UNIVERSITE CATHOLIQUE DE LOUVAIN

Secteur des Sciences de la Santé

Faculté de Médecine

de DUVE INSTITUTE

Research Unit for Tropical Diseases

Characterization of proteins involved in turnover of glycosomes in *Trypanosoma brucei*

Thèse présentée en vue de l'obtention
du grade de Docteur en Sciences
Biomédicales et Pharmaceutiques

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Acknowledgements

I would like to start by thanking my PhD supervisor Paul Michels. I am very happy for have being your student and I have learned a lot. I could never thank you enough for all you did for me. Also to Fred Opperdoes, for being always friendly and helpfull, I am very proud to have been part of TROP. Many more thanks to Fraçoise Mylle, for being very friendly, competent, also for making us feel in a family at TROP. To Nathalie Chevalier, for the help in the laboratory and the patience to order materials whenever I needed. To Melisa Gualdron for being a great labmate, always having time to explain to me whatever question I had. To Veronique Hannaert, for the many rides and french translations. To Freddy Abbrasart, for the help around the lab, and the football discussions. Many thanks to all past labmates from TROP, specially to Shredhara Gupta, Muriel Mazet, Emilie Verplaetse, Nathalie Galland, Murielle Herman, Mariana Igoilo and many others. To Eva Rico, for being a friend in autophagy. To the « neighbours » from HORM, for sharing the floor, the drinks and the pHmeter. To Daniel Rigden, for the many collaborations in ATGs and PEXs. To Patrick Van Der Smissen from CELL and Pierre Courtoy, for the enthusiast help with the acquisition of the electron microscopy and confocal images. To my good friends in Brazil (specially for the ones who came visit! Each visit was a gift) and to the new friends I made in Belgium, life is a little less gray with you all. To Opa, Oma, Greet, Mike and the Bamps family, for making me fell like Gent is a little like home. To my parents, brothers, sister and my family in Brazil, for encouraging me to come to Belgium and for all the support during these five years of thesis. To Michel, for everything, specially for buying me cheetos. To my cats, for being so cute and cool.

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List of Abbreviations

ATG	autophagy(-related gene/protein)
ASCT	acetate:succinate CoA-transferase
BF	(long-slender) bloodstream form of *Trypanosoma brucei*
bp	basepairs
BSA	bovine serum albumine
CATT	card agglutination test for trypanosomiasis
cGAPDH	cytosolic glyceraldehyde-3-phosphate dehydrogenase
CHAPS	3-[(3-Cholamidopropyl)dimethylammonio]-1- propanesulfonate
CMA	chaperone-mediated autophagy
CVT	cytoplasm to vacuole targeting pathway
DAPI	4',6-diamidino-2-phenylindole
DTM	differentiating trypanosome medium
EGFP	enhanced green fluorescent protein
EGTA	ethylene glycol tetraacetic acid
ENO	enolase
ES	expression site
ESAG	expression site-associated gene
FCS	foetal calf serum
GFP	green fluorescent protein
HAT	human African trypanosomiasis
HXK	hexokinase
IPTG	isopropyl β-D-thiogalactanopyranoside
kDa	kilodalton
LS	long-slender form
PAGE	polyacrylamide gel electrophoresis
PARP	procyclic acidic repetitive protein
PBS	phosphate buffer saline
PE	phosphatidylethanolamine
PEX	peroxin
PF	procyclic form of *Trypanosoma brucei*
PFK	phosphofructokinase
PGK	phosphoglycerate kinase
PI3K	phosphatidylinositol 3 kinase
PI3P	phosphatidylinositol 3-phosphate
PMN	piecemeal microautophagy of the nucleus
PPDK	pyruvate phosphate dikinase
PTS	peroxisome targeting sequence
PX	Phox homology domain
PYK	pyruvate kinase
RNAi	RNA interference
rpm	rotations per minute
RT	room temperature
RT-PCR	reverse transcription PCR
SDS	sodium dodecyl sulfate
SIF	stumpy-inducing factor
SNX	sorting nexin
SS	short-stumpy form

Tet	tetracycline
TLF	trypanolytic factor
TPI	triosephosphate isomerase
UTR	untranslated region
VSG	variant surface glycoprotein
VPS	vacuolar protein sorting
ZFP3	zinc-finger protein 3
ZFK	zinc-finger kinase
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).
The classification and nomenclature for kinetoplastids is used as proposed by Moreira *et al*. (2004).

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Abstract

Sleeping sickness is an infectious disease affecting humans in sub-Saharan Africa. It is caused by the protist *Trypanosoma brucei* which is transmitted by blood-feeding tsetse flies. This parasite possesses many unique features, including the organisation of the major part of the glycolytic pathway in peroxisome-like organelles known as glycosomes. This compartmentalisation has been shown to be essential for the viability of the parasite. During its life cycle, *T. brucei* proceeds through several stages. In its mammalian host, the parasite presents itself as a bloodstream form that, for its ATP supply, is dependent on glucose from the host's blood and in the tsetse fly's midgut it occurs as the procyclic form. In this latter stage the parasite prefers the less available glucose, but can also feed, for its energy, on amino acids such as proline and glutamine. The enzyme composition of glycosomes from each of these two stages is associated to the parasite's metabolic needs. Bloodstream trypanosomes have 90% of the glycosomal enzyme content dedicated to glycolysis, while in glycosomes from procyclic cells this number drops to 40-50%, and the parasites develop a more diversified metabolism. Experiments performed in our laboratory have shown an increase in colocalisation of markers for glycosomes and the lysosome during the steps by which trypanosomes differentiate from the bloodstream to the procyclic form. This indicates that autophagy plays a role in the metabolic adaptation of the parasites during differentiation. It has been shown in yeast cells that peroxisomes reach a certain stage where they are no longer able to import matrix proteins from the cytosol. These organelles then, can be considered unable to adapt their enzyme content to changes in carbon source. We propose that a similar situation may happen in *T. brucei* where mature glycosomes that are rich in glycolytic enzymes and no longer able to import matrix proteins will be specifically degraded when the parasite differentiates into the procyclic form and requires the expression of a new set of glycosomal enzymes.

Our project had two objectives: the characterization of proteins that participate in the degradation as well as a protein implicated in the growth of glycosomes, being respectively, autophagy-related proteins (ATGs) involved in the organelle degradation and a peroxin (PEX), involved in the organelle's biogenesis, more specifically in matrix protein import.

Here we present the characterisation of a *T. brucei* protein whose yeast counterpart participates in specific autophagy, ATG24, also present in mammalian cells where it is known as SNX4. This protein is part of a putative complex that is involved in autophagy induction and the switch between general autophagy and specific autophagy, including pexophagy, mitophagy, the cytoplasm-to-vacuole pathway and piecemeal microautophagy of the nucleus. TbATG24 seems to be required for the maintenance of the lysosomal morphology in bloodstream and procyclic cells and also participates in endocytosis, as shown by the effect of its RNA interference-dependent partial depletion on transferrin import in a bloodstream-form cell line. This latter function is evolutionary conserved, as it is also found for its orthologues in mammalian cells and yeasts. Moreover the interaction of TbATG24 with endosomal membranes has been shown to be dependent on TbVPS34 and to be sensitive to wortmannin treatment, in agreement with observations reported for its yeast counterpart. Interestingly, its downregulation leads to an increase in autophagosome numbers in autophagy-inducing conditions and an increase of the rate of *in vitro* differentiation of monomorphic *T. brucei* cells.

Another ATG protein characterized in this study is ATG8, the autophagy marker. Using GFP-tagged constructs, we observed that two different isoforms of ATG8, TbATG8.1 and TbATG8.2, both locate to puncta under different autophagy-inducing conditions and therefore can both be considered as autophagosomal markers. Moreover both isoforms of the protein colocalise with the glycosomal marker triosephosphate isomerase during induction of *in vitro* differentiation, indicating that both are involved in pexophagy for glycosome turnover. Intriguingly, we observed a different rate of differentiation for cells expressing the GFP-tagged ATG8.2 or cells with GFP-ATG8.1.

The *T. brucei* glycosomal matrix-protein import machinery is similar to that described for peroxisomal protein import in yeasts, with some of the proteins conserved. So far about a dozen proteins that participate in glycosome biogenesis and matrix protein import (called peroxins or PEX proteins) have previously been characterised in our laboratory. Among the peroxins characterised is TbPEX13, a transmembrane component of a complex on which dock TbPEX5 and TbPEX7, cytosolic receptors for proteins with a peroxisomal-targeting signal (PTS). In addition to binding the receptors, TbPEX13 interacts also with TbPEX14, another component of the docking complex. TbPEX13 has been shown to be essential for the parasites and to be involved in the import of PTS-containing proteins. Despite its conserved role and some structural features in common with PEX13s from other organisms, this protein also lacks important PEX13 signatures and has other remarkable features.

We describe the identification and characterisation of a second TbPEX13, named TbPEX13.2, that also lacks some PEX13 traits, such as a SH3 domain, but in other respects is more similar to the PEX13 of other organisms than the previously found TbPEX13 (now called TbPEX13.1). We show that this second TbPEX13 is associated with glycosomes, where it interacts with PEX13.1 and other peroxins. It is essential for bloodstream-form cells and participates in the import of PTS proteins. Moreover, as seen for TbPEX13.1, this second isoform is also involved in the association of PEX14 to the glycosomal membrane. Trypanosomatids are the first organisms where two distinct isoforms of PEX13 have been described.

Résumé

La maladie du sommeil est une maladie infectieuse qui affecte l'homme en Afrique subsaharienne. Elle est causée par le protiste *Trypanosoma brucei* qui est transmis par la mouche tsé-tsé lors d'un repas sanguin. Ce parasite possède de nombreuses caractéristiques uniques, y compris l'organisation d'une grande partie de sa voie glycolytique dans des organites de la famille des peroxisomes, appelées glycosomes. Il a été démontré que cette compartimentation est essentielle pour la viabilité du parasite. Au cours de son cycle de vie, *T. brucei* passe par plusieurs stades. Chez l'hôte mammifère, il est sous la forme sanguicole qui, pour son approvisionnement en ATP, est dépendante du glucose présent dans le sang de l'hôte. Dans l'intestin de la mouche tsé-tsé, il est présent sous la forme procyclique. A ce stade, le parasite préfère le glucose même s'il est peu disponible, mais peut également utiliser, pour produire son énergie, des acides aminés comme la proline et la glutamine. La composition enzymatique des glycosomes dans ces deux stades est liée aux besoins énergétiques du parasite. Dans la forme sanguicole des trypanosomes, 90% du contenu enzymatique est dédié à la glycolyse ; tandis que dans les glycosomes des cellules procycliques, cette proportion tombe à 40-50% et le parasite développe un métabolisme plus diversifié. Des expériences réalisées dans notre laboratoire ont montré une augmentation de la co-localisation des marqueurs des glycosomes et des lysosomes durant les étapes de différenciation de la forme sanguicole à la forme procyclique. Ceci indique que l'autophagie joue un rôle dans l'adaptation métabolique de ces parasites durant la différenciation. Dans les cellules de levure, il a été montré que les peroxysomes atteignent un certain stade au delà duquel ils ne sont plus capables d'importer des protéines matricielles en provenance du cytosol. Ces organites peuvent alors être considérées comme incapables d'adapter leur contenu enzymatique aux changements de source de carbone. Nous proposons qu'une situation similaire pourrait exister chez *T. brucei* où les glycosomes matures, qui sont riches en enzymes glycolytiques et incapables d'importer des protéines matricielles, seraient dégradés spécifiquement quand le parasite se différencie en forme procyclique et nécessite l'expression d'un nouveau groupe d'enzymes glycosomales.

Notre projet avait deux objectifs: la caractérisation de protéines qui participent à la dégradation ainsi qu'une protéine impliquée dans la croissance des glycosomes, soit respectivement les protéines liées à l'autophagie ("autophagy-related proteins" ou ATGs) impliquées dans la dégradation des organites et une peroxine (PEX), impliquée dans la biogenèse des organites et plus particulièrement dans l'importation des protéines matricielles. Nous présentons ici la caractérisation d'une protéine de *T. brucei* dont l'équivalent chez la levure participe à l'autophagie spécifique, ATG24, également présente dans les cellules mammifères où elle est connue comme la SNX4. Cette protéine fait partie d'un probable complexe impliqué dans l'induction de l'autophagie et le changement entre l'autophagie générale et spécifique, incluant la pexophagie, la mitophagie, la voie cytoplasme-vacuole et la microautophagie du noyau. TbATG24 semble être nécessaire pour le maintien de la morphologie des lysosomes dans les cellules sanguicoles et procycliques et participe également à l'endocytose, comme montré par l'effet de son élimination partielle par interférence à l'ARN sur l'importation de la transferrine dans une lignée cellulaire de cellules

sanguicoles. Cette dernière fonction est conservée au cours de l'évolution, comme c'est également le cas pour ses orthologues dans les cellules mammifères et les levures. De plus, il a été montré que l'interaction de TbATG24 avec les membranes des endosomes est dépendante de TbVPS34 et est sensible au traitement par la wortmannin, en accord avec les observations rapportées pour l'équivalent chez la levure. Il est intéressant de noter que cette dérégulation conduit à une augmentation du nombre d'autophagosomes en condition d'induction de l'autophagie et à une augmentation de la vitesse de la différenciation *in vitro* des celles monomorphiques de *T. brucei*.

Une autre protéine ATG caractérisée dans cette étude est l'ATG8, le marqueur d'autophagie. En utilisant des constructions marquées par la GFP, nous avons observé que les deux isoformes d'ATG8, TbATG8.1 et TbATG8.2, sont localisées de manière ponctuelle sous différentes conditions d'induction de l'autophagie et peuvent dès lors être considérées comme des marqueurs d'autophagosomes. De plus, les deux isoformes de la protéine sont co-localisées avec le marqueur glycosomal, la triosephosphate isomérase, durant l'induction de la différenciation *in vitro*, indiquant que toutes les deux sont impliquées dans la pexophagie pour le renouvellement des glycosomes. Etonnement, nous avons observé une vitesse de différenciation différente pour les cellules exprimant l'ATG8.2 marquée à la GFP ou pour les cellules avec la GFP-ATG8.1.

La machinerie d'importation des protéines matricielles des glycosomes est semblable à celle décrite pour l'importation des protéines peroxisomales chez les levures et certaines protéines sont conservées. Jusqu'à présent, une douzaine de protéines participant à la biogenèse des glycosomes et à l'importation des protéines matricielles (appelées peroxines ou protéines PEX) ont été caractérisées dans notre laboratoire. Parmi ces peroxines caractérisées, TbPEX13, est un composant transmembranaire du complexe sur lequel viennent se fixer TbPEX5 et TbPEX7, deux récepteurs cytosoliques pour les protéines possédant un signal de ciblage vers les peroxisomes (PTS). En plus de lier les récepteurs, TbPEX13 interagit aussi avec TbPEX14, un autre composant du complexe d'amarrage. Il a été montré que TbPEX13 est essentielle pour les parasites et est impliquée dans l'importation des protéines porteuses d'un signal PTS. Malgré son rôle conservé et certaines caractéristiques structurales en commun avec les PEX13 d'autres organismes, cette protéine manque certaines signatures importantes des PEX13 et possède d'autres caractéristiques remarquables.

Nous décrivons l'identification et la caractérisation d'une seconde TbPEX13, appelée TbPEX13.2, à qui il manque certains traits des PEX13, comme le domaine SH3, mais qui par d'autres aspects est plus similaire aux PEX13 d'autres organismes que la PEX13 trouvée précédemment (appelée maintenant TbPEX13.1). Nous montrons que cette seconde TbPEX13 est associée aux glycosomes, où elle interagit avec PEX13.1 et d'autres peroxines. Elle est essentielle pour les cellules de la forme sanguicole et participe dans l'importation des protéines avec un PTS. De plus, comme montré pour TbPEX13.1, cette seconde isoforme est aussi impliquée dans l'association de PEX14 avec la membrane glycosomale. Les trypanosomatidés sont les premiers organismes où deux isoformes distinctes de PEX13 ont été décrites.

Chapter 1
Introduction

1.1 *Trypanosoma brucei* and sleeping sickness

Trypanosoma brucei is the parasitic protist responsible for sleeping sickness or Human African Trypanosomiasis (HAT) and a similar disease called 'nagana' in animals in sub-Saharan Africa. The parasite belongs to the clade Kinetoplastea, order Trypanosomatida (Moreira *et al*., 2004), along with the Chagas disease causing agent, *Trypanosoma cruzi*, and *Leishmania* spp., responsible for a group of diseases collectively known as leishmaniases. *T. cruzi* is found in Central and South America where it causes a chronic disease characterised by severe hypertrophy of the heart (megacardio) and/or parts of the digestive system (megaesophagus, megacolon) known as American trypanosomiasis. The parasite is transmitted between humans and other vertebrates by triatomine insects known as 'kissing bugs' because they often bite the face of people during their sleep. *Leishmania* species can affect the skin (causing a cutaneous form of the disease), skin and mucosa (mucocutaneous form) or internal organs (visceral form, also known as 'kala azar') and are found in tropical and subtropical regions. *Leishmania* parasites are transmitted between vertebrates by female sandflies.

T. brucei is a flagelated organism (Figure 1a) that cycles between the mammalian host, where it is found extracellularly in the bloodstream and lymph, and the tsetse (*Glossina* spp.) fly's digestive system (Figure 1b). It is transmitted between mammal and fly when the latter takes a bloodmeal. The parasite has a single elongated mitochondrion with a special small round compartment called kinetoplast that contains the mitochondrial or kinetoplast DNA. This kinetoplast DNA or kDNA, characteristic for all organisms of the Kinetoplastea clade, has a unique complex structure and mode of expression involving RNA editing. Another characteristic of trypanosomes is that all its endo- and exocytosis occurs through a specialized part of the surface, the so-called flagellar pocket at the base of the single flagellum, that is attached to the cell body.

A) B)

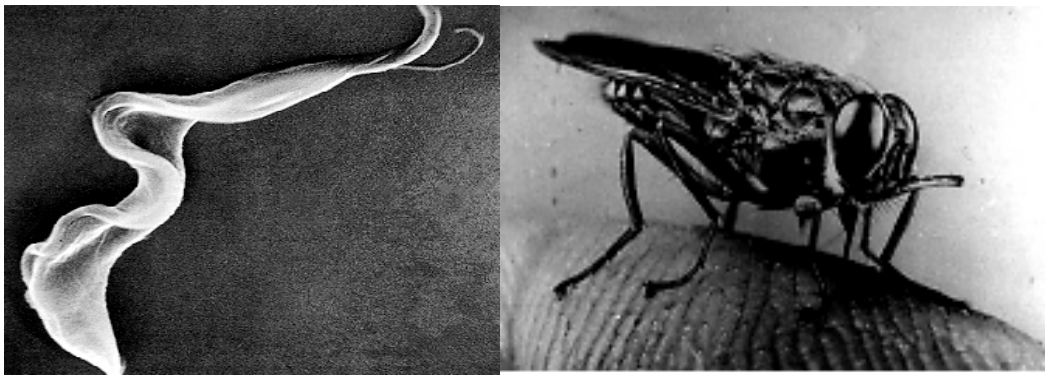

Figure 1: A) Scanning electron micrograph of a *T. brucei* bloodstream form parasite. B) Picture of a tsetse fly, the vector of sleeping sickness.

Two subspecies of *T. brucei* affect humans, *T. b. gambiense* and *T. b. rhodesiense*. The first one is mainly present in the western and central regions of Africa. It causes a chronic form of the disease and is responsible for the vast majority of sleeping sickness cases. The second subspecies is found in the eastern region of the continent and often causes a more acute form of the disease that represents less than 5% of the cases. Another subspecies, *T. b. brucei,* essentially identical to *T. b. rhodesiense*, is infective for domestic livestock but cannot infect humans and for this reason is preferably used in research.

The disease is characterised by two stages. The first stage (haemolymphatic) presents itself with nonspecific symptoms, including fever, fatigue, prostration, anemia, weakness, headache, cardiac disfunction, splenomegalia and swollen cervical lymph nodes (known as Winterbottom's sign in *T. b. gambiense* infections). It can take a few weeks to many months to appear. The disease develops to its second stage when the parasites reach the central nervous system by crossing the blood-brain barrier by an as yet poorly understood mechanism (Grab *et al*., 2009). This second stage of the disease is characterised by a vast number of neurological symptoms including a change in the sleep-wake cycle that gives the name to the disease, mood-swings, confusion, followed by coma and ultimately death for all untreated cases. Sleeping sickness was until recently found in 36 countries, putting 60 million people at risk of developing the disease

in an area larger than the United States (WHO, http://www.who.int/trypanosomiasis_african/en/). For the first time in 50 years, the number of new cases reported in 2009 and 2010 has dropped to less than 10,000 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). It is estimated that currently there are some 30,000 infected people and that 70% of the cases are found in the Democratic Republic of Congo (Figure 2).

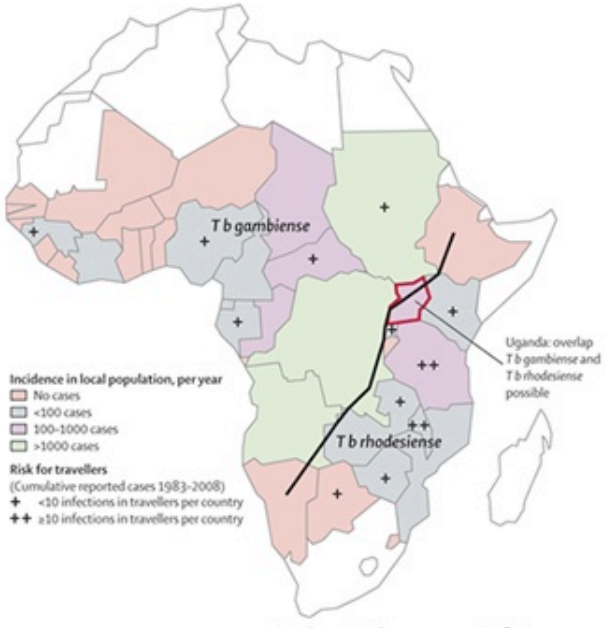

Figure 2 : Map of Africa indicating the distribution of sleeping sickness as of 2008. Taken from http://medicine.yale.edu/ysph/emd/research/tsetse/index.aspx.

The tsetse fly feeds during the day and is only found in rural areas. It is often able to feed through one's clothes and its bite can be painful. Nagana, the animal form of the disease, can affect cattle, horses, pigs, dogs, goats and sheep. It is caused by *T. congolense, T. vivax* and different subspecies of *T. brucei.* Its economic impact renders most of the region where the fly is present unable to be used for raising livestock. Since wild animals can also be infected by the two subspecies that cause HAT, these are considered important reservoirs for the

human disease, especially for *T. b. rhodesiense*, the most aggressive form. The constant presence of reservoirs associated to the vast region where the fly can be found affects severely the chances for controlling the disease.

1.2 Diagnosis and treatment

Diagnosis of sleeping sickness can be done by microscopic examination of bloodsmears. This technique is cheap and can be very specific but somewhat subjective, its sensitivity depends on the person performing the observation. Moreover *T. b. gambiense* can have very low levels of parasites in circulation, so methods to concentrate the samples might be needed. Serological methods such as CATT (card agglutination test for trypanosomiasis) and LATEX-based agglutination assays are only available for *T. b. gambiense*. Since the treatment for the second stage of the disease requires toxic drugs that can cross the blood-brain barrier, it is also essential to identify the stage of the disease. This requires a lumbar puncture and examination of the cerebro-spinal fluid for the presence of the parasite (Kennedy, 2008).

Sleeping sickness treatment is dependent on the subspecies and stage of the disease (Table 1). The main treatment options in use today are intravenous injection of pentamidine (introduced in 1940) and suramin (1920) for the first-stage of the disease caused by *T. b. gambiense* and *T. b. rhodesiense*, respectively. As mentioned before, treatment of second-stage sleeping sickness requires the use of toxic drugs that are able to pass the blood-brain barrier. Currently melarsoprol (1949) is used for both subspecies and eflornithine (also known as DMFO) (1990), is used for second stage *T. b. gambiense* infections. Development of resistance has created the need for combined therapy. One such option currently in use is the combination of nifurtimox and eflornithine (NECT or nifurtimox eflornithine combined therapy), that allows for half of the dose and half of the treatment length of the expensive eflornithine when combined with 10 days of oral treatment with nifurtimox. Along with the increasing development of resistance, the toxicity and the difficulty to administer the drugs are reasons why the search for new drugs has to be continued.

Species	stage	drug	year	drawbacks
T. b. gambiense	First	Pentamidine	1940	Kidney, heart, liver, neurologic side-effects
T. b. gambiense	second	Eflornithine*	1990	Difficult/expensive treatment, hair loss, hearing loss
T. b. rhodesiense	First	Suramin	1920	Causes urticarial rash, adrenal cortical damage
T. b. rhodesiense; T. b. gambiense	second	Melarsoprol	1949	Causes reactive ansenical encefalopathy, convulsion, loss of consciousness, high failure rate

Table 1: Table of drugs currently used as treatment for each stage of sleeping sickness. Eflornithine*: also used as a combination with oral nifurtimox (see section 1.2).

1.3 The life cycle of *T. brucei*

The *T. brucei* life-cycle (Figure 3) involves the sequential infection of mammals and tsetse flies (here described as observed for the *T. brucei brucei* infection of *Glossina morsitans morsitans* teneral flies). When a fly feeds on blood from an infected person or animal, it will take up the trypanosomes, present in the blood, as well. These trypanosomes occur in two different developmental forms: proliferating trypanosomes with a long, slender form and non-proliferating short-stumpy forms. The vast majority of these cells will die, only some short-stumpy cells will survive and differentiate into procyclic forms once in the fly's midgut. These procyclic forms multiply and after establishment of the infection in the fly's midgut, the cells start to migrate to the salivary glands while they differentiate successively into other developmental stages. The procyclics differentiate into long epimastigotes; these cells reach the foregut and proboscis and differentiate into an asymmetric dividing epimastigote. This is a very mobile

stage, that migrates from the tip of the proboscis via the hypopharynx where the entry into the salivary glands is located. This cell will divide into the long and short epimastigotes. Whereas the long epimastigotes die, the short epimastigotes attach to the salivary gland's wall, multiply and differentiate into the infective metacyclic tripomastigote stage, that will be transmitted to a new mammalian host during a next bloodmeal (Van Den Abbeele *et al*., 1999; personal communication).

In the mammalian bloodstream the metacyclics differentiate into long-slender bloodstream forms that multiply. At a certain point, dependent on the parasites' density but for which the extracellular molecular trigger ('stumpy induction factor' or SIF) has not been elucidated although it seems to involve cAMP sensing, some long-slender cells will differentiate, via intermediate forms, into short-stumpy cells that are unable to multiply. The transformation into non-proliferating short-stumpy forms appears to serve two purposes: (1) to control the population of the parasite in the bloodstream of the host and (2) to pre-adapt the cells for the fly; these transformed cells have been reprogrammed metabolically in a way that allows them to survive in the midgut of the tsetse fly before differentiation to procyclics. In contrast, the long-slender forms, also ingested by the fly during a bloodmeal, will die in the midgut.

It takes 12 to 20 days after a bloodmeal for the parasites to reach the salivary glands (Van Den Abbeele *et al*., 1999). At this point the fly will be able to transmit the trypanosomes at every feed, which occurs two to three times a week. Interestingly it has been shown that the presence of the parasites in the midgut affects the feeding behaviour of the flies. It causes them to take longer time for feeding, thus increasing the chances of transmission.

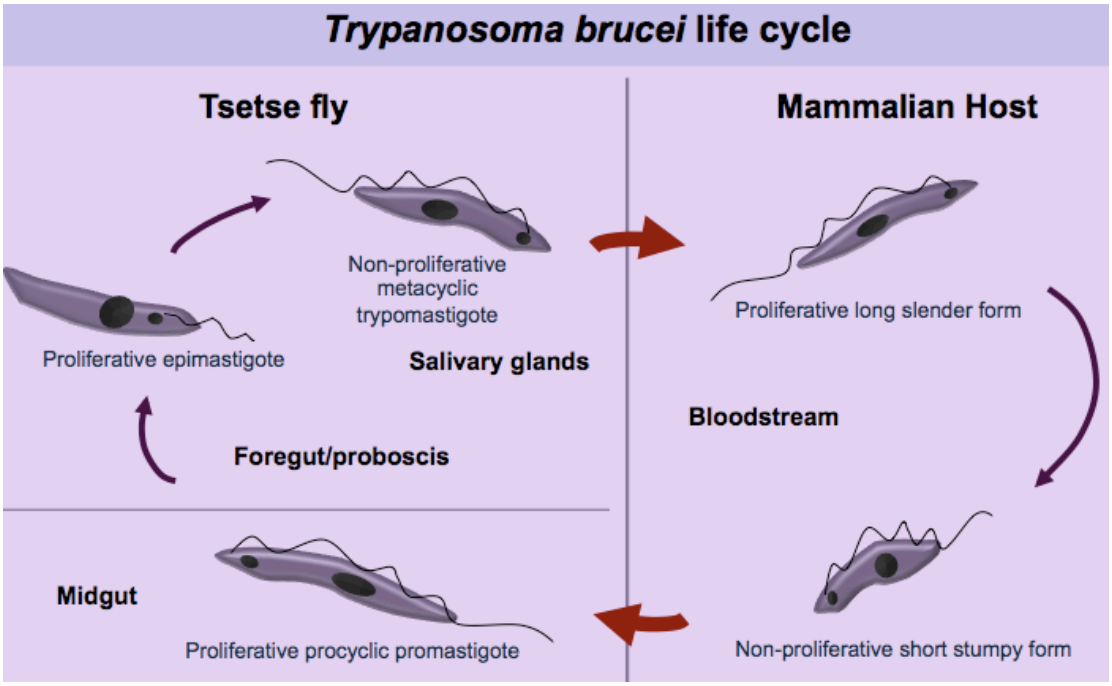

Figure 3: Scheme of the life-cycle of *T. brucei*.

1.4 Unique features of trypanosomes

Trypanosomatids are relatively well-studied organisms. A major reason for that is the fact that they are the causative agents of serious diseases affecting many people and forming a risk for a large part of the population in the tropical and subtropical areas of the world. Since these diseases were largely outside the interest of pharmaceutical industry, because the poverty of the people affected precluded financial returns of investments for drug development, research on the parasites was initiated in academic laboratories in the 1970s-1980s, encouraged by the WHO and with support of institutions such as the European Commission, the Wellcome Trust and the US National Institutes of Health. Moreover, these parasites have proved to be scientifically very interesting, because they posses many features that are unique to these organisms.

A first intriguing aspect concerns how the trypanosomes change regularly their surface coat. Upon infection this extracellular parasite uses a very sophisticated mechanism to evade the host immune system, known as antigenic variation. The bloodstream form parasite is covered by a uniform coat of densely packed molecules of a glycosylated protein known as Variant Surface

Glycoprotein (VSG). During an infection, each trypanosome expresses only a single VSG, and within the whole population only a very limited number of VSGs is expressed. By the time that the host cell is able to recognize these VSGs and attack the parasite cells, some trypanosomes will have switched to the expression of another VSG from the very large repertoire of hundreds of VSG genes. The majority of trypanosomes, which have not switched, will be destroyed by the host defence system, but the switched ones survive and form a new population. This process that is very debilitating for the host immune system, also in conjunction with the immune suppression exerted by the parasites, can continue for a long time and sustain the infection. Moreover there is a high frequency of recombination, continuously remodelling the VSG repertoire and producing entirely new mosaic variants to be expressed, that consequently contribute importantly to the antigenic variation. The possibility to express such a huge –in principle even infinite– number of different VSGs also makes the development of vaccines extremely difficult.

The high level of expression of the VSG genes, as well as the fact that only one VSG gene should be expressed at the time in a cell to make the process of antigenic variation efficient, justify the special location of the expressed VSG genes in the chromosomes and in the nuclear organization. The trypanosome has a repertoire of hundreds of so-called basic copy (BC) VSG genes which are largely clustered in the genome. These genes are silent. The active VSG gene is found near the telomere of a chromosome, in a region known as expression site (ES). Next to each version of the expressed VSG, in the ES, there are genes known as ESAG (ES-associated genes) that are transcribed together with the VSG (Hertz-Fowler *et al*., 2008). These genes often code for proteins whose expression is required at high levels for the metabolism of the bloodstream form of the parasite. Among the 12 ESAGS known are ESAG6 and ESAG7 which together form a heterodimeric transferrin receptor. There are about 20 telomeric ESs in the trypanosome, but only one is active at a time. The switch to the expression of another VSG is done either by switching the transcription from one ES to another, that will have a different VSG or, more commonly, by translocation (by duplicative gene conversion) of one of the silent BC genes into the active ES. The active ES is also located in a special region of the nucleus, in a so-called ES-body,

while partially active ESs are located in the nucleolus and inactive ESs are excluded from these regions. Silencing of inactive ESs also involves telomere-binding proteins and chromatin-remodelling proteins (for a review see Navarro *et al.*, 2007).

Procyclic cells don't express a VSG, but other surface proteins, known as procyclins or PARPs (procyclic acidic repetitive protein). The trypanosomes have a very small repertoire of such proteins which are rich in Glu-Pro (EP procyclin) or Gly-Pro-Glu-Glu-Thr (GPEET procyclin) repetitions, that, like VSGs but different from all other protein-coding genes, have a dedicated promoter, used often in molecular biology for controlled expression of proteins of interest. The EP procyclins are expressed throughout the procyclic infection in the tsetse midgut while the GPEETs seem to be downregulated as the parasites start to differentiate in the midgut of the fly (Vassella *et al*., 2000). The procyclin genes are concentrated in chromosomes 6 and 10. Both the genes in the active ES during the bloodstream stage and the PARP genes in procyclic stage are transcribed by a RNA polymerase I, very unique for transcription of protein-coding genes.

Another interesting feature of trypanosomatids is that they have very little regulation of transcription initiation in the expression of their genes. The vast majority of the genes are transcribed, by RNA polymerase II, as polycistronic units and the resulting long precursor RNAs are then processed into the individual mRNAs. The stability and expression is dependent on their specific untranslated regions (UTRs) (reviewed by Haile and Papadopoulou, 2007). In recent years, it has become clear that several epigenetic mechanisms also play a major role in the expression of proteins in trypanosomes. Some of these mechanisms are similar or reminiscent to those found in other eukaryotes, but others are unique to trypanosomes. A kinetoplastid-specific mechanism is the DNA modification involving the modified base J, that initially was thought to plays an important role in antigenic variation, being one of the mechanisms involved in the silencing of inactive ESs. This unique base is enriched at the telomeres of chromosomes of trypanosomatids, but absent in the procyclic form of *T. brucei*. Its role is yet unclear, however in *Leishmania* non-telomeric J is

associated to transcription termination (for a review, see Borst and Sabatini, 2008).

1.5 Endocytosis in *T. brucei*

Another unique feature, related with antigenic variation, has been revealed recently. It has been shown that, when an antibody binds to the VSG, the movement of the parasite and the cell's membrane fluidity cause that the antibody gets carried into the flagellar pocket, that is located at the posterior end of the cell, and is endocytosed quickly (Engstler *et al*., 2007). While the antibody is degraded, the VSG is cycled back to the cell's surface. The bloodstream form parasite has a remarkable endocytosis rate that is 10 times higher than in procyclic cells, causing that the VSG coat can be fully replaced in just 12 minutes (Engstler *et al*., 2004). The rate of formation of clathrin-coated vesicles and vesicle traffic at this stage is thought to be the highest among eukaryotes (reviewed by Morgan *et al*., 2002).

From the flagellar pocket inwards, the distribution of organelles is quite polarized. Next to the flagellar pocket are early, late and recycling endosomal vesicles, the Golgi system, lysosome and endoplasmic reticulum (ER) which are found organized in an area between the flagellar pocket and the nucleus that occupies 30 to 40% of the cell volume (Figure 4). This proximity between the different compartments seems to favour a quick interaction, required to maintain the high levels of vesicle transport in the cell.

Interestingly in short-stumpy cells the flagellar pocket becomes enlarged, indicating an increase in the rate of endocytosis at this stage. There are many proteins that are present at the flagellar pocket or endosomal system in *T. brucei* but absent in higher eukaryotes. Clathrin organisation seems similar to that in higher eukaryotes, however there is no homologue for Adaptin 2, a protein that participates in clathrin-mediated endocytosis, indicating the possibly of a somewhat unusual uptake mechanism in the parasite.

Curiously, endocytosis participates in human resistance to infection by parasites of the subspecies *T. b. brucei*. This subspecies takes up a so-called trypanolytic factor (TLF) from the host blood that causes its death after just a

few hours. The trypanolytic factor 1 is a component of HDL identified as apolipoprotein L1 (apoL1), a human specific serum protein. HDL particles also contain another human-specific protein termed 'haptoglobin-related protein' (Hpr) that has no known role. Following the binding of haemoglobin (Hb) to Hpr, the apoL1-bearing HDL particles bind to a membrane receptor for the Hp-Hb complex in the flagellar pocket, enter into the trypanosomatid cell where they will cause lysosomal membrane disruption and death of the parasite. TLF-1 resistance has been associated to a gene known as serum resistance associated (SRA) gene. This gene codes for a truncated VSG expressed in *T. b. rhodesiense* that interacts with TLF and blocks its interactions with the lysosomal membrane (reviewed by Pays and Vanhollebeke, 2008). Resistance to TLF in *T. b. gambesiense*, that does not express SRA, could be caused by a decreased expression of the receptor for Hp-Hb complex (Kieft *et al*., 2010).

