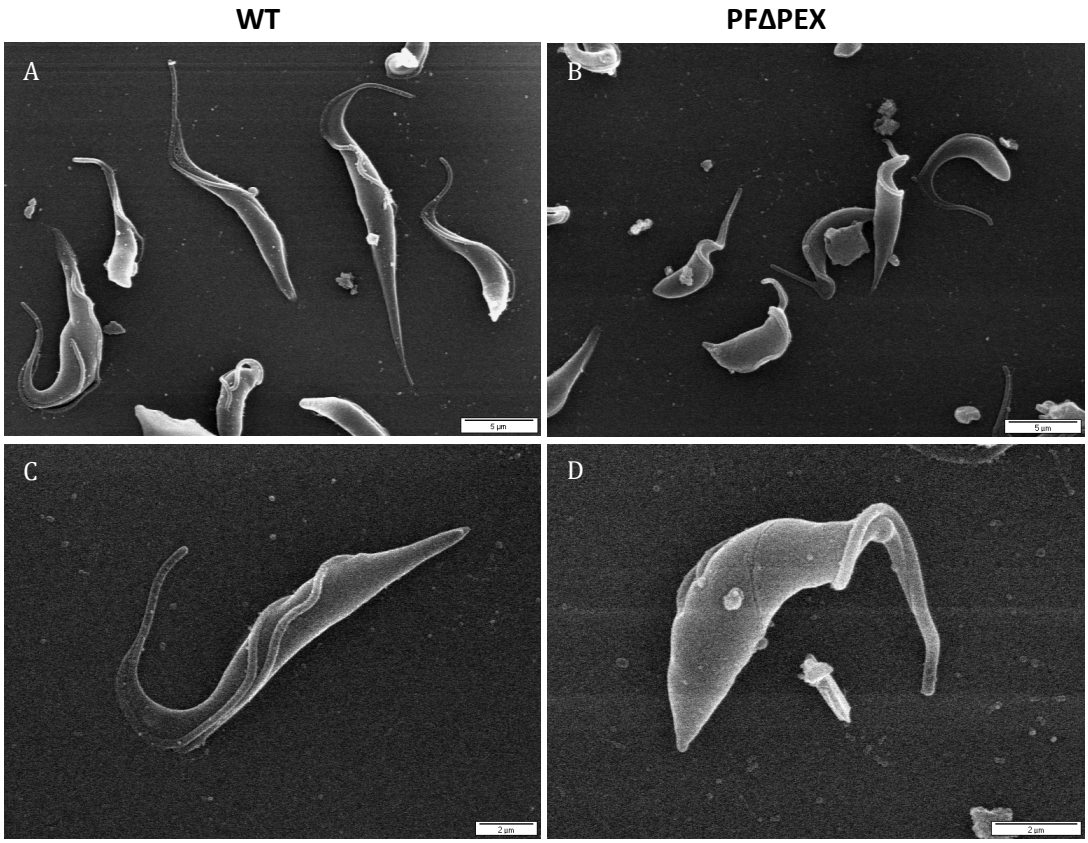


Figure 52. ***Scanning electron-microscopy of T. brucei wild-type and ΔPEX4 procyclic form cells.*** 100% of the wild-type cells presented a typical elongated thin shape (**A** and **C**). Procyclic ΔPEX4 trypanosomes formed a heterogeneous population in which approximately 85% of the cells (**B** an **D**) presented an aberrant cell-shape (shorter cell body and reduced cellular size). About 15% of the trypanosomes displayed an apparent normal morphology (not shown). Cells in panels C and B show two flagella indicating cell division.

Table 5. ***SYSTAT analysis of the projected area of T. brucei wild-type and ΔPEX4 procyclic cells*****.** 100 cases and 6 variables were processed.

	WT_AREA	KO_AREA
N° of analysed cells	100	100
Minimum	3982.000	2000.000
Maximum	14638.000	10130.000
Range	10656.000	8130.000
Sum	877393.000	584090.000
Median	8488.500	5866.000
Mean	**8773**	**5840**
Std. Error	240.458	176.186
Standard Dev	2404	1761
Variance	5782021.541	3104135.141
C.V.	0.274	0.302

Table 6. ***Kolmogorov-Smirnov Two Sample Test of the projected area of T. brucei wild-type and ΔPEX4 procyclic cells***. Maximum differences for pairs of variables (not parametric test).

Sample 1	Sample 2	Maximum differences of the relative distribution	P value
WT_AREA	WT_AREA	0.000	#
ΔPEX4_AREA	ΔPEX4_AREA	0.000	#
WT_AREA	ΔPEX4_AREA	0.560	>0.000

To address this question we have further analyzed by transmission electron-microscopy the ultrastructural features of the knockout cells and compared them with wild-type trypanosomes. In Figure 51 is shown a representative selection of pictures that summarizes the results obtained. In wild-type cells we found a typical ultrastructural organization of procyclic trypanosomes; in the majority of the analyzed images a well-defined nucleus, structures belonging to the parasite's elongated mitochondrion, acidocalcisomes, flagellum, and several glycosomes were observed (Fig. 53A, 53B and 53C). In the ΔPEX4 cells we detected again a heterogeneity in the population. In nearly all images obtained, glycosomes recognized as vesicles with a single lipid bilayer and an electron-dense matrix were abundant (Fig. 53D and 53E). The size and structure was comparable to that found for glycosomes of wild-type cells. Remarkable was the presence in some cells of intriguing, unidentified vesicles which should probably be attributed to an 'illness' of the cells (opinion by Dr Isabelle Coppens, Johns Hopkins University, USA; an expert in electron-microscopy of protist parasites, who kindly collaborated with us in the analysis of the images) (Fig. 53F). The apparent cause of the formation of such structures remains currently unknown. Although no important structural differences at the flagellar components (paraflagellar rod, FAZ and axoneme) were found, it was intriguing to observe that at least the majority of ΔPEX4 cells seemed to move differently in culture when compared to the wild-type trypanosomes.

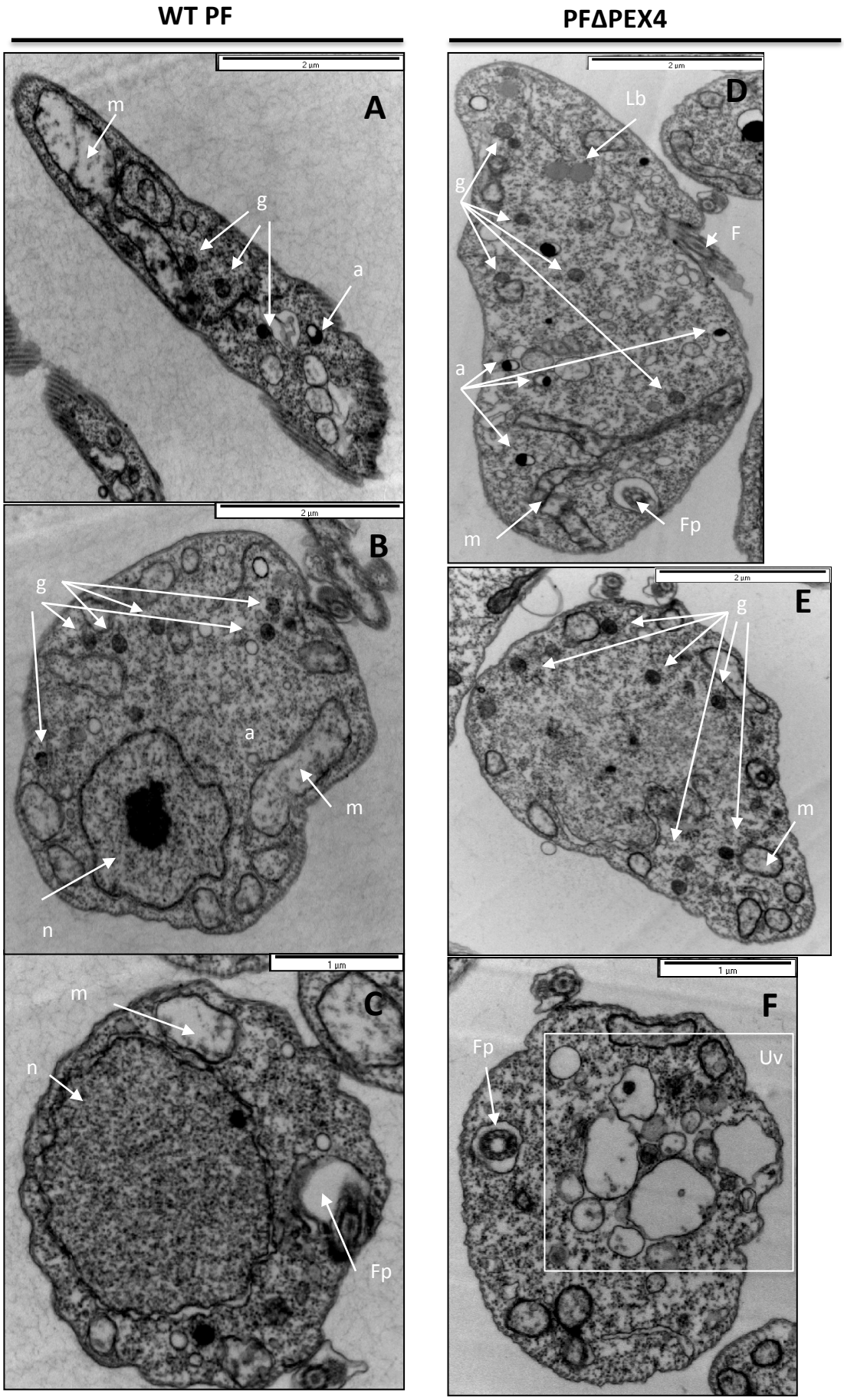

WT PF
PFΔPEX4
2 µm
A
m
g
a
2 µm
B
g
a
m
n
1 µm
C
m
n
Fp
2 µm
D
Lb
g
F
a
m
Fp
2 µm
E
g
m
1 µm
F
Fp
Uv

Figure 53. ***Transmission electron-microscopy of T. brucei wild-type and ΔPEX4 procyclic form cells.*** **A**, **B** and **C** are wild-type cells showing a well defined nucleus (n), mitochondrion (m), flagellar pocket (Fp), acidocalcisomes (a) and several electron-dense particles identified as glycosomes. **D**, **E** and **F** correspond to ΔPEX4 cells showing similar structures as wild-type cells and in addition lipid bodies (D). The cells in D and E present signs of cell division as two apparently distinct flagellar structures can be recognized (indicated as F and Fp in D). The cell in F shows undefined vesicles (Uv), which are considered as signs of illness. Notably these types of vesicles were only observed in some ΔPEX4 cells.

In an attempt to obtain, at least by rough approximation, evidence for this subtle but interesting defect, the cell displacement in culture was measured. We performed time-lapse image capturing of procyclic wild-type and ΔPEX4 cells in culture and analyzed the changes in the position of individual random cells (An example of a representative analyzed image of each sample is shown in Figure 54). Table 7 presents the measured distance (μm) and velocity (μm/s). It was interesting to see a great variability in the speed between cells of the wild-type culture, indicating that, in general, procyclic trypanosomes are able to swim in liquid cultures both slow and fast. Nevertheless, we found a higher proportion of cells in the ΔPEX4 culture that seemed to swim less fast, making effective displacement less frequent than observed for wild-type cells. Unfortunately, we could not analyze this motility phenotype in more detail, since the focus of our research was on the study of the function of PEX4 in glycosome biogenesis. Cytokinesis and flagellar function were beyond the scope of this thesis project. In spite of that, we would like to speculate that it would not be surprising that a possible disregulation of a ubiquitination process as a result of the overexpression of ubiquitin-conjugating enzymes (notably putative enzymes involved in the control of cell cycle such as Tb09.211.0050, Tb927.8.6090 and Tb927.2.3720), may have caused the observed cell shape and motility defect in the mutant ΔPEX4. It is now well known that cell cycle and flagellum formation are intimately related in *T. brucei* and that a defect in either one or the other process may lead to altered motility patterns and size (Vaughan, 2010). It is also worth mentioning here that it was recently reported that a small-ubiquitin modifier (SUMO), one of the members of the ubiquitin-like family, is required for mitosis of procyclic-form *T. brucei* (Liao *et al.,* 2010), opening the question whether a controlled function of UBC proteins may also be essential for proper cell morphogenesis in this parasite.

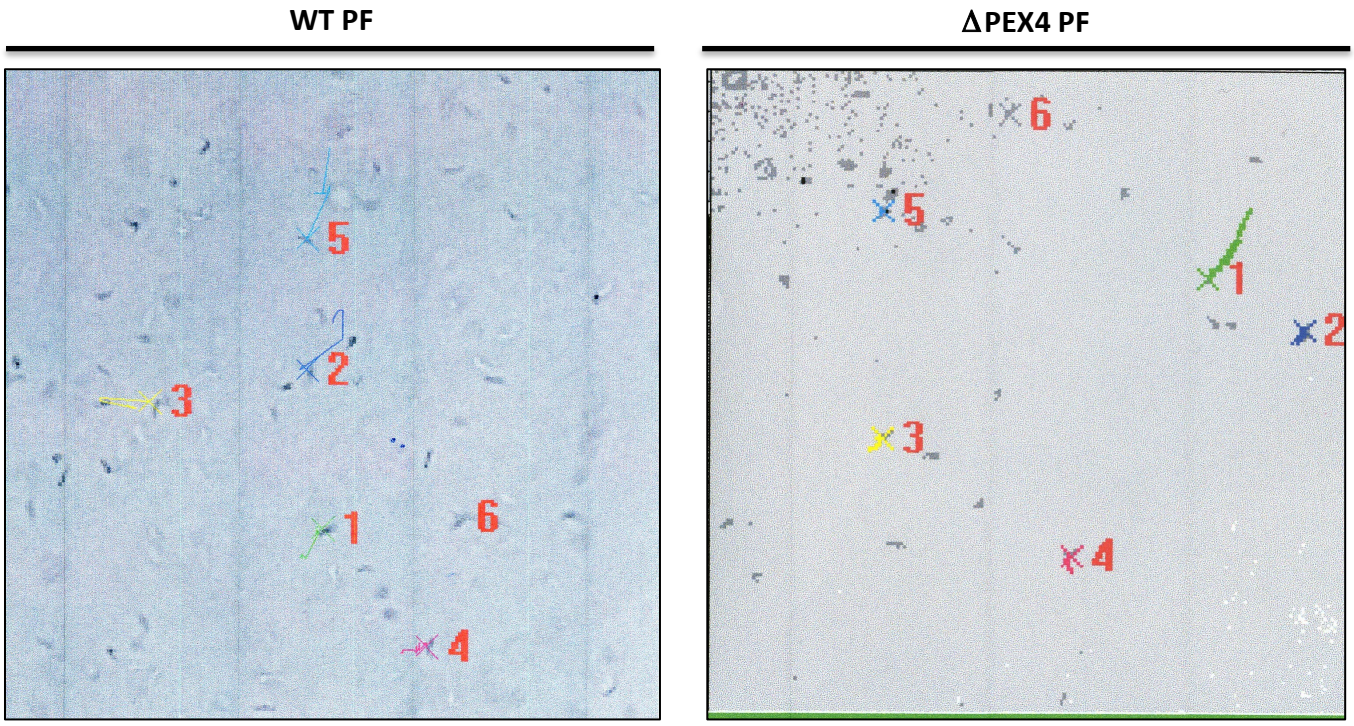


Figure 54. ***Motility analysis of T. brucei wild-type and ΔPEX4 procyclic form cells.*** Time-lapse images were taken from cells in culture. A track object analysis was performed using MetaMorph software on the images and statistic parameters (mean distance and standard deviation of the distance) were calculated in 6 random objects/image. Images in this figure show an example of the tracked objects in wild-type and ΔPEX4 procyclic-form cells which correspond with the measurements presented in Table 7.

Table 7. ***Motility parameters obtained of T. brucei wild-type and ΔPEX4 procyclic form cells***. Distances are expressed in μm (conversion analysis indicates that pixels = μm. Mean velocity is expressed in μm/s. Values highlighted in yellow indicate that ΔPEX4 cells perform less effective displacements.

WT	***Object 1***	***Object 2***	***Object 3***	***Object 4***	***Object 5***	***Object 6***
Mean X	94.323	97.9598	37.7045	126.425	97.6333	144.287
Mean Y	145.246	82.7935	103.055	178.811	38.5357	138.231
Mean Distance	**1.36592**	**3.19942**	**5.62374**	**0.681299**	**2.70794**	**3.06819**
STD Distance	2.19093	4.91184	6.38747	0.452419	5.32643	4.79332-
Mean Angle	-46.8792	-42.9816	-30.2335	-57.5651	-89.7103	-81.0602
Mean Angular velocity	0.366619	0.236018	0.362569	0.214926	0.253861	0.0586356
Angular Deviation	64.4868	70.8238	64.6926	71.7949	69.9919	78.6171
Mean Velocity	**1.65466**	**2.56072**	**2.97278**	**1.30388**	**4.09124**	**5.82692**

ΔPEX4	***Object 1***	***Object 2***	***Object 3***	***Object 4***	***Object 5***	***Object 6***
Mean X	160.941	183.321	51.9293	111.675	52.7852	92.1603
Mean Y	55.3146	81.8001	115.887	154.24	43.2099	12.1496
Mean Distance	**3.72867**	**0.597398**	**0.651794**	**0.865805**	**0.543439**	**0.775349**
STD Distance	5.47948	0.590052	0.433493	0.666705	0.278109	0.462787
Mean Angle	134.786	-14.7912	-63.1127	32.6675	-2.21411	-158.58
Mean Angular velocity	0.426003	0.0924738	0.121318	0.30183	0.195225	0.961289
Angular Deviation	61.3893	77.1912	75.9546	67.6047	72.6901	15.9425
Mean Velocity	**8.04641**	**1.17864**	**1.30766**	**1.14872**	**1.13776**	**1.02441**

4. Ubiquitination of TbPEX5 in mutants of the recycling complex.

The role of several peroxins involved in peroxisomal matrix protein import, more precisely in the recycling of PEX5, has been elucidated through the physiological and molecular characterization of yeast mutants with single and/or combined gene deletions of the different peroxins identified, making possible the establishment of the sequential events that take place during the process. It was elegantly demonstrated that the AAA$^+$-ATPases PEX1 and PEX6 are responsible for the translocation of the receptor PEX5 from the peroxisomal membrane to the cytosol, after the delivery of PTS-1 proteins to the peroxisomal matrix (Platta *et al.,* 2005). This step was shown to be essential for matrix protein import. Although it is not totally clear yet how these enzymes recognize the PEX5 molecules that should be cycled back, a considerable amount of evidence suggests that this signal is provided by the monoubiquitination of the receptor. Importantly, the N-terminal domain of PEX1 displays similarities to the corresponding adaptor-binding domains of other AAA proteins, like p97 and Ufd1, in which it has been identified a double-ϕ β-barrel fold as a ubiquitin-binding domain with binding sites for both mono- and poly-ubiquitin. (Platta *et al.,* 2008)

On the other hand, the ubiquitination process comprises a cascade of actions by several enzymes: an E1 (Ubiquitin-activating enzyme), E2 (Ubiquitin-conjugating enzyme) and E3 (Ubiquitin-ligase enzyme), all necessary to properly modify the substrate. The nature of the enzymes responsible for the E2 and E3 activity that leads to PEX5 ubiquitination in yeast (E2 and E3) and in mammalian cells (E2) has been recently determined: PEX4 (yeast)/UBCH2 (mammals) and UBC4/UBC5/UBC1 as the E2 enzymes and the RING-finger complex comprising PEX2/PEX10/PEX12 as the E3 ligases (yeast).

The molecular characterization of TbPEX6, TbPEX10 and TbPEX12 was previously performed in our laboratory. It was found that, as in yeast cells, these peroxins are involved in *T. brucei* bloodstream and procyclic glycosome biogenesis. More importantly, the depletion of these peroxins by RNAi caused a

deleterious effect on cell growth, confirming once more the essentiality of a correct compartmentalization of glycolytic enzymes for the viability of the parasites. However, the molecular function of these peroxins was not yet addressed in that study.

Since we have proved that TbPEX5 is ubiquitinated in wild-type cells and, moreover, that this posttranslational modification is disturbed when the TbPEX4 gene is knocked out, we took the advantage of the availability of RNAi constructs for TbPEX6 and TbPEX12 to explore how the ubiquitination process is affected when the cellular levels of these two peroxins have dropped as the result of RNAi induction.

We decided to transfect procyclic cells with the constructs pHD677_TbPEX6 and pHD677_TbPEX12, previously described by Krazy and Michels (2006). The reason why this molecular study was restricted to procyclic cells is the fact the *T. brucei* bloodstream form is highly susceptible to even a slight mislocalization of glycolytic enzymes, making it almost impossible to obtain large amounts of parasites suitable for biochemical analysis. After transfection with the respective constructs, positive clones were selected and checked by PCR for the genomic integration of the hygromycin/stem-loop cassette. We initially studied the response of the RNAiTbPEX6 and RNAiTbPEX12 cells to RNAi by analysis of the growth behaviour (Fig. 55), the glycosomal protein import by confocal immunomicroscopy (Fig. 56) and confirmed the depletion of the peroxin by western blotting [only in the case of TbPEX6 for which an antiserum was available (data not shown)]. It was evident that the growth of the RNAi-induced cell lines was impaired when compared to the wild-type cells. More precisely we observed in the case of RNAiTbPEX6 that growth stopped at 72 h of induction and the morphology of the cells started to become affected. For RNAiTbPEX12 the effect was even more dramatic since cells started to die massively after 72 h, leaving only very few live cells, all with a round shape, in the culture at 96 h and abundant cell debris.

Samples of induced and non-induced RNAi cell lines, as well as wild-type and ΔPEX4 trypanosomes were taken and fixed daily for analysis of glycosomal protein import by confocal immunomicroscopy. The localization of glycosomal matrix protein markers gGAPDH and TPI was used as the criterion of proper protein import.