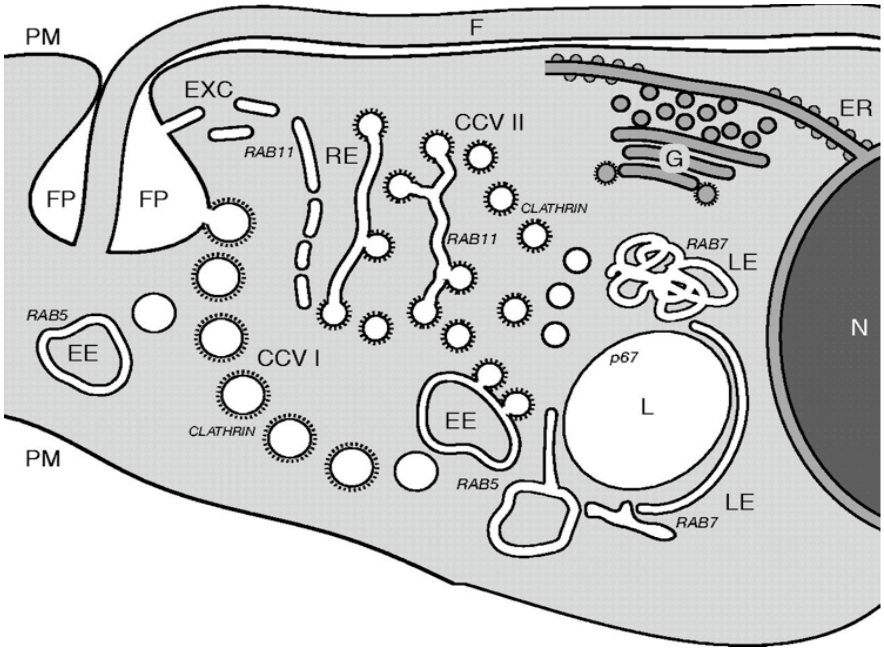

Figure 4: Schematic view of the organization of intracellular vesicles in *T. brucei*. PM – plasma membrane, ER – endoplasmic reticulum, N – nucleus, F – flagellum, FP- flagellar pocket, EE – early endosomes, RE – recycling endosomes, LE - late endosomes, L – lysosome, G – Golgi, EXC – exocytic carrier vesicle, CCV I – class I clathrin-coated vesicles, CCV II – class II clathrin-coated vesicles. Marker proteins for the different compartments are also mentioned. Taken from: Engstler *et al*., 2005. J. Cell Sci. 118, 2105-2118.

1.6 Glycosomes and metabolic characteristics of bloodstream and procyclic stage trypanosomes

Trypanosomatids, like all Kinetoplastea, possess organelles having similar morphology, biogenesis and role as peroxisomes in other eukaryotes. These peroxisome-like organelles of kinetoplastids are called glycosomes (reviewed by Gualdrón-López *et al*., 2012). Both types of organelles have no DNA and, for their biogenesis, rely on peroxins (acronym PEX), proteins that participate in the recognition of target proteins that are synthesized in the cytosol to be imported into the organelle, as well as proteins that are involved in controlling the size and number of the organelles. Both peroxisomes and glycosomes possess enzymes for ether-lipid and pyrimidine biosynthesis, purine salvage and the pentose-phosphate pathway. Unlike most peroxisome-like organelles, glycosomes lack catalase and interestingly, they possess the majority of the enzymes of the glycolytic pathway. While in any other organism the whole pathway is found in the cytosol, in bloodstream parasites seven (and in procyclics, six) of these reactions take place inside glycosomes (Figure 5). The reason why this compartmentalisation may have evolved has been debated and was recently reviewed (Gualdrón-López *et al*., 2012).

Glycolysis is the only source of energy for the bloodstream stage of the parasite and it has been shown that the compartmentalisation of the major part of glycolysis within glycosomes is essential for both bloodstream and procyclic cells when cultured in the presence of glucose (Furuya *et al*., 2001; Bakker *et al*., 1999; Haanstra *et al*., 2008). This essentiality of compartmentalisation could be attributed to the lack of regulation of the hexokinase (HXK) and phosphofructokinase (PFK) activities in trypanosomatids. Since ATP consumption and production in glycolysis are in balance inside the glycosomes, there is no ATP-dependent boost of the kinases at the beginning of the pathway that has to be compensated by feed-back inhibition, as occurs in other organisms without glycosomes, to prevent an uncontrolled accumulation of glycolytic intermediates. The generation of net ATP by glycolysis occurs in the cytosol where the last three enzymes of the pathway are located, including the ATP

producing pyruvate kinase. The glycosomal production of NADH by glyceraldehyde-3-phosphate dehydrogenase is counterbalanced by a metabolite (glycerol 3-phosphate/dihydroxyactetonephosphate) shuttle that involves a glycosomal glycerol-3-phosphate dehydrogenase and the mitochondrion –via its glycerol-3-phosphate oxidase– and restores the redox balance inside the organelle. Moreover, in the bloodstream stage the regular, cytochromes-containing mitochondrial respiratory chain is not expressed, while in the procyclics it is well developed, as well as the TCA cycle enzymes. However the functioning of these proteins is somewhat unusual in the parasite, as they do not seem to work as a proper circle but rather parts of the TCA cycle take place to produce metabolic intermediates.

As mentioned before, the bloodstream form is completely dependent on glucose from the host and relies on glycolysis to generate all the energy it uses. The parasite takes glucose from the mammalian bloodstream and excretes pyruvate. To achieve this, the glycosome is almost totally devoted to glycolysis; it has been found that 90% of the enzymatic content of glycosomes from bloodstream stage trypanosomes is made up of glycolytic enzymes. In contrast, in the procyclic form glycosomes dedicate 40-50% of their enzymatic content for glycolytic enzymes, since at this stage the parasite has little access to glucose; it has a more versatile metabolism that can take up glucose as well as amino acids for its energy metabolism (Hart *et al*., 1984; Aman *et al*., 1985).

Bloodstream form	Procyclic form
Glucose metabolism	Diversified metabolism
Mitochondrion repressed	Developed mitochondrion
PGK-c in glycosomes	PGK-b in cytosol
High endocytosis rate	Low endocytosis rate

Table 2: Table of relevant metabolic differences between the two main stages of *T. brucei*.

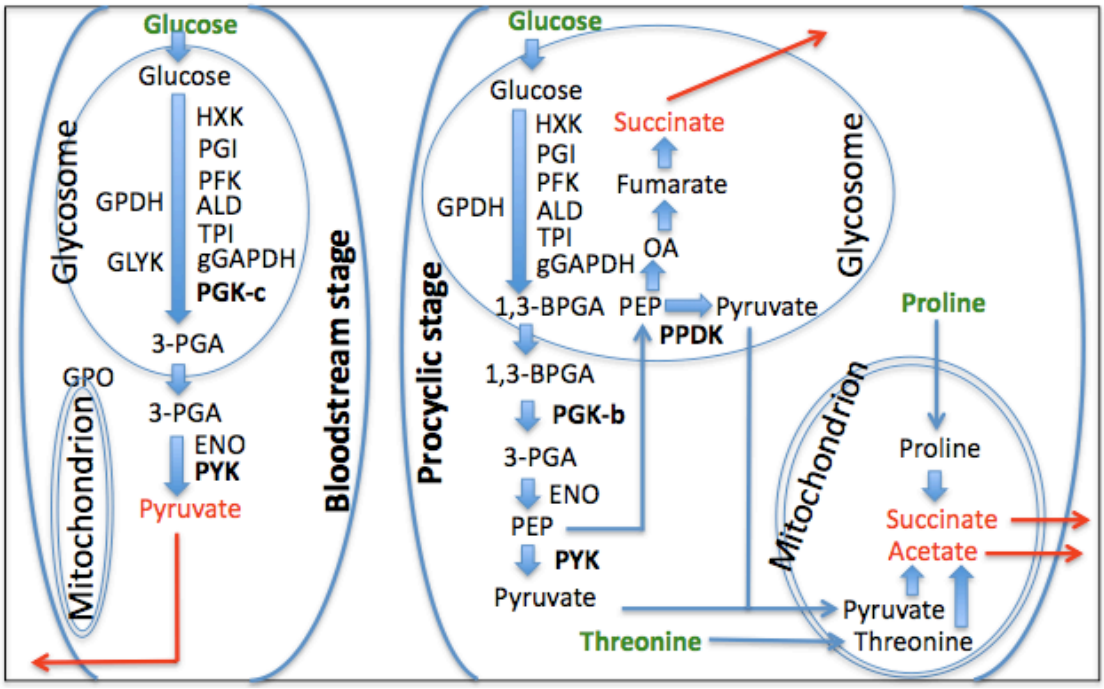

Figure 5: Scheme of the compartmentalization of enzymes in glycosomes and mitochondrion of bloodstream and procyclic stage trypanosomes. Substrates are shown in green and excreted products are shown in red. Thick arrows indicate enzymatic reactions while thin arrows indicate transport of nutrients between different cell compartments. HXK – hexokinase, PGI – phosphoglucose isomerase, PFK – phosphofructokinase ALD - aldolase, TPI – triose-phosphate isomerase, GPDH – glycerol-3-phosphate dehydrogenase, gGAPDH – glycosomal glyceraldehyde-3-phosphate dehydrogenase, GLYK – glycerol kinase, PGK – phosphoglycerate kinase, ENO - enolase, PYK – pyruvate kinase, GPO – glycerol-3-phosphate oxidase, 3PGA – 3-phosphoglycerate , 1,3BPGA – 1,3-bisphosphoglycerate, PEP - phosphoenolpyruvate, PPDK – pyruvate phosphate dikinase, OA - oxaloacetate.

1.7 Peroxisome protein import

During their formation, peroxisomes import newly synthesized matrix proteins from the cytosol by a mechanism that involves peroxins or PEX proteins (Figure 6). Studies performed with peroxisome-deficient yeast mutants showed that peroxisomes can arise *de novo* from the ER. This process involves the peroxisomal membrane proteins PEX3 and PEX16, that can be inserted in the ER membrane, as well as the soluble PEX19 that functions as a chaperone to mediate the insertion of proteins possessing a mPTS (membrane Peroxisomal-Targeting Signal) into the peroxisomal membrane.

Studies with yeast have also shed light on how growth, division and degradation control the number of peroxisomes in a cell, according to its metabolic needs. Peroxisomes are normally created by growth and division of pre-existing organelles (Motley and Hettema, 2007). The daughter organelles are import competent as they grow, until they reach a certain size and are no longer able to import enzymes. This proliferation occurs by elongation and fission in a process that involves PEX11, a peroxin that participates in the control of organelle size and number.

The import of matrix proteins requires the cytosolic matrix-protein receptors PEX5 and PEX7, in yeast and fungi also co-receptors for PEX7-dependent import, and several complexes present in the peroxisomal membrane and permanently or transiently associated with it. The matrix proteins are recognized by the receptors through their peroxisomal-targeting signal (PTS). There are different

kinds of PTS. PTS1 proteins possess a C-terminal tripeptide -SKL or a sequence of residues with similar physicochemical properties, while PTS2 proteins have a nonapeptide sequence near their N-terminus with the sequence -(R/K), (L/V/I), X_5, (H/Q), (L/A)-, where X can be any amino acid. A third means by which a protein can be imported into the peroxisomes is by a non-consensus, internal sequence or I-PTS. It seems that at least some I-PTS proteins can be imported by a piggy-back mechanism, by association with a PTS1- or PTS2-protein, but it may well be a rather general mechanism.

PEX5 binds PTS1- and PEX7 PTS2-proteins in the cytosol. The receptor-cargo complexes then interact with a docking complex, composed of the integral membrane protein PEX13 and the membrane-bound protein PEX14, and in yeasts also PEX17 (for a review, see Galland and Michels, 2010). The molecular mechanism by which PTS proteins are imported seems to involve PEX5 oligomerisation to produce a transient pore in the peroxisomal membrane. Also PEX14 is involved in the pore formation (Erdmann and Schliebs, 2005; Meinecke *et al*., 2010). The PTS protein is released into the peroxisomal matrix, while the receptor is recycled. The AAA^+-ATPases PEX1 and PEX6 provide energy by ATP hydrolysis for the retrieval of receptor back to the cytosol. The RING-finger complex that has E3-ubiquitin ligase activity and PEX4 that acts as an E2-ubiquitin-conjugating enzyme modify the receptor by mono- or poly-ubiquitination. The monoubiquitinated receptor released at the cytosol is deubiquitinated by a still unknown enzyme and can participate in more cycles of import, while polyubiquitinated receptors are routed for proteasomal degradation. The overall mechanisms involving peroxisome biogenesis and protein import are quite conserved and have been characterised also in trypanosomatids (Moyersoen *et al*., 2004; Galland and Michels, 2010; Gualdrón-López *et al*., 2012).

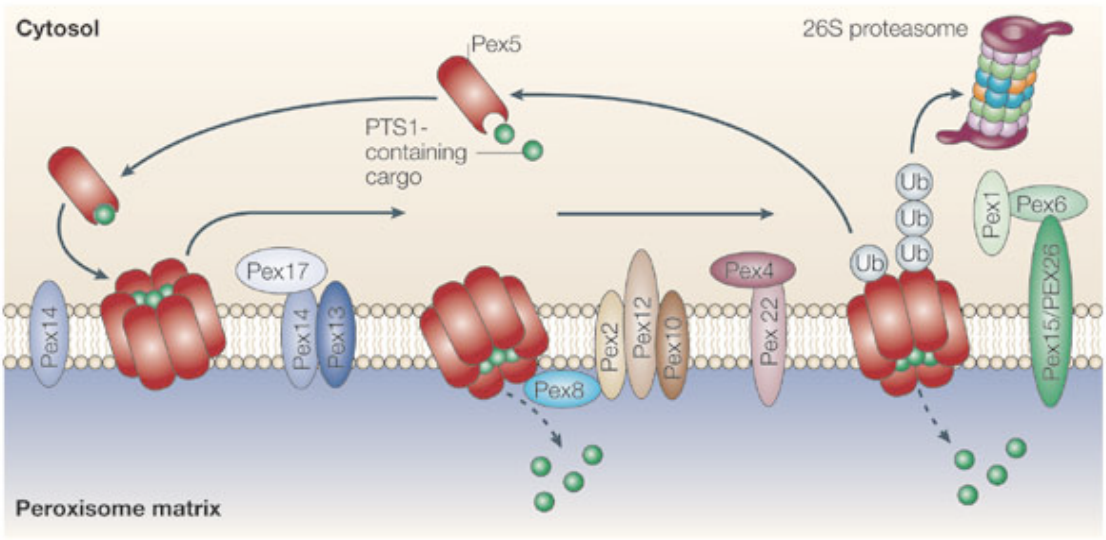

Figure 6: Schematic view of the peroxins participating in protein import from the cytosol into the peroxisome of yeast cells. Taken from: Erdmann and Schliebs, 2005. Nat. Rev. Mol. Cell Biol. 6, 738-742.

1.8 Autophagy

First described by Christian de Duve, in the 1960s, autophagy is the process of delivering material into the lysosome or vacuole, as the lysosome equivalent in yeasts is called, for degradation and recycling of the building blocks. Autophagy is observed at a basal level under normal growth conditions in almost all organisms. It is increased during specific processes such as starvation, sporulation, differentiation and infection. It has been linked to neurological diseases, cancer and metabolic disorders.

Autophagy can be divided into macro- and microautophagy. During macroautophagy an isolation membrane is formed and elongates in the cytosol until it isolates the material to be degraded within a double-membrane bounded autophagosome, that will fuse its outer membrane with the lysosome and deliver its contents to be degraded (Figure 7a). The vesicle expansion step requires at least one of the two ubiquitin-like complexes to elongate the isolation membrane (Figure 7b).

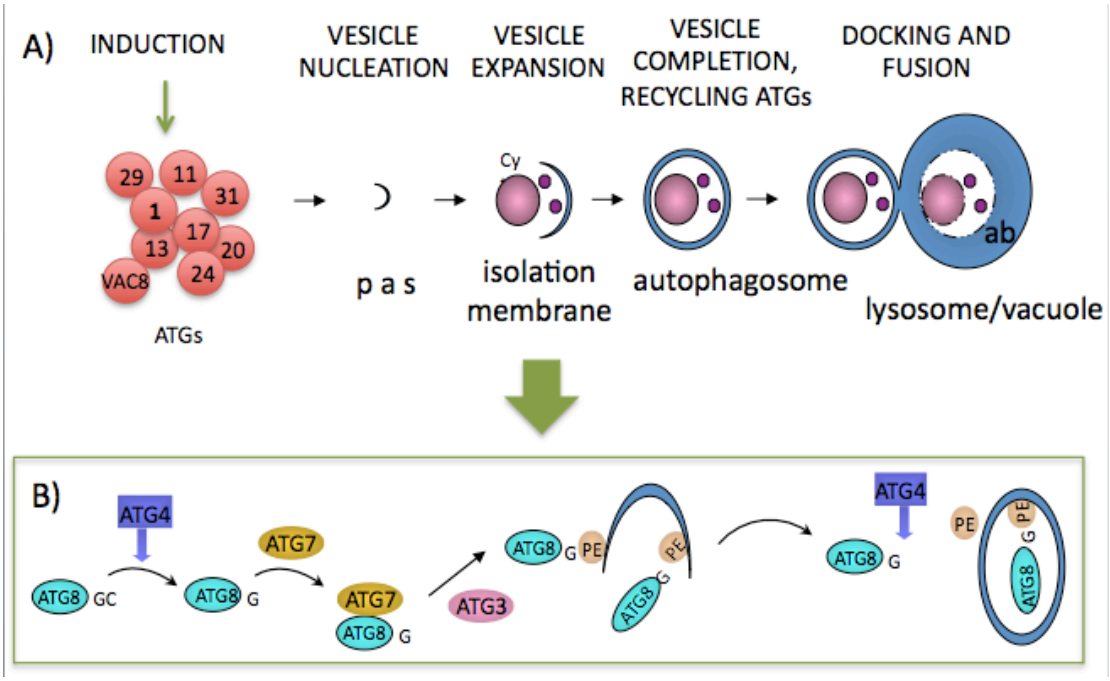

Figure 7: A) Scheme of the different steps (top, upper case) and structures (bottom) of macroautophagy as observed in different organisms. B) Sequence of interactions between ATG proteins required for the attachment of ATG8 to phosphatidylethanolamine (PE) residues in the expanding isolation membrane.

Microautophagy happens when the lysosomal membrane itself surrounds and engulfs the material to be degraded. These processes can also be directed to specific organelles; in these cases the processes are called pexophagy (see next section), mitophagy, ribophagy or ER-phagy. Autophagy has also been shown to remove unwanted parts of the nucleus (piecemeal microautophagy of the nucleus - PMN).

A mechanism similar to autophagy is also used to deliver hydrolytic enzymes to the vacuole in yeasts and called 'Cytoplasm to vacuole targeting' (Cvt) pathway. The Cvt pathway operates in a few yeast species under normal growth conditions, when the occurrence of autophagy is low or absent. It serves to deliver large complexes of pre-aminopeptidase, a vacuolar resident protein that folds and oligomerizes while still in the cytosol. Therefore it requires an autophagy-like vesicle to be delivered into the vacuole, where it will be processed into the mature form. In conditions where autophagy is upregulated

Cvt is considered to stop and pre-aminopeptidase oligomers are then delivered to the vacuoles in autophagosomes, together with organelles or proteins that are targeted for degradation. There are several ATGs that participate specifically in the Cvt pathway and the pathway also shares many ATGs with that of general autophagy. In mammalian cells there is also a process called Chaperone-Mediated Autophagy (CMA) that is involved in the clearing of protein aggregates from the cytosol (reviewed by Kaushik and Cuervo, 2008) by delivering them directly into the lysosome without the participation of intermediary membranes. For a detailed view on the current knowledge on autophagy processes in general, the proteins involved and autophagy in trypanosomatids in particular, see Chapter 2.

Until recently, there was no information regarding autophagy pathways operating in trypanosomatids. However, from many electron micrographs made in the 1960s-1970s by Vickerman's group in Glasgow, it could with hindsight be concluded that the process of autophagy does also occurs in *T. brucei* (K. Vickerman, personal communication), thus indicating that the pathway is probably also conserved in kinetoplastids. In 2006 our group performed the first search for ATG homologues in trypanosomatids. It was found that many ATGs were conserved in these organisms (Herman *et al*., 2006). In 2008 it was shown that autophagy takes place in starvation conditions in procyclic cells (Figure 8a, Herman *et al*., 2008) and during *in vivo* differentiation from long-slender to short-stumpy forms and, even more dramatically, during the synchronous *in vitro* differentiation of short-stumpy to procyclic forms (Herman *et al*., 2008). Autophagy has also been described and shown to be important during differentiation in *Leishmania* spp. and *T. cruzi* (Williams *et al*., 2006; Alvarez *et al*., 2008; Barquilla *et al*., 2008).

1.9 Peroxisome degradation

Methylotrophic yeasts growing on ethanol or methanol as carbon source develop very large peroxisomes that may take most of the volume of the cells. When these cells are transferred to a glucose-rich environment, the peroxisomes are degraded in a specific manner, by pexophagy (reviewed by Till *et al*., 2012).

The triggering of the specific degradation of peroxisomes involves at least two peroxins whose primary function is in peroxisome biogenesis, PEX14 and PEX3. It has been shown in *Hansenula polymorpha* (Bellu *et al*., 2002; Van Zutphen *et al*., 2008) that on the surface of peroxisomes that are no longer import-competent, PEX3 is in contact with PEX14. This seems to block PEX14's interaction with the cytosolic PEX proteins that carry the peroxisomal enzymes to be imported. During pexophagy-inducing conditions, PEX3 is first removed from the surface of peroxisomes and then the peroxisome is degraded.

Pexophagy-specific genes have been identified and characterised in *P. pastoris*, ATG30, and very recently in *S. cerevisiae,* ATG36 which curiously is a peroxisomal transmembrane protein. The interaction of the two proteins with PEX3 targets the autophagosomal marker ATG8 to the peroxisomal membrane, a signal for its degradation (Farré *et al*., 2008; Motley *et al*., 2012). Homologues of these proteins might not exist in the trypanosomatid genomes since it is known that, while the main core of autophagy is relatively conserved during evolution, there are many specific-autophagy genes that seem to be exclusive even to only a single yeast species (Meijer *et al*., 2007). While several peroxins that participate in the glycosome's biogenesis have been identified and in part structurally and/or functionally characterised in trypanosomes (For reviews see Moyersoen *et al.*, 2004; Galland and Michels, 2010), the molecular mechanisms and identity of the proteins involved in the organelle's degradation are still almost completely unknown.

1.10 Molecular biology aspects of *T. brucei* differentiation from the bloodstream to the procyclic stage

T. brucei differentiation from long-slender to short-stumpy bloodstream forms occurs when the cell density in the host bloodstream reaches a certain limit (Reuner *et al*., 1997). This differentiation step has been shown to be induced by hydrolysis products of cAMP analogues (Laxman *et al*., 2006), The so-called 'stumpy-inducing factor' (SIF) seems to be released by the parasites, but its precise identity remains to be established. It induces cell-cycle arrest, characteristic of short-stumpy forms. Aspects of this and other steps of *T. brucei*

differentiation are reviewed in (Fenn and Matthews, 2007). The nature of the trigger for differentiation from the short-stumpy to the procyclic form that takes place in the tsetse fly's midgut is better known. It is thought that several factors participate. It has been shown *in vitro* that a drop in temperature (Engstler and Boshart, 2004) as well as the presence of millimolar concentrations of the TCA-cycle intermediates citrate and cis-aconitate increase the efficiency of differentiation (Overath *et al*., 1986). About further differentiation steps that take place inside the tsetse fly virtually nothing is known.

In trypanosomatids, proteolysis has been described and is increasingly being associated with parasite virulence and differentiation. It has been shown to be upregulated during differentiation, as it seems to participate in the clearance of VSG that takes place in the bloodstream short-stumpy to procyclic differentiation step while procyclin expression starts in *T. brucei* (Mutomba and Wang, 1998). Proteolysis via lysosomal proteases is also essential for differentiation in *L. major* (Besteiro *et al*., 2006), *L. mexicana* (Williams *et al*., 2006) and *T. cruzi* (Santos *et al*., 2005). As mentioned above, trypanosomatids may be considered to have a more simple form of autophagy since many ATGs of yeasts and mammalian cells do not have homologues in these parasites, but other trypanosomatid-specific proteins may be involved. Irrespective whether the autophagy system in these parasites is elaborate or not, autophagy is important because it has been found to be involved in the differentiation of all three major trypanosomatid species. Previous work performed in our laboratory has shown that autophagy is quickly upregulated when procyclic cells are incubated under starvation conditions and during *in vivo* differentiation of long-slender *T. brucei* to short-stumpy forms. During differentiation one can observe an increase in the colocalisation of a lysosomal membrane marker (p67) with a glycosomal marker (aldolase). Before differentiation there is a basal level of colocalisation observed in 10% of the pleomorphic cells, that increased to 40% during the shorty-stumpy formation, indicative of an upregulation of glycosomal degradation by pexophagy.

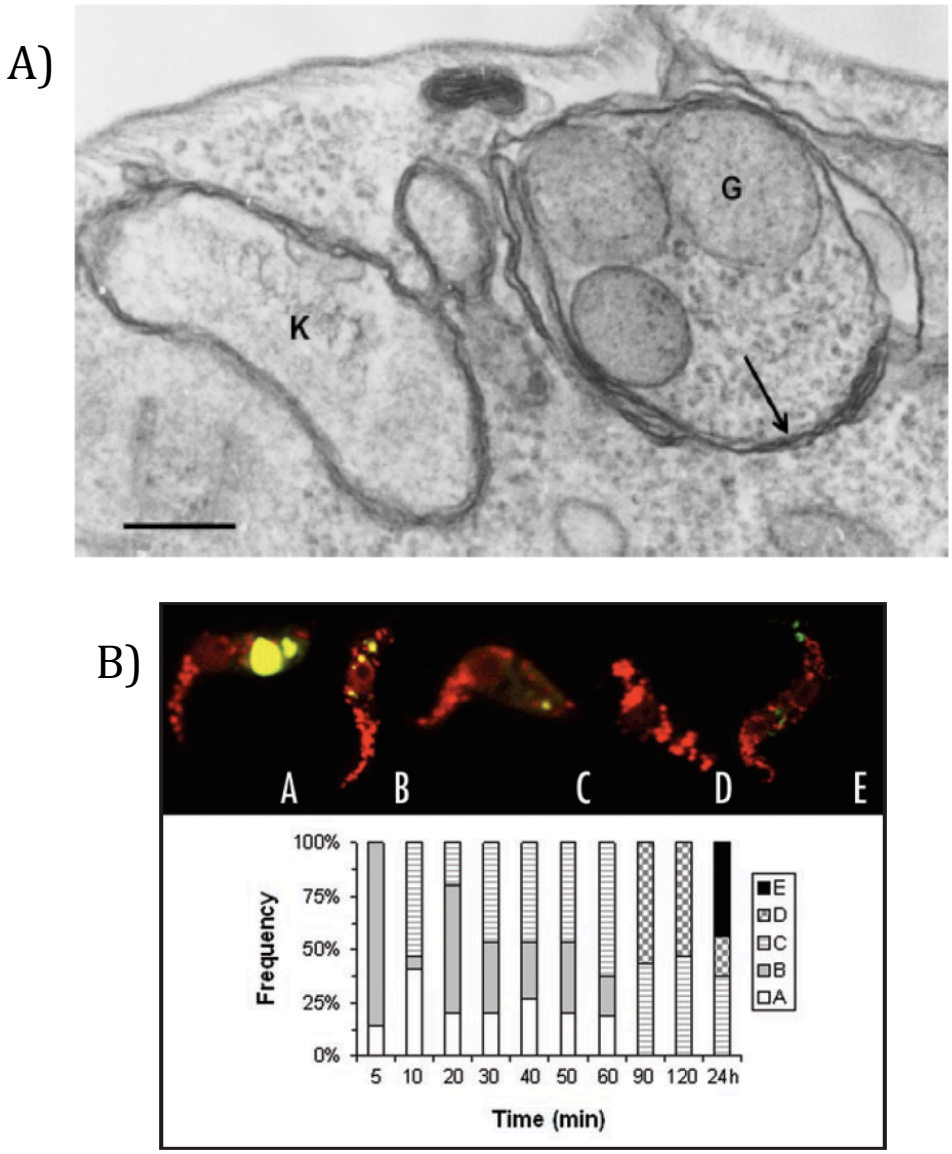

Figure 8: A) Transmission electron micrograph of procyclic *T. brucei* where 3 glycosomes (G, shown in one of the three) can be seen surrounded by a double-membrane vesicle (arrow). K - kinetoplast, G – glycosomes. Bar is 300 nm. Taken from Brennand *et al*., 2011. Mol. Biochem. Parasitol. 177, 83-99. B) Results from Herman *et al*., 2008. Autophagy 4, 1-15, obtained by confocal immunofluorescence microscopy. The upper panel of the figure shows images of different cell types observed during short-stumpy to procyclic *in vitro* differentiation, stained for the glycosomal marker aldolase (in red) and the lysosomal marker p67 (in green). It shows the distribution of glycosomes in the cell and their position with respect to the lysosome (co-localization) and the variable size of the lysosome. In the bottom panel is presented a histogram of the frequency at which each type of cell morphology (as shown at the top, indicated as type A, type B, type C or type D) was observed at different time points during the *in vitro* differentiation of short-stumpy to procyclic forms.

Moreover, *in vitro* differentiation of the short-stumpy to the procyclic form showed a colocalisation in all cells observed up until 60 minutes (Figure 8b, types of lysosome A, B and C, shown by yellow colour on merge image). after placing the parasites in procyclic medium in the presence of 6 mM cis-aconitate at 28°C (Herman *et al*., 2008). This was associated with an increase in the size of the lysosome (Figure 8b, type A, lysosome shown in yellow), also indicative of an increase in autophagy levels Autophagy is also upregulated and essential during differentiation in *L. major* (Besteiro *et al*., 2006) and *T. cruzi* (Alvarez *et al*., 2008).

1.11 Role of ATG24 in other organisms

T. brucei ATG24 is an important topic in this thesis. ATG24, previously known as CVT13 has been initially identified and well characterised in the yeast *S. cerevisiae*, where it has been shown to be present in the cytosol and in puncta near the vacuole. It precipitates with membranes, where its Phox-homology domain (from ph agocytic ox idase) interacts with phosphatidylinositol-3-phosphate (PI3P). This interaction is essential for the Cvt pathway but not for general autophagy. The protein interacts with ATG20 (probably forming a dimer) and ATG17 (Nice *et al*., 2002). It also has been shown to participate in mitophagy although its deletion does not abolish mitophagy completely (Kanki and Kliosky 2008; Mendl *et al*., 2010) and in piecemal microautophagy of the nucleus or PMN (Krick *et al*., 2008). In *Pichia pastoris* the protein is essential for macro- and micropexophagy; its absence affects vacuolar morphology and causes peroxisomal clustering. During macropexophagy ATG24 concentrates in membranes between autophagosomes and the vacuole and during micropexophagy in wild-type cells the protein concentrates in the tips of fusing elongating arms of the vacuole membrane (Ano *et al*., 2005). The yeast ATG24 is part of a putative complex centred at ATG1 that participates in the induction of autophagy and the switch between general and specific autophagy (Figure 9 – see Chapter 2 for more details). In *S. cerevisiae* ATG24 interacts with ATG20 and ATG17. In starvation conditions, the latter interacts directly with ATG1, the kinase responsible for the autophagy induction signal, that is under the control

of TORC1. The association of this protein with ATG13 and ATG17 seems to be required for the induction of the pathway. The interaction of ATG24 with intracellular membranes requires the VPS34 complex, that interestingly seems to be formed after ATG1 is activated.

The mammalian homologue of ATG24 is sorting nexin 4 (SNX4), a protein that requires VPS34 for its subcellular localisation and is involved in the transport of ricin from endosomes to the Golgi system (Skånland *et al.*, 2007); it forms complexes with clathrin and dynein, and binds to PI3P in endosomes (Skånland *et al.*, 2009). Moreover, it has a role in transferrin receptor retrieval from the lysosomal degradation route back to the cell surface, as its depletion has been shown to decrease the levels of the transferrin receptor in the cells (Traer *et al.*, 2007). However an earlier study has shown opposite results with the overexpression of a myc-tagged version of SNX4, that inhibited transferrin receptor endocytosis, while its downregulation was not inhibitory (Leprince *et al.*, 2003).

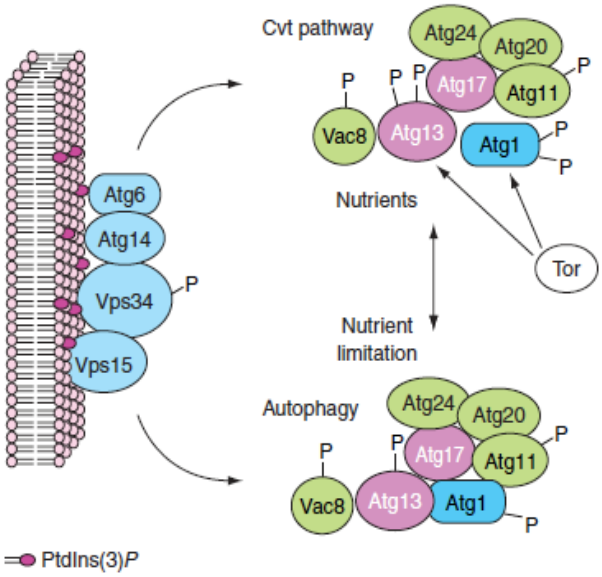

Figure 9: Interactions between the putative ATG1 complex during autophagy or Cvt pathway as described in yeast cells. Taken from Klionsky 2005. J. Cell Sci. 118, 7-18.

Chapter 2
Autophagy in parasitic protists: unique features and drug targets

This chapter has been published as a review paper:

Brennand A., Gualdrón-López M., Coppens I., Rigden D.J., Ginger M.L., Michels P.A.M.
Autophagy in parasitic protists: Unique features and drug targets.
Molecular and Biochemical Parasitology 177(2011) 83-99.

Summary:

This chapter reviews the current knowledge on autophagy with focus on major parasitic protist species (Trypanosomatids, *Giardia, Trichomonas, Entamoeba* and Apicomplexans) and elaborates on the possibility of using the parasite autophagic pathway as a target for the development of new drugs.

Molecular & Biochemical Parasitology 177 (2011) 83–99

Contents lists available at ScienceDirect

Molecular & Biochemical Parasitology

Review

Autophagy in parasitic protists: Unique features and drug targets☆

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ARTICLE INFO

Article history:
Received 27 November 2010
Received in revised form 30 January 2011
Accepted 2 February 2011
Available online 21 February 2011

Keywords:
Autophagy
Evolution
Life-cycle differentiation
Trypanosomatidae
Plasmodium
Drug discovery

ABSTRACT

Eukaryotic cells can degrade their own components, cytosolic proteins and organelles, using dedicated hydrolases contained within the acidic interior of their lysosomes. This degradative process, called autophagy, is used under starvation conditions to recycle redundant or less important macromolecules, facilitates metabolic re-modeling in response to environmental cues, and is also often important during cell differentiation. In this review, we discuss the role played by autophagy during the life cycles of the major parasitic protists. To provide context, we also provide an overview of the different forms of autophagy and the successive steps in the autophagic processes, including the proteins involved, as revealed in recent decades by studies using the model organism *Saccharomyces cerevisiae*, methylotrophic yeasts and mammalian cells. We describe for trypanosomatid parasites how autophagy plays a role in the differentiation from one life cycle stage to the next one and, in the case of the intracellular parasites, for virulence. For malarial parasites, although only a limited repertoire of canonical autophagy-related proteins can be detected, autophagy seems to play a role in the removal of redundant organelles important for cell invasion, when sporozoites develop into intracellular trophozoites inside the hepatocytes. The complete absence of a canonical autophagy pathway from the microaerophile *Giardia lamblia* is also discussed. Finally, the essential role of autophagy for differentiation and pathogenicity of some pathogenic protists suggests that the proteins involved in this process may represent new targets for drug development. Opportunities and strategies for drug design targeting autophagy proteins are discussed.
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Abbreviations: ATG, autophagy-related; Cvt, cytoplasm-to-vacuole targeting; DHA, dihydroxyacetone; EC_{50}, effective concentration to cause 50% inhibition of growth; ER, endoplasmic reticulum; HAT, human African trypanosomiasis; 3-MA, 3-methyladenine; MDC, monodansylcadaverine; MVT, multivesicular tubule; PAS, phagophore assembly site; PE, phosphatidylethanolamine; PEX, peroxin; PMN, piecemeal microautophagy; PRB1, peptidase B; PtnIns3K, phosphatidylinositol 3-kinase; PtdIns(3)P, phosphatidylinositol 3-phosphate; RNAi, RNA interference; TOR, target-of-rapamycin.

☆ *Note:* Different nomenclature rules exist for acronyms of proteins and genes in mammals, yeasts and protists. To avoid confusion, the nomenclature system for trypanosomatids (upper case for proteins, italicized upper case for genes) has been used throughout in this paper, also for proteins and genes from other organisms [127].

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0166-6851/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.molbiopara.2011.02.003

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1. Introduction

In 1955 de Duve and coworkers reported the existence of organelles with an acidic interior in mammalian cells that contain a large number of hydrolases [1,2]. These organelles were called 'lysosomes', because they were shown to be responsible for both the degradation of macromolecules taken up into the cells by endocytosis and the degradation of the cell's own macromolecular constituents. In 1963 de Duve named the latter process self-eating or 'autophagy' [3,4]. Later research showed that lysosomes and autophagy are almost ubiquitous among eukaryotes. Initially, the process of autophagy was only studied at the morphological level [5], demonstrating the sequestering of cytoplasm by a membrane of unknown origin to form autophagosomes that fuse with lysosomes or, in yeast, with the corresponding organelles called vacuoles [6]. In the last decade however, research on autophagy has expanded rapidly, resulting in a quite detailed understanding of the process at the molecular level as well as in an appreciation of the multiple functions it plays within a cell [5].

Initially two major functions were attributed to autophagy. First, it would serve as a survival mechanism under starvation; recycling of cytoplasmic constituents by non-specific autophagy would allow the cell to survive during nutrient deprivation. Second, a cell may, by a more selective form of autophagy, remove damaged, abnormal or redundant cellular constituents including cytosolic proteins as well as organelles. Ample experimental evidence for these two functions, both in unicellular and multicellular eukaryotes, has been obtained, as well as evidence for additional functions. These latter functions include assisting cellular survival under other stress conditions, such as hypoxia and high temperatures, but also major roles in the remodeling of a cell's morphology and metabolic machinery during changes of nutritional conditions or developmental differentiation. Autophagy is also considered a mechanism that, alongside apoptosis and necrosis, plays a role in cell death. In multicellular eukaryotes, autophagy is also involved in tissue remodeling and combating diseases such as cancer, neurodegenerative disorders, lysosomal storage diseases, aging and infections, be they caused by parasites, bacteria or viruses. Conversely, however, the autophagy machinery is also actively manipulated by some infectious agents in order to promote their successful invasion of and survival within host cells.

In evolutionary terms, the origin of autophagy pre-dates the divergence of the lineages from which all known extant eukaryotes are descended [7,8]. Autophagy is therefore an ancient and conserved feature of eukaryotic cell biology, but as with many other elements of eukaryotic biology, it is also a process that in various parasitic protists does not always follow the paradigms identified in well-characterised yeast and animal models. In this review, we summarise the occurrence of autophagy in parasitic protists. We describe the role played by autophagy – in its different manifestations – in the survival, life-cycle differentiation and pathogenicity of different parasites and discuss the possibility for chemotherapeutic interference to combat the diseases caused by the pathogens. We also discuss the evidence that points very clearly to the divergence in apicomplexan parasites and the secondary loss in *Giardia* of the canonical autophagy pathway described in the next section. Host autophagy also plays a role in the pathogenicity, either to combat the infection or – when derailed by the invading protist – allowing spread of the infection. For a discussion of these latter aspects, we refer to a recent detailed review about autophagy in protists published elsewhere [8]. We start this review with an overview of the molecular basis for autophagy in yeast.

2. Autophagy-related pathways

Autophagy has been studied in most detail in *Saccharomyces cerevisiae*, largely thanks to the tractable genetics of this organism, and to a lesser extent in other yeasts and mammalian cells. The information thus obtained has guided research on autophagy in many other eukaryotes and is used here to introduce major features of the process. For a more detailed description, with references to many papers reporting the original findings, see the reviews by Nakatogawa et al. [9] and Yang and Klionsky [10]. Different autophagic pathways occur in cells (Fig. 1), mediated in part by proteins that are shared by these different processes and in part by proteins that are specific for each different pathway. Some proteins are also shared with other cellular processes not related to autophagy. Proteins involved specifically in one or more autophagy pathways are called AuTophaGy-related proteins or ATGs [11]. The most common form of autophagy is a non-selective process called macroautophagy in which portions of the cytoplasm, containing bulk cytosol and other cytoplasmic constituents such as organelles, become randomly surrounded by a cup-shaped structure formed as a double-membrane and called phagophore. This structure develops into a double-membrane vesicle, the autophagosome. The outer membrane of this vesicle fuses with the lysosomal/vacuolar membrane, resulting in an autolysosome. The single-membrane vesicle now present in the lumen of the lysosome is called autophagic body. Its membrane is ruptured by lysosomal hydrolases and its contents degraded. The degradation products are transported back to the cytosol via permeases in the lysosomal membrane. In an alternative, equally non-selective process called microautophagy, cytoplasmic constituents are directly taken up by the lysosome as a result of engulfment by the membrane of this organelle.

A selective form of routing proteins to the vacuole by a process related to macroautophagy, is the cytoplasm-to-vacuole targeting (Cvt) pathway [12]. It is a constitutive process used for sorting the resident vacuolar hydrolases, aminopeptidase and α-mannosidase to the organelle. So far, it has only been described for yeasts and filamentous fungi. This process too involves the encapsulation of proteins in the cytoplasm – in this case specifically hydrolases – by a phagophore that gives rise to a double-membrane Cvt vesicle, which subsequently fuses with the vacuole and, after rupture of the single membrane of the Cvt body within the vacuolar lumen, releases the hydrolases.

Other autophagy-related processes play a role in selectively or non-selectively sorting redundant or damaged organelles to lysosomes/vacuoles for their degradation. The selective processes route peroxisomes to degradation in a process called pexophagy, mitochondria by mitophagy, ribosomes by ribophagy, parts of the

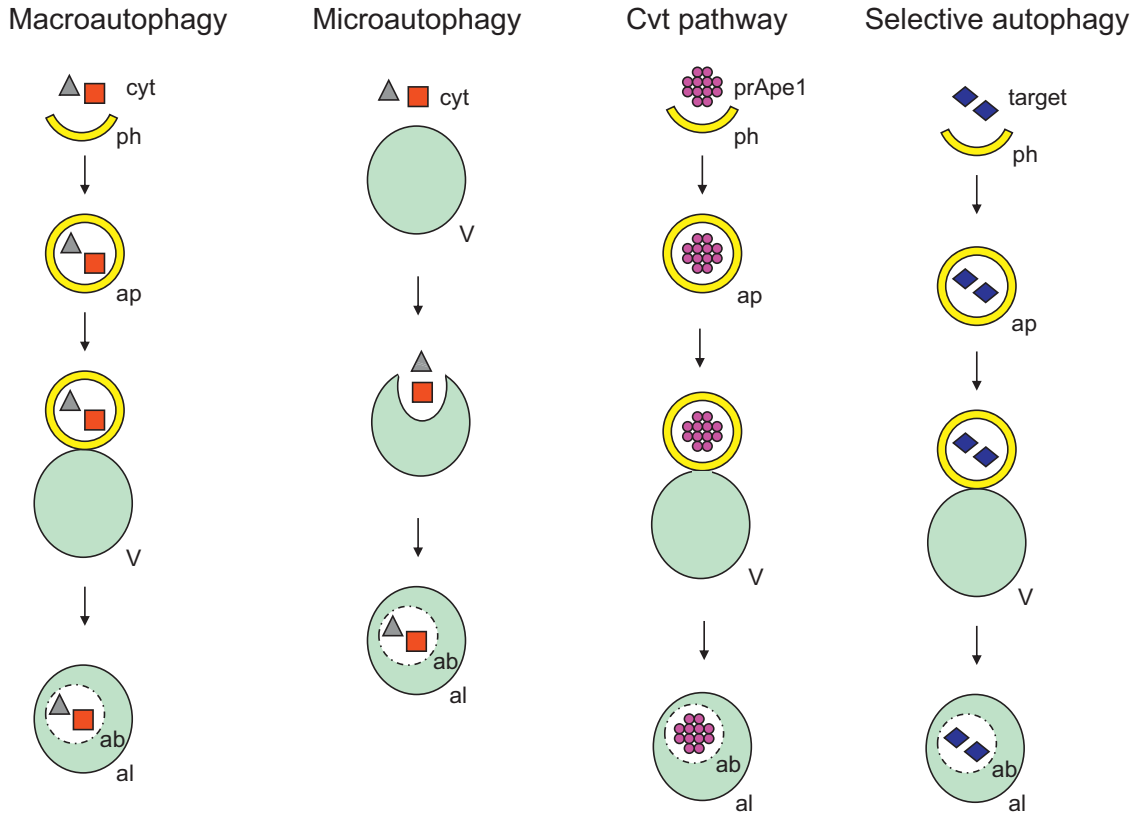


Fig. 1. Diagram presenting the different autophagy-related pathways in yeast. Abbreviations: cyt, cytosolic constituent to be degraded; ph, phagophore; ap, autophagosome; V, vacuole (yeast equivalent of lysosomes in mammalian cells and trypanosomatids); al, autolysosome; ab, autophagic body; prApe1, pre aminopeptidase 1; target, organelle to be degraded. The different forms of the cell constituents enclosed by the phagophore indicate the differences between non-selective macro- and microautophagy and selective autophagy. For details, see the main text of the paper.

endoplasmic reticulum (ER) by ER-phagy or reticulophagy and even the pinching off and degradation of part of the nucleus by piecemeal microautophagy (PMN) or micronucleophagy. For selective pexophagy and mitophagy both macro- and micro-processes have been described, respectively involving phagophore and autophagosome formation followed by the fusion of the latter with the vacuole/lysosomes, and the direct engulfment of the organelles by lysosomes. For selective ribophagy and ER-phagy only macroautophagy has been reported. Concerning pexophagy, it has been reported for methylotrophic yeasts that the degradation of the peroxisomes occurs by macro- or micropexophagy depending on the carbon source and ATP level of the cell [13]. Another distinction is that macropexophagy often involves the sequestering of a single peroxisome, micropexophagy a cluster of these organelles.

3. Proteins involved in autophagy – lessons learnt from yeast and mammals

3.1. ATG proteins

Macroautophagy and the related pathways can conceptually be understood as a number of successive steps (Fig. 2): (1) signal sensing and induction of autophagy; (2) cargo selection and packaging; (3) vesicle nucleation, *i.e.*, formation of the phagophore; (4) vesicle expansion and completion, *i.e.*, formation of the autophagosome or Cvt vesicle; (5) retrieval of ATGs used in the earlier steps for re-use; (6) docking of the autophagosome/Cvt vesicle at the lysosome/vacuole and fusion; (7) vesicle breakdown in the lysosome/vacuole. The molecular mechanisms underlying each of these different steps have been studied most intensively in *S. cerevisiae*, and will only be summarized briefly here. For a detailed review, see Nakatogawa et al. [9].

To date, over 30 ATG proteins have been described; most of them for *S. cerevisiae*, but orthologues of several of these, with similar function, have also been identified in several other organisms [14]. There are also a few ATGs found so far only in other organisms such as the methylotrophic yeasts *Pichia pastoris* and *Hansenula polymorpha* [15] and *Caenorabditis elegans* [16]. About half of the known ATG proteins play a role in the core machinery involved in most autophagy-related pathways, where they act in the formation of autophagosomes and Cvt vesicles; the others are specifically required in distinct steps of only one or some of the pathways. The major molecular events in the different steps are as follows:

(1) Signal sensing and induction. Autophagy occurs constitutively at a basal level, but it can be dramatically enhanced in response to stimuli from within the cell or from outside. The process may be stimulated by depletion of essential nutrients or other forms of stress, physiological signals such as growth factors or hormones or infection by pathogenic agents. The stimulation then results in signaling cascades similar to those involved in other cellular processes. A crucial factor in setting off the signaling is the Ser/Thr kinase TOR ("target of rapamycin") [17,18]. This protein kinase is responsible for the balance between protein synthesis and degradation through autophagy. It occurs in two multiprotein complexes, TOR complex 1 (or TORC1) and TORC2, involved in modulating different signaling branches and so exerting different functions in the physiology of the cell. Only TORC1 is sensitive to the antibiotic rapamycin. It controls temporal aspects of growth; under nutrient-rich conditions it promotes ribosome biogenesis, translation and nutrient

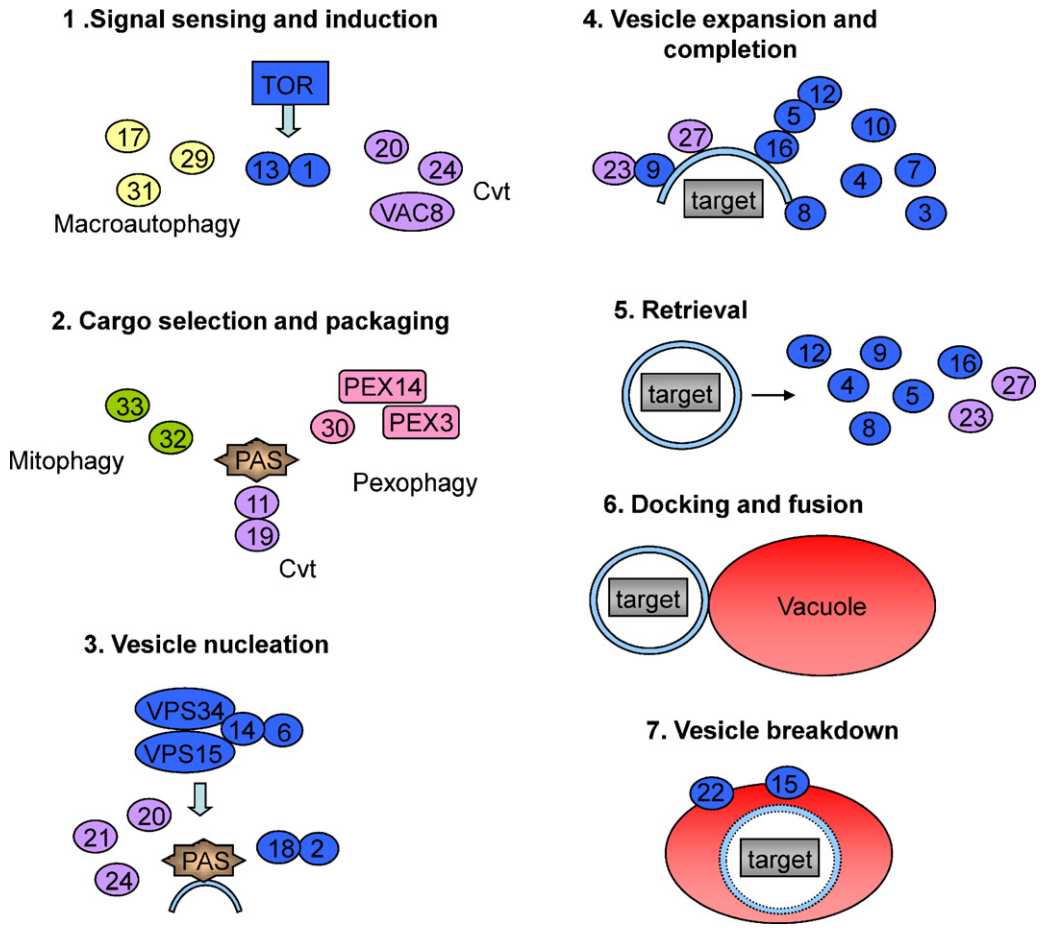


Fig. 2. The seven successive steps of selective autophagy pathways in yeast. The ATG proteins involved in the successive steps are represented by their numbers: non-ATG proteins that play a role in selective autophagy are indicated by their acronym and number. The involvement of proteins in corresponding steps of different autophagic pathways is indicated by colouring: Those involved in all pathways are blue; macroautophagy, yellow; Cvt, mauve; mitophagy, green, pexophagy, pink. PAS, phagophore assembly site. For details, see the main text of the paper.

uptake. Under starvation, or when inhibited by rapamycin, it down-regulates protein synthesis and activates autophagy. In contrast, TORC2 controls spatial aspects of growth by regulation of actin cytoskeleton polarization.

Downstream of TOR acts a complex (called ATG1 complex) with the Ser/Thr kinase ATG1 and ATG13 forming the core and ATG17, ATG29 and ATG31 as transiently associated proteins primarily involved in the non-selective process of macroautophagy, and ATG20, ATG24 and VAC8 in the selective Cvt pathway. In the presence of nutrients, TORC1 keeps ATG13 in a hyperphosphorylated state, with a low affinity for ATG1. Upon starvation, ATG13 is partially dephosphorylated resulting in an increased affinity for ATG1 and consequently an initiation of macroautophagy. The physiological protein substrate of ATG1 is still unknown. Although the ATG1 complex is also essential for the Cvt pathway, a particular phosphorylation state of ATG13 is not; this pathway is constitutively active. Components of the ATG1 complex have also been shown to be important for initiation of other selective forms of autophagy such as pexophagy, mitophagy and PMN.

The activation of the ATG1 complex upon induction of autophagy is essential for the organization of a perivacuolar membranous structure called the phagophore assembly site (PAS, also known as the preautophagosomal structure), the proposed nucleation site for sequestering vesicles [19]. Although the PAS exists constitutively, it is re-organized upon autophagy induction, from its mode for the constitutive Cvt pathway to that of autophagy. A considerable number of autophagy components are transiently recruited onto the PAS during autophagy; it probably functions as a dynamic organizing center for the process. In yeast, only a single PAS is found, whereas in mammalian cells it is likely that multiple foci with equivalent – but yet to be defined – function exist.

(2) Cargo selection and packaging. In contrast to nonselective macroautophagy, where bulk cytoplasm is sequestered, a defined cargo is encapsulated in selective forms of autophagy. In the case of the Cvt pathway, ATG11 and ATG19 play a role in cargo selection. ATG11 brings the ATG19–cargo complex to the PAS. Different proteins have been identified as essential for cargo selection in other selective processes, such as ATG30, together with ATG11, for pexophagy in *P. pastoris* [20]. Two peroxins, PEX3 and PEX14, peroxisomal membrane proteins whose principal function is in the biogenesis of the organelles, are also involved in the selection process in this yeast [20]. ATG30 interacts with a phosphorylated form of PEX14 and subsequently with PAS to target the organelles for degradation. Two proteins have been identified that are involved in selection of *S. cerevisiae* mitochondria for mitophagy: ATG32 and ATG33, both present in the outer membrane of the organelles [21]. ATG11 binds to ATG32, resulting in recruitment of mitochondria to the PAS, a similar process to that involving ATG11 and ATG19 in the Cvt pathway. The role of ATG33 is less clear. It seems to select mitochondria for degradation when a culture reaches the stationary growth phase.

(3) Vesicle nucleation. This step involves the initiation of growth of a membrane vesicle (Cvt vesicle or autophagosome). The origin of the lipid bilayer for these vesicles is a source of debate. Proposals include recruitment of lipids from existing intracellular membranes or plasma membrane or, alternatively, localized synthesis or transport resulting in new membrane material. No indications are available for *de novo* lipid synthesis at the site of vesicle formation. Recent studies with mammalian cells strongly suggest that these membranes may derive from ER, mitochondria and plasma membrane [22–25], while studies with yeast suggest a role for the Golgi complex [26]. Nucleation of the phagophore involves enrichment of membranes in phosphatidylinositol 3-phosphate [PtdIns(3)P] [27]. This is achieved by the activity of a PAS- and vacuolar membrane-associated class III phosphatidylinositol 3-kinase (PtdIns3K) complex comprising the Ser/Thr protein kinase VPS15, the PtnIns3K VPS34, as well as ATG6 and ATG14. The PtdIns(3)P thus produced then recruits other autophagy effectors – ATG18, ATG20, ATG21 and ATG24, which all bind this lipid molecule – to the PAS; in addition, ATG18 forms a complex with ATG2.

(4) Vesicle expansion and completion. During this process, at the PAS, the phagophore expands to form the autophagosome or Cvt vesicle. Two ubiquitin-like conjugation systems are involved in this process (Fig. 3A and B) (reviewed in Refs. [9,28]). In the first one, the ubiquitin-like modifier ATG8 is conjugated to phosphatidylethanolamine (PE), through the activity of ATG7, ATG3 and the complex ATG12-ATG5 as E1, E2 and E3-like proteins, respectively. In the second system the ubiquitin-like protein ATG12 is linked to substrate ATG5 by the E1 and E2-like activities of ATG7 and ATG10, respectively; here the E3 function is unknown. ATG16 can oligomerize and binds to the ATG12–ATG5 conjugate, resulting in the formation of a dimeric complex. In order to achieve the conjugation, ATG8 is first proteolytically processed at its C-terminus by ATG4, exposing a Gly residue. This ATG8 as well as ATG12, also having a C-terminal Gly, are then activated independently by ATG7, in an ATP-dependent reaction, forming a thioester bond with it and are subsequently transferred, via ATG3 or ATG10, respectively, to membrane-associated PE or ATG5. ATG8, covalently linked to PE, becomes so anchored to both sides of the phagophore membrane. The dimeric ATG12–ATG5–ATG16 complex covers predominantly the outer surface of the curved organelle. Both ATG8–PE and ATG12–ATG5–ATG16 are essential to drive the enlargement of the phagophore membrane. During this process, the integral membrane proteins ATG9 and ATG27, as well as the ATG9-associated ATG23 are brought to the phagophore, probably via membrane vesicles that fuse with it. These three ATG proteins are observed to continuously cycle between the phagophore and other, ill-defined membranous structures. After closure of the expanded phagophore membrane, the resulting double membrane-bounded vesicle loses its ATG12–ATG5–ATG16 coating. ATG4 then acts again on ATG8, cleaving off the covalently attached PE and thereby liberating it from the outer surface. The resulting cargo-filled autophagosome or Cvt vesicle, containing ATG8–PE only on its inner membrane surface, is now ready to fuse with the vacuole.

(5) Retrieval of ATG proteins used in the earlier steps for re-use. As mentioned in the previous section, ATG8, ATG5, ATG12 and ATG16 are released from the outside of the newly formed autophagosome and Cvt vesicle. These soluble or peripheral membrane proteins are easily retrieved to the PAS for re-use in further cycles. Also ATG9, ATG27 and ATG23 are continuously retrieved to the PAS from the expanding phagophore. However, retrieval of ATG9 requires PtdIns3K, the PtdIns3K-binding protein ATG18 and several other ATGs including ATG27 and ATG23.

(6) Docking and fusion. Docking of the autophagosome and Cvt vesicle at the vacuole/lysosome, and the fusion of membranes involve proteins that are not specific to autophagy, but are shared with other processes in the cell involving docking and membrane fusion events. Therefore, details of these steps of the autophagy pathways will not be further discussed here. For a review of the mechanism of membrane fusion in cells and proteins involved, see Ref. [29].

(7) Vesicle breakdown in the vacuole. Fusion of the outer membrane of the autophagosome/Cvt vesicle with the vacuolar membrane results in the appearance of the single membrane-bounded autophagic body/Cvt body within the vacuole. The body is lysed, in *S. cerevisiae*, by the activity of ATG15, an integral membrane protein with presumed lipase activity. The autophagic body's content is released and degraded as a result of the vacuole's acidic pH and the activity of peptidase B (PRB1). This peptidase probably activates various vacuolar zymogens responsible for further breakdown processes. Also operating in this last step is ATG22, an integral protein of the vacuole's membrane which shows sequence similarity to permeases of the major facilitator superfamily. It is probably involved in export of amino acids resulting from intra-vacuolar peptidase activity to the cytosol [30].

3.2. Ubiquitination and selective autophagy

Already for years, the small protein ubiquitin is known to play a major role in the turnover of soluble proteins in the eukaryotic cell and the degradation of damaged or misfolded proteins. Such proteins are tagged by addition of chains of ubiquitin moieties (polyubiquitination) and then routed to the proteasome for degradation. In the last decade, it has become clear that post-translational modification by ubiquitin also serves in a considerable number of other cellular processes such as cellular trafficking, DNA repair, immune response, and chromatin remodeling. Very recently, ubiquitination has also been shown to participate in a special form of selective autophagy, but so far only in mammalian cells (for a review, see Ref. [31]). This process begins with the polyubiquitination of substrates, which may be protein aggregates such as inclusion bodies and aggresomes, organelles such as mitochondria, peroxisomes or ribosomes, or even intracellular bacteria. These tagged substrates are then recognized by ubiquitin-binding receptors p62 and NBR1 (Fig. 3C). These proteins can simultaneously bind to ubiquitin, through their C-terminal UBA domain, and to the autophagosome-associated ubiquitin-like molecules LC3 or GABARAP, mammalian proteins belonging to two ATG8 subfamilies, through a short peptide (LC3-interacting region or LIR) somewhat upstream of their UBA domain. A similar motif is present in yeast ATG19, where it mediates the interaction with ATG8 in the Cvt pathway. As a result, the ubiquitinated substrates become sequestered in the autophagosome and are then routed to the lysosomes for degradation. No functional p62 or NBR1 orthologues have as yet been confirmed in non-mammalian organisms. However, recent data for a *Dictyostelium discoideum* protein sharing a similar domain structure to p62 and NBR1 are at least consistent with its having a similar function [32]. There are also gene models predicted for several other free-living protists (*e.g.*, the amoeboflagellate *Naegleria gruberi*), but not the parasites discussed here, that exhibit similar domain architecture to p62 and NBR1 [8].

Whether the proteasomal or the autophagosomal degradation route will be followed for an ubiquitinated substrate appears to be largely dependent on the architecture of the polyubiquitin chain. Substrates with branched K48-linked ubiquitin chains are mainly degraded in proteasomes. The UBA domain of p62 can bind both branched K48-linked and linear K63-linked ubiquitin chains, but has a higher affinity for the latter so that such conjugates are pref-

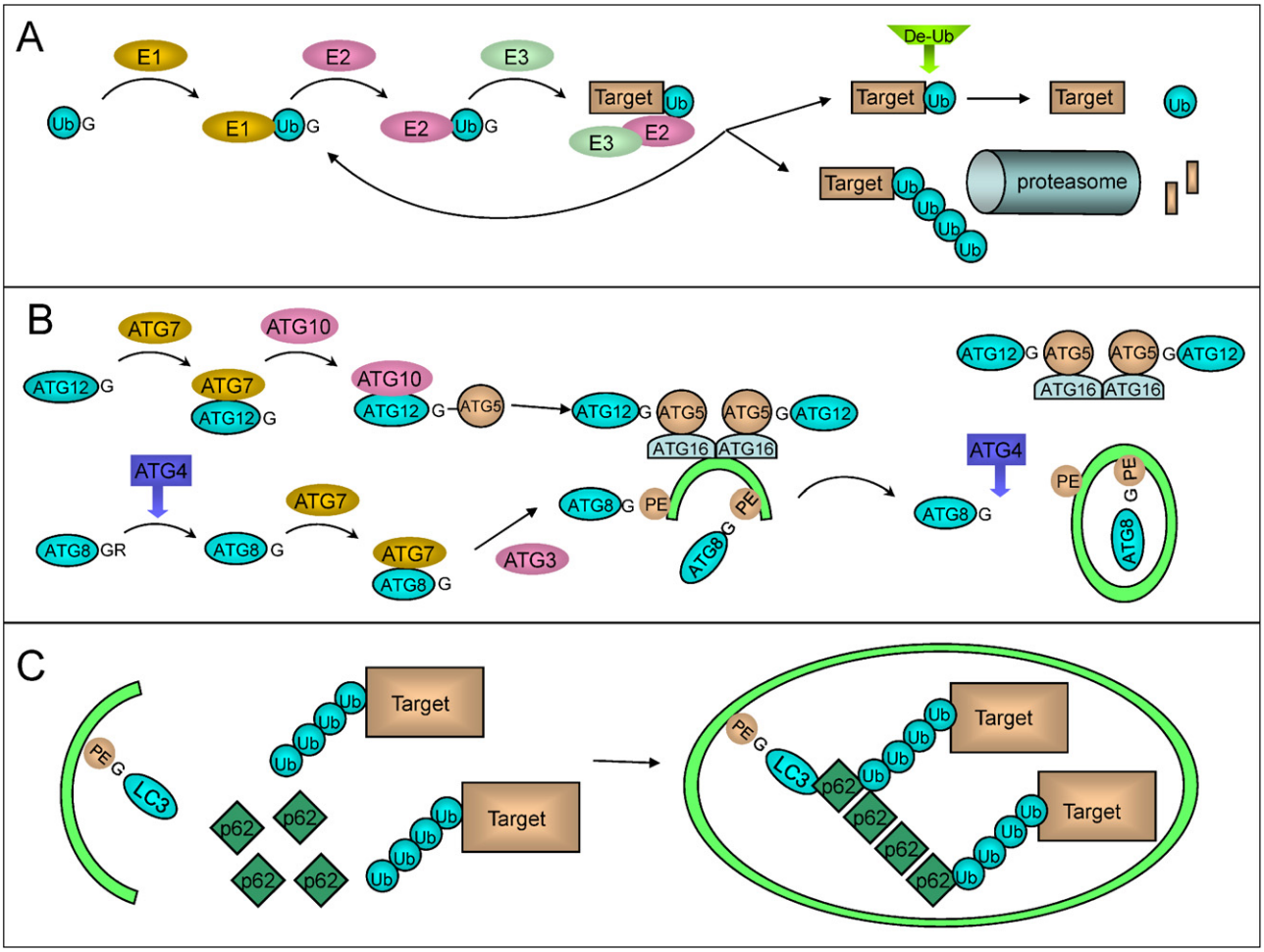


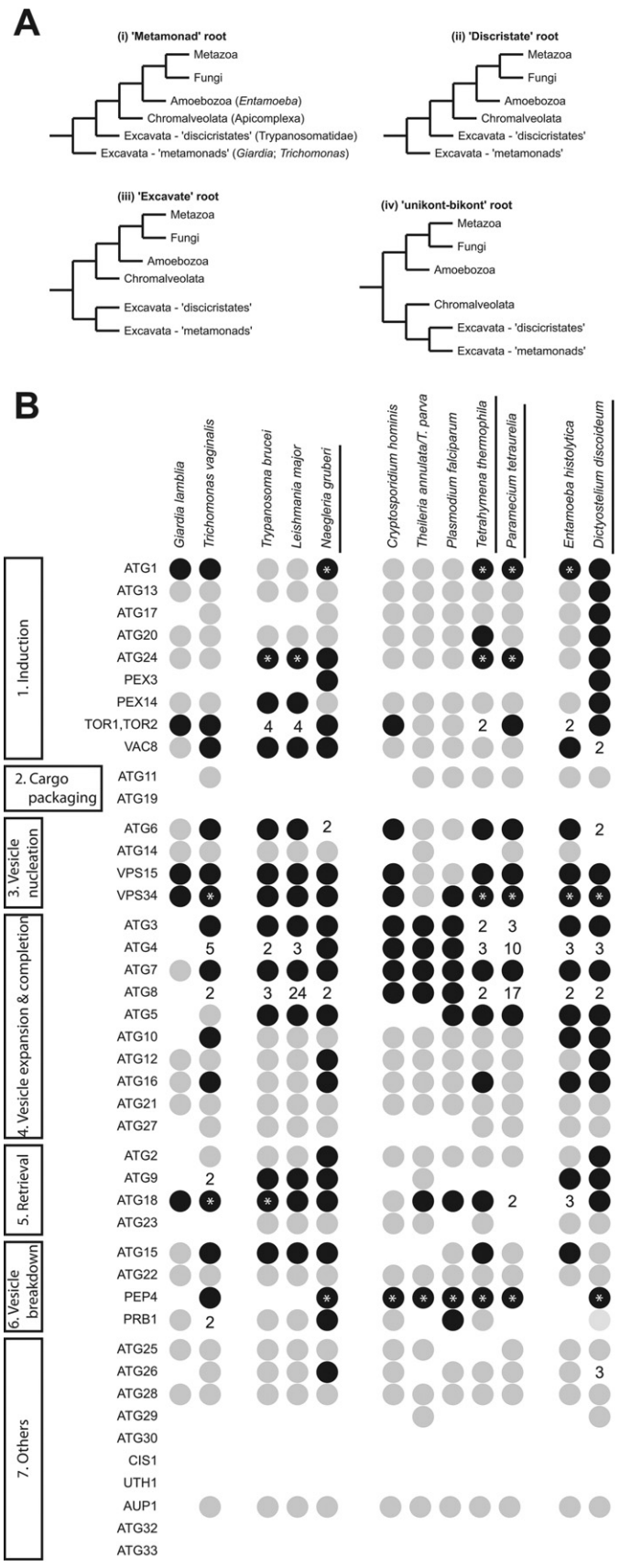
Fig. 3. Scheme of ubiquitin-like and ubiquitin-dependent conjugation systems involved in autophagy. (A) The classical ubiquitin-proteasome pathway. (B) The ATG12–ATG5–ATG16 and ATG8–ATG7–ATG3 systems. (C) The ubiquitin-p62-NBR1 system. Note that the colours indicate the similar functions executed in the different systems by the different proteins: blue: ubiquitin/ATG8/ATG12; yellow: E1/ATG7; pink: E2/ATG10/ATG3; green: E3; brown: Ub-conjugated target/ATG5/PE. ATG4 is an additional protein only functioning in the ATG8–ATG7–ATG3 pathway, in the C-terminal processing of ATG8 to expose a Gly residue (as is permanently present in ubiquitin and ATG12 in the other pathways) necessary for subsequent activation by E1. Moreover, after the autophagosome formation has been completed, ATG4 releases ATG8 from the external face of the organelle by cleaving the Gly-phosphatidylethanolamine linkage. The membrane of the phagophore and autophagosome are given in green. In C, for simplicity, only the role of p62 is given, but NBR1 acts similarly. For details, see the main text of the paper.

erentially routed to autophagosomes. However, the distinction is not absolute; on the one hand p62 has also been shown to compete for K48-linked ubiquitinated cargo with classical proteasomal receptors and on the other hand, cargo with K63-linked ubiquitin chains can also be degraded at proteasomes. Additional regulatory mechanisms are thus probably involved.

p62 can also bind single ubiquitin moieties, but only with low efficiency, probably because polyubiquitinated substrates can bind more p62 molecules. Nevertheless, mono-ubiquitination has been shown to be involved in pexophagy [33]. The attachment of a single ubiquitin moiety to the peroxisomal membrane protein PMP32 was sufficient to efficiently target mammalian peroxisomes, via p62 for autophagy.

4. The occurrence and role of autophagy in parasitic protists

In recent decades, awareness has grown that the diversity among eukaryotes is much larger than initially appreciated, particularly among protists. The classical division of these organisms into the four Kingdoms of animals, plants, fungi and protists has been superseded by the placement of known taxa into one of six eukaryotic 'supergroups' [34]. Well-known parasitic protists are found in some of these supergroups: Amoebozoa (*Entamoeba histolytica*), Chromalveolata (apicomplexan parasites), and Excavata (*Giardia*, *Trichomonas*, and the trypanosomatids). Relationships between some eukaryotic supergroups and the order with which each group diverged from the last common ancestor of all extant eukaryotes are unresolved, but this is not important in the context of this review. Rather, phylogenomic profiling recently revealed that putative orthologues of many core ATGs are encoded in species belonging to each eukaryotic supergroup, thereby providing very clear evidence to the ancient appearance of autophagy at an early point during eukaryotic evolution [7]. Moreover, in contrast to the uncertainty regarding relationships between some eukaryotic supergroups, relationships within each supergroup are generally better understood and more widely accepted. The identities of free-living relations to different parasitic protists are therefore known, and with genome sequences at hand, changes in autophagy pathways that are coincident with the adaptation to parasitism can be predicted. Bioinformatics-based predictions for the presence or absence of ATGs in the major parasitic protists and their nearest characterized free-living relatives are shown in Fig. 4. In the text below, these bioinformatics-based predictions are discussed in relation to experimental evidence pertaining to the function and regulation of autophagy-based pathways during parasite life



cycles. We start with the trypanosomatids, which are parasites for which a molecular basis for autophagy, its function and regulation is currently best understood.

4.1. Trypanosomatidae

4.1.1. The occurrence of autophagy and ATG proteins

Various trypanosomatid parasites are responsible for serious tropical diseases of mankind such as *Trypanosoma brucei* for human African trypanosomiasis (HAT) or sleeping sickness, *Trypanosoma cruzi* for Chagas' disease and various species of *Leishmania* for a variety of symptoms collectively known as leishmaniases and which occur in tropical and subtropical regions worldwide. All these parasites have complicated, digenic life cycles, involving insects as well as humans and other mammals as hosts. In addition, within each host, different life-cycle stages can be distinguished. The successive life-cycle stages are characterized by different morphologies and – at least for some of them – major differences in their catabolic and biosynthetic capacities as adaptations by the parasites to changes in their environment such as available nutrients, temperature, etc. It has been appreciated already for a long time that the morphological changes and metabolic reprogramming would involve an increased turnover of structural and enzymatic constituents of the cells. Indeed, electron microscopy studies of *T. brucei*, by Vickerman and colleagues in the 1970s, provided indications for the involvement of autophagy during differentiation of the parasites from one life-cycle stage to the next one [35,36]. Also supporting the notion that autophagy is important during differentiation of parasites is the observation that the morphology and activity of the lysosome in *Leishmania* species [37] and the corresponding organelle, called reservosome, in *T. cruzi*, are dependent on the developmental stage [38]. Moreover, the findings by Coombs and coworkers, in the 1980–1990s, of multiple genes for different peptidases in trypanosomatids and their life-cycle dependent regulation are indicative of an important role of protein degradation during differentiation (*e.g.*, see Refs. [39,40]; and for a recent review Ref. [41]). Indeed, it was later shown that peptidase inhibitors inhibit the differentiation of *T. cruzi* and *Leishmania* species [42,43].