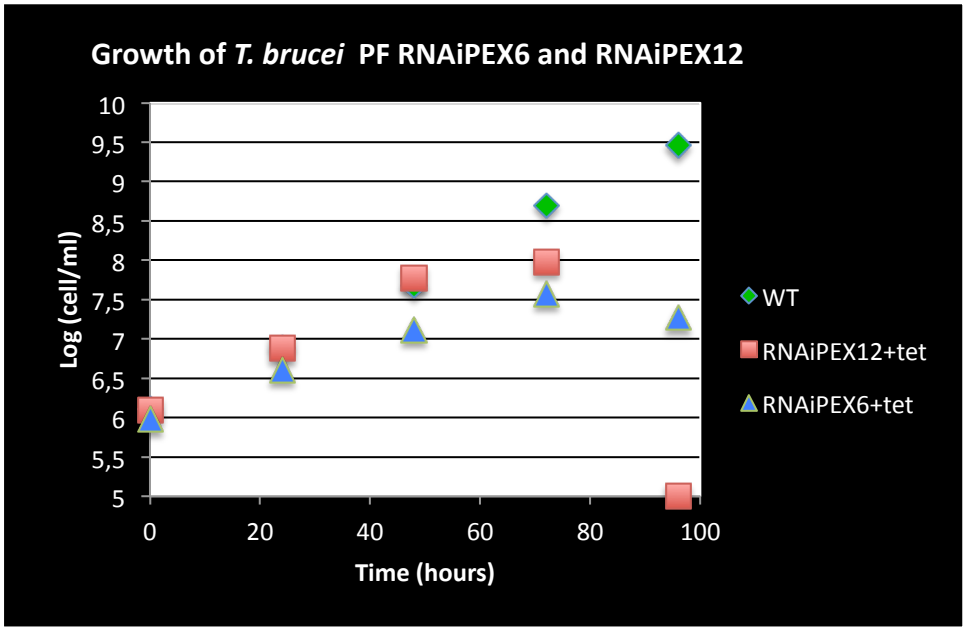


Figure 55. ***Growth behaviour of the procyclic T. brucei RNAi PEX5-recycling mutants TbPEX6 and TbPEX12***. Induced RNAi cell lines show a growth rate decrease after 72 h of induction, followed by massive cell death (RNAiTbPEX12) or a cell growth arrest (RNAiTbPEX6).

As shown in Figure 56, after the induction of RNAi for 96 h in the RNAiTbPEX6 and RNAiTbPEX12 the majority of gGAPDH (green) and TPI (red) signals were distributed along the cell body suggesting a cytosolic localization. This contrasted with the nice punctate pattern of the immunolabelled proteins in the wild-type and non-induced cells. This result is in agreement with the previous report about the phenotype of similar RNAi cell lines (Krazy and Michels, 2006). Importantly, we once more observed a slight mislocalization of gGAPDH in the ΔPEX4 cell line, supporting our previous data on the phenotype of glycosomal protein import in the TbPEX4 knock-out mutant.

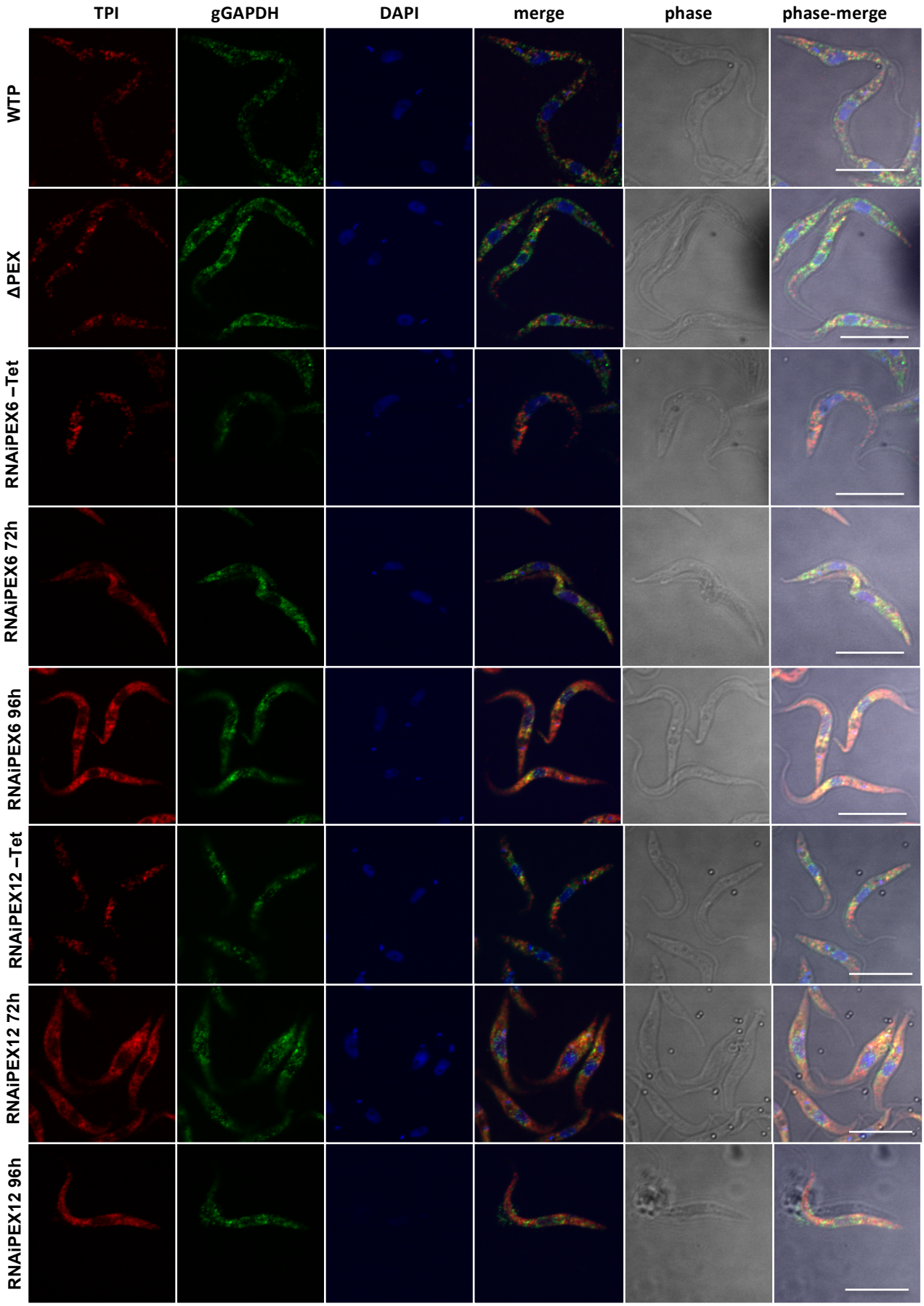

TPI
gGAPDH
DAPI
merge
phase
phase-merge
WTP
ΔPEX
RNAiPEX6 –Tet
RNAiPEX6 72h
RNAiPEX6 96h
RNAiPEX12 –Tet
RNAiPEX12 72h
RNAiPEX12 96h

Figure 56. ***PTS-protein import defect in PEX5 recycling mutants of procyclic T. brucei***. Confocal immunomicroscopy was used to analyse the glycosomal matrix-protein import in RNAi mutants of TbPEX6 (AAA$^+$-ATPase) and TbPEX12 (RING-finger peroxin = Ubiquitin-ligase) previously characterized (Krazy and Michels, 2006). The phenotype of trypanosomes of these RNAi-induced cell lines was compared with that of wild-type (WTPF) and ΔPEX4 cells. An important mislocalization of two glycosomal markers, gGAPDH (PTS-1) and TPI (I-PTS), was observed in the TbPEX6 and TbPEX12 RNAi mutants confirming the previously reported results. Trypanosomes of the ΔPEX4 cell line show only a very minor mislocalization of the glycosomal matrix proteins. Scale bar: 10 μm.

We managed to obtain sufficient material of the induced RNAiTbPEX6 and RNAiTbPEX12 cell lines for performing experiments to study the ubiquitination of TbPEX5. We have used as control wild-type as well as ΔPEX4 cells in order to establish similarities or differences in the ubiquitination pattern of samples from the different cell lines run in parallel on the same gel. In this case, we considered it worth treating the cells with the proteasome inhibitor lactacystin in order to evaluate the ubiquitination pattern of the recycling mutants RNAiTbPEX6, RNAiTbPEX12 and ΔPEX4 under conditions that possible PEX5 proteasomal degradation was arrested. The approach followed also involved a cellular fractionation by selective permeabilization of the plasma membrane with a low concentration of digitonin in the presence of NEM. Figure 57 shows the results obtained after western blot analysis of the cell fractions. We found once more that in wild-type cells a slow migrating form of TbPEX5, identified as a monoubiquitinated form, is present in the cytosolic fraction as it colocalizes with the cytosolic marker Enolase. Also evident was an important decrease of this TbPEX5 form in the ΔPEX4 cell line, in full agreement with our previous results, where we found exactly the same pattern in the absence of lactacystin (Fig. 50). In the case of the RNAiTbPEX12 cell line, we observed that the band corresponding to the monoubiquitinated TbPEX5 had decreased to become undetectable, while a new prominent band of ≈120 kDa had appeared. We hypothesize that this might correspond to TbPEX5 conjugated with several chains of ubiquitin. The fact that, as in the ΔPEX4 cell line, in the RNAiTbPEX12 cells the monoubiquitinated form of TbPEX5 is affected, might indicate that these two proteins could be acting together as the successive E2-E3 enzymes responsible for the monoubiquitination of the receptor, similar as has been shown to be the case in *S. cerevisiae* (Platta *et al*., 2009). On the other hand, it

was shown that in the induced RNAiTbPEX6 cell line some TbPEX5 monoubiquitination still occurred, since the corresponding 110 kDa form was detected. Moreover, this cell line seems to also accumulate PEX5 modified by several chains of ubiquitin, migrating similarly to the detected band in the RNAiTbPEX12 mutant. In all cases the TbPEX5 modification was found to be prominent in the cytosolic fraction and not detectable in the organelles (Fig. 57).

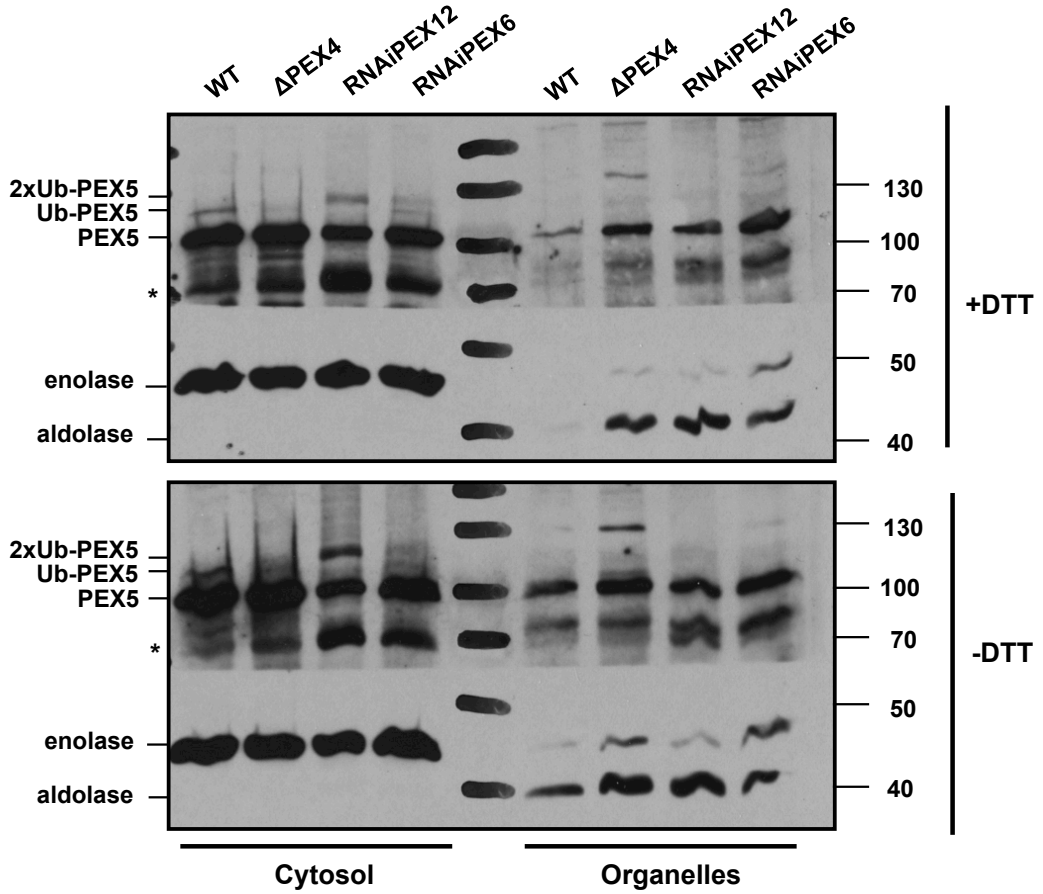


Figure 57. ***Polyubiquitination of TbPEX5 forms was detected in the RNAi PEX5-recycling mutants TbPEX6 and TbPEX12***. Cells were treated in culture with lactacystin for 6 h prior to collecting samples for western blot analysis. RNAi induction was performed for 96 h. Cytosolic and organellar proteins were obtained by selective permeabilization of the plasma membrane with digitonin in the presence of 20 mM NEM. Western blot analysis was performed and the TbPEX5 ubiquitination pattern in these mutant trypanosomes was compared with that in wild-type (WT) and ΔPEX4 cells. The TbPEX12 RNAi cell line shows a slower migrating TbPEX5 form, which might correspond to polyubiquitinated TbPEX5. No monoubiquitinated form was detected. The TbPEX6 RNAi cell line shows a monoubiquinated form and additionally the polyubiquitinated form as in the TbPEX12 RNAi cells. The ubiquitination pattern of the ΔPEX4 cell line was as shown in Figure 50.

It should be mentioned that the signal obtained in the organellar fraction reflects some technical problems related to (i) a minor contamination with cytosol as is evident from the presence of a small amount of enolase and (ii) the amount of protein loaded on the gel since in the lane containing the sample of wild-type cells the glycosomal marker aldolase was barely detectable. This precluded us from drawing definitive conclusions about the abundance of the receptor TbPEX5 in the organelle upon RNAi of TbPEX12 and TbPEX6 as well as in the ΔPEX4 cell line under the conditions used. Not shown are data in which we analyzed the TbPEX5 ubiquitination profile in the PEX5-recycling mutants not treated with lactacystin. These data were not different from the results obtained with this proteasome inhibitor as presented in this manuscript, indicating that this form of the receptor TbPEX5 accumulates in the cytosol of the mutants irrespective of a functional proteasome. Once more it was intriguing to find that not-ubiquitinated forms of the receptor were associated with the organellar fraction, especially in the case of RNAiTbPEX6 RNAi cell line. It has been reported that PEX1 and PEX6 are not redundant proteins and they have different degrees of conservation at their AAA domain, meaning that, in principle, they cannot complement the function of their partner. Intriguingly, when individual *S. cerevisiae* mutants of PEX1 and PEX6 where analysed for *in vitro* PEX5 export capacity, it was found that PEX5 was easily released from the peroxisomal membrane of these mutants (Platta *et al.,* 2005). We can argue that this situation is also reflected in the TbPEX6 RNAi mutant where PEX1 is still present. Eventually the observation that ubiquitinated TbPEX5 is released from the membrane in the TbPEX6 RNAi mutant can also been explained by a not-completed depletion of the protein upon RNAi induction, making possible that a basal level of remaining TbPEX6 (even not detectable by western blot) might be responsible for a still functional recycling of TbPEX5. The modified TbPEX5 forms were present irrespective of the presence or absence of DTT, suggesting once more that these modifications occur likely at lysine residues in TbPEX5.

Chapter 6

Channel-Forming Activities in the Glycosomal Fraction from the Bloodstream Form of *Trypanosoma brucei*

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Abstract

Background: Glycosomes are a specialized form of peroxisomes (microbodies) present in unicellular eukaryotes that belong to the Kinetoplastea order, such as *Trypanosoma* and *Leishmania* species, parasitic protists causing severe diseases of livestock and humans in subtropical and tropical countries. The organelles harbour most enzymes of the glycolytic pathway that is responsible for substrate-level ATP production in the cell. Glycolysis is essential for bloodstream-form *Trypanosoma brucei* and enzymes comprising this pathway have been validated as drug targets. Glycosomes are surrounded by a single membrane. How glycolytic metabolites are transported across the glycosomal membrane is unclear.

Methods/Principal Findings: We hypothesized that glycosomal membrane, similarly to membranes of yeast and mammalian peroxisomes, contains channel-forming proteins involved in the selective transfer of metabolites. To verify this prediction, we isolated a glycosomal fraction from bloodstream-form *T.brucei* and reconstituted solubilized membrane proteins into planar lipid bilayers. The electrophysiological characteristics of the channels were studied using multiple channel recording and single channel analysis. Three main channel-forming activities were detected with current amplitudes 70–80 pA, 20–25 pA, and 8–11 pA, respectively (holding potential +10 mV and 3.0 M KCl as an electrolyte). All channels were in fully open state in a range of voltages ±150 mV and showed no sub-conductance transitions. The channel with current amplitude 20–25 pA is anion-selective (P_{K+}/P_{Cl-} ~0.31), while the other two types of channels are slightly selective for cations (P_{K+}/P_{Cl-} ratios ~1.15 and ~1.27 for the high- and low-conductance channels, respectively). The anion-selective channel showed an intrinsic current rectification that may suggest a functional asymmetry of the channel's pore.

Conclusions/Significance: These results indicate that the membrane of glycosomes apparently contains several types of pore-forming channels connecting the glycosomal lumen and the cytosol.

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Introduction

Trypanosoma brucei is a parasite that belongs to the Trypanosomatidae family of the Kinetoplastea order of protists. The biology of *T. brucei* is under intensive investigation because of the medical and economical importance of these parasites as the causative agents of African trypanosomiasis, also known as sleeping sickness in humans and Nagana disease in cattle [1–3]. The complex life cycle of *T. brucei* involves its alternation between the insect vector (tsetse fly), where the replicative stage of the parasite is called procyclic form, and the blood of the mammalian host where the parasites differentiate into the so-called long-slender bloodstream form. The parasite's life cycle requires drastic metabolic changes in order to adapt to the environments encountered in the respective hosts [1]. It has been demonstrated that the glycolytic pathway is essential for *T. brucei*'s bloodstream form, since glycolysis is its only source of ATP synthesis. Remarkably, the first seven enzymes of glycolysis in the *Trypanosoma* species are localized in specific cellular organelles, glycosomes, where these enzymes may represent up to 90% of the total protein content [1,4–6]. This is in contrast to cells of higher eukaryotes where all glycolytic enzymes are found in the cytosol. Glycosomes are members of the microbody family of organelles that also includes peroxisomes from mammals, plant leaves and yeasts as well as glyoxysomes from oil seeds [1,6,7]. All microbodies share common morphology and biogenesis, as well as some other properties, such as the absence of DNA and involvement in the metabolism of certain lipids [7]. However, the overall enzyme composition of the particles is different and in many cases varies depending on the nutritional source.

Usually in cells, the enzymes catalyzing the two steps in which ATP is invested at the beginning of the glycolytic pathway,

hexokinase and phosphofructokinase, are allosterically regulated by their reaction products or other effectors. This regulation limits the so-called 'turbo effect', *i.e.* the uncontrolled activation of glycolysis by the net ATP that is produced at later steps of glycolysis. In contrast, an allosteric regulation of the activity of glycolytic ATP-consuming enzymes in Trypanosomatidae has not been detected [5,6,8,9]. Instead, as has been shown recently [10], compartmentalization of glycolytic enzymes within the glycosomes of Trypanosomatidae prevents from the detrimental 'turbo effect' of an uncontrolled consumption of ATP at the initial steps of glycolysis. This is apparently achieved by formation of the two pools of ATP – glycosomal and cytosolic. The glycosomal pool of ATP needs to be strictly balanced by action of glycolytic enzymes consuming and producing ATP in glycosomes. The net production of ATP in glycolysis is catalyzed by the last enzyme of the pathway, pyruvate kinase, which is located in the cytosol [1,2,6,10]. Separation of the first and second part of the glycolytic pathway between the two compartments thus predicts an important role for the glycosomal membrane in preventing free diffusion of ATP between the cytosol and the glycosomal lumen. How the glycosomal membrane is involved in the transfer of different metabolites, including ATP and other solutes such as glycolytic intermediates, is an unresolved issue.

As has been shown recently, some representatives of the microbody family, such as peroxisomes from plants, mammals, and yeasts, contain proteins that are able to form a general diffusion pore in the membrane [11–15]. In addition, experiments *in vitro* have revealed that the mammalian peroxisomal membrane is open to small solutes such as inorganic ions and most hydrophilic cellular metabolites, but selectively prevents diffusion of 'bulky' solutes, including cofactors (NAD/H, NADP/H and CoA) and ATP [16,17]. One of the mammalian peroxisomal membrane channels, formed by the protein Pxmp2 from rodents, has been characterized at the molecular level [18]. The diameter of the channel's pore was estimated at 1.4 nm. This size is lower than the dimensions of 'bulky' metabolites but far above the diameter of small solutes. Therefore, the sieve properties of the peroxisomal membrane may be well determined by the pore size of the channel molecules. Considering the evolutionary relationship of peroxisomes and glycosomes one can predict the presence of channel-forming proteins also in the glycosomal membrane. These channels may allow an easy movement across the membrane of small solutes, including all intermediate metabolites of glycolysis, but prevent transfer of ATP and cofactors and thus promoting the maintenance of separate pools of these compounds in the cytosol and the glycosomal lumen.

Here we report results of an investigation of the channel-forming activities in purified glycosomal preparations isolated from bloodstream-form *T. brucei*. Three distinct activities were detected and their electrophysiological characteristics were analyzed using the planar lipid bilayer technique.