The availability of genome sequences provided confirmation of the occurrence of autophagy in the trypanosomatids. Genome database searches in conjunction with more elaborate analyses have revealed that half of the proteins known to be involved in one or more of the autophagy pathways in *S. cerevisiae* have orthologues in the trypanosomatids [7,44–47]. However, it should be noted that for a number of the yeast ATG proteins – ATG19, ATG21, ATG22, ATG23, ATG25, ATG28 ATG29, ATG30 and ATG31 (or CIS1) – no counterparts have been found so far in organisms other than fungi, suggesting these are proteins that have evolved only in fungi. Similarly, novel lineage-specific autophagy-related proteins have also been found in animals [16]. By inference, it is therefore likely that

Fig. 4. The distribution of ATG homologues in parasitic protists. (A) The evolutionary positions of parasitic protists in four alternative views of eukaryotic phylogeny (i–iv) [124–126] indicates the absence of core components of macroautophagy pathways is a consequence of secondary loss, gene displacement or gene divergence, rather than a primitive absence. (B) Occurrence of ATG homologues in parasitic protists and, where genome information is available, near free-living relations (taxa underlined). Genome sequences were subject to iterative and reciprocal BLAST analyses as described previously [7]. Black circles identify putative orthologous sequences detected using ATG queries from *Saccharomyces cerevisiae* and/or other organisms – see Rigden et al. [7] and Duszenko et al. [8] for a breakdown of putative orthologues not detected by a *S. cerevisiae* query. Grey circles, prediction of homologous, but not orthologous sequence(s) to ATG queries. No circle, no hit with *e*-value cut-off of 0.000001 was detected. All putative orthologues were subject to manual scrutiny to check for expected domain architecture. Numbers denote the number of isoforms present in paralogous gene families that place the original query sequence top by reciprocal genome BLAST and include the putative orthologue. Asterisks denote gene families where the number of paralogues of the predicted orthologue was unclear.

lineage-specific autophagy-related proteins also await discovery in parasites and other protists. In trypanosomes, such proteins are likely to be found through forward genetic screens – increasingly possible now due to the advent of RNA interference (RNAi) libraries [48,49] – or through the analysis by mass spectrometry of biochemically isolated complexes. With reference to the ATG proteins known from yeast and other organisms, a comparison between the trypanosomatids and their nearest (although still very distant) free-living relation for which a genome sequence is available, the heterolobosean amoeboflagellate *Naegleria gruberi* [50], suggests that the trypanosomatid autophagy pathway has not been subject to extensive streamlining during the adaptation to parasitism (Fig. 4).

Both ubiquitin-like conjugation systems are present in the trypanosomatids. Orthologues of the components of the yeast ATG8–ATG7–ATG3 system could easily be detected [44–47], but the identification of candidate components from the other system, involving ATG12, ATG5 ATG10 and ATG16, was less obvious. Possible candidates were found for ATG5 and ATG10, but with low sequence similarity to their orthologues in other organisms [51]. Nevertheless, their functional identity was confirmed by complementation of respective yeast mutants. Interestingly, an *L. major* ATG12 candidate was also identified [51]. On the one hand, this protein fails to cluster reliably with yeast and mammalian ATG12 sequences by phylogenetic analyses, shows more similarity to ATG8 sequences, notably in the N-terminal region (DJR, unpublished) and, unlike other orthologues of yeast ATG12, does not terminate at its scissile glycine. Nevertheless, it complements a yeast mutant – although complementation requires expression in a form truncated after the scissile glycine – and has accordingly been named ATG12. In agreement with this function, it is localized in part to ATG8-containing puncta in *L. major* promastigotes.

The gene encoding ATG8, the classic autophagy marker, appears to have undergone multiplication in some trypanosomatids, notably in *Leishmania*. Paralogue expansion of ATG8 genes has been seen in other organisms too – for instance in mammals where at least eight different copies may occur belonging to two subfamilies; all are essential for autophagy, but act at different stages of the process [52]. In contrast, yeast possesses only one ATG8 gene. In *L. major* 25 genes are annotated as coding for ATG8-like proteins. These proteins are classified in four families: an apparently canonical ATG8 present as a single gene copy per haploid genome, ATG8A (encoded by three annotated genes), ATG8B (eight gene copies) and ATG8C (13 gene copies). The four families are also conserved in other *Leishmania* species although, with the exception of the single copy *ATG8* gene, numbers of copies are likely to differ [51]. As with mammalian cells, the different ATG8 subfamilies in *Leishmania* have different functions (see Section 4.1.3). Whether the multiple isoforms within each family are functionally redundant or have evolved distinct functions remains to be determined.

In the African trypanosome *T. brucei*, the genes encoded by Tb927.7.5900 (designated TbATG8.1) and Tb927.7.5910 (TbATG8.2) are, by phylogenetic analysis, both most closely related, among *Leishmania* proteins, to *L. major* ATG8, although genomic synteny is not conserved between any of the *Leishmania* and *Trypanosoma* sequences in the EuPath database (http://tritrypdb.org) [53]. Recently, the expression of a *YFP:TbATG8.2* gene fusion was used to suggest the likely presence of autophagosomes in procyclic cells exposed to ER stress [54]. The crystal structure of TbATG8.2 [55] exhibits the characteristic ubiquitin fold, and is very similar to that of the human ATG8/LC3. Unusually, the peptide sequence of TbATG8.1 ends atypically with a Gly residue, suggesting it is probably active without a need for processing by ATG4. The gene encoding a third *T. brucei* ATG8 homologue, Tb927.7.3320, is syntenic with the *L. major* gene encoding the ATG8-like protein that functionally resembles yeast ATG12. As homology combined with the conservation of genomic synteny often provides a good predictor of orthology in different trypanosomatid species, the current annotation of Tb927.7.3320 as 'TbATG8.3', while reflecting its closer sequence relationship to ATG8 sequences than to ATG12 proteins, could be said not to fairly reflect a probable ATG12-like function.

In the American trypanosome *T. cruzi* there is a single *ATG8* homologue (described as *TcATG8.1* in Ref. [46]) that exhibits synteny with the tandemly duplicated pair of *TbATG8.1* and *TbATG8.2*, and this gene can therefore be predicted to be orthologous to *L. major ATG8*. This *T. cruzi* protein complements yeast *atg8*Δ mutants, is processed by both *T. cruzi* ATG4 proteins (see below), and the HA-tagged protein localizes with a punctuate pattern in the cytoplasm of epimastigotes [46]. A *T. cruzi* gene annotated by Alvarez et al. [46] as *TcATG8.2* is syntenic with *L. major ATG12*. Since the *T. cruzi* protein was unable to complement a yeast *atg8*Δ mutant and shows, when expressed with a N-terminal HA-tag, a different localization pattern to HA-tagged TcATG8.1 [46], the combined biochemical and genomic evidence points towards an alternative to a typical ATG8-like function for this trypanosomatid protein, too. Curiously, although the enzyme responsible for processing of *L. major* ATG12 is unknown [51], the probable *T. cruzi* orthologue is processed at its scissile glycine by *T. cruzi* ATG4 homologues [46]. Orthologues of the *Leishmania ATG8A*, *ATG8B*, and *ATG8C* sub-families are not predicted in *Trypanosoma* species. The identification of the likely developmental patterning and/or ecological pressure responsible for the paralogue expansion of the ATG8-family in *Leishmania* or a closely related ancestor represents an intriguing avenue for future study.

For ATG4, two forms were detected in each of the three trypanosomatids. Some functional studies have been performed for the *T. cruzi* and *L. major* ATG4s. Both *T. cruzi* genes, *TcATG4.1* and *TcATG4.2* complemented, with similar efficiency, a yeast deletion mutant and restored the Cvt and autophagy pathways [46]. But only TcATG4.1 could efficiently process both *T. cruzi* ATG8 homologues (TcATG8.1 and the perhaps misleadingly labeled TcATG8.2 – see above) *in vitro*, suggesting that this isoform participates predominantly in these pathways in *T. cruzi*, with the function of TcATG4.2 still to be determined. In *L. major*, LmATG4.1 is able to proteolytically process ATG8, and proteins of the subfamilies ATG8B and ATG8C, whereas LmATG4.2 only exerts activity towards ATG8A proteins [51]. As pointed out earlier, the peptidase responsible for processing the *Leishmania* ATG8-like protein with ATG12 function remains to be determined.

Animals studied thus far have a single TOR, participating in the two different TOR complexes TORC1 and TORC2 that coordinate cell growth, but many unicellular eukaryotes, including yeast and *Trypanosoma* have two TOR proteins, TOR1 and TOR2 [45,56,57]. In addition, two TOR-related proteins (TOR-like 1 and TOR-like 2) have been identified in *T. brucei* [56]. For *L. major* three TOR kinases have been reported, designated TOR1, TOR2 and TOR3, with TOR3 corresponding to TOR-like 1 in *T. brucei* [58]. This latter protein contains a PDZ domain, characteristic for protein-protein interactions. The TOR-like 1 and TOR3 proteins were recently shown to be required for control of polyphosphate levels and acidocalcisome maintenance in *T. brucei* [59] and acidocalcisome biogenesis in *Leishmania* and infectivity of these latter parasites in the mammalian host [58]. TOR1 and TOR2 of *T. brucei* are part of the complexes TORC1 and TORC2, respectively [56,57]. Like in other organisms, these complexes contain other characteristic proteins like the orthologues of yeast KOG1/raptor in complex 1 and AVO3/rictor in complex 2. As in other organisms, the TOR complexes are found at different localizations within the trypanosome: TORC1 is predominantly in the nucleus, whereas TORC2 is cytoplasmic with association with the ER and mitochondrion.

Usually in eukaryotes, only TORC1, which controls temporal cell growth including the involvement of autophagy, is sensitive

for the macrolide antibiotic rapamycin, a compound known to induce autophagy in many organisms; TORC2 that regulates spatial cell growth, is rapamycin resistant. The situation is dramatically different in *T. brucei*, rapamycin has no effect on TORC1, even at concentrations up to 1 μM, whereas it caused an important inhibition of cell proliferation, with an EC_{50} value of 152 nM, by preventing formation of the TORC2 complex. The depletion of TOR1 by RNAi resulted in a reduction of protein synthesis, cell size, but not overall cell morphology, and cell proliferation by arresting cell cycle progression in the G_1 phase. Moreover, a role in triggering autophagy in trypanosomes, despite its insensitivity to rapamycin, was suggested by the appearance of electron-dense vacuoles and double-membrane vesicles containing cytoplasmic material reminiscent of autophagosomes when RNAi mutants were analysed by transmission electron microscopy [56,57].

4.1.2. Compounds triggering autophagy

Several compounds have been shown to induce autophagy in trypanosomatids. Duszenko and coworkers reported that rapamycin, at an EC_{50} = 6.5 μM, causes in *T. brucei* the formation of vacuoles stained with monodansylcadaverine (MDC), an autofluorescent compound initially thought to label autophagosomes but now assumed to be more specific for acidic vacuoles such as autolysosomes [60]. Moreover, electron microscopy revealed structures reminiscent of phagophores, autophagosomes and autolysosomes and densely packed membrane clusters associated with the kinetoplast similar to those found upon starvation but not under other stress conditions. Even though the rapamycin concentrations required were at least 10-fold higher than those necessary to induce autophagy in yeast, this finding is surprising because *T. brucei* TORC1 is entirely insensitive to rapamycin. Therefore it is important to establish how the observed effects are caused and if indeed, they represent death by an authentic autophagy. No indications for apoptosis or necrosis were noticed.

The same group also showed that dihydroxyacetone (DHA) induces autophagy in *T. brucei* [61,62]. The compound enters the parasites via aquaglyceroporins but, because of the lack of DHA kinase activity in the trypanosomes, cannot enter the glycolytic pathway as dihydroxyacetone phosphate. As a result, it causes cell cycle arrest in the G_2/M phase and morphological changes indicative of autophagy: formation of multivesicular bodies and vacuoles and an alteration of the mitochondrial structure.

Gonzalez-Rey and coworkers [63,64] discovered that some neuropeptides cause death of bloodstream-form trypanosomes by disrupting the energy metabolism with an apparent involvement of autophagy. This is different from the typical mechanism by which antimicrobial peptides act, *i.e.*, forming pores or causing membrane damage. Instead, these neuropeptides are endocytosed by trypanosomes after which they reach the lysosome which may be disrupted. Moreover, the peptides can be released from late endosomes or lysosome and then accumulate in various intracellular compartments. Their interaction with glycosomes causes relocalization of glycosomal enzymes with consequent disruption of the ATP supply of the cell. This causes cell death involving progressive formation and fusion of multiple lysosome-like vesicles and other vacuoles that can be labeled with anti-mammalian LC3 (ATG8) antibodies indicating that they are autophagosomes.

In *T. cruzi*, growth inhibition combined with ultrastructural changes indicative of autophagy, such as the formation of autophagosome-like bodies, appearance of vacuoles containing damaged membranes and the occurrence of concentric membrane structures in the cytosol have been observed in different life-cycle stages of the parasites after their treatment with compounds as diverse as protein kinase and PtdIns3K inhibitors [65], lysophospholipid analogues alone or combined with an ergosterol biosynthesis inhibitor [66], microtubule-disrupting taxol and dinitroanilines (trifluralin and chloralin) [67], and a variety of natural products, or derivatives of them, of which the precise molecular targets in the cell remain to be identified [68–72]. For a more detailed description of the effects caused in the parasites by these different compounds, we refer to the original publications or the reviews by Menna-Barreto et al. [73] and Duszenko et al. [8].

Also for various life-cycle stages of *Leishmania* species it has been reported that autophagy could be induced by treatment with various compounds. Antimicrobial peptides caused induction of autophagic cell death in promastigotes of *L. donovani* [74]. MDC-labeled vesicles appeared and electron microscopic studies showed extensive degeneration of the cellular organization with cytosolic vacuolization without disruption of the plasma membrane. Also different types of inhibitors of ergosterol synthesis with antiproliferative effects caused ultrastructural changes suggestive of autophagic cell death in both pro- and amastigotes of *L. amazonensis* [75,76].

Together, these data suggest that probably a large variety of compounds that have the capacity to interfere with the functioning of one of the important metabolic pathways of these parasites and affect their homeostasis causing stress could trigger an autophagic response.

As mentioned above, several compounds cause autophagic cell death in trypanosomatids. Together with necrosis and apoptosis it is considered a mechanism of 'programmed cell death' (PCD) [77]. Each of these three processes is characterized by a sequence of specific morphological and biochemical events. Although necrosis and apoptosis are typical processes of PCD, both in multicellular organisms and in protists, it should be stressed that autophagy is generally considered as a process that is not primarily meant to cause cell death but rather to support cell survival under conditions of starvation or to remodel the cell during differentiation. Only when starvation continues or autophagy has been triggered by mechanisms that simultaneously block apoptosis, may autophagy continue and result finally in cell death. For a further discussion of the occurrence and role of the three different PCD mechanisms in parasitic protists, we refer the reader to the recent review by Van Zandbergen et al. [78].

4.1.3. Autophagy in differentiation

Trypanosomatids have complicated life cycles during which the parasites change their morphology, subcellular organization and metabolic capacity. As mentioned above, already in the 1970s morphological indications were obtained that autophagy plays a role in the transition between successive differentiation steps of the life cycles. Indeed, in recent years convincing molecular evidence has been provided that supports the essential role of autophagy in differentiation of trypanosomatid parasites.

In *T. cruzi*, the transition of epimastigotes to metacyclics is triggered by nutritional stress within the rectum of the insect vector. This can be reconstituted *in vitro*, by starvation of cultured epimastigotes. Epimastigotes can endocytose proteins and lipids as nutrients and store them in the reservosome, an organelle unique to this stage that is part of the lysosomal system. Upon starvation, in culture or the insect's rectum, the reservosomal content is degraded, notably by cruzipain, the most abundant cysteine peptidase of the lysosome, and whose activity is critical for differentiation [46,79]. The reservosome then shrinks and disappears and the epimastigotes differentiate to metacyclic trypomastigotes. Such differentiating epimastigotes display autophagosomal structures that are recognized by anti-TcATG8.1 [46]. The staining decreases considerably in metacyclics where, during differentiation, the ATG8 signal colocalizes with that of serine carboxypeptidase, indicative of delivery of the autophagosome content to the lysosomal system.

More molecular information about the role of autophagy in differentiation is available for *Leishmania*. The importance of protein turnover for differentiation of this parasite, and the role of its unusual lysosome, has been known for several decades (reviewed in Ref. [41]). In promastigotes, the lysosome is present as a large single vesicular structure also known as 'multivesicular tubule' (MVT) with a relatively high lumenal pH value and low lytic capacity (reviewed in Ref. [37]). Upon differentiation of metacyclic promastigotes to intracellular amastigotes the size of the organelle increases considerably. The form and the number of lysosomes in amastigotes depend on the species. Initially, this organelle was called a 'megasome' because of its large size: it may occupy 5–15% of the total cell volume [37,80]. Furthermore, the lytic capacity of the organelle also increases, notably that of the two main lysosomal peptidases, the cysteine peptidases CPA and CPB. These peptidases have been shown to be involved in cellular reshaping and metabolic reprogramming during differentiation and also nutrient acquisition by the different life-cycle stages [41]. Inhibition of these enzymes, or deletion of both genes, results in promastigote cells that are more susceptible to nutrient withdrawal, fail to differentiate and do not degrade GFP-ATG8 labeled autophagosomes within the lysosome [44].

Besteiro and coworkers [81] analyzed the phenotype of a *L. major* dominant-negative mutant of the yeast VPS4 orthologue, an ATPase known to be involved in a late step of the endosomal system. *Leishmania* cells expressing the mutant form of VPS4 appeared to be deficient in endosome sorting, as predicted, but interestingly, autophagy and differentiation of promastigotes to obligate infective metacyclic promastigotes were also affected. In wild-type cells, the GFP-ATG8 staining is most intense, and autophagosomal structures are most abundant, during the differentiation of procyclic promastigotes to infective metacyclic promastigotes. In metacyclic parasites the signal has moved to the MVT-lysosome. However, in the mutant cells, which failed to undergo metacyclogenesis, the number of autophagosomal structures that are stained remains high even in early-log phase promastigotes and the level of ATG8–PE conjugate is consistently higher than in wild-type cells. These data point both to an inability of the autophagosomes to fuse with the endosomal/MVT-lysosomal compartment and to the importance of autophagy and late endosomal function for the transformation to infective metacyclics. Further support for the notion that autophagy plays a role in differentiation is the observation that promastigotes of a *L. major ATG4.2* null mutant appeared to be defective in transforming into amastigotes [81].

L. major ATG8 and representatives of each of the three other *ATG8* families of this species are able to complement a *S. cerevisiae atg8*Δ mutant, despite the low level of sequence conservation. This indicates that each of them can fulfill the ATG8 function in the Cvt pathway [51]. However, only LmATG8, not the paralogues, LmATGA, B and C, seems to possess the complete ATG8 physiological function because it is the only isoform for which also an involvement in autophagosome formation during both differentiation and starvation could be demonstrated, thus far [51]. None of the three other isoforms, when expressed as a protein fused to GFP, resulted in the appearance of fluorescent puncta during metacyclogenesis; in the case of GFP-ATG8A such puncta were only observed upon starvation. A single punctum, located close to the flagellar pocket, was found for GFP-ATG8B and GFP-ATG8C.

Trypanosomatidae possess, like many other eukaryotes, peroxisomes. However these organelles of Kinetoplastida are unique in that they contain the majority of the glycolytic enzymes, hence their naming as glycosomes in these species [82,83]. In some instances, *e.g.*, bloodstream-form *T. brucei*, glycolytic enzymes may contribute over 90% of the protein content of the organelles. Enzymes of some other important catabolic and anabolic pathways can also be found in glycosomes, such as those involved in gluconeogenesis, the pentose-phosphate pathway, β-oxidation of fatty acids, purine salvage and the biosynthesis of pyrimidines, ether lipids and squalenes, as well as typical peroxisomal enzymes responsible for peroxide metabolism.

Glycosomes are present at 10–100 copies per cell, dependent on the species. Furthermore, the importance of the different metabolic pathways or enzymes inside the glycosomes varies with the environmental conditions encountered during the life cycle of the parasites. Indeed, glycosomes of *T. brucei* exhibit considerably different enzyme content among successive life-cycle stages [84–86], and pexophagy seems to play an important role in these changes of the metabolic capacity by increasing glycosome turnover during the differentiation of trypanosomes from one stage to another [87] (Fig. 5). Strong indications for such a role of pexophagy were obtained by immunofluorescence microscopy studies which revealed a tendency of glycosomes to associate with the lysosome in slender bloodstream forms transforming into stumpy forms: colocalization was observed in 40% of the trypanosomes, whereas less than 10% of non-differentiating cells display such colocalization. A fast and drastic increase in the association of the organelles was observed in all trypanosomes during their transition from stumpy to procyclic forms. Additionally, the lysosome undergoes a dramatic transient size increase during this latter differentiation process. Immunoelectron microscopy confirmed the pexophagy of glycosomes and showed the appearance of glycosomal enzymes within lysosomes during the differentiation steps [87]. Interestingly, the observed encircling of glycosomes by the lysosome is suggestive of micropexophagy, whereas the size increase of the lysosome may instead suggest macropexophagy involving the formation of autophagosomes. In contrast, in *Leishmania* only macroautophagy has been observed so far [44,81]. Whether African trypanosomes and *Leishmania* species use different degradation pathways, or if both pathways can function in all trypanosomatids but the induction of either depends on the conditions encountered, as observed for *H. polymorpha* and *P. pastoris* [88], remains to be determined.

Leishmania, like *T. brucei*, encounters highly different nutritional conditions between the extracellular promastigote stage in the sandfly and the amastigote stage inside the phagolysosome of the mammalian macrophage. Consequently, metabolism of promastigotes and amastigotes differs considerably. Gluconeogenesis rather than glycolysis is more important in amastigotes than in promastigotes [89,90]. However, there are no conclusive data suggesting that the metabolic reprogramming during promastigote-to-amastigote differentiation involves an increased turnover of glycosomes by pexophagy, as occurs during differentiation of *T. brucei*. It seems probable that, in *Leishmania*, the altered metabolic fluxes result largely from the different nutrient availability and/or post-translational regulation of enzyme activity, rather than from a different enzyme network [91]. Nevertheless, pexophagy may play a role during this differentiation, since, with the considerable reduction of the size of parasites, the number of glycosomes per cell is also decreased in such a way that the relative glycosomal volume remains rather constant [80,92]. Pexophagy thus may be involved in adjusting the glycosome number, consistent with other observations on the role of autophagy in cellular remodeling.

4.2. Micro-aerophilic parasites (Giardia, Trichomonas, and Entamoeba)

Although orthologues of only around half of the known *S. cerevisiae ATG*s have been identified in the trypanosomatids, the core components required for initiation of autophagy and expansion of the autophagosome are unmistakably present (see above; Fig. 4). Ubiquitination-like conjugation systems are also seen in the

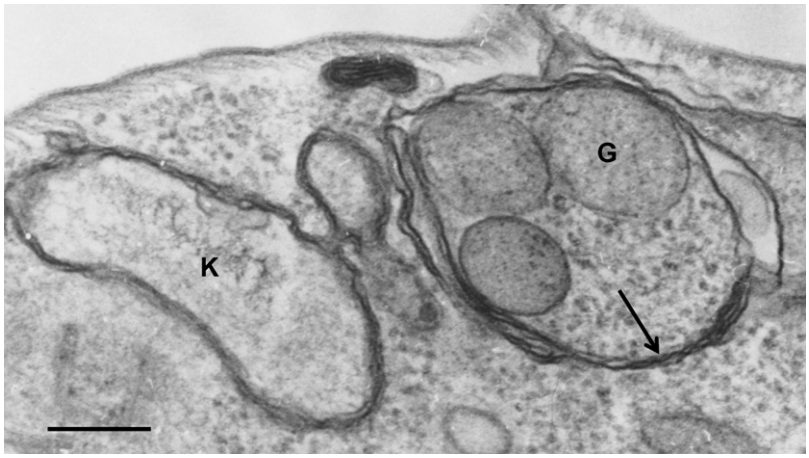


Fig. 5. Pexophagy in *Trypanosoma brucei*. Transmission electron micrograph of a *T. brucei* procyclic form cell showing an autophagosome-like structure (arrow) sequestering three glycosomes (G). K, kinetoplast. Bar is 300 nm.

microaerophilic parasites *Entamoeba histolytica* and *Trichomonas vaginalis*, which are well known for their reductive evolution of central energy metabolism. In these parasites mitochondrial function has degenerated through the loss of oxidative phosphorylation, TCA cycle activity and other pathways, and microbodies of the peroxisome class are absent [93,94]. However, despite the simplification of central metabolism in these protists, the genomic signature of autophagy is easily recognised and the available experimental evidence points towards the involvement of autophagy in encystment of *E. histolytica* [95] and the possibility that autophagy might be involved in the enigmatic presentation of glycolytic and hydrogenosomal enzymes on the cell surface of *Trichomonas* following contact with endothelial cells has been discussed briefly [96,97]. It is therefore intriguing that ATG8- and ATG12-dependent conjugation systems cannot be detected in the genome sequence of *Giardia lamblia*, another micro-aerophile with a streamlined central metabolism [98], indicating that in this parasite autophagy, at least according to known paradigms, does not operate [7].

Clearly, the examples of *Trichomonas* and *Entamoeba* indicate that a simplification of central metabolism is not automatically a prelude to loss of autophagy. The absence of autophagy in *Giardia* is also unexpected because the parasite undergoes extensive intracellular re-modeling during encystment. However, many aspects of giardial cell biology are subject to minimalism [98,99] – most widely accepted views of eukaryotic evolution indicate this is the consequence of secondary loss rather than primitive absence – and the lack of canonical autophagy perhaps correlates with the absence of typical lysosomes in *Giardia*. Instead of 'classic' lysosomes, peripheral vacuoles which sit beneath the plasma membrane appear to provide a function that is analogous to that provided by lysosomes, but intriguingly these vacuoles form a contiguous, dynamic interface with a tubulovesicular network that extends throughout the cell and serves an ER-like function [100]. Interestingly, increased vacuolation is evident at the periphery of the giardial cells following nutrient starvation [101] or treatment with sterol biosynthesis inhibitors 22,26-azasterol and 24(*R*,*S*),25-epiminolanosterol [102]. Autophagosomal like structures are also evident following azasterol treatment [102], although the biological mode of action of azasterol treatment is uncertain since *Giardia* neither makes sterols *de novo* nor is known to modify host-acquired sterols. Given these preliminary data, the possible role of the putative TOR kinase orthologue (Fig. 4) in the regulation of intracellular re-modeling and the response to nutritional or xenobiotic stress represents an interesting avenue for future study.

4.3. Malarial parasites

The phylum Apicomplexa comprises about 5000 species, and some of them are life-threatening parasites for humans and animals. Among these pathogens, four species of *Plasmodium*, notably *P. falciparum*, are responsible for human malaria. *Toxoplasma* and *Cryptosporidium* can cause fatal encephalitis and diarrhoea respectively, in immunocompromised individuals, and *Theileria* is a major animal pathogen in Africa. Transmission is either vector-borne (malaria, theileriosis) or follows from environmental sources (cryptosporidiosis) and/or ingestion of infected tissues (toxoplasmosis). All of these parasites have complex life cycles, alternating between sexual and asexual stages. Other shared characteristics include a mode of active invasion involving unique parasite organelles [103], an intracellular lifestyle in mammalian cells, and in parasites other than *Cryptosporidium* a relict plastid, known as the apicoplast, that contains essential anabolic pathways [104]. This section focuses on the potential role of ATG homologues in the life cycle and differentiation of malaria parasites. Very little is known, as yet, about autophagy in other Apicomplexa genera.

Before a victim ever succumbs to the clinical symptoms of the malaria disease, which present themselves during the blood stage of infection, the clinically silent sporozoites invade the body and develop in the liver. *Plasmodium* sporozoites make a remarkable and rapid journey from the skin, in which they are deposited by an infected mosquito, to the liver. Following penetration into hepatocytes, the sporozoite converts from an invasion-competent, motile and elongated form to a metabolically active, round trophozoite. Subsequent to this metamorphosis, a mitotic phase begins, transforming the parasite into a giant multi-nucleate schizont. After a few days, thousands of merozoites are released from the liver into the bloodstream, whereon they invade and replicate within erythrocytes. The sporozoite-to-trophozoite differentiation is accompanied by the elimination of organelles unnecessary for the schizogony and production of merozoites in liver cells [105]. Organelles that are cleared from sporozoites include (i) the inner membrane complex that is composed of flattened cisternae beneath the plasma membrane and is essential for the maintenance of the parasite shape and motility [106]; and (ii) micronemes and rhoptries that are secretory vesicles involved in hepatocyte adhesion and penetration [107,108]. The inner membrane complex is expelled 'en bloc' from the parasite into the parasitophorous vacuole, thus suggesting a mechanism of organelle exocytosis that is operational in sporozoites [105]. In contrast, micronemes and rhoptries are compartmentalized in the cytoplasm of converting sporozites and sequestered in double-membrane structures

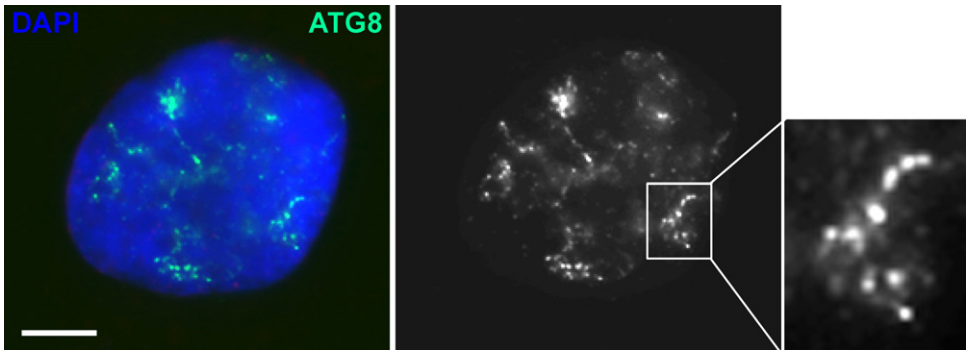


Fig. 6. Expression of ATG8 in *Plasmodium* infecting liver cells. Immunofluorescence microscopy of *Plasmodium berghei* 43-h post-invasion of hepatoma cells labeled with anti-PbATG8 antibodies and DAPI. This picture illustrates the morphology of the ATG8-containing compartments during schizogony (post-conversion) as abundant vesicles (inset) organized in a reticulate network. Scale bar is 6 μm.

resembling autophagosomes. This suggests a process of functional autophagy present in the malaria parasite during the metamorphosis (B. Jayabalasingham and I. Coppens, unpublished data). This suggestion was substantiated by the effect of 3-methyladenine (3-MA), a known autophagy inhibitor that targets VPS34 within the PtdIns3K complex [109]. Treatment of the parasites with 3-MA resulted in a significant delay in the sporozoite differentiation process [110].

In addition to VPS34 [110], *Plasmodium* genomes encode autophagy-related proteins. Of the 31 ATG proteins present in yeast, 9 ATG putative orthologues have been clearly identified in malaria parasites: ATG1, ATG17, ATG18 of the ATG9 cycling system, ATG12 of the ATG12 conjugation system, and ATG3, ATG4, ATG7 and ATG8 of the ATG8 conjugation system. The malaria parasite is thus another protist having a limited repertoire of *ATG*-related genes. In contrast to Trypanosomatidae that contain several *ATG8* genes, only one *ATG8* gene is present in each *Plasmodium* species, and the TOR complex machinery is absent from the *Plasmodium* genome. Comparison of the inventories of ATG homologues present in apicomplexan parasites and near free-living relatives, the ciliates *Paramecium tetraurelia* and *Tetrahymena thermophila*, indicate that absence or divergence of TOR and associated complexes acting in autophagy induction has occurred after the divergence of these close evolutionary groups. Curiously, however, the otherwise well-conserved ATG9 is missing detectable homologues in both ciliates and apicomplexans.

Putative ATG orthologues of the human malaria parasite *P. falciparum* and the rodent malaria parasite *P. berghei* are 50–87% identical to each other. The single *P. berghei* orthologues of ATG8 and ATG3 share close structural similarity with their yeast and mammalian counterparts (B. Jayabalasingham and I. Coppens, unpublished data). The PbATG8 sequence predicts two N-terminal α-helices tethered to an ubiquitin fold core. It is predicted to have two hydrophobic patches as well as a number of conserved basic residues on one face of the protein that form the scaffold for binding to other ATG proteins. Many of the surface-exposed residues located on the opposite face of the protein exhibit low conservation, which reflects a reduction in evolutionary constraints perhaps to mediate unique and diverse interactions among ATG8 homologues. To this point, *Plasmodium* ATG8 has a unique inserted loop consisting of 9 amino acids between α-helix 3 and α-sheet 3 which is absent in yeast and mammalian ATG8/LC3 homologues, suggesting that such binding partners may exist. A functionally important residue conserved among all ATG8 homologues is a C-terminal Gly. In most homologues of ATG8, this Gly is followed by additional amino acids that are cleaved from the protein by ATG4. In this respect, the *Plasmodium* ATG8 sequences contain a C-terminal glycine that is not followed by additional residues, suggesting that the processing of ATG8 by ATG4 in *Plasmodium* is not necessary.

The predicted sequence of PbATG3 contains the catalytic cysteine as found in other ATG3 proteins. Other conserved regions include: the 'handle region', which mediates the binding between ATG3 and ATG8, and the 'flexible region', which is implicated in the enzyme's conjugation activity and binding to ATG7 [111]. The N-terminal region of yeast ATG3 interacts with ATG8 to form an intermediate that appears to be essential for the formation of the ATG8-PE moiety [112]. Recombinant *Plasmodium* ATG8 and ATG3 also physically interact (B. Jayabalasingham and I. Coppens, unpublished data), however, unlike the yeast ATG3-ATG8 interaction, the interaction between the *Plasmodium* proteins is not dependent on any single region of ATG3. Indeed, PbATG8 is able to interact with the 'flexible region' of PbATG3, as well as with PbATG3 lacking either the 'flexible region' or the C-terminal region encompassing the catalytic cysteine residue. Unsurprisingly, PbATG8 cannot interact with yeast ATG3 and is unable to complement a yeast *atg8*Δ mutant probably due to the structural disparities between *Plasmodium* and yeast ATG3. Additionally, when expressed in mammalian cells, *Plasmodium* ATG8 never associates with autophagosomes, but remains cytosolic (B. Jayabalasingham and I. Coppens, unpublished data). It is possible that the specific conformation of *Plasmodium* ATG8 is responsible for the lack of interaction of this protein with the mammalian autophagy machinery, and that in general the interactions of *Plasmodium* ATG8 and partners have evolved in a species-specific manner.

ATG8 is expressed by *Plasmodium* blood and liver forms, but is absent from insect stages (B. Jayabalasingham and I. Coppens, unpublished data). Genetic ablation of *Plasmodium ATG8* results in a lethal phenotype, indicating that ATG8 is essential for mammalian stage development. Upon urea-SDS–PAGE separation of *Plasmodium* lysates to detect the lipidated form of ATG8, only a single band corresponding to ATG8 is detected. One possible explanation is that *Plasmodium* homologues of ATG8 are predominantly present in a lipid-conjugated state, in agreement with the presence of a C-terminal Gly of ATG8, making this protein 'ready' to bind to membranes following its synthesis. In support of this proposal, *Plasmodium* ATG8 is not present in the parasite cytoplasm but is located in the peripheral membrane of large vesicles. PbATG8-containing structures are prominently present in converting parasites, and some of them colocalize with micronemes. This suggests physical interactions between PbATG8-labeled compartments and micronemes, and potentially implicates PbATG8 in facilitating microneme disposal. Interestingly, after sporozoite metamorphosis, PbATG8-labeled structures are still visible within the parasite and persist during the replication phase of development (Fig. 6). Thus, in addition to a role in organelle degradation via autophagy, it is plausible that *Plasmodium* ATG8 has additional functions *e.g.*, for promoting the vesicular trafficking between organelles during the anabolic phase of intra-

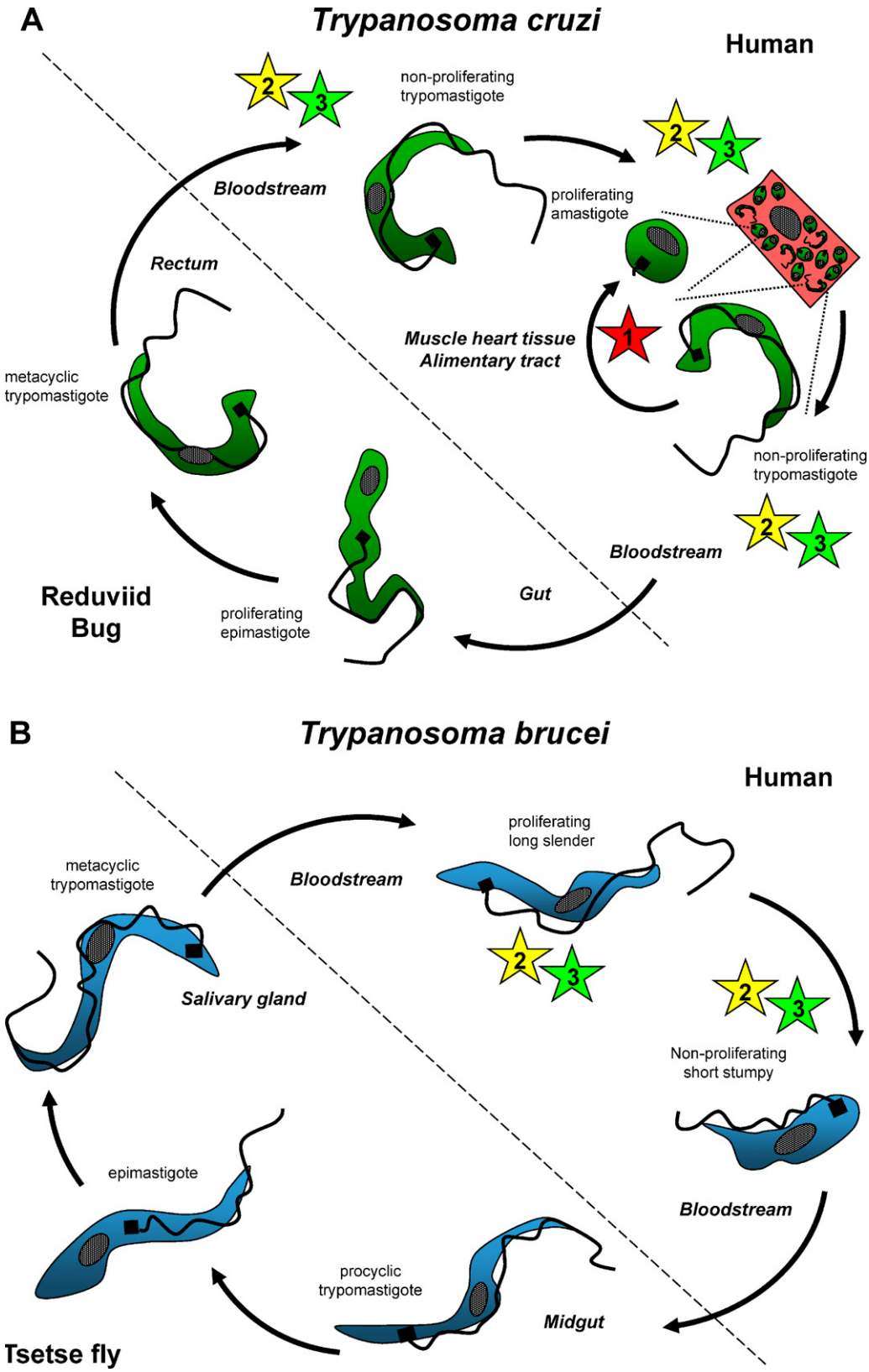


Fig. 7. Life cycles of *Trypanosoma cruzi* (A), *Trypanosoma brucei* (B), *Leishmania* spp. (C) and *Plasmodium* spp. (D) and the possibilities for interference with autophagy-specific, parasite-selective inhibitors to be developed as drugs against the diseases caused by the different parasites. Inhibitors are given as coloured, numbered asterisks at the stages of the life cycle where they would act. 1. Compounds that inhibit autophagy during differentiation and metamorphosis; 2. Compounds that may induce autophagic cell death; 3. Compounds that inhibit the parasitic autophagic response against cell damage produced by others drugs. This third option implies a combination of drugs in which the autophagy inhibitor may be used to enhance the efficacy of a drug targeting another process and/or decrease the risk of development of resistance against the latter. For a detailed explanation, see the text of Section 5 ('closing perspectives – autophagy as a drug target').

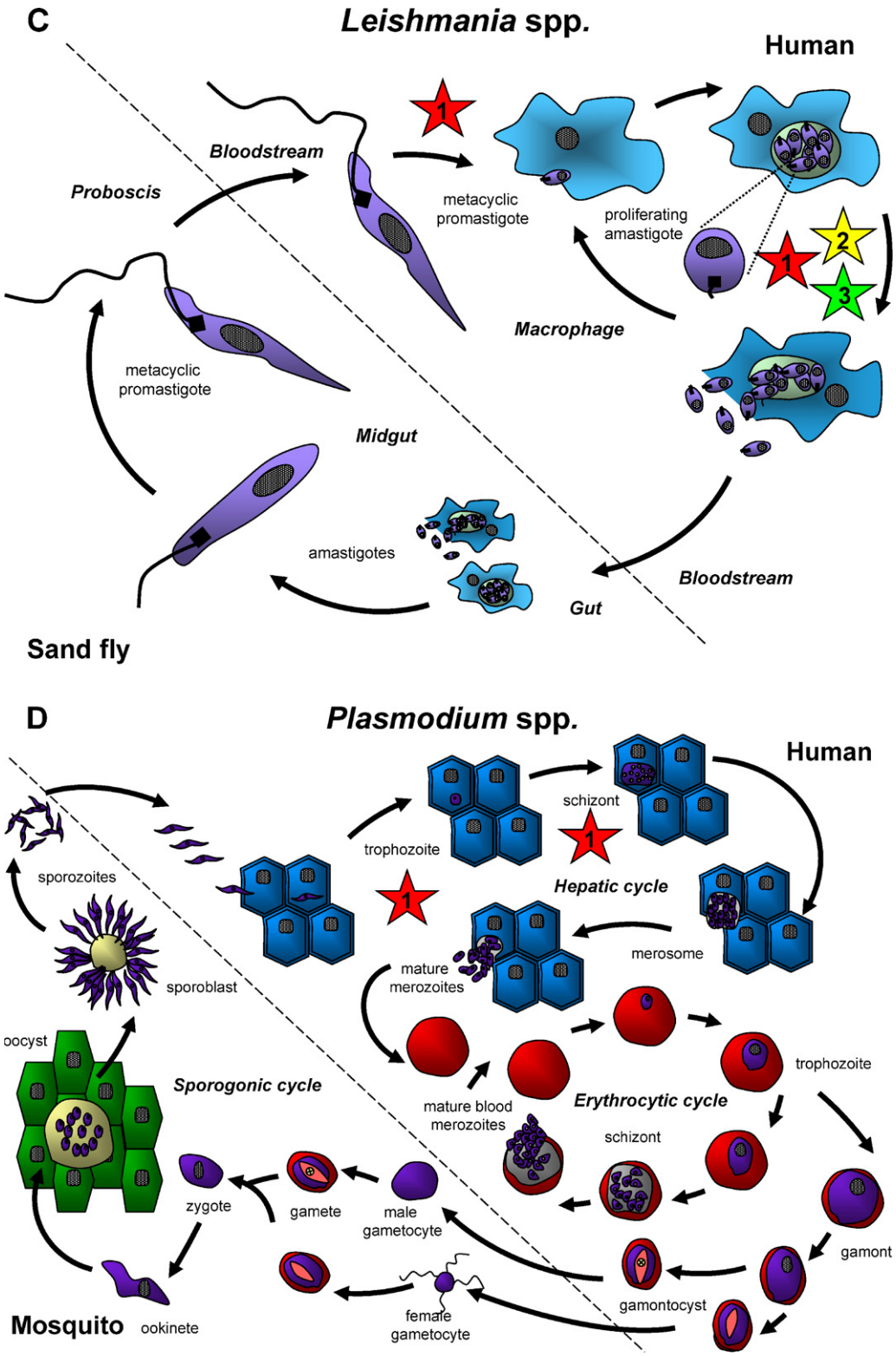


Fig. 7. (*Continued*).

hepatic development. To this point, functional divergence of ATG8 homologues has been observed in mammals, in which LC3 and GATE-16/GABARAP subfamilies act differently in autophagosome formation. Even the function of LC3 does not seem to be limited to autophagy, as this protein is also able to enhance translation [52,113,114].

5. Closing perspectives – autophagy as a drug target

As described above, research in the last few years has revealed that autophagy plays an important role in parasitic protists: in their adaptation to starvation conditions and/or other forms of stress, and in their morphological remodeling and metabolic reprogram-

ming during progress through their life cycle when they have to adapt to the different nutritional environments pertaining in different hosts or host cells. Thus far, the emphasis has generally been a cytological characterization of autophagy and functional characterisation of known ATG homologues in the process. Given the discovery of species-specific autophagy proteins in fungi and animals [14–16], it would not be surprising if lineage-specific proteins contribute to autophagy-related pathways in parasites, too. The possibility that ubiquitination contributes to selective autophagy pathways in parasites is also worthy of investigation. In the case of each of the three human-infective trypanosomatid parasites, *T. brucei*, *T. cruzi* and *Leishmania* spp., the essentiality of autophagy for cellular differentiation has been demonstrated. Also for *Plasmodium* the evidence for the involvement of ATG proteins in essential cellular remodeling seems to be strong. Moreover, by targeting specific autophagy-related proteins using either reverse genetics or specific inhibitors, it was shown that not only could differentiation be affected, but also the morphology, virulence or viability of the parasites. Thus, the possibility that autophagy-related proteins could be exploited for the discovery of new parasite-specific drugs should be considered. Important in this respect is that ATGs and other proteins involved in autophagy generally display a low level of sequence conservation, creating possibilities for developing compounds that selectively interfere with the function of an essential parasite protein but not with that of the host counterpart. In some cases inhibition of autophagy in the parasite would affect the viability or virulence of the human pathogenic form of the parasite, and so offer the possibility to cure the disease.

In the case of the American trypanosome *T. cruzi* interference with the activity of proteins that are essential for differentiation may indeed offer possibilities for chemotherapy [115–119]. Following the invasion of host cells, these parasites develop from extracellular trypomastigote forms, into intracellular amastigotes. The immotile intracellular *T. cruzi* amastigotes, after several rounds of multiplication, differentiate again into flagellate trypomastigotes that burst out of the first infected cells to enter other host cells. Blocking of autophagy may prevent these differentiation steps (see Fig. 7A) and thus decrease pathogenicity.

In contrast, in *T. brucei*, autophagy appears to play its major role during the transition from the stumpy form found in the human bloodstream to the procyclic form. This occurs after ingestion by the tsetse fly, when the trypanosome undergoes major morphological and metabolic changes. Autophagic activity seems to occur too, but on a much more limited scale, during differentiation between the successive bloodstream forms, from long-slender, via intermediate to short-stumpy, which indeed are associated with much less dramatic morphological and metabolic changes [87]. Therefore, the effectiveness of autophagy inhibitors as a possibility to cure sleeping sickness may be questionable (Fig. 7B). One could speculate, however, that in African trypanosomes, as well as in the other parasites, autophagy inhibitors may be helpful in combination with other drugs when the parasites try to overcome the deleterious effects caused by such drugs by an autophagic response. In that case, a combined administration with autophagy inhibitors may be both effective and offer possibilities to prevent the development of drug resistance.

Given that the diagnosis of leishmaniasis occurs long after the initial infection by and intracellular differentiation of metacyclic promastigotes, the possibility that autophagy could be targeted for drug development against *Leishmania* parasites could appear to be dependent on whether the developmental cycle of intracellular amastigotes during a chronic infection is dependent upon autophagy (Fig. 7C). The inhibition of cysteine peptidase function in amastigote replication and the development of infectivity [120] point towards a possibility that autophagy-related proteins, or at least lysosomal enzymes, represent possible targets for antiparasitic chemotherapy [43,120,121].

An alternative chemotherapeutic strategy may be found in inducing prolonged autophagy leading to cell death. As shown in section 4.1.2., treatment of *T. brucei* and *T. cruzi* with certain chemicals resulted in autophagic cell death; administration of DHA or neuropeptides resulted into killing of African trypanosomes [61–64], while lysophospholipid analogues and naphthoimidazoles had the same effect on American trypanosomes [73]. No such data about chemically induced autophagic cell death have been reported as yet for *Leishmania*.

Apicomplexan parasites such as *Plasmodium* also undergo major remodeling of their morphology during cell differentiation and, during the transition to different environments, their metabolic enzymatic machinery, too. During the sporozoite-to-trophozoite conversion of the rodent malarial parasite *P. berghei*, dramatic interior remodeling was recently observed in which many organelles are eliminated [105]. This organelle elimination is through extrusion which seems to involve the autophagy machinery, as supported by morphological analysis, immuno-labeling studies with anti-ATG8 and the fact that the conversion is delayed by administration of the autophagy inhibitor 3-MA. However, the essentiality of autophagy for this metamorphosis remains to be established by genetic means. Nonetheless, these observations strongly suggest that it may be worth considering the feasibility of combating infections by apicomplexan parasites through specifically interfering with components of their autophagy machinery (Fig. 7D).

In recent years, autophagy has received considerable interest as a target for therapeutic intervention in a variety of human pathologies, including cancer, neurodegenerative diseases and some viral and bacterial infections [122]. Research to explore the possibility of targeting autophagy in parasitic protists is still at a very preliminary stage, but the development of inhibitors specifically interfering with proteins of the autophagy process in parasitic protists and not with those of the host cells seems feasible because the sequences of these proteins are poorly conserved. Future studies should lead to structure determination of parasite ATG proteins that are already known to be essential or which future studies show to be so and/or to the development of sensitive binding or activity assays for them. This information will allow, respectively, for the *in silico* design and synthesis of selective inhibitors, and for high-throughput screening of compound libraries for the *in vitro* selection of inhibitors of these proteins. Such efforts could even benefit from the medical 'piggybacking' that has become increasingly prominent in numerous drug discovery initiatives aimed against tropical disease parasites [123].

Acknowledgements

AB, MGL and PAMM gratefully acknowledge the 'Fonds de la Recherche Scientifique' (F.R.S-FNRS) and associated foundations (FRSM and FRIA) and the Belgian Interuniversity Attraction Poles-Federal Office for Scientific, Technical and Cultural Affairs for support of their research on autophagy in trypanosomes; MLG acknowledges the Royal Society for an University Research Fellowship. The recent literature about autophagy is vast; we apologize to many colleagues for the fact that, due to space limitations, we were unable to cite original papers of many authors and therefore referred to some recent review articles where the citations can be found.

References

[1] de Duve C, Pressman BC, Gianetto R, Wattiaux R, Appelmans F. Tissue fractionation studies. 6. Intracellular distribution patterns of enzymes in rat-liver tissue. Biochem J 1955;60:604–17.

[2] de Duve C. The lysosome turns fifty. Nat Cell Biol 2005;7:847–9.
[3] de Reuck AVS, Cameron MP. Ciba foundation symposium on lysosomes. London: JA Churchill Ltd.; 1963.
[4] Klionsky DJ. Autophagy revisited: a conversation with Christian de Duve. Autophagy 2008;4:740–3.
[5] Yang Z, Klionsky DJ. Eaten alive: a history of macroautophagy. Nat Cell Biol 2010;12:814–22.
[6] Armstrong J. Yeast vacuoles: more than a model lysosome. Trends Cell Biol 2010;20:580–5.
[7] Rigden DJ, Michels PAM, Ginger ML. Autophagy in protists: examples of secondary loss, lineage-specific innovations, and the conundrum of remodeling a single mitochondrion. Autophagy 2009;5:784–94.
[8] Duszenko M, Ginger ML, Brennand A, et al. Autophagy 2011;7:127–58.
[9] Nakatogawa H, Suzuki K, Kamada Y, Ohsumi Y. Dynamics and diversity in autophagy mechanisms: lessons from yeast. Nat Rev Mol Cell Biol 2009;10:458–67.
[10] Yang Z, Klionsky DJ. An overview of the molecular mechanism of autophagy. Curr Top Microbiol Immunol 2009;335:1–32.
[11] Klionsky DJ, Cregg JM, Dunn Jr WA, et al. A unified nomenclature for yeast autophagy-related genes. Dev Cell 2003;5:539–45.
[12] Lynch-Day MA, Klionsky DJ. The Cvt pathway as a model for selective autophagy. FEBS Lett 2010;7:1359–66.
[13] Ano Y, Hattori T, Kato N, Sakai Y, Intracellular ATP. correlates with mode of pexophagy in *Pichia pastoris*. Biosci Biotechnol Biochem 2005;69:1527–33.
[14] Klionsky DJ, Codogno P, Cuervo AM, et al. A comprehensive glossary of autophagy-related molecules and processes. Autophagy 2010;6: 438–48.
[15] Meijer WH, Van der Klei IJ, Veenhuis M, Kiel JAKW. ATG genes involved in non-selective autophagy are conserved from yeast to man, but the selective Cvt and pexophagy pathways also require organism-specific genes. Autophagy 2007;3:106–16.
[16] Tian Y, Li Z, Hu W, et al. *C. elegans* screen identifies autophagy genes specific to multicellular organisms. Cell 2010;141:1042–55.
[17] Díaz-Troya S, Pérez-Pérez ME, Florencio FJ, Crespo JL. The role of TOR in autophagy regulation from yeast to plants and mammals. Autophagy 2008;4:851–65.
[18] Jung CH, Ro SH, Cao J, Otto NM, Kim DH. mTOR regulation of autophagy. FEBS Lett 2010;584:1287–95.
[19] Suzuki K, Ohsumi Y. Current knowledge of the pre-autophagosomal structure (PAS). FEBS Lett 2010;584:1280–6.
[20] Manjithaya R, Nazarko TY, Farré JC, Subramani S. Molecular mechanism and physiological role of pexophagy. FEBS Lett 2010;584:1367–73.
[21] Kanki T, Klionsky DJ. The molecular mechanism of mitochondria autophagy in yeast. Mol Microbiol 2010;75:795–800.
[22] Hayashi-Nishino M, Fujita N, Noda T, Yamaguchi A, Yoshimori T, Yamamoto A. A subdomain of the endoplasmic reticulum forms a cradle for autophagosome formation. Nat Cell Biol 2009;11:1433–7.
[23] Ylä-Anttila P, Vihinen H, Jokitalo E, Eskelinen E-L. 3D tomography reveals connections between the phagophore and endoplasmic reticulum. Autophagy 2009;5:1180–5.
[24] Hailey DW, Rambold AS, Satpute-Krishnan PP, et al. Mitochondria supply membranes for autophagosome biogenesis during starvation. Cell 2010;141:656–67.
[25] Ravikumar B, Moreau K, Jahreiss L, Puri C, Rubinsztein DC. Plasma membrane contributes to the formation of pre-autophagosomal structures. Nat Cell Biol 2010;12:747–57.
[26] Van der Vaart A, Griffith J, Reggiori F. Exit from the Golgi is required for the expansion of the autophagosomal phagophore in yeast *Saccharomyces cerevisiae*. Mol Biol Cell 2010;21:2270–84.
[27] Burman C, Ktistakis NT. Regulation of autophagy by phosphatidylinositol 3-phosphate. FEBS Lett 2010;584:1302–12.
[28] Geng J, Klionsky DJ. The Atg8 and Atg12 ubiquitin-like conjugation systems in macroautophagy. 'Protein modifications: beyond the usual suspects' review series. EMBO Rep 2008;9:859–64.
[29] Leabu M. Membrane fusion in cells: molecular machinery and mechanisms. J Cell Mol Med 2006;10:423–7.
[30] Yang Z, Huang J, Geng J, Nair U, Klionsky DJ. Atg22 recycles amino acids to link the degradative and recycling functions of autophagy. Mol Biol Cell 2006;17:5094–104.
[31] Kirkin V, McEwan DG, Novak I, Dikic I. A role for ubiquitin in selective autophagy. Mol Cell 2009;34:259–69.
[32] Calvo-Garrido J, Escalante R. Autophagy dysfunction and ubiquitin-positive aggregates in *Dictyostelium* cells lacking Vmp1. Autophagy 2010;6: 100–9.
[33] Kim PK, Hailey DW, Mullen RT, Lippincott-Schwartz J. Ubiquitin signals autophagic degradation of cytosolic proteins and peroxisomes. Proc Natl Acad Sci USA 2008;105:20567–74.
[34] Keeling PJ, Burger G, Durnford DG, et al. The tree of eukaryotes. Trends Ecol Evol 2005;20:670–6.
[35] Brown RC, Evans DA, Vickerman K. Developmental changes in ultrastructure and physiology of *Trypanosoma brucei*. Trans R Soc Trop Med Hyg 1972;66:336–7.
[36] Vickerman K, Tetley L. Recent ultrastructural studies on trypanosomes. Ann Soc Belg Med Trop 1977;57:441–57.
[37] Waller RF, McConville MJ. Developmental changes in lysosome morphology and function *Leishmania* parasites. Int J Parasitol 2002;32:1435–45.
[38] Sant'Anna C, Parussini F, Lourenço D, de Souza W, Cazzulo JJ, Cunha-e-Silva NL. All *Trypanosoma cruzi* developmental forms present lysosome-related organelles. Histochem Cell Biol 2008;130:1187–98.
[39] North MJ, Coombs GH. Proteinases of *Leishmania mexicana* amastigotes and promastigotes: analysis by gel electrophoresis. Mol Biochem Parasitol 1981;3:293–300.
[40] Mottram JC, Robertson CD, Coombs GH, Barry JD. A developmentally regulated cysteine proteinase gene of *Leishmania mexicana*. Mol Microbiol 1992;6:1925–32.
[41] Besteiro S, Williams RA, Coombs GH, Mottram JC. Protein turnover and differentiation in *Leishmania*. Int J Parasitol 2007;37:1063–75.
[42] Coombs GH, Hart DT, Capaldo J. Proteinase inhibitors as antileishmanial agents. Trans R Soc Trop Med Hyg 1982;76:660–3.
[43] Franke de Cazzulo BM, Martínez J, North MJ, Coombs GH, Cazzulo JJ. Effects of proteinase inhibitors on the growth and differentiation of *Trypanosoma cruzi*. FEMS Microbiol Lett 1994;124:81–6.
[44] Williams RA, Tetley L, Mottram JC, Coombs GH. Cysteine peptidases CPA and CPB are vital for autophagy and differentiation in *Leishmania mexicana*. Mol Microbiol 2006;61:655–74.
[45] Herman M, Gillies S, Michels PA, Rigden DJ. Autophagy and related processes in trypanosomatids: insights from genomic and bioinformatic analyses. Autophagy 2006;2:107–18.
[46] Alvarez VE, Kosec G, Sant'Anna C, Turk V, Cazzulo JJ, Turk B. Autophagy is involved in nutritional stress response and differentiation in *Trypanosoma cruzi*. J Biol Chem 2008;283:3454–64.
[47] Kiel JAKW. Autophagy in unicellular eukaryotes. Philos Trans R Soc Lond B Biol Sci 2010;365:819–30.
[48] Morris JC, Wang Z, Drew ME, Englund PT. Glycolysis modulates trypanosome glycoprotein expression as revealed by an RNAi library. EMBO J 2002;21:4429–38.
[49] Schumann Burkard G, Jutzi P, Roditi I. Genome-wide RNAi screens in bloodstream form trypanosomes identify drug transporters. Mol Biochem Parasitol 2011;175:91–4.
[50] Fritz-Laylin LK, Prochnik SE, Ginger ML, et al. The genome of *Naegleria gruberi* illuminates early eukaryotic versatility. Cell 2010;140:631–42.
[51] Williams RA, Woods KL, Juliano L, Mottram JC, Coombs GH. Characterization of unusual families of ATG8-like proteins and ATG12 in the protozoan parasite *Leishmania major*. Autophagy 2009;5:159–72.
[52] Weidberg H, Shvets E, Shpilka T, Shimron F, Shinder V, Elazar Z. LC3 and GATE-16/GABARAP subfamilies are both essential yet act differently in autophagosome biogenesis. EMBO J 2010;29:1792–802.
[53] Aslett M, Aurrecoechea C, Berriman M, et al. TriTrypDB: a functional genomic resource for the Trypanosomatidae. Nucleic Acids Res 2010;38:D457–62 [Database issue].
[54] Goldshmidt H, Matas D, Kabi A, Carmi S, Hope R, Michaeli S. Persistent ER stress induces the spliced leader RNA silencing pathway (SLS), leading to programmed cell death in *Trypanosoma brucei*. PLoS Pathog 2010;6: e1000731.
[55] Koopmann R, Muhammad K, Perbandt M, Betzel C, Duszenko M. *Trypanosoma brucei* ATG8: structural insights into autophagic-like mechanisms in protozoa. Autophagy 2009;5:1085–91.
[56] Barquilla A, Crespo JL, Navarro M. Rapamycin inhibits trypanosome cell growth by preventing TOR complex 2 formation. Proc Natl Acad Sci USA 2008;105:14579–84.
[57] Barquilla A, Navarro M. Trypanosome TOR as a major regulator of cell growth and autophagy. Autophagy 2009;5:256–8.
[58] Madeira da Silva L, Beverley SM. Expansion of the target of rapamycin (TOR) kinase family and function in *Leishmania* shows that TOR3 is required for acidocalcisome biogenesis and animal infectivity. Proc Natl Acad Sci USA 2010;107:11965–70.
[59] Leandro de Jesus TC, Tonelli RR, Nardelli SC, et al. Target of rapamycin (TOR)-like 1 kinase is involved in the control of polyphosphate levels and acidocalcisome maintenance in *Trypanosoma brucei*. J Biol Chem 2010;285:24131–40.
[60] Denninger V, Koopmann R, Muhammad K, et al. Kinetoplastida: model organisms for simple autophagic pathways? Methods Enzymol 2008;451:373–408.
[61] Uzcátegui NL, Denninger V, Merkel P, Schoenfeld C, Figarella K, Duszenko M. Dihydroxyacetone induced autophagy in African trypanosomes. Autophagy 2007;3:626–9.
[62] Uzcátegui NL, Carmona-Gutiérrez D, Denninger V, et al. Antiproliferative effect of dihydrroxyacetone on *Trypanosoma brucei* bloodstream forms: cell cycle progression, subcellular alterations, and cell death. Antimicrob Agents Chemother 2007;51:3960–8.
[63] Delgado M, Anderson P, Garcia-Salcedo JA, Caro M, Gonzalez-Rey E. Neuropeptides kill African trypanosomes by targeting intracellular compartments and inducing autophagic-like cell death. Cell Death Differ 2009;16:406–16.
[64] Campos-Salinas J, Gonzalez-Rey E. Autophagy and neuropeptides at the cross-road for parasites: to survive or to die? Autophagy 2009;5:551–4.
[65] Braga MV, de Souza W. Effects of protein kinase and phosphatidylinositol-3 kinase inhibitors on growth and ultrastructure of *Trypanosoma cruzi*. FEMS Microbiol Lett 2006;256:209–16.
[66] Santa-Rita RM, Lira R, Barbosa HS, Urbina JA, de Castro SL. Antiproliferative synergy of lysophospholipid analogues and ketoconazole against *Trypanosoma cruzi* (Kinetoplastida: Trypanosomatidae): cellular and ultrastructural analysis. J Antimicrob Chemother 2005;55:780–4.

[67] Dantas AP, Barbosa HS, De Castro SL. Biological and ultrastructural effects of the anti-microtubule agent taxol against *Trypanosoma cruzi*. J Submicrosc Cytol Pathol 2003;35:287–94.
[68] Dantas AP, Salomão K, Barbosa HS, De Castro SL. The effect of Bulgarian propolis against *Trypanosoma cruzi* and during its interaction with host cells. Mem Inst Oswaldo Cruz 2006;101:207–11.
[69] Docampo R, Lopes JN, Cruz FS, Souza W. *Trypanosoma cruzi*: ultrastructural and metabolic alterations of epimastigotes by β-lapachone. Exp Parasitol 1977;42:142–9.
[70] Menna-Barreto RFS, Henriques-Pons A, Pinto AV, Morgado-Diaz JA, Soares MJ, De Castro SL. Effect of a β-lapachone-derived naphthoimidazole on *Trypanosoma cruzi*: identification of target organelles. J Antimicrob Chemother 2005;56:1034–41.
[71] Menna-Barreto RF, Corrêa JR, Pinto AV, Soares MJ, de Castro SL. Mitochondrial disruption and DNA fragmentation in *Trypanosoma cruzi* induced by naphthoimidazoles synthesized from β-lapachone. Parasitol Res 2007;101:895–905.
[72] Menna-Barreto RFS, Corrêa JR, Cascabulho CM, et al. Naphthoimidazoles promote different death phenotypes in *Trypanosoma cruzi*. Parasitology 2009;136:499–510.
[73] Menna-Barreto RFS, Salomão K, Dantas AP, et al. Different cell death pathways induced by drugs in *Trypanosoma cruzi*: an ultrastructural study. Micron 2009;40:157–68.
[74] Bera A, Singh S, Nagaraj R, Vaidya T. Induction of autophagic cell death in *Leishmania donovani* by antimicrobial peptides. Mol Biochem Parasitol 2003;127:23–35.
[75] Rodrigues JC, Attias M, Rodriguez C, Urbina JA, Souza W. Ultrastructural and biochemical alterations induced by 22,26-azasterol, a $\Delta^{24(25)}$-sterol methyltransferase inhibitor, on promastigote and amastigote forms of *Leishmania amazonensis*. Antimicrob Agents Chemother 2002;46:487–99.
[76] Rodrigues JC, Concepcion JL, Rodrigues C, Caldera A, Urbina JA, de Souza W. In vitro activities of ER-119884 and E5700, two potent squalene synthase inhibitors, against *Leishmania amazonensis*: antiproliferative, biochemical, and ultrastructural effects. Antimicrob Agents Chemother 2008;52:4098–114.
[77] Kroemer G, Galluzzi L, Vandenabeele P, et al. Nomenclature Committee on Cell Death 2009 Classification of cell death: recommendations of the Nomenclature Committee on Cell Death 2009. Cell Death Differ 2009;16:3–11.
[78] Van Zandbergen G, Lüder CG, Heussler V, Duszenko M. Programmed cell death in unicellular parasites: a prerequisite for sustained infection? Trends Parasitol 2010;26:477–83.
[79] Cazzulo JJ, Stoka V, Turk V. Cruzipain, the major cysteine proteinase from the protozoan parasite *Trypanosoma cruzi*. Biol Chem 1997;378:1–10.
[80] Coombs GH, Tetley L, Moss VA, Vickerman K. Three-dimensional structure of the *Leishmania amastigote* as revealed by computer-aided reconstruction from serial sections. Parasitology 1986;92:13–23.
[81] Besteiro S, Williams RA, Morrison LS, Coombs GH, Mottram JC. Endosome sorting and autophagy are essential for differentiation and virulence of *Leishmania major*. J Biol Chem 2006;281:11384–96.
[82] Opperdoes FR, Borst P. Localization of nine glycolytic enzymes in a microbody-like organelle in *Trypanosoma brucei*: the glycosome. FEBS Lett 1977;80:360–4.
[83] Michels PAM, Bringaud F, Herman M, Hannaert V. Metabolic functions of glycosomes in trypanosomatids. Biochim Biophys Acta 2006;1763:1463–77.
[84] Opperdoes FR, Szikora JP. In silico prediction of the glycosomal enzymes of *Leishmania major* and trypanosomes. Mol Biochem Parasitol 2006;147:193–206.
[85] Colasante C, Ellis M, Ruppert T, Voncken F. Comparative proteomics of glycosomes from bloodstream form and procyclic culture form *Trypanosoma brucei brucei*. Proteomics 2006;6:3275–93.
[86] Vertommen D, Van Roy J, Szikora JP, Rider MH, Michels PAM, Opperdoes FR. Differential expression of glycosomal and mitochondrial proteins in the two major life-cycle stages of *Trypanosoma brucei*. Mol Biochem Parasitol 2008;158:189–201.
[87] Herman M, Pérez-Morga D, Schtickzelle N, Michels PAM. Turnover of glycosomes during life-cycle differentiation of *Trypanosoma brucei*. Autophagy 2008;4:294–308.
[88] Bellu AR, Kiel JA. Selective degradation of peroxisomes in yeasts. Microsc Res Tech 2003;6:161–70.
[89] Naderer T, Ellis MA, Sernee MF, et al. Virulence of *Leishmania major* in macrophages and mice requires the gluconeogenic enzyme fructose-1,6-bisphosphatase. Proc Natl Acad Sci USA 2006;103:5502–7.
[90] Rodríguez-Contreras D, Landfear SM. Metabolic changes in glucose transporter-deficient *Leishmania mexicana* and parasite virulence. J Biol Chem 2006;281:20068–76.
[91] Saunders EC, D.E. Souza DP, Naderer T, et al. Central carbon metabolism of *Leishmania* parasites. Parasitology 2010;137:1303–13.
[92] Hart DT, Opperdoes FR. The occurrence of glycosomes (microbodies) in the promastigote stage of four major *Leishmania* species. Mol Biochem Parasitol 1984;13:159–72.
[93] Loftus B, Anderson I, Davies R, et al. The genome of the protist parasite *Entamoeba histolytica*. Nature 2005;433:865–8.
[94] Carlton JM, Hirt RP, Silva JC, et al. Draft genome sequence of the sexually transmitted pathogen *Trichomonas vaginalis*. Science 2007;315:207–12.
[95] Picazarri K, Nakada-Tsukui K, Nozaki T. Autophagy during proliferation and encystation in the protozoan parasite *Entamoeba invadens*. Infect Immun 2008;76:278–88.
[96] Hirt RP, Noel CJ, Sicheritz-Ponten T, Tachezy J, Fiori PL. *Trichomonas vaginalis* surface proteins: a view from the genome. Trends Parasitol 2007;23:540–7.
[97] Collingridge PW, Brown RW, Ginger ML. Moonlighting enzymes in parasitic protozoa. Parasitology 2010;137:1467–75.
[98] Morrison HG, McArthur AG, Gillin FD, et al. Genomic minimalism in the early diverging intestinal parasite *Giardia lamblia*. Science 2007;317:1921–6.
[99] Ankarklev J, Jerlström-Hultqvist J, Ringqvist E, Troell K, Svärd SG. Behind the smile: cell biology and disease mechanisms of *Giardia* species. Nat Rev Microbiol 2010;8:413–22.
[100] Abodeely M, DuBois KN, Hehl A, et al. A contiguous compartment functions as endoplasmic reticulum and endosome/lysosome in *Giardia lamblia*. Eukayot Cell 2009;8:1665–76.
[101] Corrêa G, Vilela R, Menna-Barreto RF, Midlej V, Benchimol M. Cell death induction in *Giardia lamblia*: effect of β-lapachone and starvation. Parasitol Int 2009;58:424–37.
[102] Maia C, Attias M, Urbina J, Gilbert I, Magaraci F, de Souza W. Azasterols impair *Giardia lamblia* proliferation and induces encystation. Biochem Biophys Res Commun 2007;363:310–6.
[103] Baum J, Papenfuss AT, Baum B, Speed TP, Cowman AF. Regulation of apicomplexan actin-based motility. Nat Rev Microbiol 2006;4:621–8.
[104] Lim L, McFadden GI. The evolution, metabolism and functions of the apicoplast. Philos Trans R Soc Lond B Biol Sci 2010;365:749–63.
[105] Jayabalasingham B, Bano N, Coppens I. Metamorphosis of the malaria parasite in the liver is associated with organelle clearance. Cell Res 2010;20:1043–59.
[106] Morrissette NS, Sibley LD. Cytoskeleton of apicomplexan parasites. Microbiol Mol Biol Rev 2002;66:21–38.
[107] Robson KJ, Frevert U, Reckmann I, et al. Thrombospondin-related adhesive protein (TRAP) of *Plasmodium falciparum*: expression during sporozoite ontogeny and binding to human hepatocytes. EMBO J 1995;14:3883–94.
[108] Sinnis P, Sim BK. Cell invasion by the vertebrate stages of *Plasmodium*. Trends Microbiol 1997;5:52–8.
[109] Seglen PO, Gordon PB. 3-Methyladenine: specific inhibitor of autophagic/lysosomal protein degradation in isolated rat hepatocytes. Proc Natl Acad Sci USA 1982;79:1889–92.
[110] Vaid A, Ranjan R, Smythe WA, Hoppe HC, Sharma P. PfPI3K, a phosphatidylinositol-3 kinase from *Plasmodium falciparum*, is exported to the host erythrocyte and is involved in hemoglobin trafficking. Blood 2010;115:2500–7.
[111] Yamada Y, Suzuki NN, Hanada T, et al. The crystal structure of Atg3, an autophagy-related ubiquitin carrier protein (E2) enzyme that mediates Atg8 lipidation. J Biol Chem 2007;282:8036–43.
[112] Hanada T, Satomi Y, Takao T, Ohsumi Y. The amino-terminal region of Atg3 is essential for association with phosphatidylethanolamine in Atg8 lipidation. FEBS Lett 2009;583:1078–83.
[113] Zhou B, Boudreau N, Coulber C, Hammarback J, Rabinovitch M. Microtubule-associated protein 1 light chain 3 is a fibronectin mRNA-binding protein linked to mRNA translation in lamb vascular smooth muscle cells. J Clin Invest 1997;100:3070–82.
[114] Cann GM, Guignabert C, Ying L, et al. Developmental expression of LC3α and β: absence of fibronectin or autophagy phenotype in LC3β knockout mice. Dev Dyn 2008;237:187–95.
[115] Cazzulo JJ. Proteinases of *Trypanosoma cruzi*: potential targets for the chemotherapy of Chagas desease. Curr Top Med Chem 2002;2:1261–71.
[116] Docampo R, Moreno SN. Current chemotherapy of human African trypanosomiasis. Parasitol Res 2003;90(Suppl.):S10–3.
[117] Steverding D, Tyler KM. Novel antitrypanosomal agents. Expert Opin Investig Drugs 2005;14:939–55.
[118] Alvarez VE, Kosec G, Sant Anna C, Turk V, Cazzulo JJ, Turk B. Blocking autophagy to prevent parasite differentiation: a possible new strategy for fighting parasitic infections? Autophagy 2008;4:361–3.
[119] Duschak VG, Couto AS, Cruzipain. the major cysteine protease of *Trypanosoma cruzi*: a sulfated glycoprotein antigen as relevant candidate for vaccine development and drug target. A review. Curr Med Chem 2009;16:3174–202.
[120] Selzer PM, Pingel S, Hsieh I, et al. Cysteine protease inhibitors as chemotherapy: lessons from a parasite target. Proc Natl Acad Sci USA 1999;96:11015–22.
[121] McKerrow JH, Engel JC, Caffrey CR. Cysteine protease inhibitors as chemotherapy for parasitic infections. Bioorg Med Chem 1999;7:639–44.
[122] Rubinsztein DC, Gestwicki JE, Murphy LO, Klionsky DJ. Potential therapeutic applications of autophagy. Nat Rev Drug Discov 2007;6:304–12.
[123] Nwaka S, Hudson A. Innovative lead discovery strategies for tropical diseases. Nat Rev Drug Discov 2006;5:941–55.
[124] Roger AJ, Hug LA. The origin and diversification of eukaryotes: problems with molecular phylogenetics and molecular clock estimation. Philos Trans R Soc Lond B Biol Sci 2006;361:1039–54.
[125] Burki F, Shalchian-Tabrizi K, Pawlowski J. Phylogenomics reveals a new 'megagroup' including most photosynthetic eukaryotes. Biol Lett 2008;4:366–9.
[126] Cavalier-Smith T. Kingdoms Protozoa and Chromista and the eozoan root of the eukaryotic tree. Biol Lett 2010;6:342–5.
[127] Clayton C, Adams M, Almeida R, et al. Genetic nomenclature for *Trypanosoma* and *Leishmania*. Mol Biochem Parasitol 1998;97:221–4.