Results

Characterization of the isolated glycosomal fraction

Glycosomes were purified from bloodstream-form cells of *T. brucei* using a two-step isolation procedure (Figure 1). Differential centrifugation was applied to obtain a large granular fraction enriched in glycosomes and mitochondria. This fraction was then subjected to Optiprep density gradient centrifugation to separate glycosomes from other cellular organelles. As has been shown previously, Optiprep is a highly effective medium for isolation of peroxisomes from different sources [17,19] and glycosomes from *T. brucei* [20]. To preserve intactness of glycosomes we used poly(ethylene)glycol PEG 1500 (PEG 1500) as an osmoprotector expecting that, like in the case of mammalian [17] and yeast [13] peroxisomes, this compound may effectively prevent damage of the particles. Indeed, our preliminary experiments revealed that addition of PEG 1500 to the isolation medium significantly reduced the leakage rate of the glycosomal matrix enzyme hexokinase from the organelles (data not shown). Localization of glycosomes in the gradient fractions was monitored just after centrifugation by measuring hexokinase activity. According to the results obtained, the particles are detected near the bottom of the gradient and are also localized in the middle gradient fractions (Figure 1A, panel a). This broad distribution of glycosomes may reflect an appearance of the glycosomal 'ghosts' which, similar to the peroxisomal 'ghosts' [17], are formed due to the partial leakage of matrix proteins from damaged particles. The expected density of the glycosomal 'ghost' is lower than that of the intact organelle [17]. Purity of the glycosomal preparations was routinely estimated by analysis of the activity of marker enzymes and by immunodetection of marker proteins for different organelles (Figure 1). A significant portion of glycosomes (fractions 2–4 of the gradient, see Figure 1A) was well separated from the other cellular organelles although the yield was relatively low. This was an expected result since our isolation protocol was aimed to achieve the maximal purity of glycosomes that inevitably resulted in the lower yield of the particles. Enrichment of the glycosomal preparations as determined by hexokinase assays was about 10-fold relative to the specific enzyme activity in the granular fraction loaded on the gradient. In the glycosomal preparations we found only traces of the activities of markers for mitochondria (FAD-dependent glycerol-3-phosphate dehydrogenase) and flagellar membranes (acid phosphatase), indicating a low contamination of glycosomes by membrane fragments of these organelles which may be considered as the main potential sources of the contaminating channel-forming activities (Figure 1A, panel c). According to the distribution of marker enzymes activity, the glycosomal preparations contain less that 2% of the total amount of lysosomes (mannosidase) and endoplasmic reticulum (α-glucosidase) loaded on the gradient (Figure 1A, panel d). Considering the latter data, it is important to emphasize that the fraction loaded on the Optiprep gradient was obtained as a result of differential centrifugation of the postnuclear homogenate (see 'Methods' section). It is known that this procedure leads to partial purification of glycosomes especially to separation of these particles from endoplasmic reticulum and to a less extent from lysosomes [4,21]. We also analyzed the purity of the glycosomal fraction by immunodetection of marker proteins for different organelles (Figure 1B, panel a). As expected, the glycosomal marker (aldolase) was detected in the bottom gradient fractions and in the other fractions containing glycosomes, while markers for mitochondria (heat shock protein 60, HSP60) and acidocalcisomes (vacuolar pyrophosphatase) were found exclusively in the top gradient fractions. The pyrophosphatase immunosignal was very weak even after long blot exposure (fractions 16–18, see Figure 1B, panel a). This is a predictable result since the bulk of acidocalcisomes was not sedimented with the large granular fraction during differential centrifugation. Indeed, immunodetection of pyrophosphatase at the same conditions of blotting revealed a strong signal at the corresponding protein size only in the postnuclear homogenate and no signal was found in the mitochondria-enriched fraction (compare lines 1 and 2 in Figure 1B, panel b). The purity of isolated glycosomes was also verified by electron microscopy (EM) (Figure 2). Consistent with the biochemical data, the EM images demonstrate that the preparations of glycosomes are highly enriched with these particles

(Figure 2A). Most glycosomes are well preserved; they are filled with an electron-dense matrix and surrounded by a single membrane (Figure 2B). In the same preparations some fragments of the flagellar apparatus (paraflagellar rods and axonemes) can be detected. As shown by a careful analysis of the images, these structures are not connected to the flagellar membranes, which corroborate the data obtained in the enzyme activity assays performed for the flagellar membrane marker, acid phosphatase (see Figure 1A, panel c). One may therefore expect that the low contamination of the glycosomal fraction by protein fragments of the flagellar apparatus should not affect the results of the detection of channel-forming activities since these activities belong to proteins which are localized in the membrane structures. We also conducted EM of fractions in the middle (fractions 8–11, see Figure 1) and the top (fractions 15–18) of the Optiprep gradient. The middle fractions showed a high enrichment of fragments of the flagellar apparatus (Figure 2C and 2D), whereas the top fractions contained a complex mixture of different membrane-bounded organelles with mitochondrial vesicles as a predominant constituent (Figure 2E and 2F).

Multiple channel recording (MCR) of the isolated organelles

MCR is a useful analytical tool to describe an overall pattern of channel-forming activities in certain membrane preparations. This method is especially suitable for samples containing several types of pore-forming proteins. It also helps to establish optimal experimental conditions for revealing activity of the channels. We initially attempted to conduct MCR using 1.0 M KCl as a bath solution. While the activity in the glycosomal fraction was evident, the amount of insertion events was relatively low and the inserted channels were mainly unstable, frequently showing an intensive flickering (data not shown). Therefore, we increased the ionic strength of the bath solution up to 3.0 M KCl. This led to the appearance of more stable channel-forming activities (Figure 3A) that allowed quantitative analysis of the whole set of insertion events using histograms which indicate insertion frequency relative to current amplitude at a certain holding potential (Figure 3B). This approach was applied for comparative analysis of pore-forming activities in the preparations of glycosomes (Figure 3B, upper panel), flagella (Figure 3B, middle panel) and mitochondria (Figure 3B, lower panel). The conductance pattern registered in the glycosomal preparations was clearly different from that of the mitochondrial fraction containing in addition to mitochondria some other membrane-bounded organelles. This may indicate that glycosomes contain their own pool of channel-forming proteins and the registered activities are not caused by contaminating proteins from other organelles. Comparative MCR of glycosomal and flagellar preparations revealed similar patterns of current amplitudes, whereas the frequency of insertion events with the flagellar fraction was much lower. Remarkably, we did not register any noticeable number of current increments with amplitudes characteristic for only flagellar preparations. These observations are in line with the prediction that the channel-forming activity in the flagellar fractions is due to their contamination with glycosomes.

Next, we studied conditions favourable for detection of the channel-forming activities and tried to register these activities using different bath solutions (NaCl, LiCl, NH_4Cl, potassium acetate, sodium glycolate, and sodium phosphate, pH 7.4). The best results were obtained using NH_4Cl as the electrolyte (Figure 3C). A high frequency of insertion events was observed not only at 3.0 M NH_4Cl (Figure 3C, upper panel), but also at 1.0 M NH_4Cl (Figure 3C, lower panel). The overall patterns of insertion events registered at +10 mV using 3.0 M KCl and 3.0 M NH_4Cl as bath solutions were similar (compare Figure 3B, upper panel and 3C, upper panel). They showed two predominant types of channel-forming activities with average current amplitudes of 20–25 pA and 70–80 pA, respectively. As expected, lowering of the ionic strength of the electrolyte from 3.0 M to 1.0 M NH_4Cl was accompanied by a corresponding decrease in the registered conductance levels (compare Figure 3C, upper panel and Figure 3C, lower panel). This is a characteristic feature of non-selective channels forming water-filled pores in the membrane [22,23].

To proof a membrane localization of the channels, the glycosomal preparations were treated by sonication and the membrane fragments were separated from matrix proteins by sucrose density gradient centrifugation. As expected, the channel-forming activity was only detected in the membrane preparations (data not shown). To analyze a potential role for protein SH-groups in modulation of the channels activity, glycosomal preparations were pre-incubated with 5 mM dithiothreitol (DTT) or the reducing agent was added into the bath solutions (2 mM DTT, final concentration). We did not detect any effect of DTT on the conductivity pattern of analyzed channels (data not shown). Nevertheless, we performed standard activity measurements in the presence of 2.0 mM DTT to preserve any SH-groups from oxidation during prolonged incubation periods. A moderate increase in the applied potentials (up to 60 mV) did not influence the conductivity pattern of glycosomal preparations, but strongly reduced the amount of high-conductance insertion events registered in the mitochondrial fraction (data not shown). More detailed investigation of this phenomenon using single channel analysis (SCA) showed that, contrary to high-conductance activity in glycosomal preparations (see below), the mitochondrial channel is spontaneously gated at holding potentials above 40–50 mV (Figure 3D). This difference in properties of high-conductance channels was routinely used in the following experiments to distinguish them from each other.

Single channel analysis (SCA) of a high-conductance channel

We applied SCA for detailed electrophysiological characterization of the channel-forming activities registered using MCR (see above). The most prominent of these activities was a high-conductance channel (Figure 4A) which comprises more than 25% of the total number of insertion events registered by MCR (see Figure 3B, upper panel). The channel inserted in the membrane was usually stable in the fully open conformation during the entire period of registration (minutes) at different holding potentials. This was confirmed using voltage-ramp (Figure 4B) and voltage-step (Figure 4C) protocols. The results indicate a near linear dependence of the channel current on the voltage with a slope conductance of $\Lambda = 9.2 \pm 0.6$ nS, $n = 8$ (3.0 M KCl on both sides of an artificial membrane). The conductance of a single channel was linearly decreased following dilution of the bath solution from 3.0 M KCl to 2.0 M then1.0 M KCl (Figure 3D). The high conductance of the channel and linear dependence on the KCl concentration suggest an electrochemical current flow through a porin-like structure forming a hole in the membrane which is filled with water [22,23]. The channel was resistant to gating even at extreme holding potentials (Figure 4E), distinguishing it from the voltage-dependent anion channel of the outer mitochondrial membrane [24]. The probability of the channel to be open was $P_{open} \approx 0.9$ between $V_{hold} = +150$ mV and $V_{hold} = -150$ mV. Only a single conductance state was detected. The reversal potential of the channel in asymmetric KCl solutions (3.0 M KCl *trans*/1.5 M

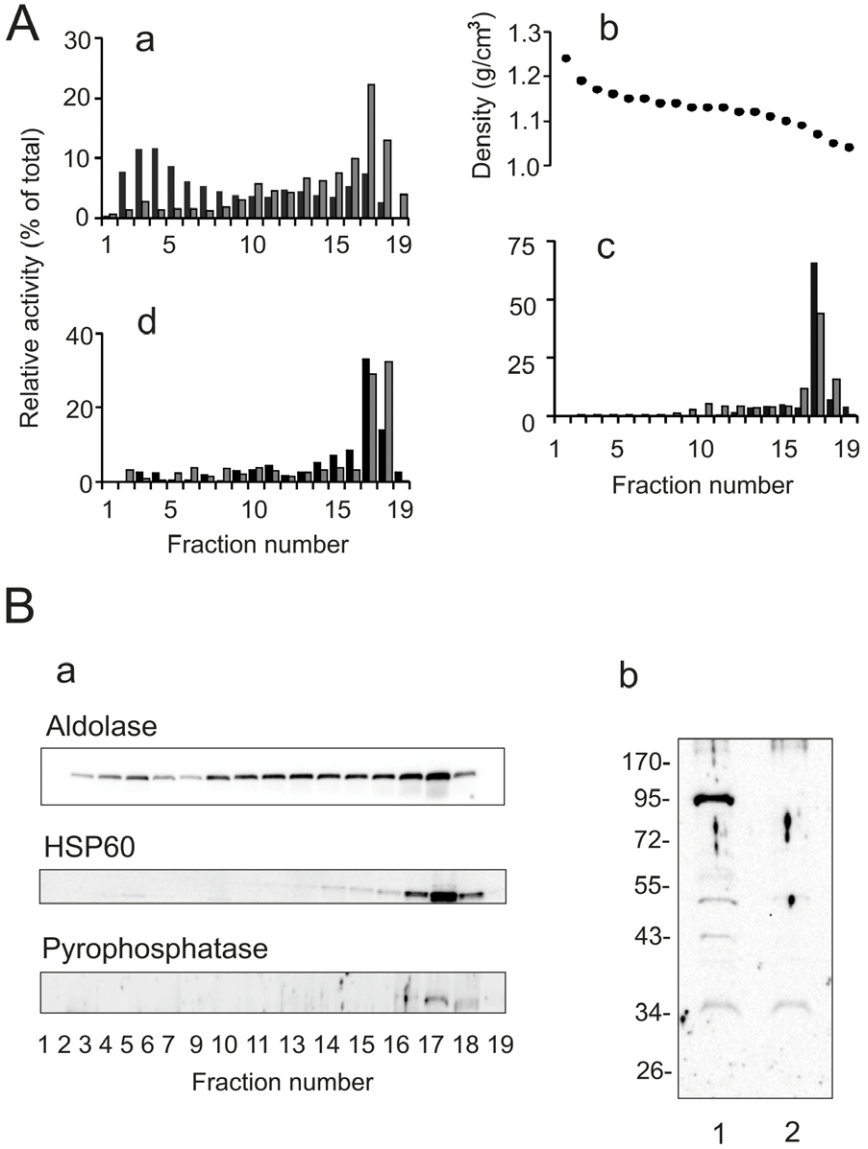

Figure 1. Isolation of glycosomes. A large granular fraction enriched in glycosomes and mitochondria as obtained by differential centrifugation was subjected to Optiprep density gradient centrifugation and the contents of the fractions obtained were analyzed by (**A**) marker enzyme activity measurements and (**B**) immunoblot analysis of marker proteins. (**A**) Activities of hexokinase (**a**, filled bars), FAD-dependent glycerol-3-phosphate dehydrogenase (**c**, filled bars), acid phosphatase (**c**, gray bars), mannosidase (**d**, gray bars), and α-glucosidase (**d**, filled bars) were measured. Protein content (**a**, gray bars) and density of the gradient (panel **b**) were also determined. The results obtained are expressed as the activity in each fraction relative to the total activity in the whole gradient. Enzyme (protein) recoveries varied between 78–112%. (**B**) Panel **a**: Proteins from equal volumes (70 μl) of each fraction (fractions 1–7, 9–11, and 13–19, see Figure 1**A**) were separated by SDS/PAGE and analyzed by western blotting using antibodies against markers for different organelles [aldolase as a glycosomal marker and heat shock protein 60 (HSP60) as a mitochondrial marker]. Panel **b**: Immunodetection of the acidocalcisome marker pyrophosphatase in the post-nuclear homogenate (line 1) and in the mitochondrial fraction (line 2). Molecular mass markers are indicated.
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KCl *cis*) was $E_{rev} = +1.14$ mV indicating that this channel has only limited preference for cations over anions, P_{K+}/P_{Cl-} ratio ~1.15.

Low-conductance channel showing current rectification

In addition to high-conductance channel-forming activity, the MCR experiments revealed an abundant low-conductance activity in glycosomal preparations (see Figure 3, upper panel). Similarly, the insertion events with current amplitudes 20–25 pA at a holding potential +10 mV and an electrolyte concentration of 3.0 M KCl were frequently detected by SCA. Surprisingly, a switch of the applied voltage from +10 mV to −10 mV was accompanied by a significant decrease in the current flow through a single channel (Figure 5, upper panel). However, in a few cases the same change in the holding potential led to the opposite result – an increase in the current amplitude (Figure 5A, lower panel). These results may indicate that the low-conductance channels show current rectification. Indeed, analysis of the current-voltage relationships using voltage-ramp (Figure 5B) and voltage-step (Figure 5C) protocols confirmed this prediction. Within the whole set of the activities registered by SCA, 48 out of 56 low-conductance channels displayed rectification at negative voltages (Figure 5B and 5C, upper panels). The mean chord conductance

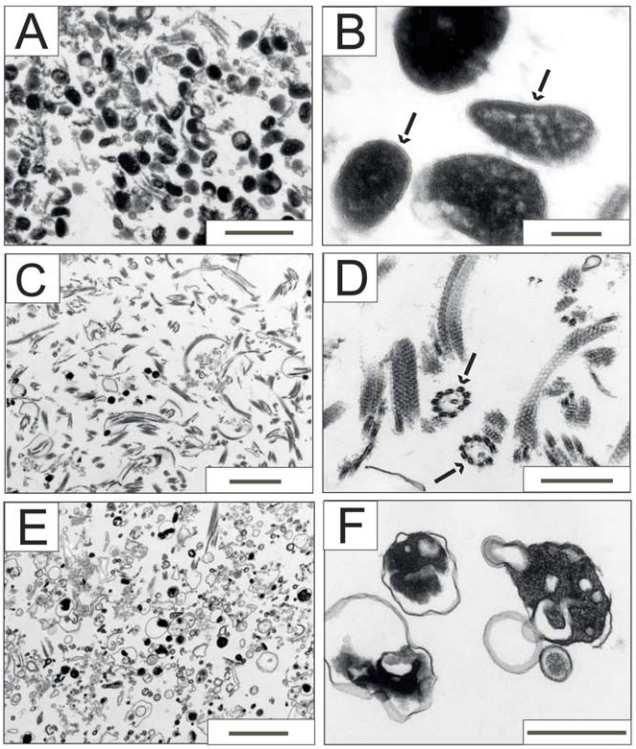

Figure 2. Electron microscopy of cellular organelles separated by Optiprep gradient centrifugation. Fractions enriched in glycosomes (fractions 2–5, see Figure 1A), fragments of flagella (fractions 8–11) or mitochondria and other organelles (fractions 15–18) were combined and processed for EM examination (see the Materials and methods section). (**A** and **B**) Isolated glycosomes shown at lower (**A**) and higher (**B**) magnifications. The fraction consists mostly of glycosomes. Some contamination by fragments of flagella is also visible. Importantly, fragments of flagella (paraflagellar rods and axonemes) show no sign of attachment to the flagellar membrane. Note the presence of intact glycosomes as electron-dense vesicles surrounded by a single membrane (marked by arrows in panel **B**). (**C** and **D**) Fractions enriched in flagella at low (**C**) and high (**D**) magnifications. One can see many paraflagellar rods in longitudinal section (**C**) and recognize flagellar axonemes (marked by arrows in panel **D**). Some glycosomes are also visible in panel **C**. (**E** and **F**) Composition of the fraction from the top of the Optiprep gradient that is enriched with mitochondria. Several types of organelles – mitochondria, lysosomes, lipid droplets, clathrin-coated vesicles, and components from the flagellar apparatus – can be observed. Note the shrinking of the mitochondrial inner membrane (see panel **F**) apparently due to osmotic misbalance. Scale bars: 2 μm (**C** and **E**); 1 μm (**A**); 0.5 μm (**D** and **F**), and 0.1 μm (**B**).
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of this channel, as deduced from the results in Figure S1A, was 2.8±0.4 nS at +50 mV and 1.4±0.3 nS at −50 mV. Most channels showed an intensive flickering at holding potentials over +40 mV. However, we did not detect any sub-conductance states of the channel that preserved an open conformation at a range of voltages ±150 mV (Figure S1B). Interestingly, at holding potentials below −100 mV the channels partially lost their rectification ability (see Figure S1, panel B2). The dependence of current amplitudes on the strength of an electrolyte solution deviated moderately from a linear curve towards lower conductance rates especially at high KCl concentrations (Figure 5D). At asymmetric electrolyte conditions (3.0 M KCl *trans*/1.5 M KCl *cis*) the reversal potential of the channel was $E_{rev} = 9.0$ mV, indicating anion selectivity with P_{K+}/P_{Cl-} ratio 0.31 (Figure 5E). Rectification by the low-conductance channels may indicate a longitudinal asymmetry of the channel's pore especially regarding the distribution of charged amino acids [22,23,25]. The fact that most channels show rectification at negative voltages suggests that incorporation of the channels is a directional process. To verify this prediction we tried to introduce the channels not from the *trans* side (standard conditions), but from the opposite, *cis* side of an artificial membrane. This was accompanied by predominant insertion of channels showing rectification at positive voltages (Figure S1C).

Electrophysiological properties of a very-low-conductance channel

The third abundant group of channel-forming activities in the glycosomal preparations besides the high- and low-conductance channels was represented by channels showing current amplitude of 8–11 pA (3.0 M KCl, +10 mV; see Figure 6A). This activity comprises more than 15% of the total number of insertion events registered by SCA in glycosomal preparations. The activity is near linearly dependent on the KCl concentration (data not shown). The current-voltage relationship measured at symmetric salt conditions (3 M KCl) revealed a slope conductance of $\Lambda = 0.98 \pm 0.4$ nS, $n = 4$ (data not shown). Contrary to the low-conductance activity (see above), this very-low-conductance channel showed no signs of current rectification. When applying both voltage-ramp (Figure 6B) and voltage-step (Figure 6C) protocols, the response of the channel's current to voltage modulations was close to linear. We did not detect gating of the channel and appearance of any sub-conductance states at low-speed linear change of the holding potential from zero to 150 mV in both, positive and negative, directions (data not shown). The reversal potential of the very-low-conductance channel in asymmetric salt concentrations (3.0 M KCl *trans*/1.5 M KCl *cis*) was $E_{rev} = +2.0$ mV that gives the P_{K+}/P_{Cl-} ratio ~1.27. Therefore, the channel is slightly cation-selective.

Super-large-conductance channel-forming activities

During MCR of glycosomal preparations at standard conditions (3 M KCl or 3 M NH_4Cl, +10 mV) channel-forming activities with current amplitudes over 180 pA were occasionally detected (Figures 3B and 3C). The activities mainly with conductance of ~24.0, 32.0, and 50.0 nS (3 M KCl) were also registered by means of SCA (Figure S2). Some of these channels were stable in their open confirmation (Figure S2A), but most of the activities displayed an irregular flickering (Figure S2B), indicating fast transition between open and closed states. The average mean lifetime of the fully open, unstable channels was remarkably low ($\tau_{open} < 50$ ms). Attempts to apply a voltage-ramp protocol to the stable super-large-conductance activities usually led to the appearance of multiple sub-conductance levels (Figure S2C), indicating that several channel-forming molecules may form clusters which are more or less resistant to treatment with detergents. Low abundance of the super-large-conductance channels precluded a detailed analysis of their properties.