2.2 Objectives

Trypanosoma brucei, the causative agent of sleeping sickness, is a well described organism with a complex life cycle. Many aspects of its metabolism and its interaction with the host immune system are being studied in the hope to develop more effective and less toxic drugs. The autophagy field has expanded vastly in the last few decades. Autophagy has been identified in almost every eukaryotic organism and it has been associated to metabolic and infective diseases, from cell survival to cell death. Recently, it has also been identified in trypanosomatids and it is becoming clear that it plays an important role in infection and parasite survival. The possibility of using autophagy as a drug target is possibly limited in the case of *T. brucei*, but may be more promising for *T. cruzi* and *Leishmania* spp. (discussed by Brennand *et al*., 2011). However, autophagy is also known as a mechanism to fight stress conditions. It is reasonable to imagine that autophagy is also used for that purpose by different infective agents when exposed to drugs, and its upregulation might be responsible for a less than optimal drug effect. Indeed, studies with *T. cruzi*, discussed by Duszenko *et al.*, 2011 and Brennand *et al.*, 2011 – (see also Chapter 2) show that, at least in some cases, autophagy is upregulated upon drug administration. Therefore, understanding the mechanisms by which parasites use autophagy in attempts to overcome the stress provoked by drugs might give an important insight into how the current chemotherapeutic options may be improved.

It is known that during differentiation there are an important number of morphological and metabolic changes occurring in the parasite. Autophagy is increased during *T. brucei in vivo* (LS-SS) and *in vitro* (SS-PF) differentiation (Herman *et al*., 2008). Of importance to us are the drastic metabolic changes that occur: from a simple form of energy and carbohydrate metabolism in bloodstream forms to an elaborate procyclic metabolic repertoire. This implies that many pathways that were repressed or expressed at low level in the bloodstream stage will be upregulated. Conversely, the levels of enzymes involved in glycolysis, the only ATP-producing process of bloodstream trypanosomes, will decrease in the procyclics that will have little access to

glucose. Also, the versatile glycosome will need to adapt. In analogy to what has been observed for peroxisomes in yeasts, it is presumed that the import of newly-produced enzymes takes place only into immature glycosomes, while older glycosomes that are no longer import-competent will be cleared; presumably the only means to degrade these organelles in bulk is by pexophagy.

Previously, our laboratory reported morphological observations that degradation of glycosomes is upregulated during differentiation and we postulate that pexophagy is an important part of the cellular adaptation to the new nutrient conditions, and consequently for the establishment of the infection in the midgut of the fly.

It was our interest to identify and characterise proteins involved in autophagy in *T. brucei*, with emphasis on those proteins that might be responsible for the controlled degradation of glycosomes. A subsequent aim was to identify if such proteins are essential for the parasite during differentiation. Some 20 putative ATGs have previously been identified in *T. brucei* by genomic searches (Herman *et al*., 2006). Of these, five are involved also, or exclusively, in pexophagy in yeasts: ATG24, VAC8, GCN2, GCN3, GCN4. While the three latter ones are transcription factors and play a more indirect role in autophagy, the former two are part of a putative complex that is involved in autophagy induction; moreover these two proteins have been implicated in many types of selective autophagy in yeast. Our objective was to describe the putative ATG24 protein and assess if it participates in autophagy especially during *T. brucei* differentiation, and if this role is essential. For this we developed tools such as RNAi cell lines, cells expressing GFP-tagged ATG proteins and antibodies against the protein. Experiments performed in an attempt to characterise VAC8 are described in Chapter 6.

Chapter 3
Materials and methods

In this chapter we describe the vectors, constructs, conditions, materials, antisera and cell lines used for each experiment, the parameters used for the phylogenetic analysis of ATG24 and Tb927.7.4500 as well as the protocols for the different methods and techniques used in the different experiments described in the following chapters.

3.1 Trypanosomes and growth conditions

For all experiments monomorphic bloodstream and procyclic form cells of *T. brucei* 427, cell line 449, were used (Biebinger *et al*., 1997). These parasites constitutively express the *Escherichia coli* tetracycline (Tet) repressor gene from a chromosomally integrated plasmid pHD449, that also endows phleomycin resistance. This cell line is metabolically indistinguishable from the wild type. Bloodstream parasites were maintained in HMI-9 medium supplemented with 10% heat-inactivated foetal calf serum (Invitrogen) as well as 0.18 µg/mL phleomycin (Cayla) and incubated at 37°C under water-saturated air with 5% CO_2. Procyclic cells were cultured in SDM79 medium (Brun and Schönenberger, 1979) containing 15% foetal calf serum and 0.5 µg/mL phleomycin, at 28°C under water-saturated air with 5% CO_2. Experiments were done with cells in the exponential phase of growth, being a density for bloodstream cells below $2x10^6$ cells/mL and for procyclics $5x10^7$ cells/mL.

3.2 Identification of the sequence for the *T. brucei* homologue of ATG24

The identification of the sequence for the trypanosomatids putative homologues of several yeast ATGs including *ATG24* has been described by Herman *et al*. (2006). The gene (Tb927.9.13380, previously Tb09.211.4240) codes for a protein of approximately 48 kDa that contains a Phox (PX) homology

domain in its N-terminal region and a BAR (Bin/Amphiphysin/Rvs) in its C-terminal domain. It is 24% similar to its yeast counterpart.

3.3 Molecular biology methods

Standard methodologies (described by Sambrook and Russell, 2001) were used for most experiments, as well as protocols provided by suppliers of biological materials (Fermentas, Roche Applied Sciences, New England Biolabs, Promega, Invitrogen, Novagen).

3.4 Construction of expression systems, protein expression, solubilisation and purification of recombinant protein and antiserum production

The complete gene sequence for *ATG24* was amplified by PCR using Phusion polymerase (Finnzymes Reagents) as well as the following pair of primers : 5'-CCACATATGTCTGAAAATGTCTTTGAGTTCCGT -3' (forward, containing a *Nde*I restriction site, shown underlined and the start codon of the gene italicized) and 5'- CCATCTGAGCTAATCGTCATCAATCAAATG -3' (reverse, containing the stop codon and a *Xho*I restriction site, shown underlined and the stop codon of the gene italicized). The amplified product was purified and ligated into the expression vector pET28a (Novagen), that allows the expression of the protein with a N-terminal $(His)_6$-tag. The recombinant plasmid was used to transform competent *E. coli* cells of both the XL-1-Blue strain (Stratagene) and the expressor strain BL21(DE3) (Novagen). Sequencing was done at Macrogen (South-Korea) using plasmid DNA purified from cloned recombinant XL1-Blue cells.

For the expression of the recombinant protein, 200 mL of a culture of *E. coli* BL21(DE3) cells expressing the plasmid was grown under agitation at 37°C until the OD_{600nm} reached 0.4, when 1 mM isopropyl β-D-thiogalactanopyranoside (IPTG) was added. Cells were then incubated for another 20 h. The cells were harvested by centrifugation and the pellet was subsequently resuspended in a lysis buffer in the presence of benzonase (Novagen) and a home-made mix of

protease inhibitors containing a final concentration of 1 mM pepstatin, 1 mM leupeptin, 10 μM AEBSF (all from Fluka) and 100 μM EDTA. After cell lysis by ultrasonication and freezing/defrosting cycles, the pellet was resuspended in a buffer containing 50 mM Tris/HCl, pH 8, and treated for solubilisation of inclusion bodies by first a series of wash steps in the presence of 1% sodium deoxycholate and 1% CHAPS, followed by a wash step with 0.25% deoxycholate and finally an overnight incubation in a small volume of the Tris/HCl buffer in the presence of 8 M urea, at 4°C under gentle agitation. After solubilisation of the inclusion bodies the concentration of urea in the solution was decreased to 6 M by dilution and the mixture was centrifuged at 12,000 x *g* for 20 min (Sorvall centrifuge, HS-4 rotor). Next, the supernatant was incubated with 1 mL HisLink Protein Purification Resin suspension (Promega) for 2 h at room temperature (RT) under gentle agitation. Then the resin with the protein suspension was transferred to a column, the flow-through was collected and the column material washed with the Tris/HCl-urea buffer. Increasing millimolar amounts of imidazole were subsequently added onto the resin in the column, while the elution was collected in aliquots. Finally the purity of the protein in the collected aliquots was checked by SDS-PAGE followed by a Coomassie Blue staining. The purest fractions were pooled and concentrated by centrifugation in a Millipore Amicon Ultra concentrating device. 800 μg of the purified recombinant protein was used for rabbit immunization to raise a polyclonal antiserum (Gentaur).

3.5 RNA interference studies

ATG24 RNAi cell lines were obtained by first creating a construct in which a region (of 486 bp) specific for the gene was placed next to its complementary region (of 435 bp) in the pHD677 vector, under the control of a tetracycline-inducible PARP promoter. Upon tetracycline addition, the genome-inserted plasmid will serve to generate a double-stranded, hairpin mRNA that will activate the RNAi machinery present in *T. brucei.* The creation of the construct was accomplished by the use of the following primers : forward sense : 5'-GTAAAGCTTCCTGCATGAATTTGGTGTTGC -3' (*Hind*III site underlined), forward antisense : 5'- TTACTCGAGTCTGCAGGTCAGCAAACAT -3' (*Xho*I site underlined),

reverse sense : 5'- TTACTCGAGCACGATGGAAGCGATGAATTT -3' (*Xho*I site underlined), reverse antisense : 5'- GTAGGATCCCCTGCATGAATTTGGTGTT -3' (*Bam*HI site underlined). The reverse fragment was PCR amplified using GoTaq DNA polymerase (Promega) and ligated into the pTZ57R vector (Fermentas). The direction of the insert was verified by restriction site analysis and then the forward fragment was ligated next to it in the plasmid. After cloning of this sense-antisense construct, the *Hind*III-*Bam*HI fragment was liberated from pTZ57R and inserted into pHD677.

Upon transfection of trypanosomes, the recombinant plasmid becomes inserted in the parasite's genome by means of homologous recombination between a region of the vector and a transcriptionally silent ribosomal RNA gene repeat spacer. The spacer is found in many copies along the genome; therefore the precise location of the insertion of the construct into the genome can be different for each clone, as well as the number of copies of the linearized vector that are inserted into the genome. Transfected cells were kept under hygromycin selection, at a concentration of 5 μg/mL for bloodstream cells and 50 μg/mL for procyclics. Induction of RNAi was done by adding 1 μg/mL tetracycline to bloodstream cultures and 5 μg/mL to procyclic cultures. For experiments in which growth rates were determined, cells were maintained in the exponential growth phase. Cell density was daily determined by counting using a Bürker-Türk cell (counting grid with a depth of 0.01 mm), subsequently diluted appropriately and recounted. Growth curves were plotted as the product of cell density and total dilution *versus* time.

The bloodstream cell line for RNAi against VPS34 was previously prepared in our laboratory (Murielle Herman, PhD thesis). It contains a hairpin-forming RNA corresponding to a region of the VPS34 gene inserted into the pHD1336 vector that also endows resistance to blasticin. We induced RNAi, collected samples at 24 h or 48 h after induction and processed the cells for immunofluorescence.

3.6 Transfection of trypanosomes

$1x10^7$ bloodstream cells were washed and resuspended in 400 µL of pre-warmed cytomix (Van den Hoff *et al*., 1992). Transfection was done with 10 µg of the plasmids linearized with *NotI*, in a 2 mm electroporation cuvette (electroporation parameters : 1250 V, 25 µF, 50 Ω) and the trypanosomes were subsequently diluted into 12 mL pre-warmed HMI-9 and incubated overnight. One day after electroporation of the cells, selection was done by adding 12 mL HMI-9 and the required resistance antibiotic to the flask: hygromycin at 5 µg/mL and/or G418 at 15 µg/mL. One mL aliquots of the 24 mL culture were then transferred to a 24 well plate and incubated for 5-7 days when positive clones were selected.

$2x10^7$ procylic cells were washed and resuspended in 500 µL cold Zimmerman post-fusion medium (ZPFM; McCulloch *et al*., 2004) and 10 µg of linearized plasmid was added to the cuvette. The cells were subsequently electroporated with the following parameters: 1800 V, 25 µF, 50 Ω, using 4 mm electroporation and then placed in 4.5 mL pre-warmed SDM79 for an overnight incubation. The next day, 4.5 mL of conditioned medium (obtained from a culture of wild-type cells in the exponential growth phase after filtering to remove the cells) were added to the flask and the selective antibiotic was added when needed at a concentration of 50 µg/mL for hygromycin and 50 µg/mL for G418. Next, aliquots of 1 mL were placed in the first row of a 24 well plate and 300 µL was diluted serially with the conditioned medium in the corresponding well of the following row with 600 µL of conditioned medium. From this well, 300 µL was taken and diluted in the wells of the third row with 600 µL of conditioned medium. Finally 300 µL of each well in this third row was diluted with 300 µL of conditioned medium in the wells of the last row. Plates were maintained for 7-10 days when positive clones were selected.

3.7 Reverse Transcription - PCR assays

Samples from wild-type, RNAi non-induced and RNA induced bloodstream and procyclic cells were collected at different time points in three independent experiments. The cell suspensions were centrifuged at 1,000 x *g* for 10 min (Beckman TJ-6 centrifuge, TH-4 rotor) and total RNA was extracted and purified

with the SV Total RNA Isolation System (Promega). 2 µg of total RNA was then used to produce cDNA with the RevertAid H Minus First Strand cDNA Synthesis Kit (Fermentas). The cDNA obtained was diluted fivefold and PCR was then performed using 2.5 µL of these samples, using GoTaq DNA polymerase (Promega) with the following primers for a small fragment of the tubulin gene (*TUB*) as control, forward : 5'- GCGCGAAATCGTCTGCGTTCAGG -3', reverse : 5'- CGACGTACGGAGTCCATTGTACC -3' and for *ATG24*, forward : 5'- CCAGCACAATGACCAACTTTC -3' and reverse 5'- TCCAACACTTTATCCCTCTGC -3'. The PCR conditions used were chosen for a T_m = 58°C, with 18 cycles for tubulin or 24 cycles for ATG24. 20 µL of the PCR product was run in a 2% agarose gel and stained with SYBR Safe DNA Gel Stain (Invitrogen) for 20 min with agitation, then photographed at a Kodak Image Station 2000MM and the intensities of the area defined as each band was calculated. To compare points, the intensity given to each band for *ATG24* by the Kodak Image Station program minus the background, were normalized for the levels of the *TUB* bands at each time point, where wild-type levels obtained for *ATG24/TUB* were considered 100%. For each of three experiments, the PCR was performed at least twice.

3.8 Tagged ATG24 expressing cell lines

An *ATG24* construct was made using the primers forward : 5'- CCCAAGCTT*ATG*TCTGAAAATGTCTTTGAGTTC -3' (*Hind*III site underlined and start codon italicized), reverse : 5'- AGGGGATCCATCGTCATCAATCAAATGGT -3' (no stop codon, *Bam*HI site underlined) and GoTaq DNA polymerase. The amplified PCR product was inserted into pGN1, a vector that can be integrated into the *T. brucei* chromosome and codes for a tetracycline-inducible Green Fluorescent Protein (GFP) that can be expressed as a tag fused to the C-terminus of the protein coded by the DNA insert. Bloodstream cells were transfected as described above. Selection of transfected cells was done by their resistance to 5 µg/mL hygromycin. Cells were maintained under hygromycin selection. Positive clones were tested by PCR of genomic DNA to check for amplification of the cassette, and immunofluorescence 24 h after induction by tetracycline, using the same concentration as described above.

Another *ATG24* construct was created with the following primers : Fw : 5'–AGAGGGCCC*ATG*TCTGAAAATGTCTTTGA -3' (*Sma*I site underlined, start codon italicized) and Rv : 5'- AGGGGATCCATCGTCATCAATCAAATGGT -3' (no stop codon, *Bam*HI site underlined). The PCR conditions used were : 2 min at 95°C, then 25 cycles of 30 sec at 95°C, 30 sec at 61°C, 1.5 min at 72°C and a final incubation of 5 min at 72°C. The PCR amplified construct was treated with the restriction enzymes *Sma*I and *Bam*HI and inserted into the vector pHD1700 that allows the expression of the ATG24 from the cloned insert with two tandem copies of the myc peptide at its C-terminus. The plasmid was then linearized and used to transfect procyclic cells as described in section 3.6. Selection of the positive clones was performed by cultivation in the presence of 50 µg/mL hygromycin, while induction of expression of the tagged protein was done by adding 5 µg/mL tetracycline to the medium.

3.9 GFP-ATG8.1 and GFP-ATG8.2 expressing bloodstream and procyclic forms

We received from Remo Schmidt and Dr Peter Bütikofer (Bern, Switzerland) two plasmids containing the genes for respectively EGFP-tagged TbATG8.1 and TbATG8.2 in the vector pG-EGFP-ΔLIIγ. The linearized vector can integrate upstream of the tandemly-arranged procyclin genes and is controlled by a procyclin promoter. Consequently, the GFP-tagged protein is constitutively expressed in procyclic cells. We transfected both bloodstream and procyclic wild-type cell lines and both ATG24 RNAi cell lines with these constructs. Positive clones were selected by their resistance to G418 (Gibco). Bloodstream cells were maintained under G418 selection at a concentration of 15 µg/mL and procyclic cells at 50 µg/mL. As expected, bloodstream cell lines expressed lower levels of the tagged proteins when compared to procyclic cells.

3.10 Differential centrifugation assay

Procyclic wild-type cells and cells expressing myc-tagged ATG24, grown to exponential phase, were collected by centrifugation at 1,000 x *g*, (Sorvall

centrifuge, rotor SM-24, inner row) washed in isotonic SIE buffer (250 mM sucrose, 3 mM imidazole, pH 7, 1 mM EGTA) and finally resuspended in 2.5 mL of the same buffer. The cells were then broken by maceration in a mortar with carborundum powder (approximately the same weight as the cell pellet). After microscopically checking that over 95% of the parasites were lysed, the mixture was centrifuged at 50 x *g* at 4°C for 3 min to pellet the carborundum powder. The supernatant constituting the cell homogenate was centrifuged at 1,500 x *g* for 10 min, the pellet (nuclear fraction) was collected and the supernatant was centrifuged at 5,000 x *g* for 10 min. The pellet (large granular fraction) was again collected and the supernatant centrifuged at 15,000 x *g* for 10 min to give a pellet (small granular fraction) and supernatant. The supernatant fraction was used for the last centrifugation step, performed with a 50 Ti rotor in an ultracentrifuge at 140,000 x *g* for 1 h, to separate the microsomal fraction in the pellet and a supernatant composed of the cytosolic fraction. The protein concentration of each fraction was measured using the Bradford assay and equal protein amounts of each fraction were charged into slots of a gel, size-fractionated by SDS/PAGE and finally analysed by western blotting. The experiment was performed three times, giving similar results for the subcellular localisation of ATG24.

3.11 *In vitro* differentiation assay

Cultures of bloodstream-form wild-type, ATG24 RNAi non-induced and 48 h induced trypanosomes, expressing or not the EGFP-ATG8.1 or EGFP-ATG8.2 constructs, were harvested in the exponential growth phase by centrifugation at 1,000 x *g* for 5 min (Beckman TJ-6 centrifuge, TH-4 rotor). The cells were resuspended in pre-warmed Differentiating Trypanosome Medium (DTM) in the presence of 3 mM citrate and 3 mM cis-aconitate (from a 300 mM stock in 25 mM Hepes, pH 7.3), and transferred from 37°C to 28°C. Maintenance of clones was assured by culturing the cells in the presence of 1 µg/mL hygromycin and/or 15 µg/mL G418 when needed. RNAi induction was done by addition of 1 µg/mL tetracycline. Samples were taken at several time points for analysis by immunofluorescence and western blot.

3.12 Western blot analysis

For western blot analysis, protein samples were boiled for 5 min in loading buffer, size-fractionated by SDS/PAGE and then transferred to Amersham Hybond-ECL (GE Healthcare) membranes. To prevent nonspecific binding of antibodies to the blots, the membranes were then blocked with 5% milk in PBS for at least 1 h at RT or overnight at 4°C under agitation, before incubation with antibodies for 1 h at RT or overnight at 4°C under agitation in 1% milk in PBS. The primary antibodies, all raised in rabbits, were used in the following dilutions: ATG24 1 :4,000, ZFP3 1 :10,000 (gift from Dr Keith Matthews, Edinburgh), cGAPDH 1 :10,000, PPDK 1 :25,000, PYK 1 :50,000, GFP 1 :10,000 (Invitrogen), myc 1 :5,000, PEX1 1:10,000, ENO 1:150,000. Secondary antibody (peroxidase-conjugated anti-rabbit IgG produced in goat; Invitrogen) was used in a dilution of 1 :10,000. Washes were done with PBS and 0.05% NP40 in PBS. Revelation was performed using Pierce ECL western blotting substrate (Thermo Scientific) and photographic films were exposed to the membranes for periods varying from 30 sec to 1 h, dependent on the intensity of the signal obtained with each antibody. Films were then developed and fixed.

3.13 Concanavalin A and transferrin uptake assays

Bloodstream wild-type and RNAi cells induced for 48 h, growing in the exponential phase were centrifuged at 1,000 x *g* for 10 min at 4°C, (VWR microcentrifuge, rotor T15A61-0806) washed in FCS-free HMI-9 with 1% BSA and resuspended in the same medium. Different samples were incubated at 4°C, 12°C and 37°C, for up to 30 min, then AlexaFluor 594-conjugated concanavalin A (5 µg/mL) or AlexaFluor 568-conjugated transferrin (total protein concentration 25 µg/mL, containing 5 µg/mL of transferrin) were added to the suspensions and the cells were kept at different temperatures for the rest of the incubation. Samples were collected at different times, placed on ice, washed in FCS-free HMI-9 with 1% BSA and processed for immunofluorescence.

To verify if the effects seen for transferrin were caused by a decreased uptake or an increased lysosomal degradation we performed the transferrin uptake experiments with wild-type bloodstream trypanosomes and cells of the ATG24 RNAi cell line induced for 48 h with tetracycline induction, after addition of 50 μg/mL leupeptin to the growth medium. The incubation in the presence of the compound was for initially done for one hour. After this the parasites were centrifuged and resuspended in pre-warmed FCS-free HMI-9 with 1% BSA with leupeptin for another 30 min. Next, 15 or 25 μL of transferrin was added to the medium. Samples were collected at 15 and 30 min after addition of transferrin and placed on ice, protected from light. Cells were then centrifuged at 4°C and resuspended in the same volume of cold FCS-free HMI-9 with 1% BSA to remove the transferrin from the medium. Subsequently cells were centrifuged again, resuspended in cold vPBS and fixed with 3% paraformaldehyde on ice for 10 min. Next, the paraformaldehyde was removed from the suspension with two rounds of centrifugation and washing of the pellet with vPBS. Cells were finally resuspended in a small volume of vPBS and kept at 4°C in the dark before processing for fluorescence studies.

3.14 Determination of the effect of wortmannin on ATG24 localisation

Procyclic wild-type cells growing in the exponential phase were incubated in SDM79 with increasing concentrations of wortmannin (0.3 μM, 3 μM or 30 μM, diluted in DMSO) for 1 h. Cells were then centrifuged, washed with PBS and processed for immunofluorescence as described below.

3.15 Immunofluorescence studies

Trypanosome cultures were centrifuged at 2,200 x *g*, (Beckman TJ-6 centrifuge, TH-4 rotor) resuspended in PBS, fixed in 8% formaldehyde at RT under gentle agitation, permeabilized with 0.1% Triton X-100 and incubated for 1 h in the presence of glycine. After centrifugation and resuspension in PBS, the cells were placed on poly-L-lysine treated slides and kept at RT for 1 h. Excess cell suspension was aspirated and blocking was performed with BSA 5% in

water at 37°C for 45 min. Incubation of the cells fixed to the slides with primary and subsequently secondary antibodies diluted in 2% BSA in water was performed for 45 min at 37°C. Excess antibodies were removed by washing several times with PBS. Anti-PGK-c was used at a 1:1,000 dilution, anti-ATG24 at 1:400, anti-ASCT at 1:750 (gift from Dr F. Bringaud, Bordeaux) and monoclonal anti-TPI at 1:5,000. As secondary antibodies were used, both at 1:500, anti-rabbit conjugated to AlexaFluo568 (red) (Invitrogen) and anti-mouse conjugated to AlexaFluo488 (green) (Invitrogen). When appropriate, also an incubation with DAPI, diluted at 1:500 in PBS, was done for 30 min at RT. Slides were mounted with Mowiol 4-88 (Calbiochem) or DAKO fluorescent mounting medium (DAKO) and observed in a COSD Spinning Disc microscope.

Samples from the concanavalin A and transferrin uptake experiments were washed in cold vPBS (Voorheis' modified PBS, containing 46 mM sucrose and 10 mM glucose) and fixed in 3% paraformaldehyde, slides were mounted with DAKO.

3.16 Electron microscopy

Different samples of trypanosomes were prepared for transmission electron microscopy (TEM): (1) bloodstream wild-type cells, (2) bloodstream wild-type cells placed for 24 h in DTM; (3) ATG24 RNAi cells induced for 72 h and (4) ATG24 RNAi cells first induced for 48 h with tetracycline for RNAi and then placed for 24 h in DTM, were RNAi was maintained. The cells were then collected by centrifugation at 1,000 x *g* for 10 min at 4°C (Sorvall centrifuge, HS-4 rotor) washed in PBS (pH7.3), pelleted and centrifuged again, resuspended in PBS-Ca/Mg (PBS, pH 7.0, with 3.6 mM $CaCl_2$ and 3.0 mM $MgCl_2$), then fixed for 30 min in 2% glutaraldehyde at 4°C in phosphate buffer. Cells were then washed in veronal buffer and post-fixed in 1% osmium tetroxide in veronal buffer for 1 h at 4°C. Next, cells were washed with veronal buffer and pelleted in 2% agar. Small blocs were coloured using 0.5% uranyl acetate in veronal buffer overnight at 4°C protected from light. Next morning, the blocs were extensively washed and dehydrated in increasing concentrations of ethanol and embedded in spur. Ultrathin sections measuring 70 nm were made using a Reichert ultramicrotome

and sections on grids were coloured with uranyl acetate and lead citrate for 10 min each. Samples were observed in a transmission electron microscope (Phillips, CM12) at 80 kV and Images were imported to and analysed in AxioVision software (4.8.2; Zeiss, Germany).

3.17 Phylogenetic analysis

(Courtesy Dr Daniel J. Rigden, University of Liverpool, UK)

To determine evolutionary relationships between the candidate TbATG24 and other proteins with both a PX and BAR domain in *T. brucei* and some other organisms (*S. cerevisiae*, human, *Drosophila melanogaster* and *Caenorhabditis elegans*), their sequences were first aligned for optimal positional identity. Terminal regions prior to the PX domain and after the BAR domain which were clearly non-homologous were trimmed. The relationships between the truncated sequences were then analysed by three different methods: the Neighbour-Joining method (Saitou and Nei, 1987), the Minimum Evolution method (Rzhetsky and Nei, 1992) and the Maximum Parsimony method (Felsenstein, 1985). The analysis involved 27 amino-acid sequences. There were a total of 629 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 (Tamura *et al*., 2011). In all three cases a bootstrap consensus tree was inferred from 500 replicates. When the evolutionary history was inferred using the Neighbour-Joining method, an optimal tree with the sum of branch length = 17.12554452 was prepared. The evolutionary distances were computed using the p-distance method (Nei and Kumar, 2000) and are in the units of the number of base differences per site. The ME tree was searched using the Close-Neighbour-Interchange (CNI) algorithm (Nei and Kumar, 2000) at a search level of 1. The Neighbour-Joining algorithm was used to generate the initial tree. In the Maximum Parsimony analysis, the most parsimonious tree with length = 5185 was used. The consistency index is (0.638254), the retention index is (0.449044), and the composite index is 0.296880 (0.286604) for all sites and parsimony-informative sites (in parentheses). The Maximum Parsimony tree was obtained using the Close-Neighbour-Interchange algorithm (Nei and Kumar, 2000) with

search level 1 in which the initial trees were obtained with the random addition of sequences (10 replicates).

Chapter 4

Analysis of *Trypanosoma brucei* ATG24

4.1 ATG24 is a protein expressed in bloodstream and procyclic *Trypanosoma brucei*, partially associated to the endosomal system

Previously, Tb09.211.4240 was identified in the *T. brucei* genomic sequence database as the most likely homologue of yeast ATG24 (Herman *et al*., 2006). We have expressed and purified full-length recombinant trypanosomal ATG24 and raised a polyclonal antiserum (see Section 3.4). This antiserum was able to specifically recognize a band of the expected size (between markers of 43 and 55 kDa) on western blots of cell lysates from both bloodstream and procyclic stages (Figure 10). Moreover, in immunofluorescence experiments with trypanosomes of both stages, the same antiserum recognized ATG24 partially in the cytosol and partially to puncta in a region between the nucleus and kinetoplast (Figure 11).

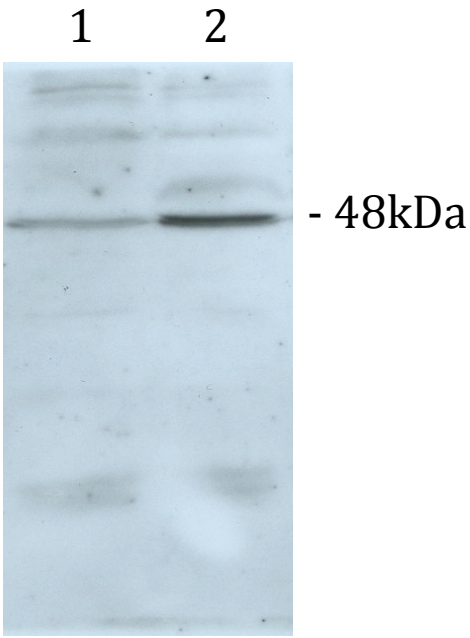


Figure 10: Specificity of the anti-ATG24 antiserum. Line 1- proteins from a lysate obtained from $8x10^6$ procyclic cells, line 2 – proteins from a lysate obtained from $8x10^6$ bloodstream cells. On the western blot one band at the expected size for the protein was detected by the antiserum raised against ATG24 in both procyclic and bloodstream cell lines.

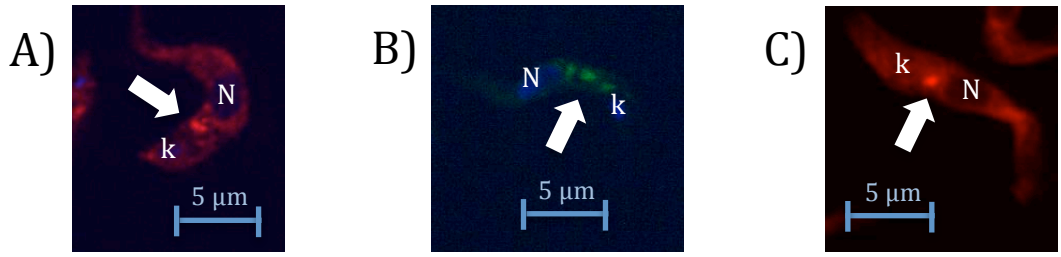


Figure 11: Subcellular localisation of ATG24 (arrows) in A) bloodstream wild-type cells (using anti-ATG24). B) Bloodstream cells expressing ATG24-GFP. C) Subcellular localisation of ATG24 in wild-type procyclic cells. Red – ATG24, green – ATG24-GFP, blue – DNA: nucleus (N) and kinetoplast (k).

Bloodstream parasites expressing a C-terminally GFP-tagged ATG24 also show fluorescence concentrated in a few puncta in the same region between nucleus and kinetoplast. The GFP-tagged ATG24 shows good colocalisation with the fluorescence observed with anti-ATG24 (Figure 12). This region is frequently described in the literature as the part of the cell where endocytic vesicles accumulate as well as where the lysosome is located, which in trypanosomes is often seen as a single structure. The GFP-tagged ATG24 also partially colocalises with concanavalin A and transferrin in bloodstream cells incubated with these molecules at 37°C for 10, 20 or 30 min, indicating that it is indeed a protein localising to the endocytic system (Figure 13).

Moreover, analysis of subcellular fractions of procyclic cells prepared in three independent differential centrifugation experiments shows consistently that the protein is mainly present in the cytosolic fraction and also, but to a lesser extent, in the microsomal fraction. The result of one of the experiments is shown in Figure 14. The microsomal fraction is composed of vesicles that are normally present as organelles in the cells (endosomes, vesicles of the rough ER and of the secretory and trans-Golgi system) as well as vesicles that are created during the preparation of cell lysates (vesicles ruptured from the plasma membrane or from organellar systems such as the Golgi apparatus and the mitochondrion).

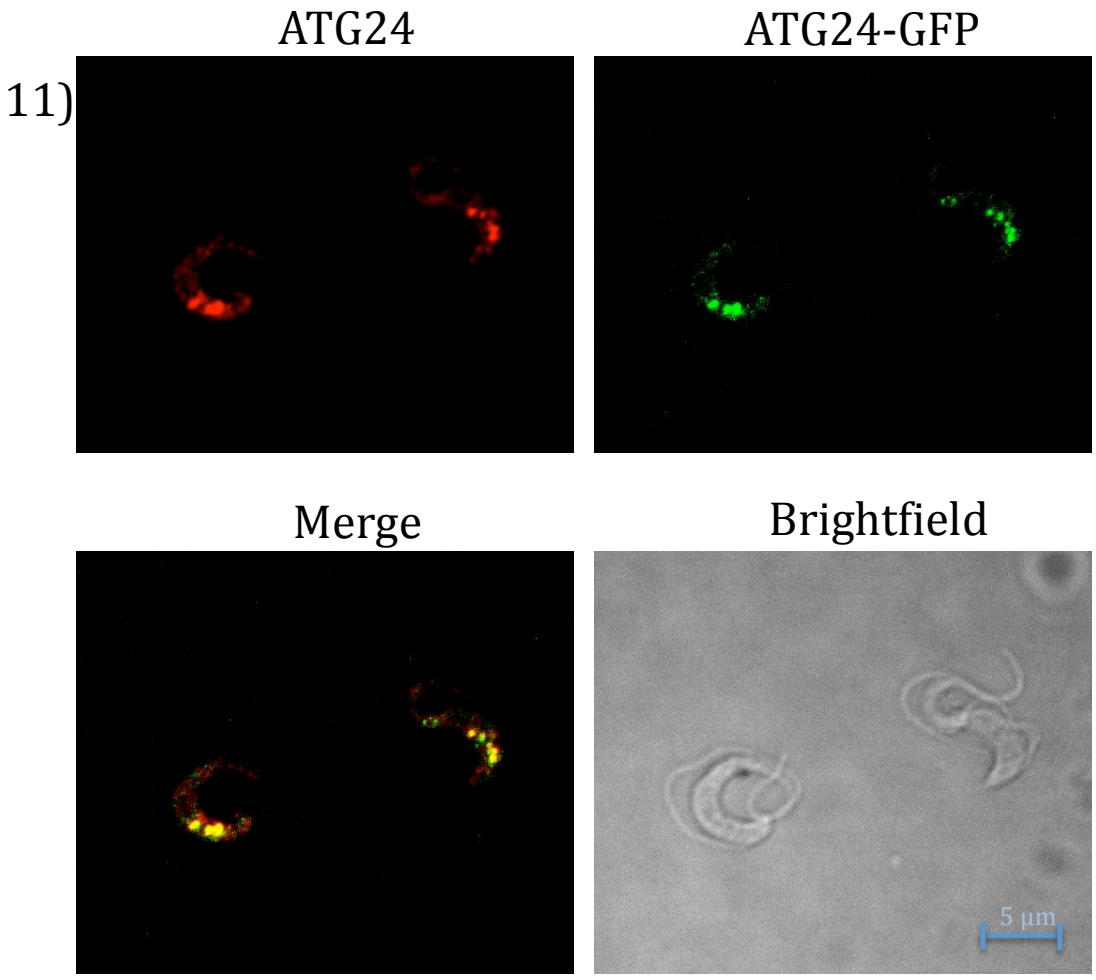


Figure 12: Confocal immunofluorescence images showing colocalisation (merge, yellow) of ATG24 (red) detected using anti-ATG24 and ATG24-GFP (green).