Discussion

Glycosomes of *T. brucei* are highly specialized subcellular organelles focused on the conversion of the main nutrient for the bloodstream form of the parasite, glucose, into 3-phosphoglycerate and under anaerobic conditions, also glycerol. The 3-phosphoglycerate is further metabolized in the cytosol to pyruvate, a metabolic end-product for this parasite, with concomitantly a net production of ATP [1]. However, the spectrum of metabolic

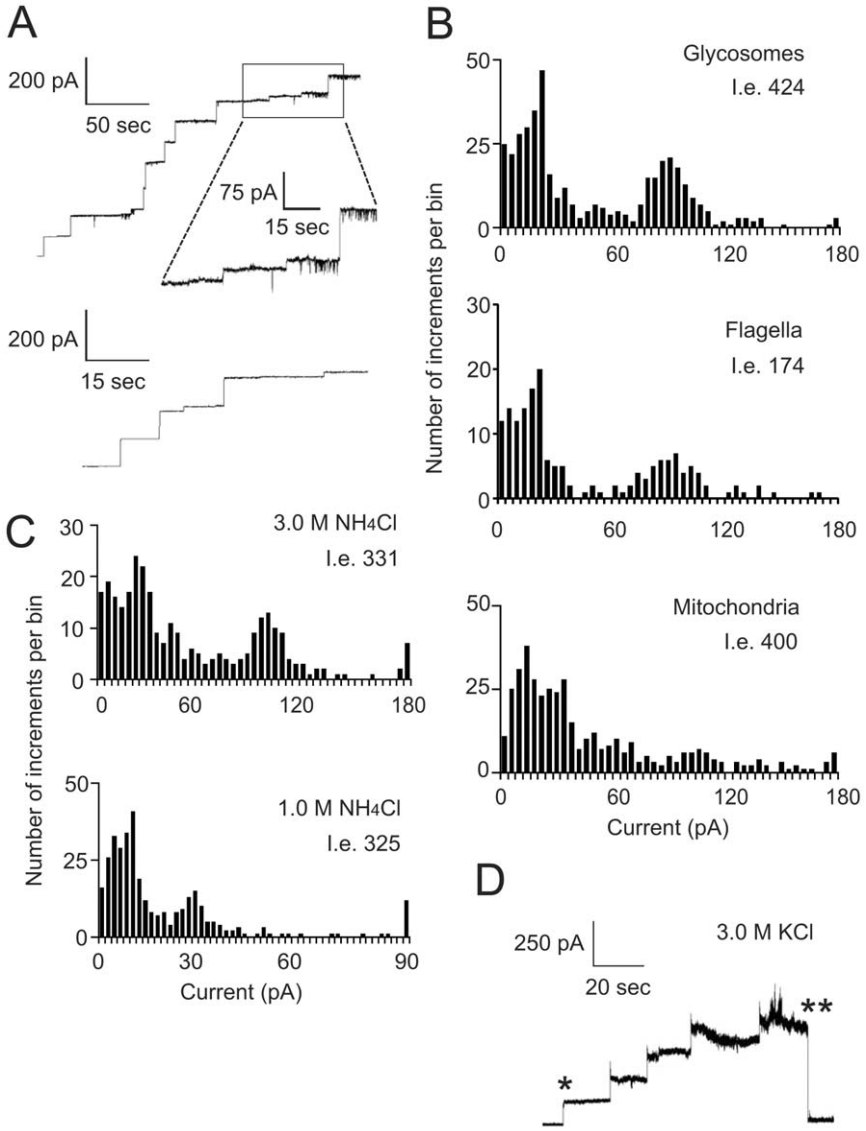

Figure 3. Detection of channel-forming activities in subcellular fractions. Fractions 2–4 (glycosomes), 8–11 (fragments of flagella), and 15–18 (mitochondria) from Optiprep density gradients (see Figure 1A) were combined and treated with Genapol X-080 to solubilize membrane proteins (see the Materials and methods section). After sedimentation of insoluble material, aliquots of the resulting supernatants were used for MCR (**A**–**C**) or SCA (**D**). (**A**) Traces of the current monitoring in the presence of glycosomal (upper panel) or mitochondrial (lower panel) preparations. The middle trace represents a timescale-expanded current recording of the upper trace. The bath solution contained 3 M KCl and the applied voltage was +10 mV. (**B**) Histograms of insertion events registered in subcellular fractions (see panel **A**). Bin size is 4.0 pA. The total number of insertion events (I.e.) is indicated. Here and in Figure 3 C (upper panel) all insertion events with current increments over 180 pA (for Figure 3C, lower panel −90 pA) are combined in one bin (180 pA or 90 pA, respectively). Note that the amount of insertion events in the flagella fraction (see **B**, middle panel) is lower than that observed in other fractions. This is mainly due to low channel-forming activity (per protein content) in the preparations of this fraction. For the sake of compatibility we used the same amounts of protein for measurements in different fractions. (**C**) Histograms of insertion events detected for glycosomal preparations using NH_4Cl as the electrolyte. Bin size: 4 pA (upper panel) or 2 pA (lower panel). See legend to Figures 3A and 3B for other details. (**D**) Trace of the current monitoring using the glycosomal fraction (initial holding potential +10 mV) indicating the insertion (marked by one asterisk) of a large-conductance channel that spontaneously closed (marked by two asterisks) after stepwise (each step is +10 mV) increase in the holding potential up to 50 mV.
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activities of glycosomes is not limited to only glycolysis but also includes such diverse pathways as β-oxidation of fatty acids, ether-lipid and squalene biosynthesis, the pentose-phosphate shunt, purine salvage, synthesis of pyrimidines, energy homeostasis, and others, although many of these non-glycolytic activities are largely or entirely repressed in the bloodstream-stage of these trypanosomes [1,26]. The diversity of metabolic pathways in glycosomes raises questions concerning the ability of the glycosomal membrane to cope with the transfer of different metabolic intermediates between glycosomal lumen and cytosol. Like other membranes impermeable to solutes, such as the inner mitochondrial or the plasma membranes, the membrane of glycosomes may contain many transporter proteins specific for certain metabolites. However, to our knowledge, only one of the 24 solute transporters of the mitochondrial carrier family of trypanosomes with still unidentified substrate specificity, MCF6, has been documented in

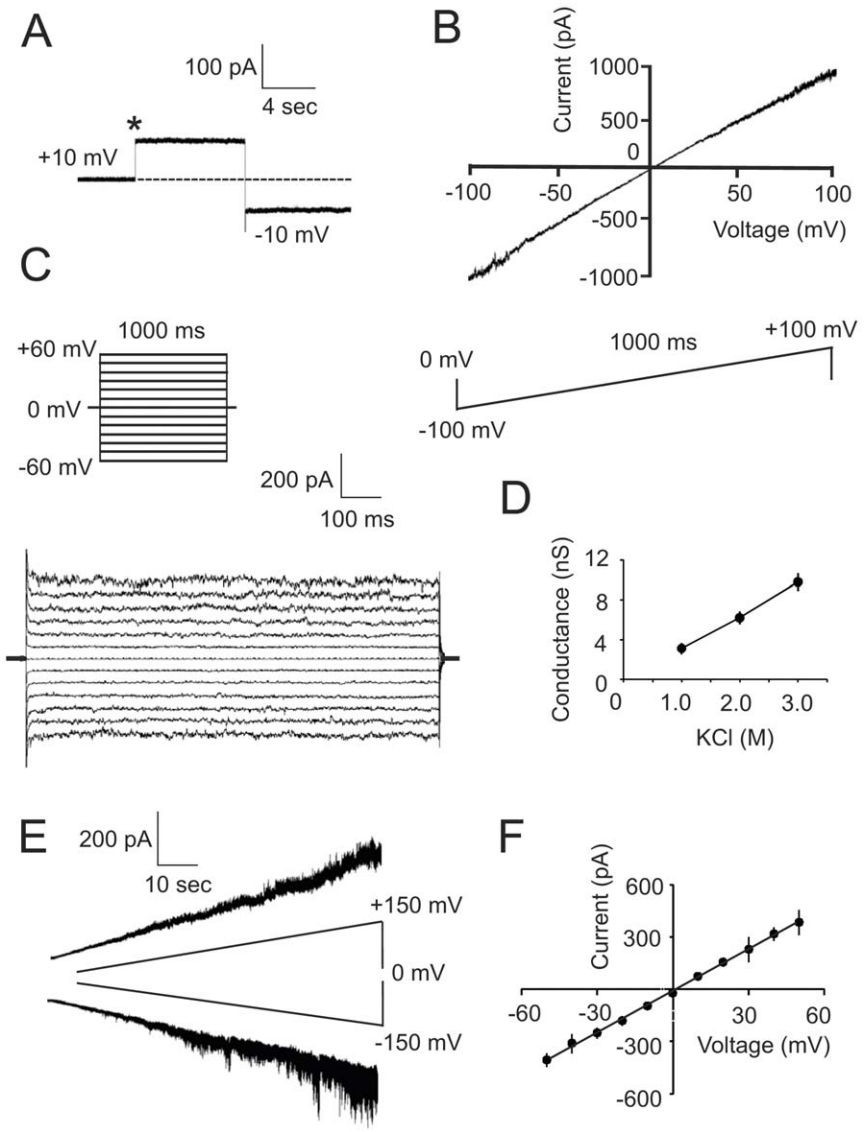

Figure 4. SCA of a high-conductance channel. (**A**) Current trace of a single high-conductance channel. The insertion event (marked by an asterisk) was registered at +10 mV and the applied voltage was then switched to −10 mV. The dashed line indicates the current level (zero) before insertion of the channel. The data in panels **A**, **B**, and **C** were collected using 3 M KCl as the electrolyte. (**B**) Current trace of the channel in response to the indicated voltage-ramp protocol. Note the near linear dependence of the current on the applied voltage. (**C**) Single channel currents in response to the indicated voltage-step protocol. (**D**) Dependence of the single channel conductance on the KCl concentration. After detection of a single channel insertion using 3 M KCl as bath solution (holding potential +10 mV), the electrolyte was diluted and registration of the current amplitudes of the same channel was conducted at 2.0 M and 1.0 M KCl, respectively. Data points are mean±SD for at least 4 independent measurements. (**E**) Current traces of a single channel in response to a low-speed linear increase (upper trace) or decrease (lower trace) of the holding potential. The bath solution contained 1.0 M NH_4Cl, 20 mM Tris-Cl, pH 7.8, and 2 mM DTT at both sides of the membrane. Note that the channel was still open even at hyperpolarizing holding potentials of ±150 mV. (**F**) Current-voltage relationship of the high-conductance channel under asymmetric salt conditions: 3.0 M KCl *trans*/1.5 M KCl *cis* compartment. The insertion of a single channel was detected at 3 M KCl at both sides of the membrane and at a voltage of +10 mV, then the electrolyte concentration in the *cis* compartment was decreased by dilution and an initial current recording was conducted at zero potential followed by stepwise (±10 mV) change of the applied voltage. Data points are mean±SD, n = 4–5. Bars in some cases are smaller than symbols.
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the glycosomal membrane of bloodstream-form cells, whereas in procyclic cells it was located in the mitochondrial membrane [27,28]. Information about some other solute transporters requires further clarification since the isolated glycosomal fraction where these transporters were detected by using a proteomic approach may have been contaminated by mitochondria [20]. In contrast, if the glycosomal membrane is open to solutes, it should, like the outer mitochondrial membrane or outer membrane of Gram-negative bacteria, contain pore-forming channels with low selectivity towards the chemical nature of the transported metabolites. Indeed, as shown in this report, such channel-forming activities were readily detected in solubilized membranes from preparations of glycosomes isolated from bloodstream-form *T. brucei*. It nevertheless should be emphasized that any researcher who use an isolated fraction of certain organelles faces a problem of contamination of this fraction by other subcellular particles.

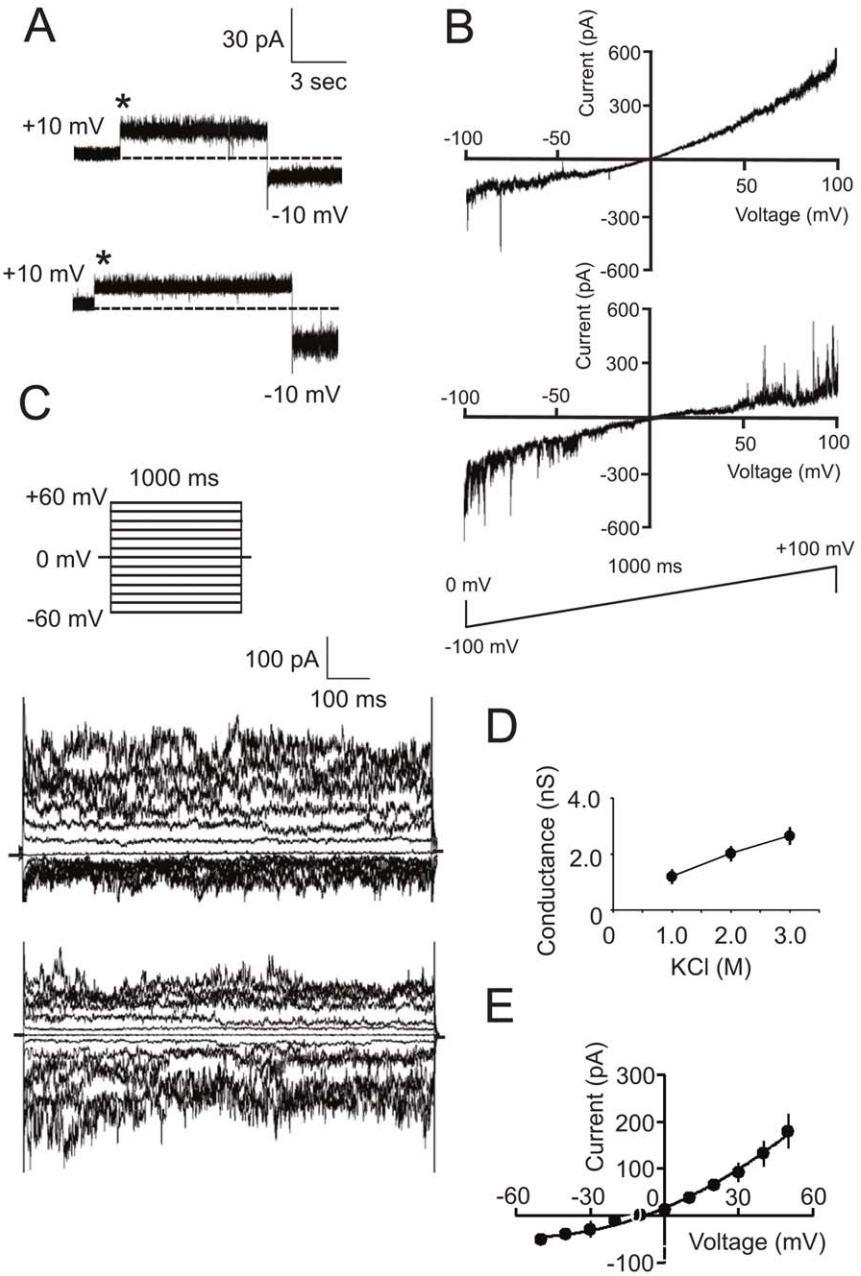

Figure 5. SCA of a low-conductance channel. (**A**) Current traces of two low-conductance channels. The bath solution (**A–C**) contained 3 M KCl at both sides of the membrane. See legend to Figure 4**A** for other details. Note that two types of channels were registered. Most of them showed a larger current amplitude at +10 mV than at −10 mV (upper trace). In contrast, some channels displayed an opposite trend (lower trace). (**B** and **C**) Current traces of the channels in response to the indicated voltage-ramp (**B**) and voltage-step (**C**) protocols. Most detected channels displayed a current rectification at negative holding potentials (upper panels). However, in a few cases the rectification was observed at positive holding potentials (lower panels). (**D**) Dependence of the low-conductance channel activity on the electrolyte concentration. The channel insertion was registered at a holding potential of +10 mV using 3 M KCl as a bath solution. After confirming that the channel shows current rectification at negative voltages by application of a voltage-ramp protocol, the electrolyte in the chambers was diluted to 2.0 M or 1.0 M KCl and the current amplitudes were measured at +10 mV. Data are mean±SD, n = 4–5. (**E**) Ion selectivity of the low-conductance channel. See legend to Figure 4**F** for details. The current-voltage relationship of channels (rectification at negative voltages) was validated using a voltage-ramp protocol.
doi:10.1371/journal.pone.0034530.g005

Therefore, we tried to exploit additional approaches to verify link between the detected channel-forming activities and glycosomes. In this context it is important that the conductivity pattern of the channels in glycosomal preparations determined by MCR was distinctly different from that obtained with membrane protein preparations from the mitochondrial fraction which harbours, among other channels, the mitochondrial outer membrane voltage-dependent anion channel (VDAC). Due to its abundance, this channel is usually considered as a major mitochondrial contaminant of different subcellular fractions. Interestingly, we detected in our mitochondrial preparations a high-conductance channel-forming activity with gating properties similar to VDAC

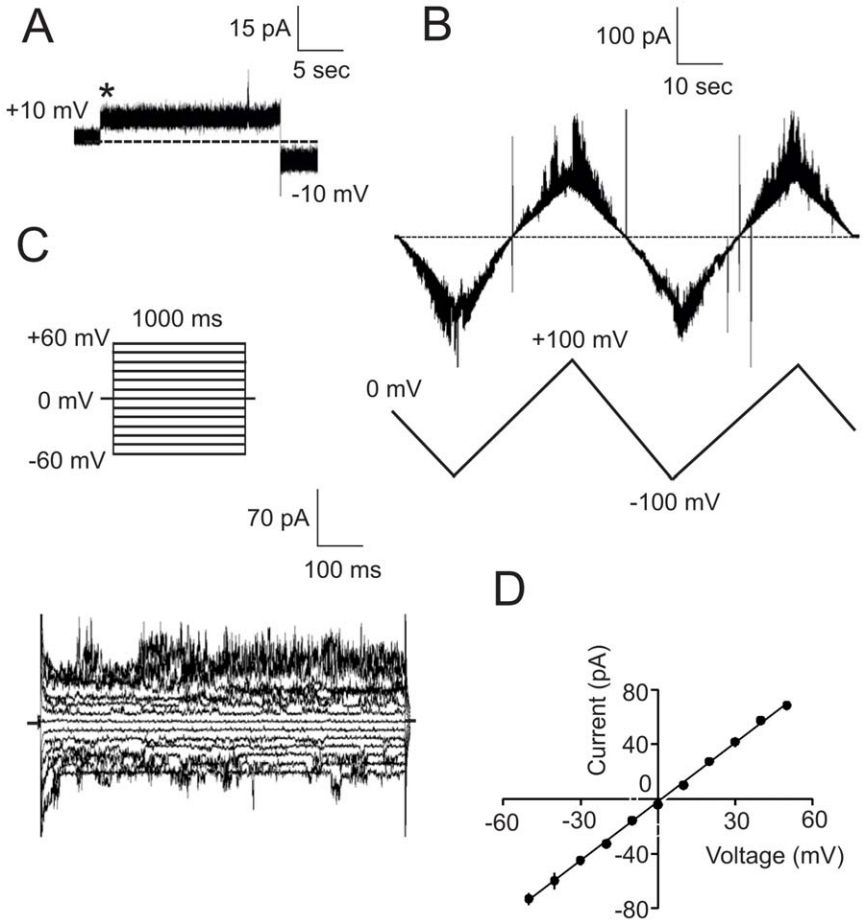

Figure 6. SCA of a very-low-conductance channel. (**A**) Current recording of a single very-low-conductance channel. The bath solution (panels **A**, **B**, and **C**) contained 3 M KCl. See legend to Figure 4**A** for other details. (**B**) Current trace of the channel in response to the shown voltage-ramp protocol. Dotted line indicates the current level at zero holding potential. Note the near linear dependence of the current on the applied voltage. (**C**) Current traces of a single channel in response to the indicated voltage-step protocol. (**D**) Ion-selectivity of the channel. See legend to Figure 4**D** for details.
doi:10.1371/journal.pone.0034530.g006

(see Figure 3D). However, only traces of this activity were found in the glycosomal preparations. This observation coincides with the results of the purity analysis of the glycosomal fraction that showed very low contamination by membrane-bounded organelles other than glycosomes such as mitochondria, lysosomes, and acidocalcisomes as well as fragments of the endoplasmic reticulum and flagellar membranes (see Figure 1). As was revealed by EM examination, the purified preparations of glycosomes contain some fragments of the flagellar apparatus (paraflagellar rods and axonemes). However, these fragments are not connected to the flagellar membrane which might have been a potential source of the channel-forming activity contamination. As a whole, our experiments have revealed the channel-forming activities in purified glycosomal preparations. This can be considered as an indication on the presence of channel proteins in glycosomal membrane. However, further investigation is required to reveal the molecular nature of the channel-forming activities described here.

Three main channel-forming activities (high-, low-, and very-low-conductance) were detected in the glycosomal preparations. We did not find evidence for the possibility that any of these activities are in fact subconductance states of the other registered channels. In particular, the activities did not show spontaneous transitions between different conductance levels and were highly resistant to gating even at extreme holding potentials. This may lead to suggestion that not only one, but several distinct pore-forming proteins are apparently localized in the glycosomal membrane. The same conclusion has been made recently after analysis of the channel-forming activities in preparations of mammalian [12,18] and yeast [13,14] peroxisomes. Similar properties have been previously attributed to the outer membranes of Gram-negative bacteria and chloroplasts. Both these membranes contain different types of pore-forming proteins [29,30]. The reason for localization of several distinct channels in the same membrane is not entirely clear. Some of them (so-called specific porins) show a preference for transfer certain compounds [29]. The function of others is under regulatory control by corresponding metabolites [30]. The latter property is especially evident for channels displaying an intrinsic rectification. Therefore it is highly probable that the intrinsically rectifying low-conductance channel detected in our experiments (see Figures 5 and S1) is formed by an allosterically regulated protein.

Our observation of the super-large-conductance channel-forming activities in glycosomal preparations is intriguing since it may be linked to the formation of a large and highly dynamic pore by the peroxisomal importomer which is involved in the

transmembrane transfer of the newly synthesized, completely folded proteins into the matrix of peroxisomes [31]. The high conductance and the rare appearance of the super-large conductance channels may indicate that they are indeed the peroxisomal importomers. On the other hands, these activities may reflect formation - natural or artificial - of the channel clusters that is a common feature of hydrophobic, membrane-bound proteins.

The most intriguing question arising from our study is if and how the apparent glycosomal channels may be involved in regulation of glycolysis. The relatively high conductance and the long-lasting open states of the channels predict that they form pores in the membrane filled with water, *i.e.* belong to family of so-called non-selective channels [22,24,29]. The sieve properties of each such channel are mainly determined by the dimension of its pore. Our previous results on mammalian [15] and yeast [13] peroxisomes revealed that the membrane of these particles is highly selective towards the size of transported molecules. The peroxisomal channels allow transmembrane diffusion of solutes with molecular mass up to 300–400 Da but are unable to transfer 'bulky' metabolites such as ATP and some cofactors (NAD/H, NADP/H, CoA) whose molecular mass exceeds 500 Da. The sieve properties of peroxisomal channels apparently determine the formation of two independent pools, peroxisomal and cytosolic, of 'bulky' metabolites. In contrast, the peroxisomal lumen and the surrounding cytosol may share a common pool of small solutes. If the same rules are applicable to glycosomes, one can suggest a physical separation of glycosomal and cytosolic pools of ATP by the glycosomal membrane that might be sufficient and important to prevent from 'turbo effect' of uncontrolled ATP production (see 'Introduction' for further details). The proposed model does not require a multitude of glycosomal transporters specific for each small solute that has to be translocated, since these solutes would be able to use the channels to overcome the membrane barrier. However, transporters specific for at least some 'bulky' solutes are necessary. We are tempted to speculate that the MCF6 protein [27], if indeed its location in the glycosomal membrane can be confirmed, is involved in the transfer of some 'bulky' solute(s).