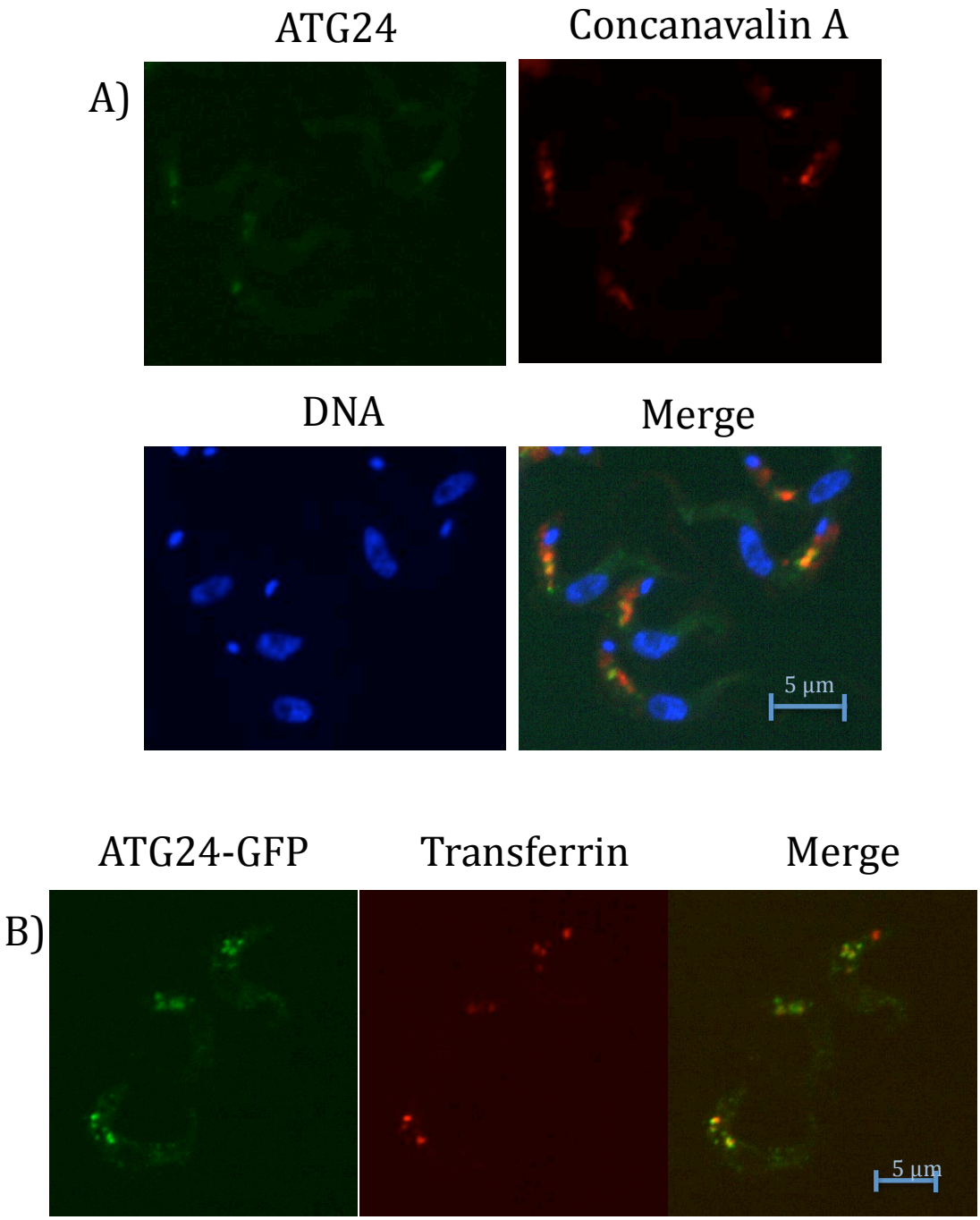


Figure 13: Wild-type bloodstream cells expressing ATG24-GFP. A) Incubated with AlexaFluor594-conjugated concanavalin A at 37°C for 30 min, shown in red. B) Incubated with AlexaFluor568-conjugated transferrin at 37°C for 30 min, shown in red. Yellow colour in the Merge channels indicate colocalisation of the two signals.

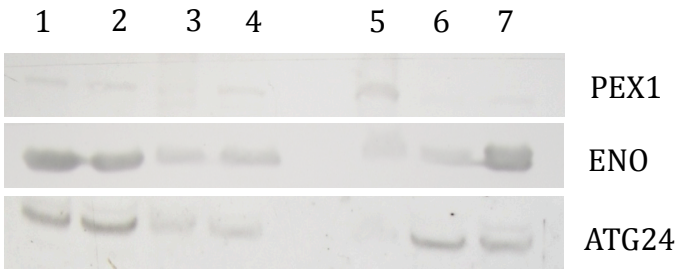


Figure 14: Western blot illustrating the localisation of proteins in fractions obtained from differential centrifugation of wild-type procyclic cells. The same amount of protein was loaded in each lane. 1 – total homogenate, 2 – post nuclear fraction, 3 – nuclear fraction, 4 – large granular fraction, 5 – small granular fraction, 6 – microsomal fraction, 7 – cytosolic fraction. PEX1 – glycosomal marker; ENO - enolase, cytosolic marker.

4.2 ATG24 interacts with intracellular membranes and its subcellular localisation is affected by VPS34

The ATG24 sequence lacks a recognisable transmembrane domain, nonetheless the protein is found enriched in microsomal fractions and in a region of the cell that is rich in endosomal membranes. The *T. brucei* ATG24, as its yeast and mammalian counterparts, possesses a N-terminal Phox (PX) homology domain that is predicted to interact with phosphatidylinositol-3-phosphate (PI3P) in intracellular membranes. It also possesses the BAR domain that has been described as a constituent of proteins that interact with strong membrane curvatures. The phosphorylated membrane lipid PI3P is known to participate in many processes by directing the localisation and function of proteins that possess at least one of three domains that are able to interact with it: the PX (Ellson *et al*., 2002), PH and FYVE domains (reviewed in Vanhaesebroeck *et al*., 2012). It has been shown in yeast cells that the protein responsible for the localisation of ATG24 to endosomal membranes is the PI3 kinase VPS34 (Nice *et al*., 2002). *T. brucei* VPS34 has been identified and characterised as an essential protein and as the only PI3P kinase (PI3K) apparently present in this parasite. It has been shown to participate also in Golgi division (Hall *et al*., 2006). We used the PI3K inhibitor wortmannin to verify its effects on *T. brucei* ATG24's association to intracellular membranes. In three

independent experiments we observed that incubation of procyclic cells with increasing concentrations of the compound for 1 h affects the subcellular localisation of the protein in a dose-dependent fashion; the ATG24 becomes less concentrated in the endosomal area and its distribution over the cytosol seems to increase (Figure 15).

To assess if ATG24 is dependent on VPS34 for its subcellular localisation, we have used a bloodstream VPS34 RNAi cell line available in our laboratory. It contains the VPS34 gene inserted in the pHD1336 vector under the control of a tetracycline-inducible promoter and is resistant to the vector-encoded selectable marker blasticidin. This cell line has been shown to have a growth effect; death of many cells was observed at 72 h after RNAi induction, but the population recovered growth, from reverted RNAi mutants, at 96 h (PhD thesis, Murielle Herman). We have performed immunofluorescence on the RNAi induced cells to verify if the decrease of VPS34 would affect the binding of ATG24 to subcellular membranes. Indeed, upon induction of RNAi against VPS34 we could see a change in the subcellular localisation of ATG24, from the concentrated puncta in the endocytic region of the parasite to a more dispersed, apparently cytosolic localisation as early as 24 h after the induction (Figure 16).

4.3 ATG24 is not essential for the parasite in the conditions tested

The essentiality of ATG24 was assessed by RNAi. A tetracycline-inducible, genome-inserted cassette that causes the transcription of a double-stranded RNA specific for ATG24 will result in the destruction of the ATG24 mRNA by the RNAi enzymatic machinery that is present in *T. brucei*. Upon RNAi induction the parasites of both life-cycle stages showed only a slight decrease in growth rate. This experiment was repeated three times, yielding similar results; one is shown in Figure 17. This indicates that ATG24 is not essential for cell growth or at least that sufficient protein remained to sustain growth under the conditions employed. The effect of the RNAi on the levels of the mRNA and protein was followed in time by Reverse Transcription-PCR, performed three times in triplicates (Figure 18) and western blot analysis (Figure 19), respectively.

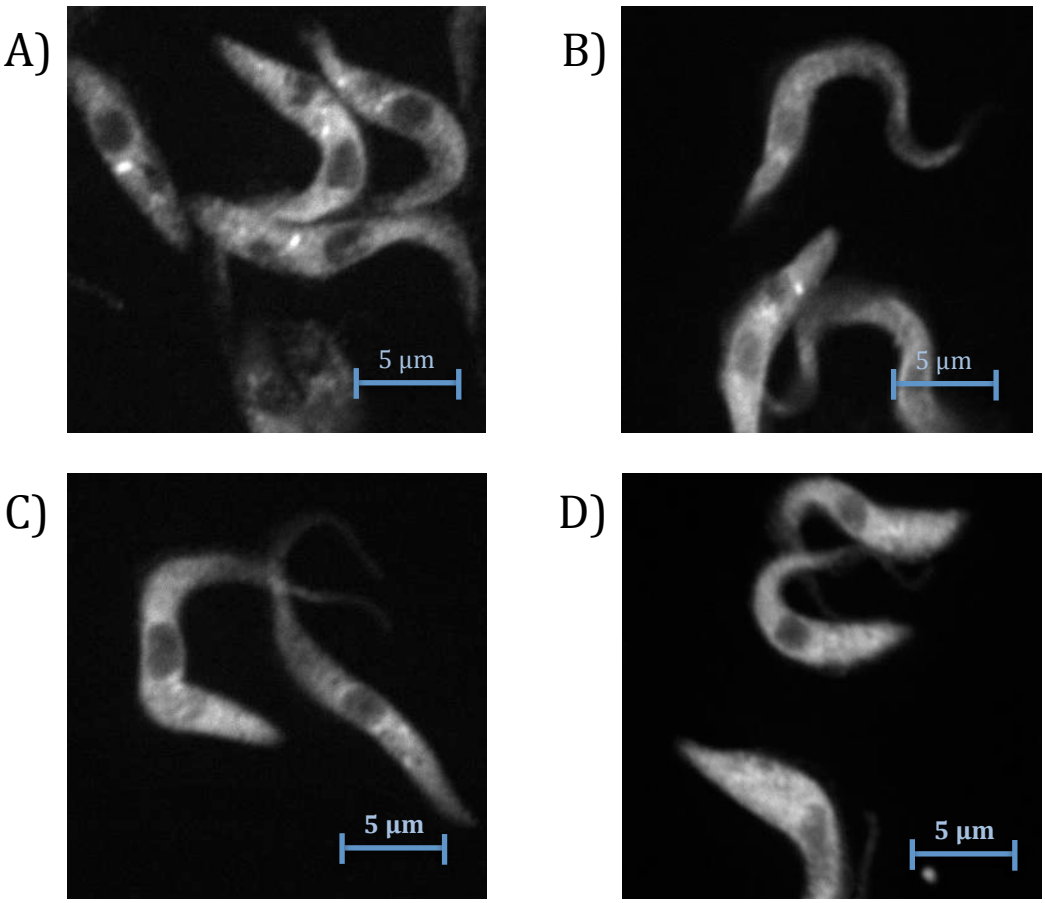


Figure 15: Subcellular localisation of ATG24 (white) in A) wild-type procyclic cells and wild-type procyclic cells incubated for 1 h with B) 0.3 µM, C) 3 µM and D) 30 µM wortmannin.

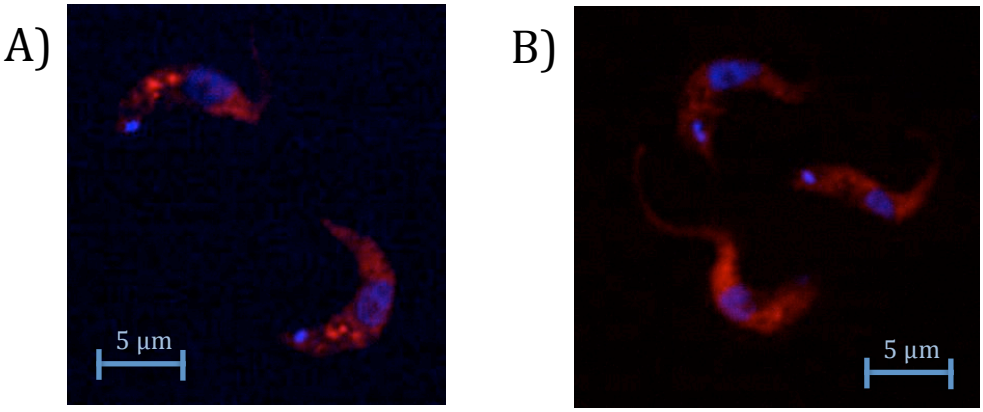


Figure 16: Subcellular localisation of ATG24 (red) in bloodstream form trypanosomes A) wild-type cells and B) VPS34 RNAi 24 h Induced cells.

We detected a maximal decrease of 40% of the ATG24 mRNA concentration in both cell lines (where samples were collected in three independent experiments and the PCR were performed three times for each one), whereas the levels of the protein were, in independent experiments, often reduced to an extent that it was barely detectable on western blots. Despite the absence of a strong growth effect, we observed morphological aberrations in bloodstream and procyclic cells treated for ATG24 RNAi. Upon RNAi induction some of the cells became swollen and eventually many of them became completely round (Figure 20). In transmission electron microscopy ATG24 RNAi bloodstream cells, induced with tetracycline for 72h, presented several morphological abnormalities: an enlarged structure (probably the lysosome, that is often observed as a single organelle) could be seen in some of the cells that also displayed an abnormal morphology of their mitochondrion, ER, and vesicles (Figure 21).

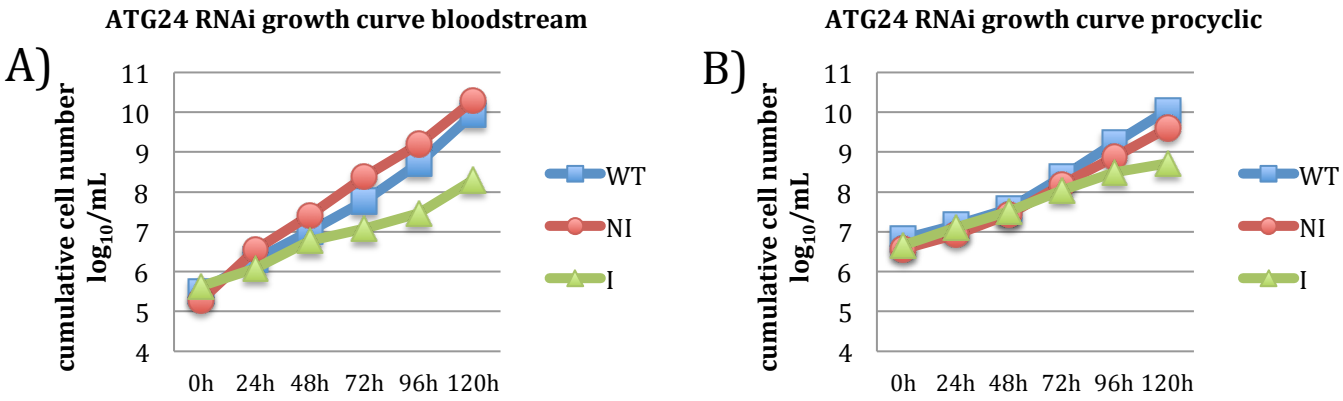


Figure 17: A) Growth curve of trypanosomes of the bloodstream ATG24 RNAi cell line. Blue squares – wild type (WT), red balls – non induced (NI), green triangles – induced cells (I). B) Growth curve of procyclic ATG24 RNAi cells. Blue squares – wild type (WT), red balls – non induced (NI), green triangles – induced cells (I).

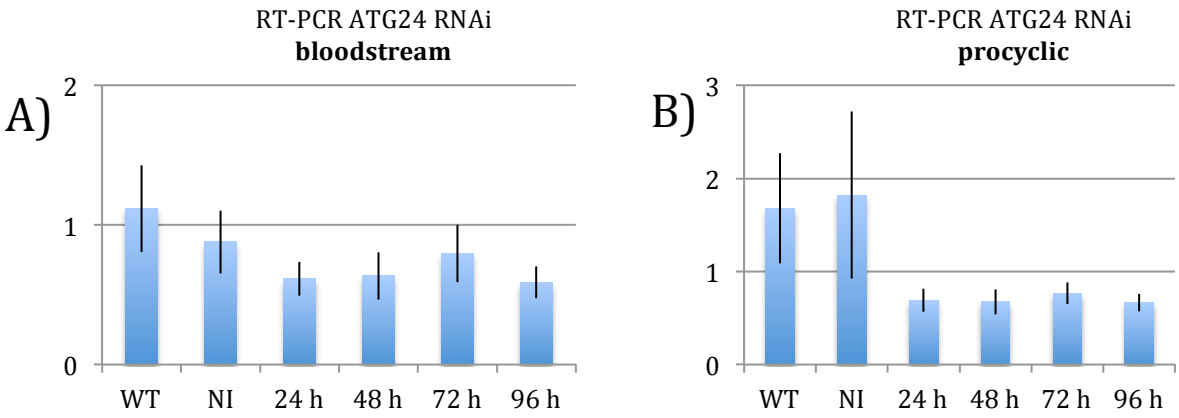
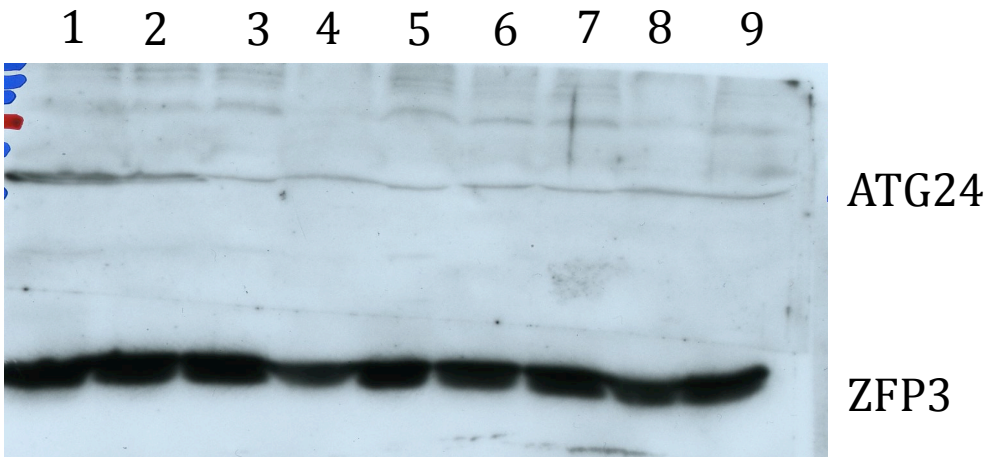


Figure 18: Knock-down of ATG24 expression in *T. brucei*. ATG24 mRNA levels were determined by Reverse Transcription PCR (RT-PCR) in A) bloodstream and B) procyclic ATG24 RNAi cells. The levels of ATG24 mRNA were normalized against tubulin mRNA, whose levels were considered arbitrarily as 1. WT – wild type. NI – non induced cells.

A)



B)

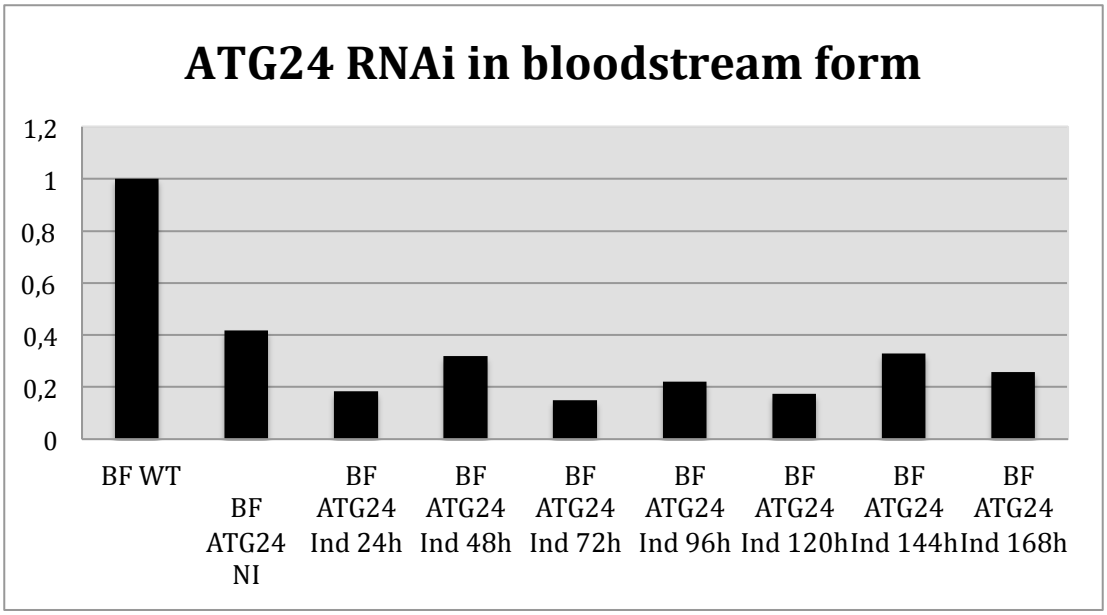


Figure 19: Decrease in levels of ATG24 upon RNAi induction. A) Bloodstream cells. 1 – Bloodstream wild-type. 2 – Bloodstream ATG24 RNAi Non-Induced. 3 – Bloodstream ATG24 RNAi Induced for 24 h. 4 – Bloodstream ATG24 RNAi Induced for 48 h. 5 – Bloodstream ATG24 RNAi Induced for 72 h. 6 - Bloodstream ATG24 RNAi Induced for 96 h. 7 - Bloodstream ATG24 RNAi Induced for 120 h. 8 - Bloodstream ATG24 RNAi Induced for 144 h. 9 - Bloodstream ATG24 RNAi Induced for 168 h. ZFP3 was used as a loading control. B) Quantification of the intensities of each band, considering ATG24 levels of the wild-type cells as 1, and using ZFP3 as a loading control.

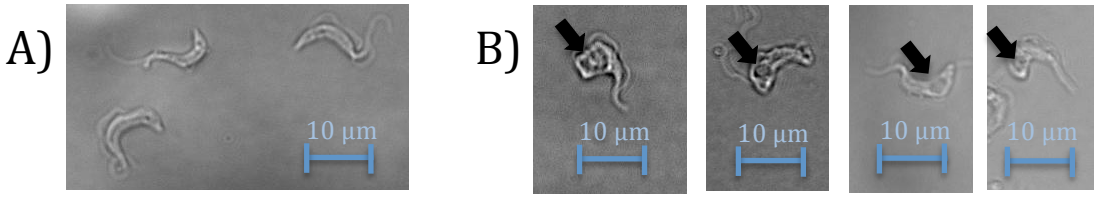
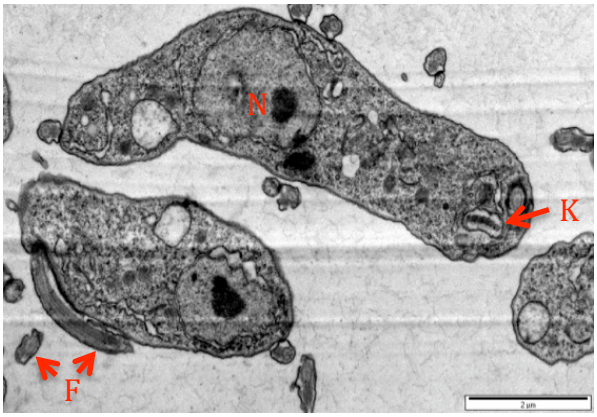


Figure 20: A) Phase-contrast microscopy images of bloodstream wild-type cells and B) bloodstream ATG24 RNAi cells after 48 h of RNAi induction. Notice the change in lysosomal morphology in the RNAi cells (arrows).

Since ATGs are rarely essential in nutrient-rich conditions, but may be essential during starvation or under otherwise stressful conditions, we followed the growth of procyclic cells during a change of their carbon source. We performed in three independent experiments the RNAi induction as usual in the glucose-rich SDM79 medium and, at 48 h, where the levels of the ATG-protein were decreased, the culture was centrifuged and resuspended in SDM80 medium, that is similar to SDM79 but lacks glucose, rendering the cells entirely dependent on amino acids as energy and carbon source. We expected that the cells, in order to adapt to the drastic change in carbon source, might need to change their glycosomal enzymatic profile via import into the glycosomes of a new set of proteins and removal of mature, import-incompetent glycosomes by pexophagy. However, also in this case, ATG24 appeared not to be essential because only a small decrease in the growth of the cells was observed during their adaptation to the change in carbon source (not shown).

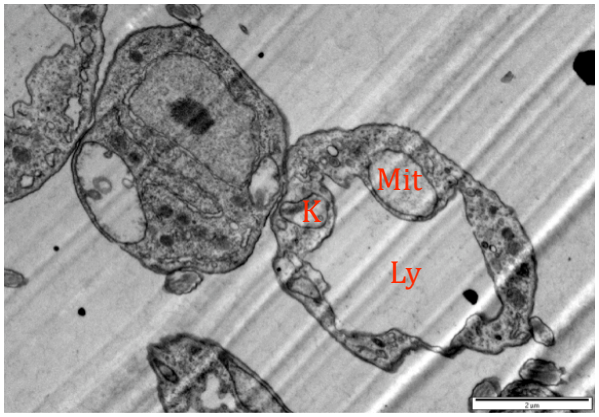
A)



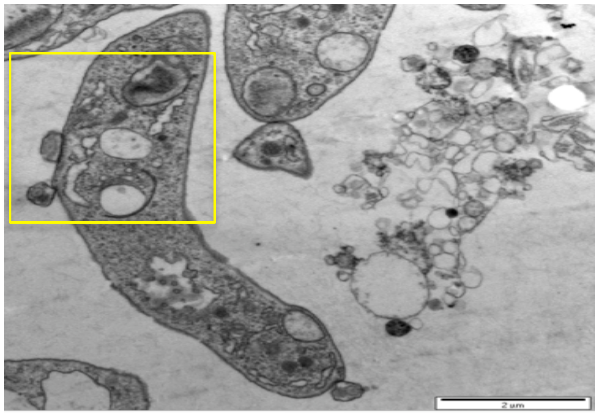
B)

C)

D)



E)



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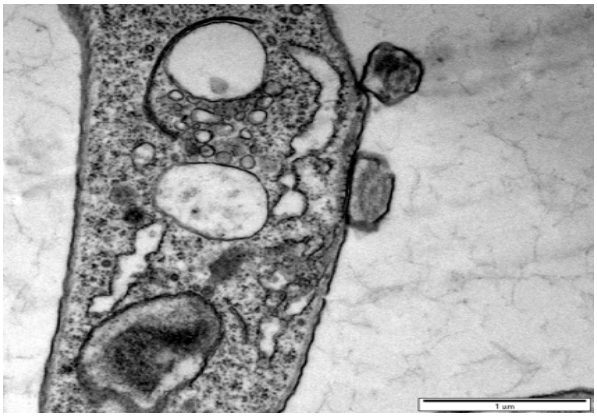
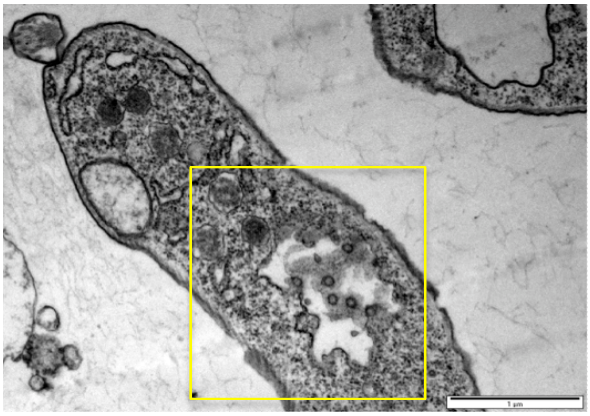


G)



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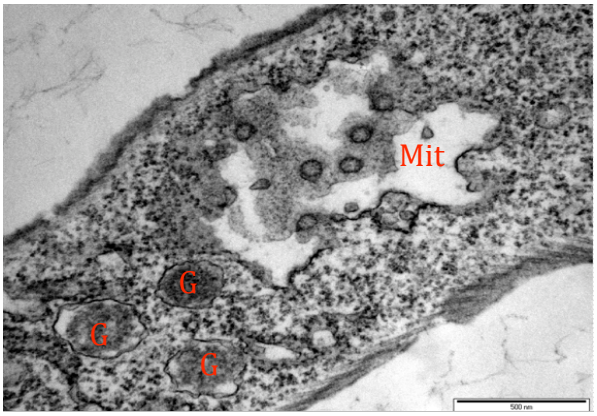


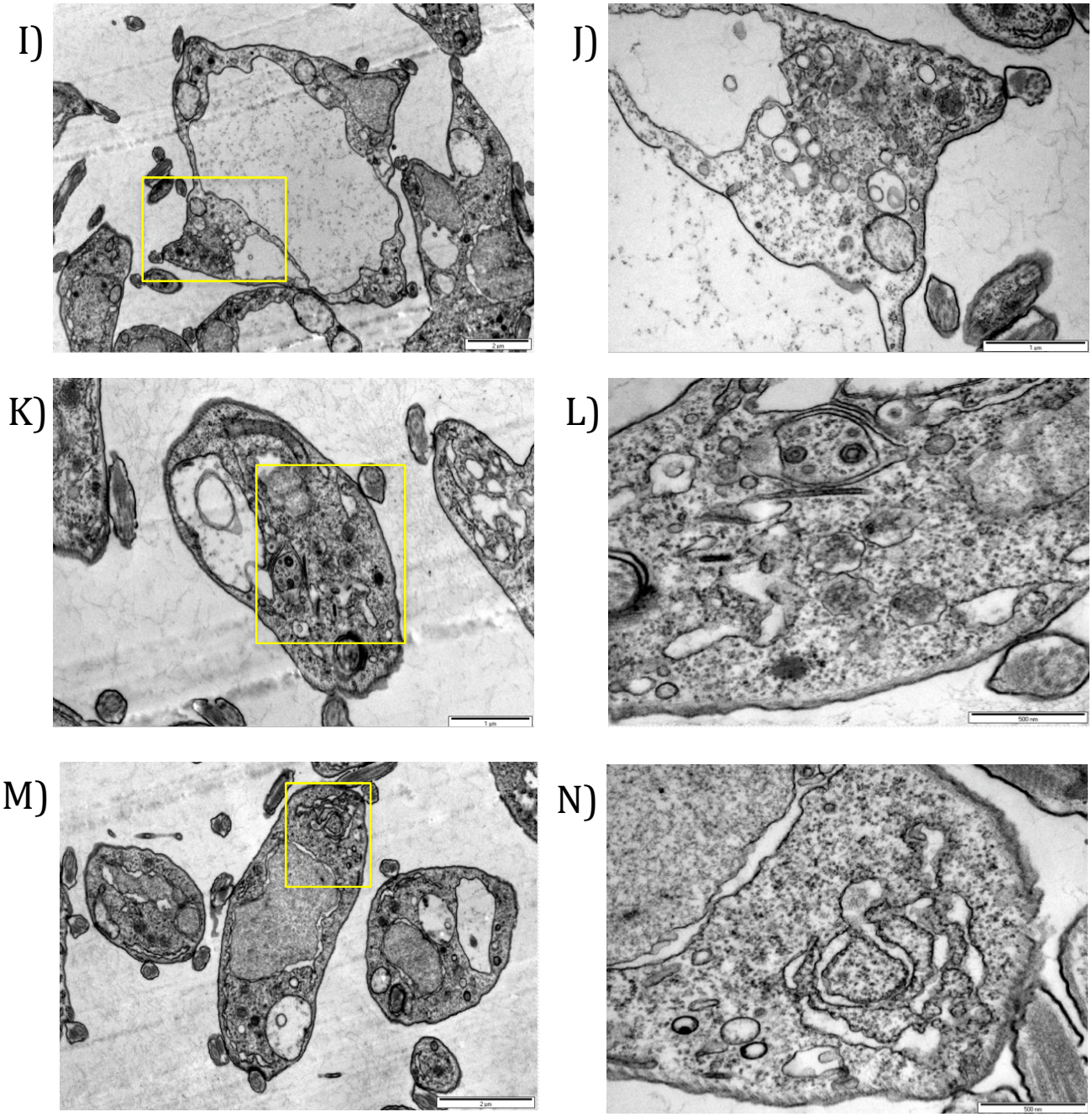


Figure 21: Depletion of ATG24 is associated with apparent morphological defects in *T. brucei.* A-C) Wild-type bloodstream cells. D-N) ATG24 RNAi bloodstream cells induced for 72 h. Images F, H, J, L and N present details of areas of trypanosomes shown in yellow in images E, G, I, K and M, respectively. Note the enlarged structure in images D and I. Images E and F show enlarged vesicles. Images G and H show a mitochondrion defect. Trypanosomes in images I and J present an increased number of vesicles. Images K and L show a cell with abnormal structures. Images M and N show a cell with a partial endoplasmic reticulum defect. Ly – lysosome, Mit – mitochondrion, N – nucleus, FP – flagellar pocket, F – flagellum, G – glycosome.

4.4 ATG24 plays a role in endocytosis

Yeast and mammalian ATG24/SNX4 play a role in endocytosis in addition to the role in autophagy reported for the yeast protein. To assess if the *T. brucei* ATG24 also participates in endocytosis, we performed transferrin and concanavalin A uptake experiments with the bloodstream wild-type and ATG24 RNAi cell lines. In wild-type bloodstream cells concanavalin A binds to saccharide-moieties of glycoproteins at the surface of the cell which become rapidly concentrated in the flagellar pocket membrane. It is subsequently translocated to endosomal vesicles, while remaining bound to the membranes, from where it is delivered to the lysosome and degraded at 37°C. Transferrin is internalized via a specific receptor, present in the flagellar pocket membrane, into early endosomes. The low pH in the endosomes releases the iron and then, unlike in humans where the apo-transferrin and the receptor are recycled back to the cell's surface, in trypanosomatids the receptor is recycled to the cell surface but the transferrin is delivered into the lysosome and there it is degraded. Confocal fluorescence microscopy images of wild-type and ATG24 RNAi cells show that, while the wild-type cells become positively stained for transferrin uptake, the RNAi cells show very little labelling, indicating a defect in the receptor-mediated import of this compound (Figure 23a and 23b). Pre-incubation of wild-type and ATG24 RNAi with the protease inhibitor leupeptin confirmed that the RNAi cell line has a defect in the uptake of the compound in three independent experiments (Figure 23c and 23d). In contrast, a concanavalin A uptake was observed in both wild-type and RNAi induced cells, but with the difference that the stain in wild-type cells is concentrated in many small puncta, whereas in the RNAi cells the staining is observed mainly as a strong signal in the lysosomal compartment that is often enlarged. These results indicate that ATG24 indeed also plays a role in endocytosis, as it appears to be involved in the receptor-mediated uptake of transferrin. In contrast, the bulk uptake of concanavalin A was not affected by RNAi, the compound is then internalized, proceeds through endosomal vesicles and accumulates in the terminal vesicle, the lysosome, that is increased in size in the RNAi cells (Figure 22).