The presence of pore-forming proteins with low selectivity towards small solutes in the glycosomal membrane seems, at first glance, inconsistent with the systems biology analysis that demonstrated moiety conservation relations within the glycosomes not only for adenine nucleotides (ATP+ADP+AMP) and nicotinamide adenine nucleotides (NAD^++NADH), but also for all the phosphorylated intermediates involved in the glycolytic reactions occurring within the organelles [32]. Moreover, pulse-labeling experiments with radioactive glucose indicated that the exchange of phosphorylated intermediates between the two cellular pools is 60 times slower than the labeling of pyruvate [33]. Furthermore, it has been experimentally shown that concentrations of intermediates can increase to very high levels within the trypanosome if glycosomal enzymes are relocated from glycosomes to the cytosol [10]. These are all indications of a low rate of release of these intermediates out of glycosomes. In view of the results presented in this paper, one may wonder if this process is not so much the result of an impermeability barrier created by the glycosomal membrane, but rather the consequence of so-called Donnan equilibrium that may exist between glycosomal lumen and surrounding cytosol. The mechanism of Donnan equilibrium in biological systems is well known [34] and apparently responsible for the formation of a pH gradient across the mammalian and yeast peroxisomal membranes [6,18] and outer mitochondrial membrane [35]. The low release of the intermediates from a multienzyme complex within the glycosomes in which substrate channeling might occur can also be considered. Indeed, there are indications for a strong association of the enzymes found at high density within the organelles, but so far channeling has not been demonstrated. This possibility is currently under investigation.

Which protein molecules determine the glycosomal channel-forming activities remains to be established. One of the channel-forming proteins in mammalian peroxisomes, Pxmp2, has recently been described [18]. The isolated protein forms homotrimer which is active as a channel in *in vitro* experiments and show a conductance of 1.3 nS in 1.0 M KCl. The channel is weakly cation-selective and shows no voltage dependence. An estimated diameter of the channel's pore is ~1.4 nm, intermediate between the dimensions of small and 'bulky' solutes. Surprisingly, deletion of Pxmp2 in mice by disruption of the corresponding gene did not lead to the development of a severe phenotype, indicating redundancy of the channel function in mammalian peroxisomes. Indeed, in the peroxisomal preparations purified from liver of $Pxmp2^{-/-}$ mice some of the channel-forming activities were still registered [18]. Therefore, channel proteins other than Pxmp2 may be expected in the peroxisomal membrane. Good candidates for this role may be members of the Pex11 family of proteins. The family consists of three proteins localized in the mammalian and yeast peroxisomal membranes [36,37]. Remarkably, trypanosomal homologues of this protein family (PEX11, GIM5A, and GIM5B) are also main components of the glycosomal membrane [38] that apparently does not contain proteins detectably homologous to mammalian Pxmp2 since we failed to find any sequence in the *T. brucei* genome with significant similarity to the *Pxmp2* gene (Antonenkov, unpublished results). The Pex11 family members are usually considered as proteins involved in the biogenesis of peroxisomes [36–38]. However, knock-out of yeast *Pex11* is accompanied by evident disruption of the transport function of the peroxisomal membrane [39]. The channel-forming activity of yeast and mammalian Pex11 proteins has recently been observed and is currently under investigation (Grunau et al., manuscript in preparation).

In conclusion, we described here channel-forming activities in purified glycosomal preparations isolated from the bloodstream form of *T. brucei*. The channels which are most probably the glycosomal membrane constituents may be involved in the transmembrane transfer of metabolic intermediates and could play an important role in creating conditions that prevent deregulation of the glycolytic pathway and energy balance in the *T. brucei* cells.

Materials and Methods

Chemicals

Optiprep density gradient medium, a 60% w/v, solution of Iodixanol in water was from Axis-shield PoC AS. A cocktail of protease inhibitors was from Fermentas. An artificial lipid bilayer former, diphytanoyl phosphatidylcholine, was obtained from Avanti Polar Lipids Inc. Genapol X-080 was from Fluka and Fos-choline-10 from Affymetrix. Reagents for electron microscopy were from Electron Microscopy Sciences. All other chemicals were from Sigma.

Growth and isolation of trypanosomes

Monomorphic long-slender bloodstream-form cells of *T. brucei* strain Lister 427 (cell line 449) were grown in 300 g Wistar rats. Blood was collected from animals showing high parasitaemia (between 10^8 and 10^9 trypanosomes/ml) by cardiac puncture under ether anesthesia. Trypanosomes were separated from blood constituents by ion-exchange chromatography on DEAE-cellulose

[40] and washed twice by centrifugation at 1000 *g* for 10 min using phosphate-buffered saline (PBS) containing 50 mM D-glucose. A final wash step prior to glycosome purification was performed using homogenization buffer (250 mM sucrose, 1 mM EDTA, 0.1%, v/v, ethanol, 5 mM MOPS, pH 7.2 and 12%, w/v, PEG 1500). PEG 1500 was used to prevent osmotic damage of glycosomes [17]. All animal care and handling procedures were approved by the committee on animal experimentation of the Université catholique de Louvain (Belgium): 2006/UCL/MD/037 BCHM (TROP) "Etude de la biochimie, de la biologie cellulaire et de la biologie moléculaire des protozoaires responsables des maladies parasitaires chez l'homme et particulièrement des protozoaires de la famille *Trypanosoma* (rats and mice)".

Purification of glycosomes

Trypanosomes were harvested by centrifugation for 10 min at 1000 *g* and the cell pellet (approximately 5.0×10^{10} cells) was subjected to grinding with silicon carbide (325 mesh) [41] in the presence of homogenization buffer supplemented with a cocktail of protease inhibitors (total volume 2 ml). The disruption of parasites was followed by phase-contrast microscopy and continued till at least 95% of the cells were broken. After dilution of the resulting mixture with homogenization buffer (final volume 10 ml) the silicon carbide was removed by centrifugation at 100 *g* for 3 min. The homogenate obtained was centrifuged twice at 1500 *g* for 10 min to remove nuclei and cellular debris. The postnuclear supernatant was then centrifuged at 17 000 *g* for 15 min to sediment mitochondria and glycosomes. The pellet was resuspended in homogenization buffer using a Dounce homogenizer and 5 ml of the suspension were loaded on two preformed linear 20%–35%, v/v, Optiprep gradients (33 ml each) mounted on top of a 3.5 ml 50%, v/v, Optiprep cushion according to the manufacturer's instruction (Optiprep Application Sheet S09, Axis-shield). The gradients were prepared with homogenization buffer containing no sucrose and PEG 1500. The samples were centrifuged in a vertical VTi-50 (Beckman) rotor at 100 000 *g* for 120 min at the slow acceleration and deceleration mode. Fractions of 2 ml were collected from the bottom of each tube and were used immediately for analysis of the activities of marker enzymes or were frozen at −70°C. Disruption of glycosomes by sonication and separation of membrane fragments from matrix proteins by sucrose density gradient centrifugation were performed as described [17].

Measurement of enzyme activities

Spectrophotometric measurements of the activity of marker enzymes for different cellular organelles were conducted according to standard procedures. Activity determination of hexokinase [42] and FAD-dependent glycerol-3-phosphate dehydrogenase [43] was used to localize in Optiprep gradient fractions the glycosomes and mitochondria, respectively. Mannosidase activity (lysosomal marker) was detected with 4-nitrophenyl-D-mannopyranoside as a substrate [44]. α-Glucosidase (substrate: 4-nitrophenyl-D-glucopyranoside) [45] and acid phosphatase (substrate: p-nitrophenylphosphate) [41,46] were used as markers for endoplasmic reticulum and flagellar membranes, respectively. Protein content was measured by the Lowry assay (Bio-Rad).

Antibodies and Western blotting

Proteins from aliquots of gradient fractions were precipitated with cold acetone and resuspended in denaturing SDS-PAGE buffer according to standard procedures. Separation of proteins by SDS-PAGE was performed under reducing conditions using 12.5%, w/v, acrylamide Criterion Precast gels containing 18 wells (Bio-Rad). For Western blotting, the proteins were transferred from gels to nitrocellulose membrane using Bio-Rad Trans-Blot Turbo equipment and Trans-Blot Turbo Transfer Packs (Midi Format, 0.2 µm nitrocellulose). The transfers were done using preprogrammed protocols. The membranes were blocked by 3%, w/v, bovine serum albumin in Tris-buffered saline (TBS) containing 0.2%, v/v, Tween 20 for at least 1 h at room temperature with gentle shaking, and incubated with the appropriate primary antibodies overnight at +4°C, followed by detection with horseradish peroxidase conjugated anti-rabbit or anti-mouse IgG (Santa Cruz Biotechnology). Polyclonal antisera, raised in rabbits, against aldolase (marker for glycosomes) and vacuolar pyrophosphatase (marker for acidocalcisomes; a gift by Dr. R. Docampo, University of Georgia, USA) were used as secondary antibodies. A monoclonal antibody against HSP60 (marker for the mitochondrial matrix) [47] was a gift by Dr. F. Bringaud (Université Bordeaux Segalen, France).

Electron microscopy

Aliquots of the corresponding fractions of an Optiprep gradient were subjected to several rounds of concentration using a 'Centriprep' filter (cut-off 10 kDa) device (Millipore) and gradual dilution by homogenization buffer. This procedure was aimed to remove the gradient former (Iodixanol) and to concentrate particles while avoiding osmotic damage of glycosomes (see ref. [17] for details). The resulting samples were mixed with an equal volume of 2%, v/v, glutaraldehyde prepared in homogenization buffer. After fixation overnight at 4°C, the particles were sedimented at 20 000 *g* for 30 min, and the pellets were processed for transmission electron microscopy as described [16,17].

Detection of channel-forming activity

Channel-forming activities of glycosomal and mitochondrial preparations were registered using a Planar Lipid Bilayer Workstation equipped with a BP-535 amplifier and 8 pole low-pass Bessel filter (Warner Instruments). Acquisition and analysis were performed using pCLAMP software (Axon Instruments). The method is based on reconstitution of solubilized membrane proteins into an artificial lipid bilayer followed by detection of an electrochemical current arising due to insertion of proteins with channel-forming capability. Fractions collected from Optiprep or sucrose gradients were diluted 3–5 fold with 20 mM MOPS buffer, pH 7.2 (final concentration of protein: 0.01–0.05 mg/ml) and treated with detergents: 0.5%, w/v, Genapol X-080 or 0.2%, w/v, Fos-choline (final concentrations) by rotating for 2 h at 4°C. After centrifugation at 100 000 *g* for 40 min, the resulting supernatant was immediately used for registration of the channel-forming activity. Two experimental approaches were exploited for activity detection: multiple channel recording (MCR) and single channel analysis (SCA) [48]. MCR allows the registration of a large number of pore-forming events with detection of their conductance at a certain holding potential. This is a useful test for 'pilot' quantitative estimation of the presence of different channel-forming activities in membrane preparations. SCA was applied for detailed characterization of the properties of a certain channel type. The method allows an electrophysiological analysis of a single channel molecule. Measurements were performed in chambers with two compartments separated by a wall containing a tiny hole which was covered by an artificial membrane. The Ag/AgCl electrodes were connected to the compartments via 3 M KCl-agar bridges. The bath solutions contained 3 M KCl, 20 mM Tris-Cl, pH 7.8, and 2 mM DTT in both compartments (unless otherwise stated). The protein sample was added only into the *trans* compartment and the membrane

currents were measured at a holding potential of +10 mV (unless otherwise stated). The other details of the measurements have been described [12,13,18] and some modifications are mentioned in the legends to Figures 3, 4, 5 and 6.

Supporting Information

Figure S1 SCA of a low-conductance channel. (**A**) Current-voltage relationship averaged from SCA of five channels showing current rectification at negative voltages. The current amplitudes were measured after stepwise changes of the voltage (±20 mV). Bath solution (panels **A**, **B**, and **C**) contained 3 M KCl in both chambers. (**B**) The current-voltage relationship of the low-conductance channel in response to the shown voltage-ramp protocol (**B1**) or low-speed linear increase (**B2**, upper panel) and decrease (**B2**, lower panel) of the holding potential. All three protocols were applied to the same single channel. Note that the channel is open at all applied potentials. (**C**) Dependence of the current-voltage profile of low-conductance channels on the side of their insertion into an artificial membrane. Upper panel: A typical current-voltage relationship of the channel inserted from the *cis*-side of the membrane. Lower panel: Relative frequency of insertion of the low-conductance channels depending on the sample application to chamber compartments facing either the *trans* or *cis* side of an artificial membrane. The current-voltage relationship of each inserted channel was verified using the voltage-ramp protocol (see Figure S1**C**, upper panel). The relative number of channels displaying current rectification at negative (filled bars) or positive (gray bars) holding potentials is shown. The total number of insertion events registered was 56 and 42 for *trans* and *cis* compartments, respectively.
(TIF)

Figure S2 Glycosomal super-large-conductance channels. (**A**) Current trace showing the insertion of two low-conductance channels (marked by asterisks) followed by the appearance of a stable super-large-conductance channel with current amplitude over 300 pA (3.0 M KCl, +10 mV). The dashed line indicates a current level (zero) before insertion of the channels. (**B**) Insertion of a highly unstable super-large conductance channel (1.0 M NH_4Cl, +10 mV). The lower trace represents a timescale-expanded current recording of the upper trace. Direct transition of the current amplitude from near maximal to zero (marked by asterisk) indicates insertion of a single channel or channel cluster rather several separate channels. (**C**) Current-voltage relationship of a single super-large-conductance channel in response to the indicated voltage-ramp protocol (1.0 M NH_4Cl at both sides of the membrane). The current amplitude of the channel before applying the voltage-ramp protocol was 120 pA at +10 mV. The appearance of multiple current amplitude transitions indicates the clustered nature of the super-large-conductance channel.
(TIF)

Author Contributions

Conceived and designed the experiments: VDA PAMM JKH. Performed the experiments: MGL MHV IJM VDA. Analyzed the data: IJM PAMM VDA. Wrote the paper: VDA.

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Chapter 7

General Discussion

Conclusions, discussions and perspectives

1. Glycosomal matrix protein import

In this thesis we have addressed an essential aspect of the glycosomal matrix protein import in *T. brucei*: the recycling of the matrix protein receptor TbPEX5.

We have proved that TbPEX5 is a cytosolic protein that also interacts with the glycosomal membrane. The slow turnover of this protein, as observed in our work, is in agreement with the concept of a cycling receptor as established recently in other organisms. The finding that also *T. brucei* PEX5 is ubiquitinated indicates that the principle of the mechanism by which the receptor is recycled has been conserved throughout eukaryotic evolution, because trypanosomatids belong to the phylogenetic supergroup of the Excavata and yeasts and mammals to that of the Opisthokonta. The common origin of these and other supergroups has been traced back to the so-called LECA or Last Eukaryotic Common Ancestor which must have possessed already a peroxisome (as discussed in Chapter 2). The data accumulated in our research and described in Chapter 5 indicate, however, that some of the peroxins involved in PEX5 recycling exhibit important differences in their primary structure and functional properties when comparing *T. brucei* with other organisms.

A considerable part of our research has been devoted to the determination of the nature of the different TbPEX5 forms detected by western blot analysis in *T. brucei* wild-type cells. This was a crucial investment for the investigation of TbPEX5 ubiquitination, since this post-translational modification generates slower migrating forms of the protein. In order to be able to distinguish native PEX5 from versions modified by ubiquitination, we needed to establish first whether different isoforms exist in the trypanosome and/or the possible occurrence of other forms of physiological or artificial processing of PEX5. TbPEX5 is encoded by a single gene. Analysis of transcripts of this gene by 5'-RACE PCR, detected only mRNAs that could be translated into a protein with a

predicted molecular weight of 72 kDa, no mRNAs with coding potential for larger PEX5 forms (data not shown). The migration of this 72 kDa protein in SDS-PAGE is aberrant as its position in the gel corresponds to that of a protein with an apparent higher molecular weight of ≈90-100 kDa. This behaviour has been previously reported also for a truncated, recombinant form of TbPEX5 (de Walque *et al*., 1999) and for PEX5 of *P. pastoris*, plants and the human long and short isoforms and has been attributed to a high density of negatively charged residues (about 20%) within the N-terminal half of the protein (Schliebs *et al.,* 1998).

We have also demonstrated that TbPEX5 is highly susceptible to *in vitro* proteolytic processing that leads to a faster migrating form with an apparent molecular weight of 72 kDa (Fig. 21). This shorter form of TbPEX5 is still able to bind PTS-1 proteins *in vitro* (Fig. 22), as well as to interact with the glycosomal membrane (data not shown). We postulate that the proteolytic cleavage occurs in the N-terminal part, upstream of the third pentapeptide motif, a region previously shown to be responsible for TbPEX5's interaction with the docking complex at the glycosomal membrane. This proteolytic process also occurred in the presence of protease inhibitors and seemed to affect – to the extent we could detect – exclusively TbPEX5. We argue therefore that, although it is an *in vitro* process, it must be the result of a specific and very active protease that acts preferably on TbPEX5 and not on other cytosolic proteins. A possible explanation is that the unstructured N-terminal domain of TbPEX5 exposes frequently, during its intramolecular motions, a specific cleavage site that does not exist or is inaccessible in most other well-structured proteins also present in cell lysates. Intriguing is the fact that in other organisms such as yeast, mammals and also *L. major* (A. Jardim, personal communication), this proteolytic processing also occurs, indicating that if it is performed by a specific protease, it must be an enzyme that has well conserved its specificity. Moreover, also the cleavage site must have been maintained throughout evolution in the otherwise highly divergent sequence of the N-terminal half of PEX5. We wonder, therefore, if this phenomenon would not reflect some unknown event, also important *in vivo*, in the PEX5 function and that has been overlooked for years. It might be an

important, still unknown key event in peroxisomal matrix protein import. The identification of the protease involved, its specificity and subcellular localization would be an interesting issue to address in a further analysis of this puzzling "artifact".

Part of TbPEX5 was found to be ubiquitinated in both bloodstream and procyclic wild-type cells (Fig. 27). This modification was always detected exclusively in the cytosolic fractions after cellular fractionation. Our results show that a minor amount of unmodified native TbPEX5 is present at steady-state levels in the glycosomal membrane, similar to what has been reported for PEX5 in mammalian cells (only about 10% of total PEX5) (Okumoto *et al.,* 2011), implying that in wild-type conditions, the protein is efficiently retrieved to the cytosol after matrix-protein import. The PEX5 ubiquitination machinery in *T. brucei* is, as in other organisms, present at the glycosomal membrane, proving that this modification must occur at this cellular location. We did not succeed to find conditions in which the membrane-bound receptor was trapped in the recycling stage with any ubiquitin molecules attached. This might indicate that in *T. brucei* the ubiquitination and export of TbPEX5 form a concerted fast process making it difficult to detect by western blot analysis the minor population of ubiquitinated TbPEX5 molecules in the glycosomal membrane. Also for yeast peroxisomes it has been reported that only a minor fraction of the membrane-bound PEX5 is ubiquitinated (Platta *et al.,* 2005). The fact that in trypanosomes ubiquitinated PEX5 was detectable in the cytosol, albeit as a minor fraction of the total TbPEX5, may also reflect an important difference with regard to the *S. cerevisiae* peroxisomal protein import, where an AAA^{+}-ATPase-associated ubiquitin hydrolase is in charge of the immediate de-ubiquitination of the receptor PEX5, shortly after or during its export to the cytosol (Debelyy *et al.,* 2011). In *T. brucei,* we demonstrated that, irrespective of the presence of de-ubiquitinase inhibitors as NEM, the ubiquitinated forms of TbPEX5 were stable in the cytosol (Fig. 28). It seems to us that this might somehow be more similar to the steady-state process as occurs in mammalian cells, where recently such stable ubiquitinated forms of PEX5 were also found to be present in the cytosol of Fao and CHO cells (Okumoto *et al.,* 2011; Miyata *et al.,* 2012). Nevertheless,

the ubiquitin hydrolase responsible of the cysteine-dependent monoubiquitination of mammalian PEX5 has been identified (Grou *et al.,* 2009). We imagine that some differences exist between different organisms in the way the PEX5 deubiquitination process is regulated.

Another intriguing aspect of the TbPEX5 ubiquitination concerns the residue that is modified by ubiquitin. In yeast and mammalian cells, the signal for PEX5 recycling is the monoubiquitination at a conserved cysteine residue present in the N-terminal half (Williams *et al.,* 2007; Grou *et al.,* 2008). Our analysis indicates that the ubiquitination occurring on TbPEX5 is not sensitive to reducing agents such as DTT (Figs. 27 and 28), which is an indication for a lysine-linked ubiquitin moiety rather than via cysteine. PEX5 in several species of yeast is also a target of lysine-linked ubiquitin chains but this kind of modification occurs only when recycling is impaired as in PEX1-PEX6 and PEX4-PEX22 deletion mutants and is not considered as a physiological step in the receptor's recycling (Platta *et al.,* 2004; Kiel *et al.,* 2005a). The yeast's lysine-linked PEX5 ubiquitination process has been associated with polyubiquitination of non-functional receptors acting as a signal for its routing to proteasomal degradation. This occurs as a quality control mechanism in yeast peroxisomal matrix-protein import (Kiel *et al*., 2005a). In *T. brucei* this seems to be unlikely the case, because 1) the lysine-linked ubiquitinated PEX5 molecules detected in the cytosol were stable in wild-type cells and did not accumulate after inhibition of the proteasome by the inhibitor lactacystin and 2) the molecular weights of the modified TbPEX5 forms do not correspond to the conjugation of a considerable number of ubiquitin moieties, they rather correspond with mono- or di-ubiquitination (Figs. 27 and 28). These two facts suggest that the detected ubiquitinated TbPEX5 is an authentic intermediate in physiological receptor recycling. The cysteine that serves as ubiquitin target in yeast and mammalian PEX5s is also conserved in the trypanosome's receptor. In order to unambiguously confirm that the TbPEX5 ubiquitination that we have reported in this thesis indeed occurs at a lysine residue and not the cysteine, it might be necessary 1) to repeat the experiments at higher concentrations of DTT to ensure the reduction of the thio-ester bond and the comparison with a positive

control of the treatment, and 2) to determine if the deleterious phenotype of the *T. brucei* RNAiPEX5 cell line can be rescued by expressing single-substitution mutant versions of the receptor in which either the conserved cysteine or some of the candidate lysines present in the N-terminal half of the protein are replaced by a residue that cannot be linked to ubiquitin and to use these cells for further PEX5 ubiquitination studies.

We have reported in this thesis the identification of the *T. brucei* orthologue of PEX4, the ubiquitin-conjugating enzyme responsible for the monoubiquitination of PEX5 in yeast and plant cells, and proved its function in the ubiquitination of TbPEX5. This protein is associated with glycosomes (Figs. 36, 37 and 38), where it is exposed at the cytosolic face of the membrane (Fig. 39), a topology also presumed for its counterpart in yeast. In spite of the low conservation of the identified TbPEX4 with other PEX4s (Fig. 30), the amino acids of ScPEX4 involved in the interaction with its membrane-anchor protein ScPEX22 are largely conserved in the *T. brucei* orthologue (Fig. 31), strongly suggesting that such an anchor protein must also exist in the trypanosomes. We have identified a candidate TbPEX22 that presented structural features found in the *S. cerevisiae* counterpart (Figs. 32 and 33). The predicted interface region was proved to interact with TbPEX4 in a yeast two-hybrid system (Table 3), supporting its identity as the anchor protein of TbPEX4. It is worth to mention that TbPEX4, as the PEX4s of yeast and plants, does not possess a predicted transmembrane region to attach it directly to the glycosomal or peroxisomal membrane, respectively. Nonetheless, in all our analyses TbPEX4 was constantly found in the organellar fraction after cell fractionation, further supporting the notion that it is anchored by the binding to another protein. Unfortunately, our functional studies on TbPEX22, as well as its subcellular localization were hampered due to technical problems, making it impossible to confirm it unambiguously as the real functional PEX22 orthologue in the trypanosome.

The function of TbPEX4 was studied first by RNAi and subsequently also by knocking out the two alleles of the TbPEX4 gene. Only the latter strategy allowed us to prove unambiguously its role in the ubiquitination of TbPEX5. The results

of the phenotypic analysis were however intriguing, since only a minor mislocalization of glycosomal proteins was found in this mutant (Figs. 49A). In *S. cerevisiae* cells, the impairment of ubiquitination of PEX5 by the absence of PEX4 leads to the accumulation of PTS-1 proteins in the cytosol, which severely affected growth in oleate based media (Platta *et al.,* 2004). In *P. pastoris* however the absence of PEX4 caused a reduction in peroxisome size, but PTS-1 and PTS-2 protein import remained functional (Collins *et al.,* 2000). Our finding that even in the absence of the large majority of ubiquitinated-PEX5 molecules as observed in the ΔPEX4 mutant, glycosomal protein import was still largely functional, and growth was only partially slowed down, surprised us. These results would suggest that receptor ubiquitination is not required for proper receptor recycling in trypanosomes, contradicting the current model of peroxisomal matrix-protein import in yeast and mammalian cells. However, further investigation of the ΔPEX4 mutant revealed peculiar aspects that may account for this unexpected result. We have observed that upon knockout of both alleles of the TbPEX4 gene in procyclic cells, several other UBC genes were importantly and significantly overexpressed (Fig. 45). This may have occurred as a compensatory mechanism to overcome the TbPEX5 ubiquitination defect. One of these UBC proteins could have taken over the function of TbPEX4, albeit with less efficiency than TbPEX4 (since only a very faint ubiquitinated PEX5 signal was detected in the ΔPEX4 mutant by western blot (Fig. 50), keeping the cells still capable of importing proteins into their glycosomes. Such a "redundant" protein should have similar features to TbPEX4 in order to be able to interact with the E3 ligase present in the glycosomal membrane. One may wonder if it should also be able to interact with the anchor protein TbPEX22. If so, it does it probably less efficiently, because there seems no reason why it should have conserved the residues for making such contacts. Moreover, permanent attachment to the membrane may not be mandatory, since in mammalian cells the functional counterpart of PEX4, UBCH5, is a soluble cytosolic protein. In addition, the putative PEX4-compensating protein in the trypanosome has probably not an orthologue in yeast cells, or at least not one that has a similar function as in the trypanosomes, since this compensatory mechanism has not emerged in the yeast ΔPEX4

mutants. Interestingly, one of the highest overexpressed UBC genes (Tb927.4.2710) is trypanosome specific and has a very similar predicted molecular weight as TbPEX4 (18.16 kDa). Sequence similarity analysis indicated a very low conservation level (16%), but remarkably some of the amino acids predicted to be responsible for the interaction with the TbPEX22 candidate are conserved in this protein, opening the possibility that it can also attach to the glycosomal membrane. In agreement with this is the fact that the TbPEX4 antibodies (both those raised against the full-length protein and against a specific synthetic peptide) recognized a protein of nearly identical molecular weight in organellar fractions after the TbPEX4 gene knockout and RNAi-dependent knockdown, suggesting that indeed another similar UBC protein is overexpressed. A very interesting step forward would be the further functional analysis of this trypanosome-specific UBC protein as well as the determination of its subcellular localization in both wild-type and ΔPEX4 cells. Together, the additional data obtained for the *T. brucei* ΔPEX4 mutant could also explain the limited phenotypic effects we observed in the characterization of the TbPEX4 RNAi cell line.

We have equally addressed another important aspect of the TbPEX5 receptor recycling, which concerns the function of the translocase TbPEX6 and the RING-finger protein TbPEX12. It has previously been established that these two proteins are essential for glycosomal protein import and that their depletion by RNAi caused a growth impairment that ended in cell death (Krazy and Michels, 2006). These observations were confirmed in our own research, supporting a very crucial function of these peroxins in glycosomal matrix-protein import as has been found in other organisms (Platta *et al.,* 2009; Prestele *et al.,* 2010; Krause *et al.,* 2006; Goto *et al.,* 2011). By studying TbPEX5 ubiquitination in these RNAi cell lines, we proved that the ubiquitination pattern found in wild-type cells was altered when these two peroxins were individually depleted.

Specifically, we could attribute a function to TbPEX12 in the monoubiquitination of TbPEX5, a role that is probably exerted in concert with the TbPEX4 that we identified, acting as respectively the E3 ubiquitin-ligase and E2-ubiquitin-

conjugating partners in this process. In addition, we found that the absence of TbPEX12 caused the accumulation of higher molecular weight species of TbPEX5, a pattern that we attribute to the possession of several ubiquitin moieties. It remains to be determined if these are real polyubiquitinated species (*i.e.* with four or more ubiquitins), to be targeted to the proteasomes for degradation). The presence of these species in the cytosol implies that also these forms of TbPEX5 are efficiently exported from the glycosomal membrane by the translocases TbPEX6/TbPEX1, similarly as has been reported for *S. cerevisiae* (Platta *et al.,* 2005). We did not observe an increase in the abundance of these TbPEX5 species upon inhibition of the proteasome by lactacystin. It will be important to investigate in the future their stability during longer treatment of the cells with this proteasome inhibitor, in order to fully address the question about the fate of these higher molecular weight forms. The results obtained with the TbPEX12 RNAi mutant are intriguing and difficult to explain. On the one hand, there is a clear phenotype of these cells with a major mislocalization of glycosomal proteins (Fig. 56), accompanied by massive cell death (Fig. 55), indicating that the formation of TbPEX5 species modified by several ubiquitin molecules somehow impairs the glycosomal protein import process, in a unknown way or as a consequence of a defect in the PEX5 recycling. On the other hand, the steady-state levels of native PEX5 seem not to have been altered. Furthermore, the presence of TbPEX5 with several ubiquitins bound in this mutant indicates that the E3 ligase activity responsible for this kind of modification is clearly not PEX12. Two other RING-finger proteins are also present in the glycosomal membrane, TbPEX2 and TbPEX10. These proteins have been functionally characterized in *T. brucei* (Guerra-Giraldez *et al.,* 2002; Krazy and Michels, 2006), assigning to them a fundamental function in the glycosomal protein import. We assume that the modification of TbPEX5 with several ubiquitins as observed in the TbPEX12 RNAi cell line may come from the activity of one or both of these proteins. Previously it has been proved that in *S. cerevisiae* the PEX2-ligase activity is in charge of the *in vivo* polyubiquitination of PEX5 together with the ubiquitin-conjugating enzyme UBC4 (Platta *et al.,* 2009), contradictory with an earlier report by another group that ScPEX10 activity together with UBC4 polyubiquitinates PEX5 *in vivo* (Williams *et al.,* 2008).

Moreover, human PEX10 was reported to act as the E3 ligase responsible for the cysteine–dependent monoubiquitination of PEX5 (Miyata *et al.,* 2012). Taking all these data together, we speculate that in principle the formation of polyubiquitinated forms of TbPEX5 (or at least the modification of TbPEX5 with several ubiquitin moieties) as a response to the depletion of TbPEX12 can be done either by TbPEX2 or TbPEX10. However, no data concerning this issue are available because it was not further addressed in this work. It is well known that the stabilization of an intact RING-finger complex is achieved by protein-protein interactions between its constituents. The depletion of one of them causes the rapid degradation of the others, suggesting that a functional ligase activity depends on the proper organization of the RING-finger complex (Platta *et al.,* 2009). By depleting TbPEX12 in *T. brucei* we had expected an abolishment of all ubiquitinating activity because the complex would be disassembled, as has been found previously in the corresponding yeast mutants (Platta *et al.,* 2009). It seems that even if such a disassembly has occurred in the TbPEX12RNAi mutant, some ligase activity that produced the polyubiquitination of PEX5 was still functional. It might be that this remaining activity is due to incomplete depletion of TbPEX12 by the RNAi machinery. We can also imagine that the strong glycosomal protein import and cell viability defect observed in the TbPEX12 RNAi cell line could be the result of a destabilization of the entire importomer supercomplex, because of the physical connection of the affected RING-finger complex with the docking complex. This may then have caused the pronounced mislocalization of glycolytic enzymes, a well-known killer effect in *T. brucei.*

We have also found ubiquitination of TbPEX5 in the AAA$^+$-ATPase TbPEX6 RNAi mutant. The pattern observed indicates the conjugation of monoubiquitin as well as several ubiquitin moieties, probably as a result of the coordinated action of TbPEX4-TbPEX12 (monoubiquitination) in a normal round of recycling and of TbPEX4-TbPEX2/TbPEX10 (polyubiquitination), the latter being a consequence of the TbPEX5 accumulation in the glycosomal membrane. This is in agreement with the phenotype observed in *S. cerevisiae* cells lacking the AAA$^+$-ATPase complex PEX6/PEX1 and its anchor protein PEX15 (Kiel *et al.,* 2005a; Platta *et al.,* 2005), where these forms accumulate in the peroxisomal membrane (Platta

et al., 2005). In other yeasts such as *P. pastoris,* the absence of PEX1/PEX6 caused, by contrast, the fast destabilization and degradation of the PEX5 receptor as a result of a faster turnover after polyubiquitination (Collins *et al.,* 2000). Our results suggest that no accumulation in the glycosomal membrane of either the native TbPEX5 or its ubiquitinated forms occurred upon depletion of the AAA$^+$-ATPase TbPEX6, indicating a still efficient export. This unexpected molecular phenotype is not in agreement with the prominent glycosomal protein import and impaired viability of the TbPEX6RNAi induced cells (Fig. 56). It has been found in yeasts that PEX1 and PEX6 are not redundant AAA$^+$-ATPases, since the ectopic expression of one of them does not restore the function of the other. This is expected because of the low conservation of the D2 domain of the two AAA$^+$-ATPases. However, in *in vitro* export reactions where single mutants of PEX1 and PEX6 were analysed, it was observed that PEX5 was efficiently released from the peroxisomal membrane (Platta *et al.,* 2005), a result that has not been clearly explained to our knowledge. We argue that up to some extent this may also be the case for the phenotype observed in the TbPEX6 RNAi cell line. The fact that we did not observe changes in the steady-state levels of TbPEX5 in this RNAi mutant argues against a fast turnover of the receptor after its accumulation in the glycosomal membrane. Support for this idea is the apparent absence of an effect of the proteasome inhibitor lactacystin on the levels of ubiquitinated TbPEX5 forms in this mutant (Fig. 57). We think that, in future experiments, it would be interesting to extend the lactacystin treatment for a longer period in order to totally exclude this possibility. The high impact on the growth observed in this mutant may also be the result of other effects not related with glycosomal matrix-protein import, but related to the other functions that are associated to this peroxin. It has been found that in *P. pastoris* and *Y. lipolytica* PEX6 plays a role in lipid and membrane transport, a process that can account for a function in the fusion of peroxisome vesicles during maturation of ER-derived pre-peroxisomal structures during *de novo* synthesis of peroxisomes. In addition, PEX6 has been associated with the localization of the GTPase Rho1 which is thought to organize actin filaments during peroxisome proliferation (Platta *et al.,* 2006). Since our immunomicroscopy data indicated a mislocalization of glycosomal enzymes, it would be very interesting to study at the ultrastructural

level the morphological features of the TbPEX6 RNAi mutant in order to assess these possibilities. We also find of extreme relevance a future characterization of the function of the identified TbPEX1 candidate. The generation of double mutants lacking both AAA$^+$-ATPases would probably further contribute to our understanding of the mechanism of TbPEX5 export from glycosomes as was the case for the *S. cerevisiae* system.

Our research on the molecular mechanism involved in the recycling of TbPEX5, together with previous work done by us and others, led us to propose the following model for the glycosomal protein import in *T. brucei* (Fig. 58): TbPEX5 is a cytosolic protein able to recognize PTS-1 proteins and participate in their import, as well as to mediate the PTS-2 protein import through its interaction with the PTS-2 receptor TbPEX7. The PTS-1 receptor docks at the peroxisomal membrane by interacting, through its N-terminal domain, with peroxins TbPEX13 and TbPEX14. The translocation of the cargo must occur as in *S. cerevisiae* cells, by the formation of a dynamic and transient pore formed by the receptor TbPEX5 and probably TbPEX14. After cargo release, the receptor TbPEX5 is ubiquitinated, likely at a lysine residue, by the glycosomal E2 enzyme TbPEX4 in concert with the E3 RING-finger protein TbPEX12 and subsequently quickly exported to the cytosol by the action of the TbPEX6/TbPEX1. The ubiquitinated TbPEX5 is stable in the cytosol. The signalling ubiquitin is probably removed by an (enzymatic) mechanism homologous to the one in mammalian and yeasts but that operates at a much lower steady-state level.

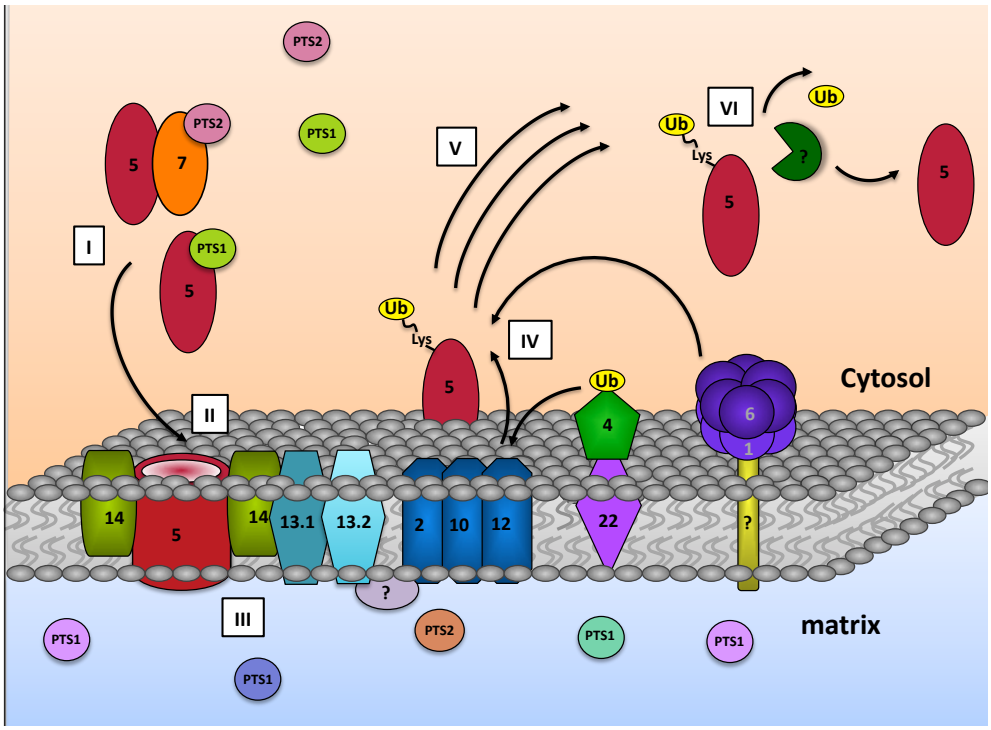

Figure. 58. ***Model of the import of glycosomal matrix proteins in Trypanosoma brucei.*** Matrix protein import is achieved by a process which involves cyclic receptors, here depicted for PEX5, the receptor of matrix proteins with a PTS-1 motif. Six distinct steps can conceptually be understood: (I) binding of proteins with a PTS-1 to PEX5 (and PTS-2 to PEX7, plus PEX7-PTS2-proteins to PEX5). (II) Docking of the receptor-cargo complex to the docking complex (PEX13.1, PEX13.2, PEX14) in the glycosomal membrane, followed by the insertion of PEX5 into the membrane, forming in conjunction with PEX14 a transient pore. (III) Release of PTS proteins, via the transient pore, into the matrix. (IV) Ubiquitination of the membrane-inserted PEX5 by the coordinated action of a cytosolic ubiquitin-conjugating enzyme PEX4, anchored to the membrane by PEX22, and an ubiquitin-ligating enzyme of the RING-finger complex, which comprises PEX2, PEX10 and PEX12. (V) Retrieval of the ubiquitinated PEX5 from the membrane by the cytosolic AAA$^+$-ATPase complex including PEX1 and PEX6, recruited to the membrane. (VI) deubiquitination of PEX5, rendering the receptor available to mediate new rounds of PTS-protein import. All peroxins identified in *T. brucei* are indicated in this scheme by the numbers attributed previously to their *S. cerevisiae* homologues.

2. Summary of surprising features of the procyclic ΔPEX4 mutant – possible explanations

Making a null mutant by knocking out both alleles of the *PEX4* gene in procyclic form *T. brucei* generated cells that were affected in their *in vitro* growth, presenting a significant decrease in their duplication time. Despite the impaired ubiquitination of the glycosomal matrix protein receptor TbPEX5, the protein import appeared not to be importantly affected, leading only to a minor mislocalization of PTS-1 and I-PTS proteins. Our further analysis of this cell line

indicated increased gene expression of some putative members of the ubiquitin-conjugating enzyme family. Three of the UBC family members that showed significantly increased mRNA levels in the mutant compared to wild-type cells, Tb09.211.0050, Tb927.8.6090, Tb927.2.3720, are firm orthologues of proteins involved in the cell-cycle regulation in yeast and in mammalian cells. More specifically, Tb927.8.6090 and Tb927.2.3720 are the corresponding orthologues of UBE2C and UBE2S, two mammalian ubiquitin-conjugating enzymes that initiate and extend Lys11-linked polyubiquitin chains in substrates of the ubiquitin ligase APC/C. This is a complex that acts during the anaphase to promote mitosis progression through the targeting of cell-cycle regulatory factors, including M and C-cyclins, for proteasome degradation, thus leading to the final separation of sister chromatids. Tb09.211.0050 is the orthologue of UBC13 in yeast and UBE2N in mammalian cells whose function is related to the formation of Lys63-linked polyubiquitin chains, a modification that is not meant to target their specific substrates for proteasome degradation but for an involvement in the activation of gene transcription as well as DNA repair, two processes that play important roles in the progression through the cell cycle. Based on this information, we can imagine that overexpression of these genes in the ΔPEX4 cell line might cause disregulation of the polyubiquitination of their targets, affecting directly the checkpoints of the cell cycle during DNA synthesis and/or during mitosis, thus offering an explanation for the retardation of growth observed in the ΔPEX4 cells. However, our preliminary ultrastructural analysis by transmission electron microscopy did not give us any clue regarding a possible defect in the division of the cells, since all analysed images showed that cells had an apparent normal mitochondrion, nucleus and flagellum (Fig. 53). Indeed, some wild-type as well as *PEX4*-knockout cells presented signs of initiation of the cell cycle, such as the presence of two flagellar structures. Relevant, however, may be the observation of some ΔPEX4 cells that were clearly "ill", as judged from the abundant presence of cytosolic vesicles that were not observed in the set of images of wild-type cells (Fig. 53F). These vesicles may reflect a late stage of cells in which such a defect in the cell cycle was caused. The scanning-electron microscopic analysis described in this thesis showed that a

large majority of the ΔPEX4 cells presented a reduced cell body with an abnormal shape (Fig. 52). We can imagine that the coordination of all cell-cycle related events including cytokinesis, to lead properly to two equally-sized similar procyclic daughter cells, also involves ubiquitination processes, which could have been affected in the ΔPEX4 cells by the disregulation of the expression and thus activity of the ubiquitin-conjugating enzymes. The finding that the ubiquitin-like protein SUMO is required for the transition through mitosis in *T. brucei* (Liao *et al.,* 2010) suggests that the ubiquitin system may indeed play an important role in the cell-cycle regulation in this parasite.

Taking into account that we are working with not-synchronized cultures and that a disregulation of the cell cycle may cause transient effects in the cytokinesis may somehow account for the heterogeneity at the morphological level found in the population of ΔPEX4 cells. Different cells of the population are each at distinct stages of the cell division, in which potential defects will be manifested differently. To analyse the morphological effects further and to assess if indeed there may be a link between on the one hand the genotype and on the other hand the cell cycle and cytokinesis would require a detailed morphometric analysis with a statistically assessed quantification of all morphological changes observed between mutant and wild-type cells and the ratio of the numbers of nuclei and kinetoplasts per cell in the ΔPEX4 population.

All these possible explanations for the unexpected phenotype of the procyclic ΔPEX4 *T. brucei* mutant remain speculative, because we have not been able to perform a detailed analysis of the molecular consequences of the overexpression of the mentioned UBC genes. Such an analysis was not only impossible within the timeframe of this PhD project, but also would have been outside its scope of glycosome biogenesis. Yet, the data presented here raise exciting prospects for information that may be obtained in a future study of the ubiquitinome of *T. brucei*. An exploration of the important functions of this posttranslational modification in the biology of this divergent eukaryote can also be of high interest in the ongoing research for development of drugs against human African trypanosomiasis.

3. Channel-forming activities in glycosomes

In this thesis we also described our investigation of a very important aspect related to the metabolic role of glycosomes in *T. brucei*, which concerns the translocation of metabolites through the glycosomal membrane. Data obtained during previous years have provided several lines of evidence to support the notion that this membrane forms a permeability barrier. First, glycosomes are bounded by a single phospholipid bilayer. Such membranes are generally considered as impermeable for ions and polar molecules such as most metabolites. Secondly, as explained in Chapters 2 and 3, under anaerobic conditions, glycerol can be formed with concomitant ATP synthesis owing to the accumulation of glycerol 3-phosphate and a lowered ATP/ADP ratio inside the glycosomes, allowing the reversal of the glycerol kinase reaction. A rapid equilibrium of glycerol 3-phosphate and adenine nucleotides across the membrane would prevent this reversal. Thirdly, an apparent low equilibrium of glycolytic intermediates across the membrane was observed upon addition of a pulse of radioactively labelled glucose to bloodstream-form trypanosomes. A small (20-30%) pool of intermediates, as well as the endproduct pyruvate were very rapidly labelled, whereas the label appeared only 60 times slower in a large (70-80%) pool of intermediates. The small pool was interpreted as the metabolites present inside the glycosomes, and the large pool as those in the cytosol (Visser *et al*., 1981). Fourth, a mathematical model of bloodstream-form glycolysis has been made based on the available kinetic data of all enzymes involved and this model has been experimentally validated (Bakker *et al*., 1997). Simulation of glycolysis using this model indicated the essentiality of a defined pool of phosphorylated glycolytic intermediates within the glycosome, separate from the cytosol, to enable a functional glycolytic flux (Bakker *et al*., 1997). Finally, several of the glycolytic glycosomal enzymes possess peculiar functional properties which restrict their proper functioning to within the glycosomal boundaries (Bakker *et al*., 2000; Haanstra *et al*., 2008).

While these observations suggest a permeability barrier between glycosome and cytosol, our investigation of the presence of channel-forming activities in the glycosomal membrane preparations of *T. brucei* strongly indicates the existence

of non-selective channels, similarly as detected in the membrane of perixosomes from other organisms (Antonenkov and Hiltunen, 2012). Together, these data present an apparent paradox. We argue that one possible explanation to accommodate the seemingly contradictory data is that the slow equilibrium of metabolites across the membrane is due to the presence of channelling of the glycolytic intermediates between the active sites of enzymes catalyzing consecutive reactions within the glycosomes (Fig. 59A). In this way the intermediate metabolites do not form part of a homogeneous pool within the entire organelle and consequently do not readily diffuse into the cytosol. Channelling may offer the advantage of decreasing transit times of intermediates and enhancing metabolic fluxes. A corollary is that it may exclude inhibitors from the active site of enzymes. A prerequisite of channelling is the need for organization of enzymes, either as stable multiprotein complexes or by transient protein-protein interactions between enzymes within a common metabolic pathway, forming what is called a metabolon (Srere, 1987). Although there is strong evidence for channelling in several pathways in different organisms (Vélot and Srere, 2000; Cleg and Jackson, 1990), it remains in general difficult to demonstrate that channelling occurs and/or that enzymes are organized as functional multiprotein complexes. There is a general consensus that many protein-protein interactions are fragile and lost by dilution either during isolation or under *in vitro* conditions (Williamson and Sutcliffe, 2010). Despite this difficulty, there is increasing evidence that, indeed, enzyme-enzyme interactions exist in many metabolic pathways of different organisms (Beeckmans and Kanarek, 1987; Meyer *et al*., 2011; Islam *et al*., 2010; Jørgensen *et al*., 2005). Moreover, structural analysis of several enzyme complexes has shown molecular evidence for channelling such as a tunnel in tryptophan synthase (Hyde *et al*., 1988) and other complexes (Miles *et al*., 1999). Another consequence of enzyme-enzyme interactions, different from channelling, is the alteration of kinetic properties of the enzymes involved (Ricard *et al*., 1998).

Evidence for associations between enzymes of glycolysis, the most prominent pathway in the glycosome, has been reported for other cells. A complex of glycolytic enzymes formed by GAPDH, aldolase, phosphofructokinase (PFK), pyruvate kinase, lactate dehydrogenase and other proteins, was found in

association with the membrane of red blood cells (Campanella *et al*., 2005). The formation of this complex is regulated by oxygenation and phosphorylation. Furthermore, six glycolytic enzymes (aldolase, TPI, GAPDH, glycerol-3-phosphate dehydrogenase, phosphoglycerate kinase (PGK) and phosphoglycerate mutase) colocalize at Z-disc and M-lines in *Drosophila* flight muscle (Sullivan *et al*., 2003). Glycolytic enzymes have also been found associated with other proteins such as tubulin (Vértessy *et al*., 1997) and calmodulin (Christova *et al*., 1996) among others. In some cases, interactions between glycolytic enzymes have been associated with modifications of their kinetic properties (Ovádi, 1988).

Glycosomal proteins are found at a relatively high concentration inside glycosomes (approximately 150 mg/ml) of bloodstream-form *T. brucei* (Misset *et al*., 1986) and need an elevated concentration of salt (0.15-0.25 M NaCl) to be solubilized. Thus, one may speculate that a special organization exists within the organelle. Indeed, cross-linking experiments with *T. brucei* glycosomes have provided evidence that the enzymes are in close proximity, possibly with an average intermolecular distance of around 20 Å (Aman *et al*., 1985).

The glycosomal enzymes have a tendency to associate and require high ionic strength during the purification process (Misset and Opperdoes, 1984). In addition, it has been shown that hexokinase (HXK) exists as different aggregated forms at low ionic strength (Cáceres *et al*., 2003). Another glycosomal enzyme, glucokinase, can be obtained as different molecular mass forms by gel filtration, dependent on the protein concentration at loading (Cáceres *et al*., 2007). These behaviours would suggest a capacity of the glycolytic enzymes to make protein-protein interactions. However, so far no evidence for channelling has been found in this organelle. On the contrary, it has been argued that channelling is absent in the glycosome since a cross-linked complex and solubilized glycosome preparations had similar kinetic features (Aman *et al.*, 1986). In all, it is clear that more studies about the interactions of the glycosomal enzymes are necessary.

Another possibility to explain the low exchange of glycolytic intermediates across the glycosomal membrane, that is not mutually exclusive with the one

discussed above, is the presence of a Donnan equilibrium over the membrane (Fig. 59B) (Antonenkov and Hiltunen, 2012). The uneven distribution of charged macromolecules across two sides of a membrane that is only permeable for small compounds will lead to an asymmetrical distribution of permeant compounds with a charge opposite to that of the macromolecules. Matrix proteins of peroxisomes have usually a higher isoelectric point (pI) than those of the average cytosolic proteins and therefore are positively charged (Brett *et al*., 2006). The pI's of many glycosomal proteins of trypanosomatids are even remarkably high (Misset *et al*., 1986; Hannaert and Michels, 1994) which may lead to a tendency to restrict the diffusion of the negatively charged glycolytic intermediates from the matrix to the cytosol.

A third alternative explanation for why diffusion of metabolites across the membrane is limited could be that the channels are associated with proteins that endow them with specificity and effectively control the passage of solutes. Such proteins may be regulatory factors or specific enzymes (Fig. 59C). For example, a specific association of HXK with a pore may make it specific for hexoses such as glucose. The glucose may then be immediately directed to the active site of HXK, resulting in a direct coupling of the translocation and ATP-dependent phosphorylation of the sugar. Similarly, an association of the glycosomal PGK with the same or another pore may make it specific for the efflux of its product 3-phosphoglycerate from glycosomes. In this respect, it may be relevant that, during purification, *T. brucei* HXK shows a strong tendency to remain associated with the glycosomal membrane (our unpublished results).

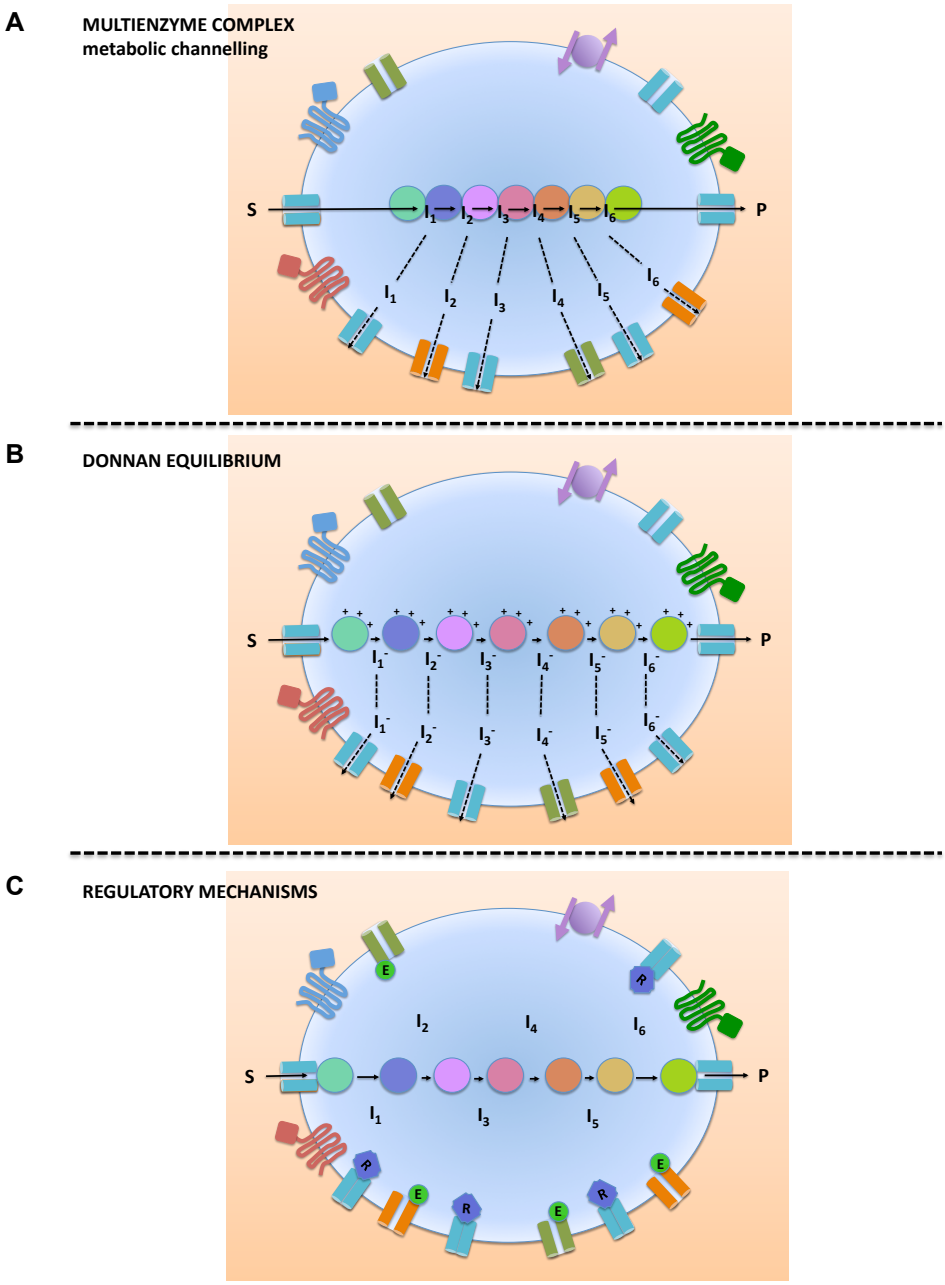

Figure 59. ***Possible explanations for the 'Glycosomal Paradox'***. Three, not mutually exclusive explanations are proposed to explain how the apparent low permeability of the glycosomal membrane for metabolites is compatible with the existence of non-selective channels in the membrane. A. Glycosomal enzymes form a multienzyme complex in which intermediates (I) in the conversion of substrate (S) to product (P) are channelled between active sites of consecutive enzymes of the pathway without major release into the lumen of the organelle and consequently through the channels into the cytosol. B. Glycosomal enzymes have on average a higher pI than cytosolic proteins and are densely packed. The net positive charge of these proteins creates a Donnan equilibrium of charged molecules/ions across the glycosomal membrane, with the – mostly negatively charged – metabolites retained within the lumen. C. Putative regulatory proteins (R) may control the opening and closing of the channels, or specific enzymes (E) at the beginning and end of the compartmentalized pathway) may be associated with channels causing a preferential flux of the substrate from the cytosol directly into the active site of the enzyme, or a flux of the product from the active site into the cytosol.

4. Drug discovery aspects

4.1. Glycosome biogenesis as drug target

The principal mechanism of matrix-protein import process of peroxisomes is widely conserved, yet significant differences exist between organisms from phylogenetically distinct groups, including mammals and trypanosomatids. Glycosome biogenesis has been proposed already previously as an interesting drug target for sleeping sickness since the human pathogenic (bloodstream) form of *T. brucei* is for its energy entirely dependent on the ATP produced by glycolysis, a process for which most enzymes are present inside glycosomes, a form of compartmentalization that has been shown to be essential for the viability of the parasites. The low sequence conservation that exists between peroxins of mammals and *T. brucei,* in addition to the important variations that have been found for their regions responsible for the essential protein-protein interactions that occur during the cascade of reactions in the import process, are evident arguments supporting this proposal (Moyersoen *et al*., 2004; Schliebs, 2006).

Galland and Michels (2010) have recently reviewed the differences that exist in matrix-protein import between peroxisomes of different organisms and glycosomes of trypanosomatids. They have discussed the different possibilities to impair the glycosomal protein import. A first evident prospect would be the impairment of the interaction of the PTS-1 and PTS-2 bearing proteins with the respective receptors TbPEX5 and TbPEX7 in the cytosol. Interfering with glycosome biogenesis at this level has the advantage that a potential inhibitor has only to cross the plasma membrane to reach its target. The TPR domain structure of human PEX5 and *T. brucei* PEX5 is very well conserved, with a highly similar PTS-1-binding pocket. But despite this high conservation several differences in the TPR structure could be exploited to design specific drugs. For example, the two TPR subdomains undergo a hinge motion upon cargo binding. This is accompanied by the creation of additional pockets at the interface. Importantly, two of the residues contributing to such a pocket are smaller in

parasite PEX5 than in human PEX5. These two residues, Met407 and Cys492 in human PEX5, are replaced by Ala421 and Ala504 in *T. brucei* PEX5. The development of small molecules that could specifically occupy this "unique double Ala pocket" may then hinder the hinge motion and consequently PTS-1 binding or release in trypanosomatids but not in humans and could thus potentially inhibit glycosomal protein import. Another residue in trypanosome PEX5 with potential for targeting is Leu523 which is Phe511 in the human receptor. This residue is near the PTS-1-binding pocket and a compound binding in the double-Ala pocket may be extended to reach to this leucine and so acquire increased affinity and selectivity (Sampathkumar *et al.,* 2008). Interestingly, the PTS-2-protein import could possibly also be a target based on the differences found in the interaction between *L. donovani* PEX5 and PEX7. Indeed, notwithstanding that PEX5 of both *T. brucei* and *L. donovani* posses a predicted PEX7-binding box, similar to that of the long isoform of human PEX5, it was found that the *in vitro* interaction between the LdPEX5 and LdPEX7 occurs through an upstream region of the N-terminal domain of PEX5. This differs considerably with the situation in humans (Pilar *et al.,* 2008). A compound with high affinity for this unique region located between residues 111 and 148 in *L. donovani* PEX5, could specifically disrupt the PTS-2-protein import in this parasite. TbPEX5 has been proved to interact with PEX7 but the region responsible for the interaction has not been yet identified. We should stress however that the sequence conservation between TbPEX5 and LdPEX5 in this N-terminal stretch is low (Galland and Michels, 2010), suggesting that a compound directed to this region could perhaps only be used as a drug against leishmaniasis

The docking step of glycosomal matrix-protein import has been proposed too as a stage with potential for drug targeting. Also an inhibitor that blocks this step would only need to cross the plasma membrane to reach its target in the cytosol. This part of the import process is also conserved between human and trypanosomatids in the sense that homologues proteins are involved. However, the maps of the regions mediating interacting between TbPEX5, TbPEX13 and TbPEX14, as well as between the pair LdPEX5 and LdPEX14, show interesting

differences that may be exploited for selective impairment of the interactions of the parasite peroxins by inhibitors. TbPEX5 interacts with TbPEX13 via its third pentapetide motif and the cytosolic SH3 domain of TbPEX13. The second and fourth pentapeptide of PEX5 are involved in the corresponding interaction in human, suggesting an interesting difference. In the case of the interaction between TbPEX14 and TbPEX5 the same domains of both proteins are responsible for the interaction in the human pair, with similar pentapeptide motifs and electrostatic interactions between negatively charged residues within and around the pentapeptide motifs and the positive surface of the PEX14 binding site also involved in the binding. However, in trypanosomes there is much less conservation of these charged residues, rendering it possible to design selective peptidomimetic inhibitors of the PEX5–PEX14 interactions (Galland and Michels, 2010). The results found for the corresponding *L. donovani* peroxins suggest that the pentapeptide motifs in LdPEX5 are not involved, but rather a region between the third pentapeptide motif and the TPR domain (Madrid and Jardin, 2008). This could also represent a specific difference that could be investigated for specific drugs directed to *L. donovani*.

The results presented by Krazy and Michels (2006) and corroborated by us in this thesis, strongly point to the potential of the RING-finger complex, as well as the AAA^+-ATPase PEX6, as possible drug targets since the depletion of these peroxins causes the mislocalization of the glycolytic enzymes in the parasite leading to an inhibition of growth followed by cell death. Our results indicate that these proteins are involved in the recycling of PEX5 similarly as their homologues in human cells, opening the possibility to explore also this step of the glycosomal protein import for drug targeting. Low conservation at the sequence levels (approximately 30% sequence identity for these peroxins between trypanosomatids and human) is already a good indication that structural differences may exist that could be exploited for the design of selective inhibitors of the activity of the *T. brucei* peroxins. So far, no experimental evidence has been acquired regarding the domains and more precisely the residues that are essential for their functional activity, as well as for the interaction performed by these peroxins in trypanosomatids. Future research

aiming to unravel details of the protein-protein interactions in which they are involved and also the acquisition of structural data, would improve our knowledge about the potentiality of these peroxins as drug targets.

In this thesis we reported the identification of TbPEX4 and showed that it is involved in a crucial step of PEX5 recycling, the receptor's monoubiquitination that very likely serves also in *T. brucei* as the signal for its export from the glycosomal membrane. This protein belongs to the ubiquitin-conjugating enzyme family, a group that comprises 15 different proteins encoded in the *T. brucei* genome. The members of this family are likely the orthologues of the ubiquitin-conjugating enzymes identified in human genome databases and found to be involved in several biological processes. The function of TbPEX4 is accomplished by a cytosolic protein of which three isoforms exist in human cells, members of the UBCH5 subfamily (Grou *et al.,* 2009), which are also involved in other processes such as the selective degradation of short-lived and abnormal proteins, the E6/E6-AP-induced ubiquitination of p53/TP53, and the auto-ubiquitination of STUB1, TRAF6 and TRIM63/MURF1 among others. Our phylogenetic analysis suggests that the firm UBCH5 orthologue in *T. brucei* is another putative UBC enzyme (Tb927.5.1000), thus clearly different from the identified TbPEX4. The overall sequence conservation between TbPEX4 and its functional counterpart in humans is relatively low (34%), with TbPEX4 notably also possessing extensions at the N- and C-termini that are absent in the human UBCH5. In addition, the majority of the residues presumed to be involved in the interaction with TbPEX22 are not conserved. In our research, we did not find a prominent glycosomal protein import defect in the TbPEX4 knockout mutant. Nonetheless, cell growth of the mutant was importantly affected. The corollary of all data together is that a possible redundancy exists between the UBC proteins and that another one could replace the function of TbPEX4, notably the trypanosome-specific putative ubiquitin-conjugating enzyme Tb927.4.2710. Taking together, our findings may indicate that, despite the facts that monoubiquitination must be essential for TbPEX5 recycling and TbPEX4 functions as the ubiquitin-conjugating enzyme in this process, TbPEX4 itself is not an interesting drug target because its function may be taken over by another

UBC protein. Further research on the function of Tb927.4.2710 in wild-type cells as well as in the context of the TbPEX4 knockout, could open an interesting opportunity to study a trypanosome-specific protein that has no orthologue in humans, and eventually discover an essential role of this protein in glycosome biogenesis and/or other cell processes that may reveal it as an interesting drug target.

4.2. Solute translocation across the glycosomal membrane as drug target

The solute transporters and channels in the glycosomal membrane may also offer perspectives as drug targets. Although still very little is known about them – only one class of transporters has recently been recognized and partially characterized, namely the GAT transporters belonging to the subfamily D of ABC transporters (Yernaux *et al*., 2006; Igoillo-Esteve *et al*., 2011; see also Chapters 2 and 3) and the protein(s) responsible for channel formation remain to be identified – they may be considered as potential drug targets (Fig. 60). Indeed, correct compartmentalization of glycosomal metabolism, or at least part of it, is essential for the parasites and therefore the translocation of the substrates and products across the membrane should be essential as well. Moreover, the glycosomal ABC transporters show very little sequence similarity with their counterparts in the peroxisomes or with other ABC transporters in human cells and probably the same will be true for the channel-forming proteins. No homologue of the currently only known peroxisomal channel protein (the mammalian PXMP2; Rokka *et al*., 2009) can be found in the trypanosomatid genome databases.

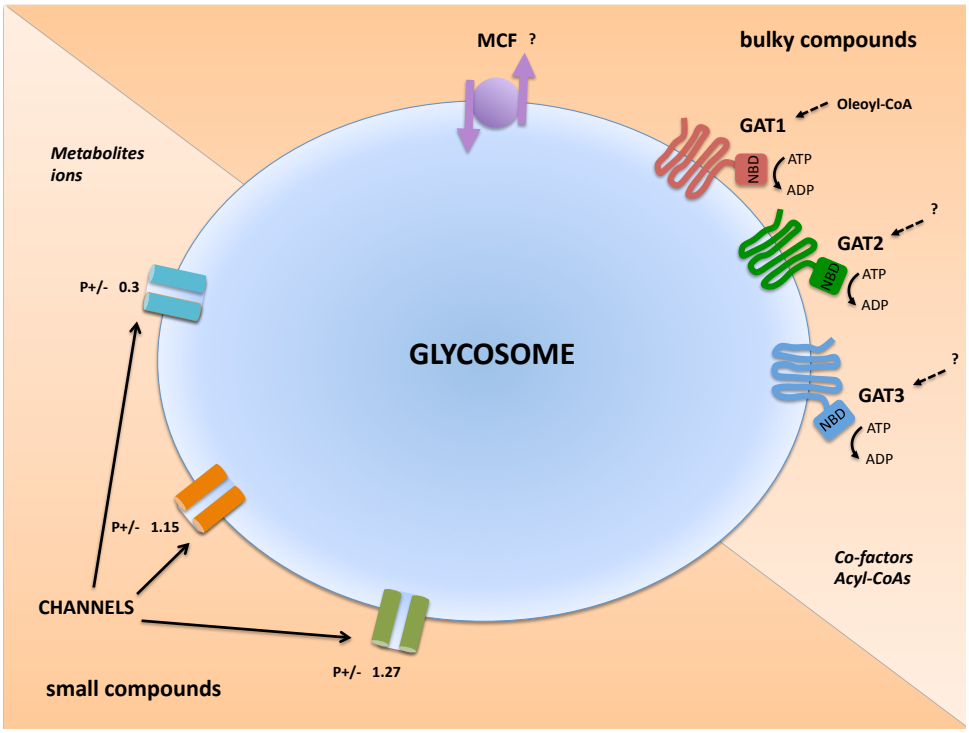

Figure 60. ***Solute transporters and channels in the glycosomal membrane of T. brucei.*** The membrane contains three half-size ABC transporters called GAT1-3, with a topology indicating their role as importers. GAT1 has been shown to transport oleoyl-CoA, the substrates of GAT2 and GAT3 remain to be identified. Probably, the membrane contains also MCF transporters, involved in transmembrane exchange of substrates. The ABC and MCF transporters are likely involved in transport of bulky (> 500 Da) substrates such as cofactors [like ATP, ADP, NAD(P)] and acyl-CoAs. Additionally, glycosomal membrane preparations contain channel-forming activities suggesting the existence of at least three distinct channels with low substrate selectivity for non-bulky molecules such as glycolytic intermediates and ions. The ratio of selectivity for anions/cations (P+/–) is different for the distinct channels. Abbreviations: GAT, glycosomal ABC transporter, MCF, mitochondrial carrier family, NBD, nucleotide-binding domain.

4.3. Glycosomal proteins as drug targets

Many glycosomal enzymes have been proposed as drug targets in *T. brucei*, but also in the other human pathogenic trypanosomatids, *T. cruzi* and *Leishmania* spp. Recently we prepared an exhaustive inventory of all validated and potential glycosomal drug targets, and the state of the art in the development of inhibitors as candidates for future anti-parasite chemotherapy. However, this will not be further discussed here, in this General Discussion, because this survey and a detailed discussion of the findings and perspectives have been presented as Chapter 3 of this thesis that will also be published as a chapter in a book about 'Drug discovery for trypanosomatid diseases'.

4.4. How drugs can reach their targets in the glycosome; their import into trypanosomes and import into glycosomes

An important aspect in targeting glycolytic and other enzymes compartmentalized in the glycosomes of trypanosomatids is the fact that the potential inhibitors have to enter the organelles. This is an aspect that should be seriously be taken into account when designing potential drugs by structure-based approaches an/or the selection of inhibitors by high-throughput screening of compound libraries with purified glycosomal enzymes, followed by medicinal chemistry approaches for the optimization of initial leads. The work presented in this thesis necessitates to reconsider the criteria for designing/selecting such inhibitors as drugs. One could consider several possibilities by which these compounds could cross the glycosomal membrane: (1) by using existing transporters; (2) by passive diffusion through the lipid bilayer; (3) via the channels of which the identity was revealed by our work described in Chapter 6. The first option is not very likely. The number of different transporter molecules in the glycosomal membrane seems very limited. Although these transporters may have relatively broad substrate specificities, the structural diversity of inhibitors that may be transported is possibly very limited. The second option would possibly only be feasible for non-polar compounds; such compounds, like those obeying the 'Lipinski rule of five' for good solubility and permeability properties as found for many drugs (Lipinski *et al.*, 2001), may enter via this route. However such properties may be difficult to combine with a high affinity of the inhibitors for their target enzymes. Most of the glycosomal enzymes catalyse reactions with negatively charged substrates and therefore have several positively charged residues in their active sites. Moreover, recent analyses suggest that even most known hydrophobic drugs don't reach their intracellular targets by passively permeating through the lipid-bilayer membranes of cells, but are internalized by transporters (Dobson *et al*., 2008; Dobson *et al.*, 2009; Lanthaler *et al*., 2011; Kell *et al.*, 2011). Also the recent finding that almost all known trypanocidal compounds seem to enter the parasites via transporters and not passively is noteworthy in this respect (Schuman-Burkard *et al*., 2011; Alsford *et al*., 2012). Therefore, as a third option, our finding that the glycosomal

membrane possesses channel-forming activities may have to be considered seriously, *i.e.* that many inhibitors of glycosomal enzymes may have to enter the organelles through the channels. This may impose little selectivity on the structure and charge of the compounds, only on their size. Assuming that the pores in the glycosomal membrane form channels with similar diameters as those in peroxisomal membranes (Antonenkov and Hiltunen, 2012), it is expected that the molecular mass of the inhibitors should be limited to 400 - 500 Da. We thus assume that most, if not all currently available inhibitors of purified glycosomal enzymes with trypanocidal activity and which have been shown to most likely kill the cells indeed via these targets *in situ*, will cross the glycosomal membrane via these channels. If indeed the channels play an important role in the targeting of glycosomal enzymes, the pore size sets a limit on the size of inhibitors to be developed in the future.

Of course, one should also take into account that drugs for glycosomal enzymes, like drugs for enzymes in other compartments of the cell, have to cross other membranes: the trypanosome's plasma membrane, for oral drugs against stage 1 sleeping sickness also the intestine of the host, and for stage 2 of the disease the blood-brain barrier (Fig. 61). Some people may conclude from this that 'Lipinski rule' compliant compounds will be optimal, because that would allow them to cross all successive membranes. However, recent measurements indicate that, contrary to general ideas, such compounds permeate very slowly through lipid membranes and, when acting as drugs, usually enter cells via transporters (Lanthaler *et al.*, 2011; Kell *et al.*, 2011). Alternatively, one could consider compounds which combine properties allowing their translocation through glycosomal channels with other properties allowing their passage over the plasma membrane such as 'Lipinski properties', or with affinity for plasma-membrane transporters or receptors that are endocytosed, or as compounds that enter passively as neutral pro-drugs of which the masked active-charged group will be unmasked by cytosolic enzymes. As mentioned, our recent identification of glycosomal channels, as well as recent findings by others that most drugs, both for trypanosomatid infections and for other, non-infectious diseases enter cells via transporters and receptors, call for a reconsideration of drug-design and targeting strategies.

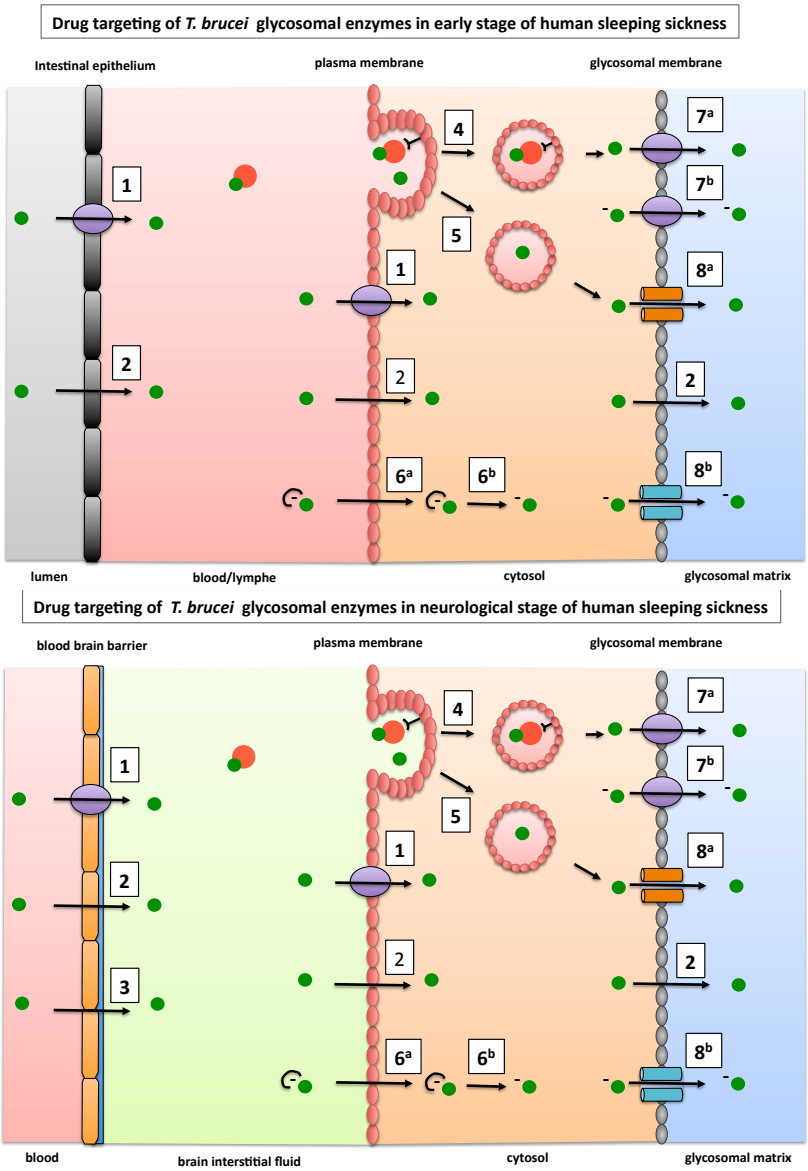

Figure 61. ***Diagrams depicting how drugs for human sleeping sickness may reach their targets inside the glycosomes***. Upper panel: drugs for stage 1 of the disease should preferably be orally administered, thus to be able to cross the intestinal epithelium. Lower panel: drugs for stage 2, when trypanosomes are present also in the interstitial fluid of the brain, should also be capable of crossing the blood-brain barrier (BBB). Green dots represent the drugs. Indicated are the different means by which the drugs may cross successive membranes. (1) By using transporter systems in the intestinal epithelium, the BBB and the plasma membrane of the trypanosome; (2) by passive diffusion through each of the successive membranes; (3) via the tight junctions between cells of the BBB; (4) in association with blood proteins (red dots) via receptor-mediated endocytosis through the flagellar pocket and the endosomal system of the trypanosome; (5) by fluid-phase endocytosis through the flagellar pocket; (6) by passive diffusion of pro-drugs (a) of which the charge is unmasked in the cytosol leading to activation of the drug (b); (7) by translocation of neutral (a) or charged (b) drugs via ABC or MCF transporters in the glycosomal membrane; (8) by translocation of neutral (a) or charged (b) drugs via one of the channels in the glycosomal membrane.

5. The origin and evolution of peroxisome and glycosomes

The origin and evolution of glycosomes has been constantly a topic of interest throughout the years of research described in this thesis. Although it has not been the central topic of our research, it has always played some guiding role 'in the background' because of the importance of comparing glycosome biogenesis and function with the homologous processes of peroxisomes. Moreover, the topic is of utmost interest for our long-term objective of selecting drug targets in the parasites and ultimately of developing drugs against sleeping sickness and diseases caused by related parasites. However, no further discussion of the origin and evolution of the organelles will be presented in this General Discussion, because the topic has been extensively dealt with in Chapter 2.

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Curriculum vitae

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Personal Information

Nationality	Venezuelan
Passport number	1440639
Date of Birth	05/05/1981
Place of Birth	Maracay, Venezuela

Education

1998	High School graduate (Sciences). Educational Unit School "La Concepción", Maracay (Venezuela).
2005	Bachelor degree in Biology, Faculty of Sciences, "Universidad de los Andes" (ULA), Mérida (Venezuela). Undergraduate thesis: "Cloning and expression of the gene that encode the hexokinase in *Leishmania mexicana.* Application of methodologies in molecular biology and biochemistry".
2007	Master degree in Cellular Biology, "Universidad de los Andes" (ULA), Mérida (Venezuela). Master thesis: "Contribution of the interaction of Enolase-Plasminogen to the infection of *Leishmania mexicana*. Application of methodologies in immunology, biochemistry and parasitology".
Currently (since February 2007)	PhD student at the "Université catholique de Louvain", Graduate School of Biochemistry, Cell Biology and Microbiology, Brussels (Belgium). PhD research topic: "Study of aspects of the molecular mechanism involved in the recycling of matrix-protein receptor PEX5: a late step of glycosome biogenesis in *Trypanosoma brucei*". Supervisor: Professor Paul Michels.

Language knowledge

Spanish:	mother tongue
English:	understanding and speaking: very good; writing: good
French:	understanding and speaking: very good; writing: good

Work experience

Research assistant, ULA. Mérida. March 1999 - February 2000.

Teacher assistant in the vegetal biology practical course, ULA. Mérida. March 2000 - March 2003.

Teacher assistant in the genetic II practical course, ULA. Mérida. March 2004 - February 2005.

Researcher in molecular biology and biochemistry of *Leishmania mexicana.* Laboratory of parasites enzymology, ULA. Mérida. January-December 2006.

Participation in Courses and Congresses

Workshop in Sequence analysis methods. CecalCULA. Mérida, Venezuela. September 2003.

Congress "New Frontiers of Bioinformatics in Latin America" BIOLAC/CecalCULA. Mérida, Venezuela. April 2003.

1st meeting "Thesis and final work-studies". Faculty of sciences. "Universidad de los Andes". Mérida, Venezuela. March 2004.

10th National meeting of scientific research. Faculty of sciences. Univerisity of Zulia. Maracaibo, Venezuela. October 2004. *(Poster presentation).*

LIV Annual convention AsoVac. University of Carabobo. Valencia, Venezuela. November 2004. *(Poster presentation).*

Workshop in glycobiology of parasites. "Universidad de los Andes", Mérida, Venezuela. September 2005.

Theoretical-practical course on protein crystallization. IDEA. Caracas, Venezuela. November 2005.

39th, 40th, 41st, 42nd and 43rd International Microsymposia on "Glycosomes, glycolytic enzymes and development of antiparasite chemotherapy". de Duve Institute. Brussels, Belgium. October 2007, September 2008, October 2009, October 2010 and October 2011. *(Oral presentations in 2007, 2008, 2010 and 2011).*

Workshop "ÄKTA user day". GE Healthcare. Eindhoven, The Netherlands. May 2008.

Workshop "Recombinant proteins". GE Healthcare. Paris, France. June 2008.

IAP Meeting on "Molecular dialogue between parasite and hosts: the trypanosome model". "Université Libre de Bruxelles". Brussels, Belgium. September 2008.

Scientific meeting "Organelle biogenesis and partitioning during the cell cycle". "Université Notre-Dame de la Paix". Namur. Belgium. October 2008.

Annual scientific meeting of the Belgian Society of Protozoology. de Duve Institute. Brussels, Belgium. March 2009. *(Oral presentation).*

The EMBO meeting 2009. RAI Center. Amsterdam, The Netherlands. August 2009.

IAP Meeting "Molecular dialogue between parasite and hosts: the trypanosome model" 2009. Prince Leopold Institute for Tropical Medicine. Antwerp. Belgium. *(Oral presentation).*

"International Meeting on Peroxisome Research". Systems Biology Institute. Seattle, USA. November 2009. *(Poster presentation).*

International meeting on "Confronting Neglected Protozoan Diseases". Pasteur Institute, Paris, France. September 2010.

Open European Peroxisome Meeting. Lunteren, The Netherlands. October 2010. *(Oral presentation).*

International meeting on "Molecular Dialogue in Host-Parasite Interaction". Royal Academy of Belgium. Brussels, Belgium. November 2010. *(Poster presentation).*

International summer course on "Biology of Parasitism, Modern Approaches". Marine Biological Laboratory. Woods Hole, Massachusetts, USA. June 17th - August, 6th 2011.

British Society for Parasitology, "Spring Meeting and Trypanosomiasis & Leishmaniasis Seminar, Celebrating 50 years in parasitology", University of Strathclyde, Glasgow, Scotland. April 2nd – 5th, 2012. (*Poster presentation*).

Academic Recognitions

Academic recognition. Faculty of sciences, Department of Biology ("Universidad de los Andes").
Periods A-2001, A-2002, A-2003.

Order "Luís María Ribas Dávila" - Highest academic achievement. Faculty of sciences, Biology school ("Universidad de los Andes"). November 2002.

Award "Bourse the Patrimoine" from "Le Secteur des Sciences de la Santé, Université catholique de Louvain", December 1st, 2011.

Award of a travel grant from the Belgian Society of Protozoology, to participate in the "Spring Meeting 2012 of the British Society for Parasitology / Trypanosomiasis & Leishmaniasis Seminar, Celebrating 50 years in parasitology", Glasgow, Scotland. April 2nd – 5th, 2012.

Publications

1. Full publications:

Pabón, M.A., Cáceres, A.J., Gualdrón M., Quiñones, W., Avilán, L., Concepción, J.L. (2007).

Purification and characterization of hexokinase of *Leishmania mexicana*.
Parasitology Research. 100:803-810.

Cáceres, A.J., Quiñones, W., Gualdrón, M., Cordeiro, A., Avilán L., Michels, P.A.M., Concepción, J.L. (2007).
Molecular and biochemical characterization of novel glucokinases from *Trypanosoma cruzi* and *Leishmania* spp.
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Trypanosoma brucei oleate desaturase. Its genetic and chemical evaluation as a candidate drug target.
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Duszenko, M., Ginger, M.L., Brennand, A., Gualdrón-López, M., Colombo, M.I., Coombs, G.H., Coppens, I., Jayabalasingham, B., Langsley, G. Lisboa de Castro, S., Menna-Barreto, R., Mottram, J.C., Navarro, M., Rigden, D.J., Romano, P.S., Stoka, V., Turk B., Michels, P.A.M. (2011).
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Autophagy in parasitic protists: Unique features and drug targets.
Molecular and Biochemical Parasitology. 177:83–99 (solicited review).
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Avilán, L.,* Gualdrón-López, M.,* Quiñones,W., González-González, L., Hannaert, V., Michels, P.A.M., Concepción, J.L. (2011).
Enolase, a key player in the metabolism and a probable virulence factor of trypanosomatid parasites- Perspectives for its use as a therapeutic target.
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Stearoyl-CoA desaturase is an essential enzyme for the parasitic protist *Trypanosoma brucei*.
Biochemical and Biophysical Research Communications. 412:286-290.

Gualdrón-López, M, Brennand, A, Hannaert, V., Quiñones, W., Cáceres, A.J., Bringaud, F., Concepción, J.L., Michels, P.A.M. (2012).
When, how and why glycolysis became compartmentalized in the Kinetoplastea. A new look at an ancient organelle.
International Journal of Parasitology. 42:1-20 (solicited review).

Gualdrón-López, M., Vapola, M.H., Miinalainen, I.J., Hiltunen, J.K, Michels, P.A.M., Antonenkov, V.D. (2012).

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The function of glycosomes in the metabolism of trypanosomatid parasites and the promise of glycosomal proteins as drug targets. In: Drug discovery for trypanosomatid diseases (Jäger, T., Koch, O., Flohé, L., eds.). Wiley-Blackwell. Accepted for publication.

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Translocation of solutes and proteins across the glycosomal membrane of trypanosomes; possibilities and limitations for targeting with trypanocidal drugs.
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2. Abstracts:

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Gualdrón-López, M., Michels, P.A.M. The mystery of the different forms of *Trypanosoma brucei* PEX5, the receptor for glycosomal matrix-protein import. Open peroxisome European meeting. Lunteren, The Netherlands. 21-22/10/2010.

Michels, P., Hannaert, V., Gualdrón-Lopez, M., Brennand, A., Mazet, M., Gupta, S., Chevalier, N. Glycosomes of trypanosomatid parasites as drug target. Open European Peroxisome Meeting. Lunteren, The Netherlands. 21-22/10/2010.

Gualdrón-López, M., Hannaert, V., Brennand, A., Mazet, M., Gupta, S., Chevalier, N., Opperdoes, F.R., Michels, P.A.M. Glycosomes of trypanosomatid parasites; biogenesis

and function offer perspectives for drug development against African sleeping sickness. International meeting on 'Molecular host-pathogen interaction'. Royal Academy of Belgium. Brussels. 18-19/11/2010.

Michels, P.A.M., Gualdrón-López, M., Igoillo-Esteve, M., Mazet, M., Antonenkov, V. Solute transport across the glycosomal membrane. Possible targets for new trypanocidal drugs? COST CM0801 Annual Meeting 'New Drugs for Neglected Diseases'. Modena, Italy, 5-7/2011. Abstract book pp. 28-29.

Gualdrón-López, M., Chevalier, N., Michels, P. PEX4 and its role in glycosomal matrix protein import of *Trypanosoma brucei*. British Society for Parasitology, Spring Meeting and Trypanosomiasis & Leishmaniasis Seminar, Celebrating 50 years in parasitology, University of Strathclyde, Glasgow, Scotland. 2-5/4/2012. Abstract book no. P179, pp. 200-2001.

Gualdrón-López, M., Antonenkov, V., Michels, P. Channel-forming activities of glycosomal membrane proteins from *Trypanosoma brucei*. British Society for Parasitology, Spring Meeting and Trypanosomiasis & Leishmaniasis Seminar, Celebrating 50 years in parasitology, University of Strathclyde, Glasgow, Scotland. 2-5/4/2012. Abstract book no. OR19, p 34.