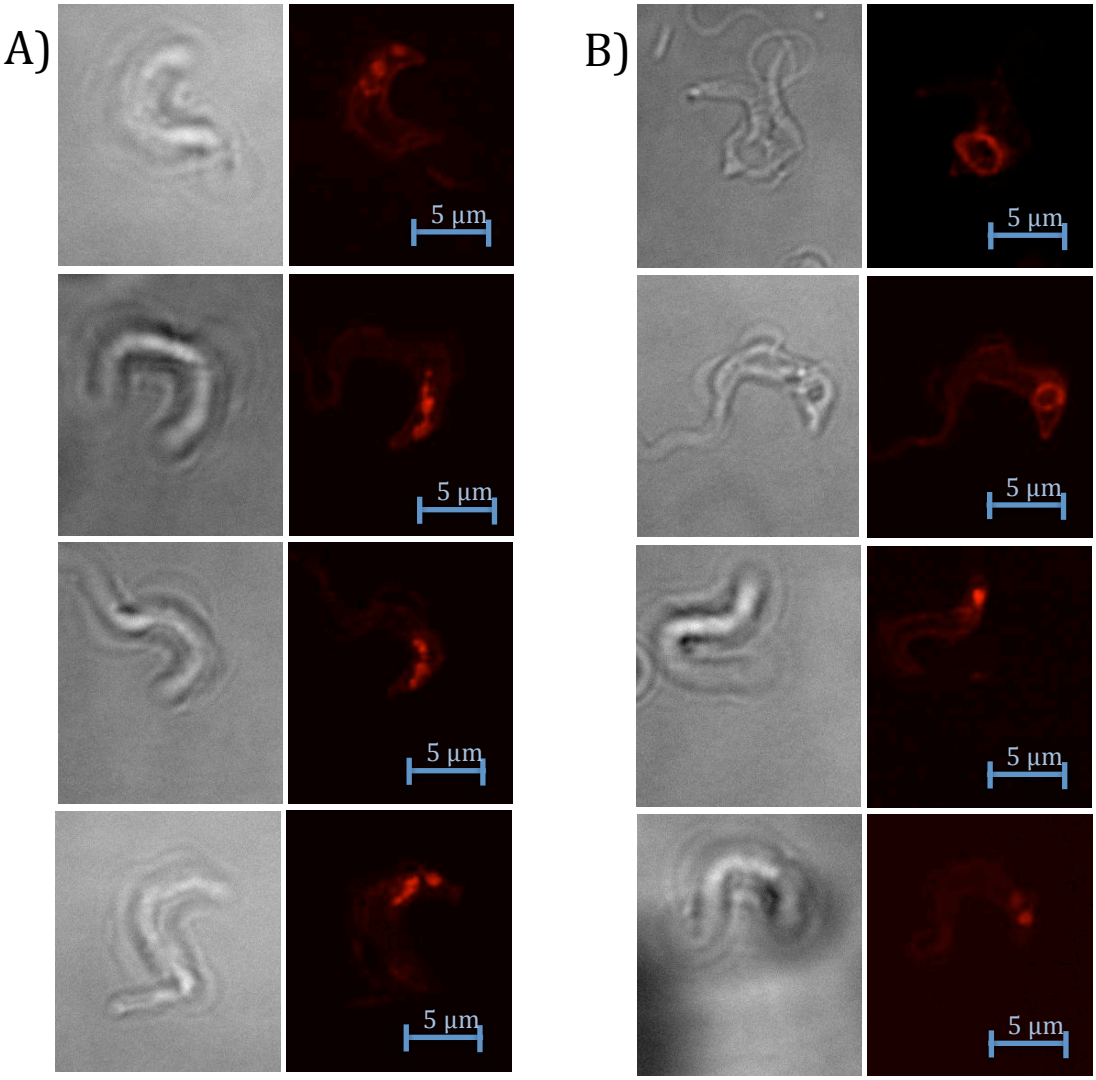
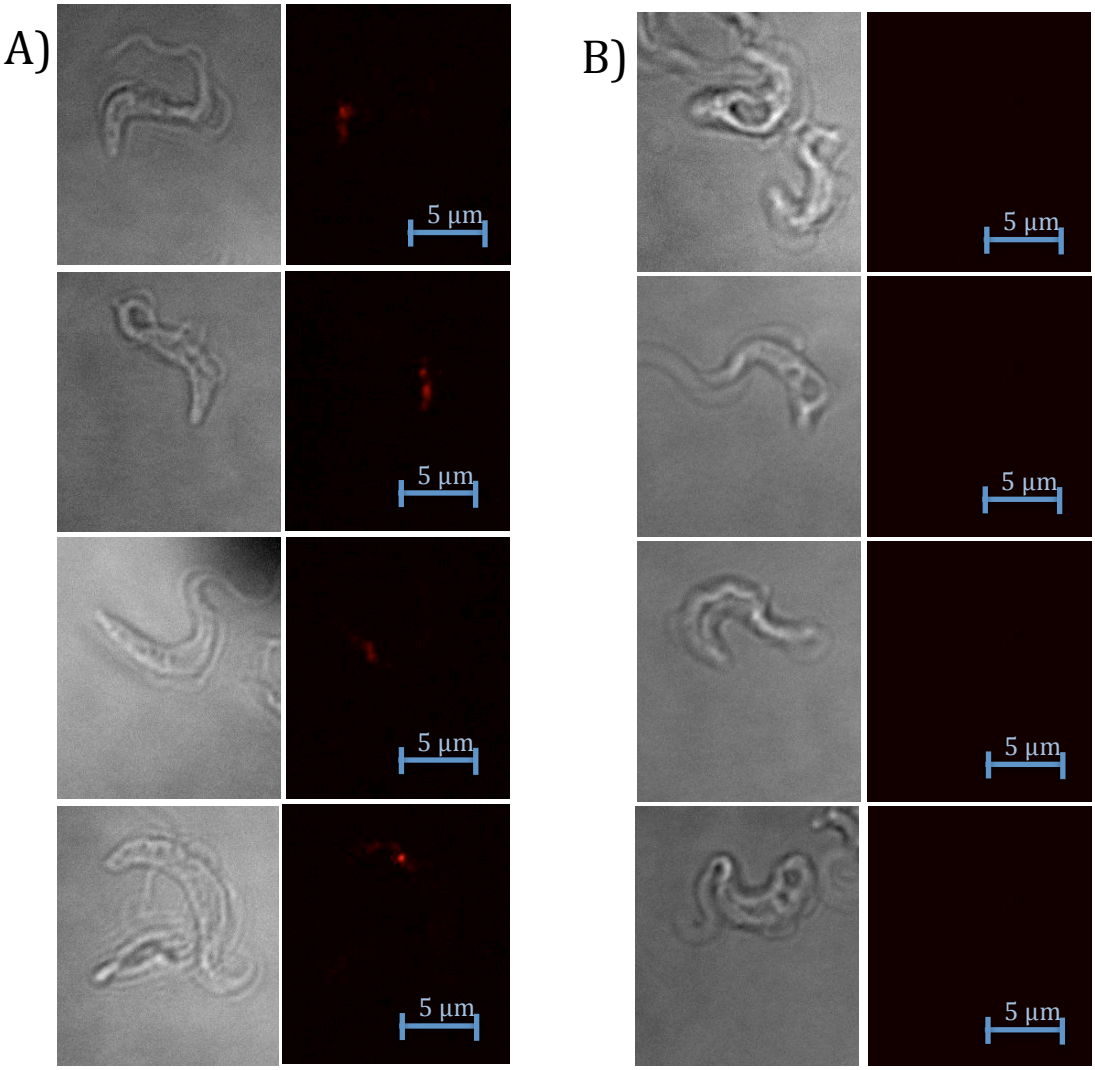


Figure 22: A) Bloodstream wild-type cells Incubated for 20 min at 37°C in the presence of AlexaFluor-594 conjugated concanavalin A. Note that all cells have taken up the compound (shown in red) into distinct structures. B) Bloodstream ATG24 RNAi cells induced for 48 h and incubated for 20 min at 37°C in the presence of AlexaFluor-594 conjugated concanavalin A. Note the strong staining for concanavalin A that colocalises with the enlarged lysosome. C) Bloodstream wild-type cells incubated for 30 min at 37°C in the presence of AlexaFluor-568 conjugated transferrin. Note that all cells have taken up the compound (shown in red) into discrete structures. D) Bloodstream ATG24 RNAi cells induced for 48 h and incubated for 30 min at 37°C in the presence of AlexaFluor-568 conjugated transferrin. The majority of the cells, as shown here, do not fluoresce.


A)
B)
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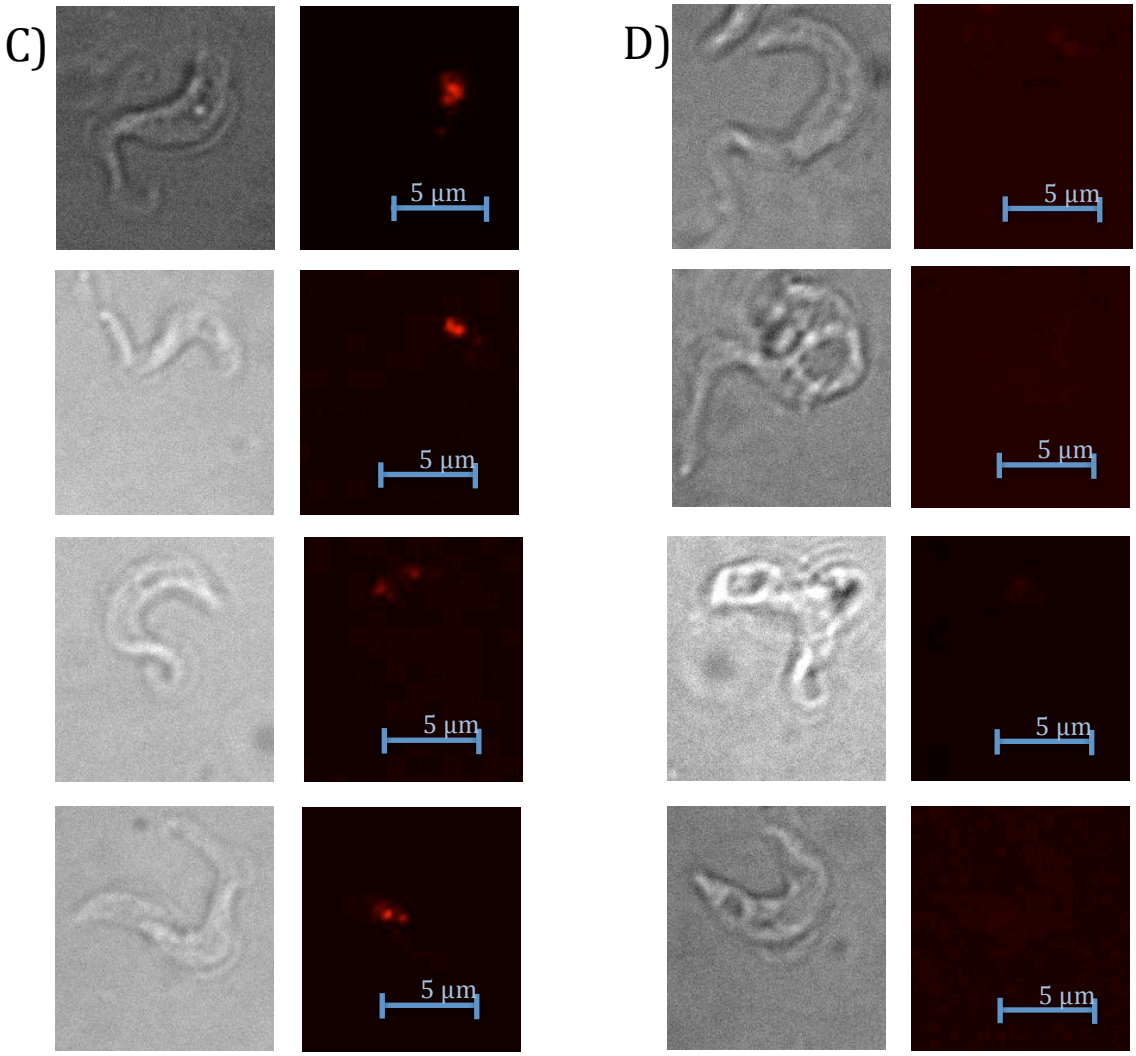

Figure 23: A) Bloodstream wild-type cells incubated for 30 min at 37°C in the presence of AlexaFluor-568 conjugated transferrin. Note that all cells have taken up the compound (shown in red) into discrete structures. B) Bloodstream ATG24 RNAi cells induced for 48 h and incubated for 30 min at 37°C in the presence of AlexaFluor-568 conjugated transferrin. The majority of the cells, as shown here, do not fluoresce. C) Bloodstream cells previously incubated with leupeptin then with transferrin. D) Bloodstream ATG24 RNAi cells induced for 48 h and incubated with leupeptin before incubation with transferrin.

4.5 ATG24 plays a role in the parasite's *in vitro* differentiation

To study the role of ATG24 in autophagy during differentiation we induced RNAi for 48 h in bloodstream cells, then centrifuged and resuspended the cells in Differentiating Trypanosome Medium (DTM) in the presence of 3 mM citrate and 3 mM cis-aconitate and moved the cultures from 37°C to 28°C, the temperature

at which procyclic forms grow optimally. These conditions have been shown to induce differentiation even in monomorphic cells, which survive and grow as procyclic-like cells for some period of time. The trypanosomes were followed until 72 h after induction of differentiation. Samples were collected once daily for western blot and immunofluorescence analyses, to check for expression of different markers specific for either bloodstream or procyclic cells. As markers, samples were checked by western blot studies for PPDK expression (a procyclic-specific glycosomal protein) and decreased levels of PYK (a cytosolic protein enriched in bloodstream forms; not shown), as well as for a change in morphology and in PGK localisation by immunomicroscopy. *T. brucei* has three genes coding for different isoforms of PGK, PGK-a, an isoform with a considerably higher molecular weight than the others due to the presence of an 80 residue long insertion, that is expressed at very low levels in both stages in the glycosomes, PGK-b that is present in procyclics in the cytosol and PGK-c that is expressed in bloodstream cells in the glycosomes (Blattner and Clayton, 1995). PGK-b and PGK-c have a very high degree of similarity and our antiserum raised against PGK-c recognizes PGK-b in immunomicroscopy slides (Figure 24a). We were therefore able to use this antiserum to distinguish between bloodstream cells and procyclic-like cells by their morphology in conjunction with the subcellular localisation of PGK. We observed that wild-type cells have a lower rate of differentiation than the ATG24 RNAi cells. While 67% of the wild-type cells achieved differentiation after 72 h, 92% of the RNAi cells appeared to be procyclic-like after 72 h, as assessed by the increase in the size of the cells and a relocation of PGK from glycosomes to the cytosol, as observable by immunomicroscopy (Figure 24b). Moreover, the ATG24 RNAi induced cells start to express the procyclic marker PPDK sooner than wild-type cells, after inducing differentiation, and are also able to restart cell growth faster. Taken together these results indicate that the downregulation of ATG24 is beneficial for the *in vitro* differentiation of monomorphic bloodstream-form *T. brucei*.

However, transmission electron microscopy images of samples collected after 24 h of incubation in differentiation medium show that both wild-type and ATG24 RNAi cells present abnormal intracellular structures, including an apparent increase in the number of non-specified vesicles, probably a sign of

cellular stress (Figure 25); this is somewhat expected as these cells differentiate with a lower efficiency than pleomorphic cells, and as they cannot adapt to the changes as quickly, cell death is often observed.

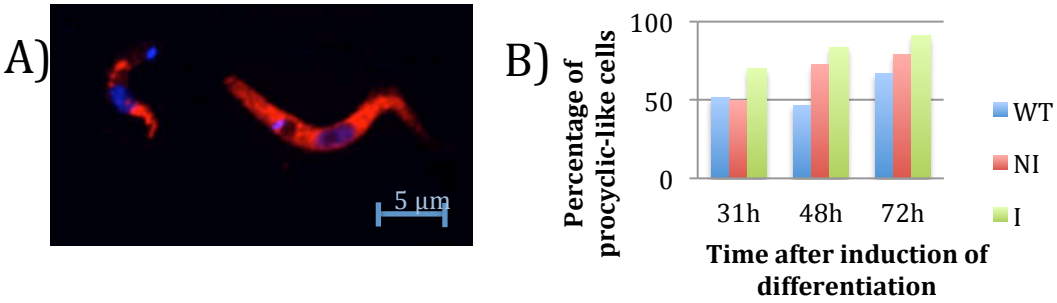

Figure 24: Alteration of ATG24 levels influences differentiation. A) Morphology and distribution of PGK (red) in a glycosomal pattern in bloodstream (cell at the left) and a cytosolic pattern in a procyclic cell (cell at the right) by confocal immunomicroscopy. B) Percentage of differentiated cells in wild-type (WT) shown in blue, Non-Induced ATG24 RNAi cells (NI) shown in red and ATG24 RNAi Induced for 48 h (I) shown in green, after induction of *in vitro* differentiation.

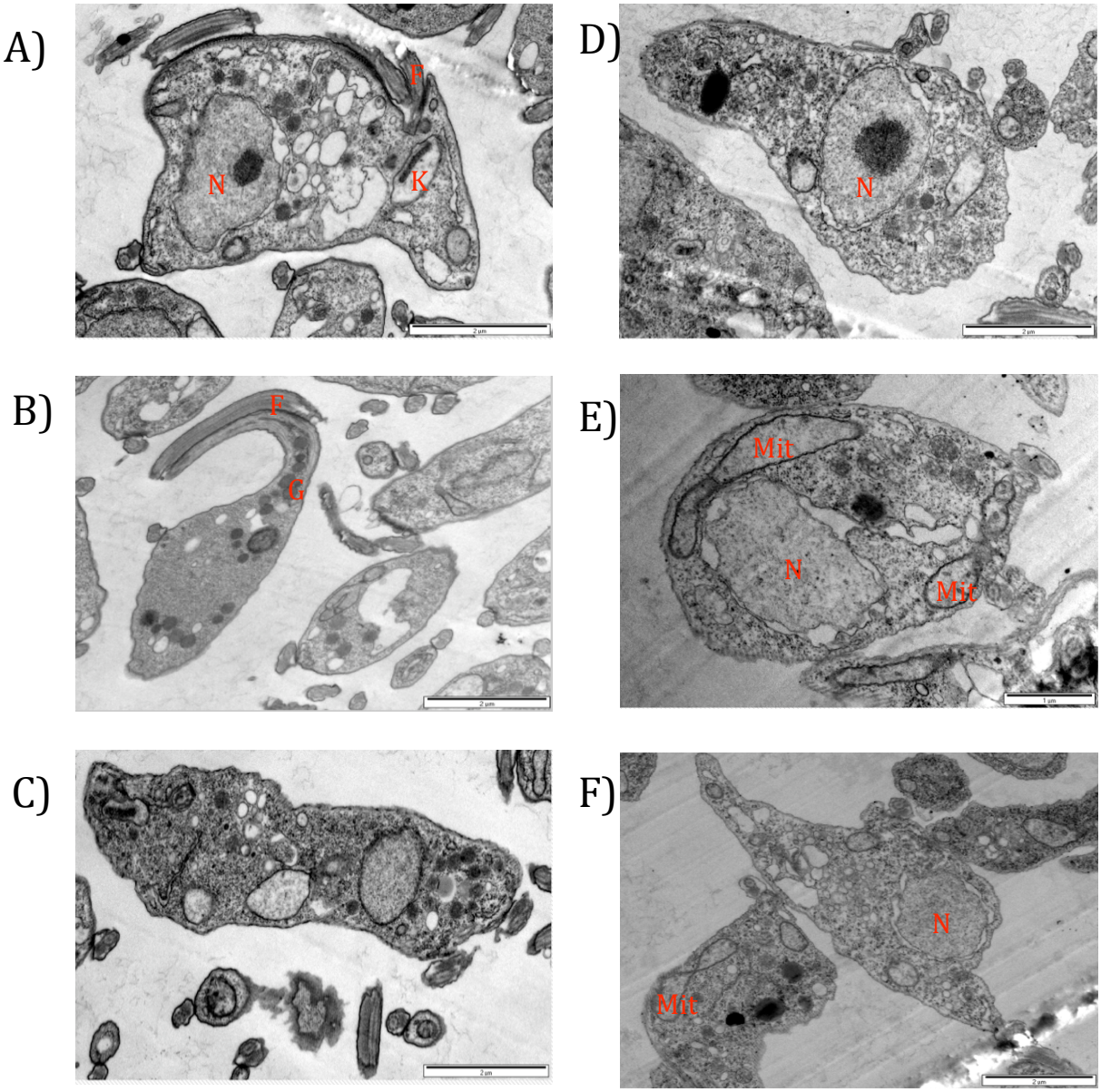

Figure 25: Bloodstream cells after 24 h in differentiation medium. A-C) Wild-type cells. D-F) ATG24 RNAi cells induced with tetracycline for 48 h prior to establishing differentiation conditions. Note that both cell types present an increased number of intracellular vesicles, indicative of cellular stress. Abbreviations: N – nucleus, K – kinetoplast, F – flagellum, Mit – mitochondrion.

4.6 Decreasing ATG24 levels by RNAi leads to an increased number of cells presenting autophagosomes

To assess the role of ATG24 in autophagy we compared the number of cells presenting autophagosomes in ATG24 RNAi non-induced and for 48 h induced procyclic cells both expressing either GFP-ATG8.1 or GFP-ATG8.2, after the cells had been incubated for 15 or 30 min in pre-warmed PBS. The results are shown in Figure 26. A small but reproducible (in two independent experiments) difference could be observed in the number of cells presenting fluorescent puncta between ATG24 RNAi non-induced and induced cells with GFP-ATG8.1, but not for cells with GFP-ATG8.2. Interestingly the number of cells containing autophagosomes is increased when the ATG24 levels are decreased: from 4-8% of the cells to 9-12% (Figure 26a). Also bloodstream wild-type and ATG24 RNAi cells showed reproducible differences with regard to the number of cells presenting autophagosomes after 24 h of *in vitro* differentiation. Puncta associated to GFP-ATG8.1 were seen in 10-14% of wild-type cells in contrast to 26-27% in ATG24 RNAi-induced cells. Autophagosomes possessing GFP-ATG8.2 were seen in 18-19% of wild-type cells and in 25% of ATG24 RNAi-induced cells at 24 h of *in vitro* differentiation (Figure 26b). These results indicate that the protein might act as a negative regulator of autophagy since its depletion increases the number of cells presenting ATG8-associated puncta.

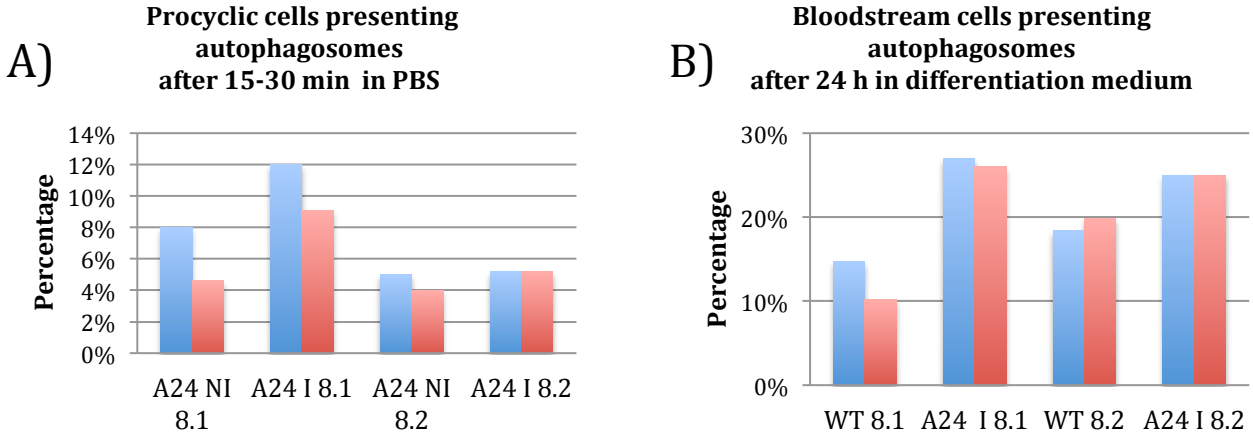

Figure 26: Percentage of trypanosomes possessing either GFP-ATG8.1 or GFP-ATG8.2 associated to autophagosomes in A) Procyclic ATG24 RNAi cells non induced (A24 NI) and induced for 48 h (A24 I) after 15 – 30 min in PBS. B) Bloodstream wild-type (WT) and ATG24 RNAi cells induced for 48 h (A24) after 24 h in differentiation medium. Blue and red indicate results obtained from two independent experiments. In each experiment, at least 100 cells were counted for each point.

4.7 GFP-ATG8 colocalises with a glycosomal marker upon *in vitro* differentiation

We have performed the same *in vitro* differentiation assay with wild-type bloodstream cells constitutively expressing GFP-tagged ATG8.1 or GFP-tagged ATG8.2 under a procyclin promoter, that is constitutively expressed in procyclic cells. Samples were collected at several time points and analysed by (immuno)fluorescence microscopy for colocalisation of the glycosomal enzyme triosephosphate isomerase (TPI) and GFP. Upon differentiation these cells start to express GFP-tagged ATG8.1 or ATG8.2 (Figure 27) under the control of a procyclic-specific promoter.

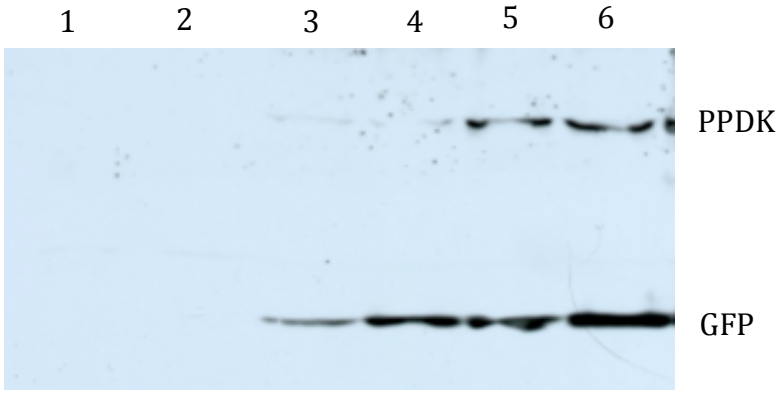

Figure 27: *In vitro* differentiation induces expression of procyclic-specific proteins. Bloodstream cells possessing the GFP-ATG8.2 construct under the procyclin promoter start to express the construct during differentiation. Approximately $8x10^6$ cells were loaded in each well. PPDK is a procyclic marker. 1 – BF GFP-ATG8.2 clone 1. 2 - BF GFP-ATG8.2 clone 2. 3 - BF GFP-ATG8.2 clone 1 in differentiating medium for 24 h. 4 - BF GFP-ATG8.2 clone 2 in differentiating medium for 24 h. 5 - BF GFP-ATG8.2 clone 1 in differentiating medium for 48 h. 6 - BF GFP-ATG8.2 clone 2 in differentiating medium for 48 h.

We could observe an increase in the colocalisation of GFP and TPI as early as 17 h after induction of differentiation until 72 h in these cells (Figures 28 and 29). Both forms of ATG8 were seen to colocalise with the glycosomal marker in an enlarged region between nucleus and kinetoplast which we assume to be the lysosome.

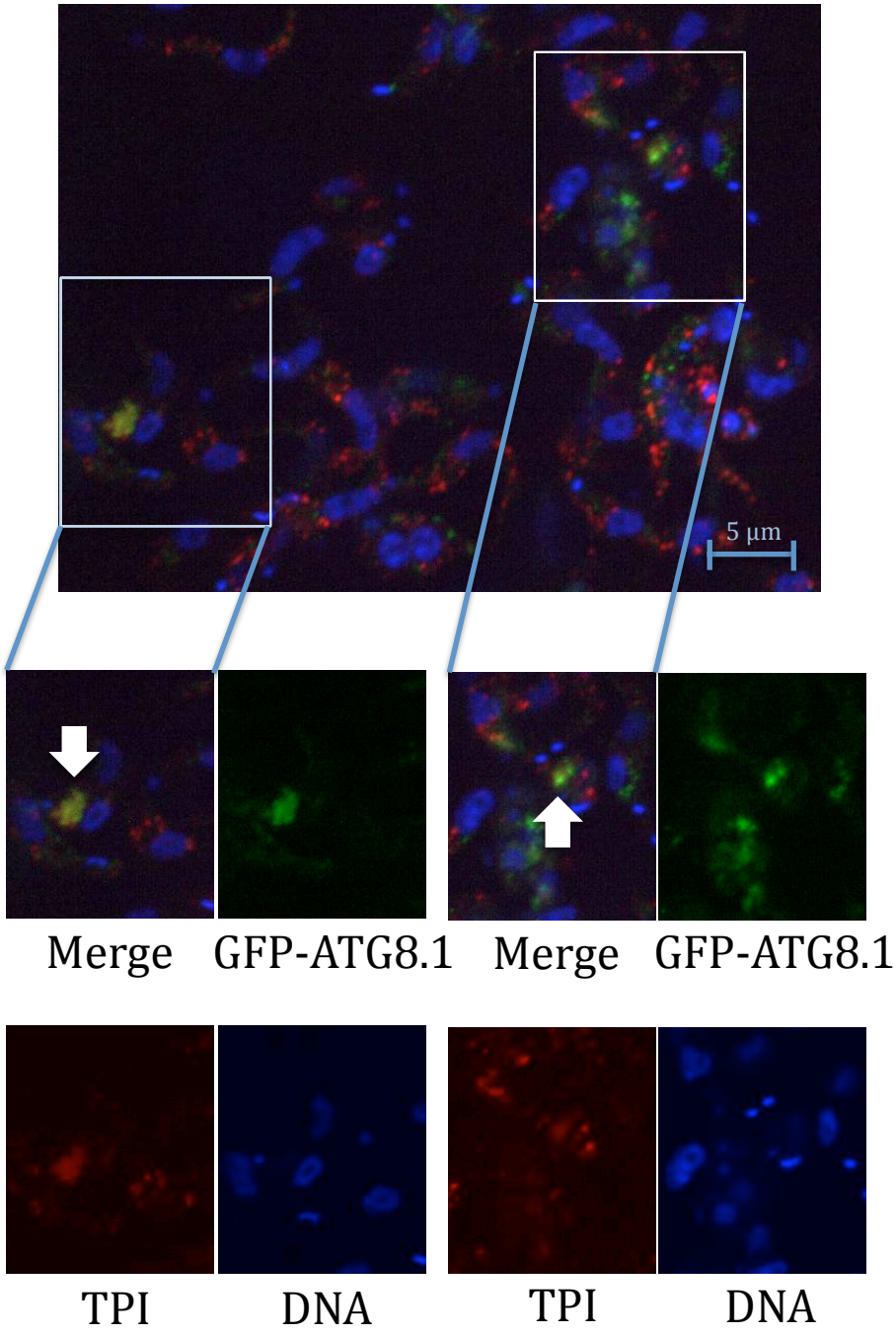

Image 28: ATG8.1 colocalises with the glycosomal marker TPI upon differentiation. Merge image of *T. brucei* bloodstream cells in differentiation medium for 36 h expressing GFP-ATG8.1. The glycosomal marker TPI is shown in red. Nuclear and kinetoplast DNA is shown in blue. Yellow areas in the merge (white arrows) indicate colocalization of ATG8.1-GFP and TPI.

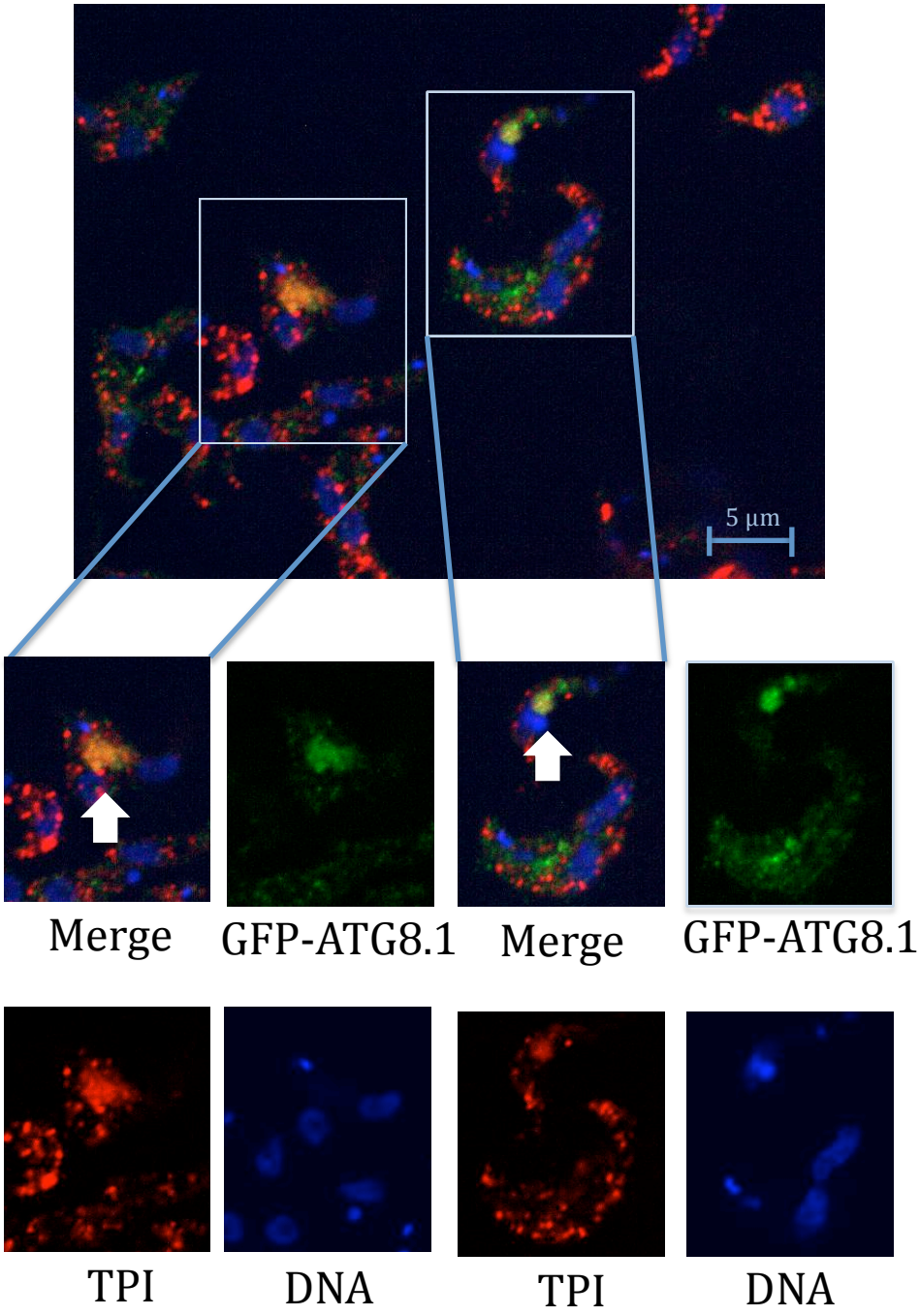

Figure 29: ATG8.2 colocalises with the glycosomal marker TPI upon differentiation. Merge image of *T. brucei* bloodstream cells in differentiation medium for 36 h expressing GFP-ATG8.2. The glycosomal marker TPI is shown in red. Nuclear and kinetoplast DNA is shown in blue. Yellow areas in the Merge (white arrows) indicate colocalization of ATG8.2-GFP and TPI.

Interestingly, wild-type cells possessing the GFP-ATG8 constructs reproducibly (in four independent experiments) differentiate at a very low rate when compared to ATG24 RNAi cells with the same GFP-ATG8 constructs and compared to wild-type cells without this construct (Figures 30 and 31). The wild-type/GFP-ATG8 cells reached a maximum of only 3,5% of procyclic-like cells 72 h after induction of differentiation (compared to 67% without the GFP construct – see section 4.5). In contrast, the ATG24 RNAi/GFP-ATG8 cell line, induced for 48 h before induction of differentiation, achieved up to approximately 90% of procyclic-like cells after 72 h of differentiation, very similar to the RNAi cell line without the GFP construct (section 4.5).

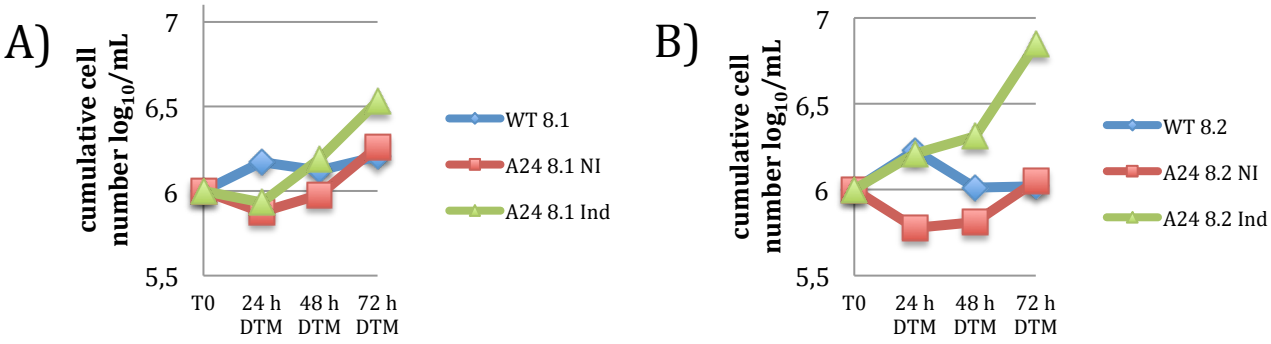

Figure 30: Knock-down of ATG24 is associated with cell growth during differentiation. Growth curve for *in vitro* differentiation experiment performed with bloodstream wild-type (WT), ATG24 RNAi non-induced (NI) and 48 h Induced (I) cells. A) Cells expressing GFP-ATG8.1 (8.1). B) Cells expressing GFP-ATG8.2 (8.2). In blue: bloodstream wild-type cells expressing GFP- ATG8.1 or GFP-ATG8.2. In red: bloodstream non-induced ATG24 RNAi cell line expressing ATG8.1 or ATG8.2. In green: bloodstream ATG24 RNAi cell line induced for 48 h expressing GFP-ATG8.1 or GFP-ATG8.2.

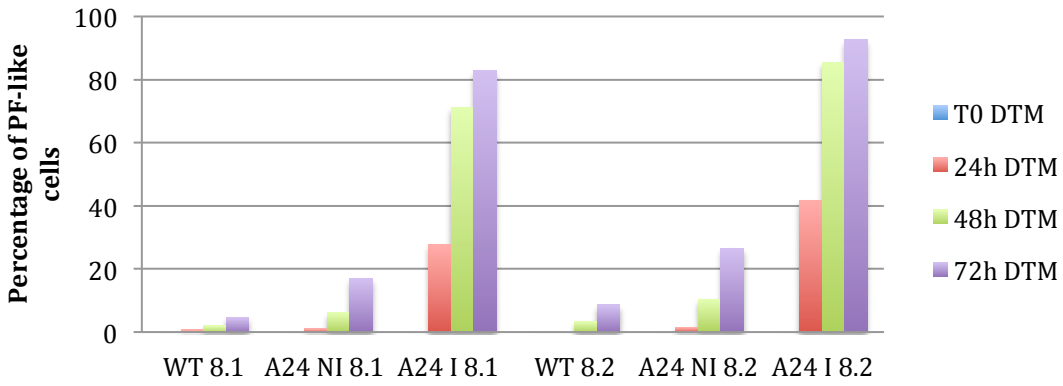

Figure 31: ATG24 knock-down cell line shows higher bloodstream-to-procyclic *in vitro* differentiation rate. Percentage of cells with procyclic morphology and PGK cytosolic distribution after induction of *in vitro* differentiation in DTM medium. Wild-type (WT), ATG24 RNAi non-induced (NI) and 48 h induced (I) cells, expressing GFP-ATG8.1 (8.1) or GFP-ATG8.2 (8.2). Samples collected after induction of differentiation: blue T0, red 24 h, green 48 h, purple 72 h.

The morphological changes observed in the differentiating trypanosomes were associated with the development of a functional mitochondrion as evidenced by labelling with antiserum against acetate:succinate CoA-transferase (ASCT) in confocal immunomicroscopy and a change in the profile of the western blot, from bloodstream (no PPDK, but PYK is present) to procyclic–like (PPDK present, low or no PYK), where ZFP3 was used as loading control (Walrad *et al.*, 2012). We noticed that often both wild-type and ATG24 RNAi cell lines expressing GFP-ATG8.2 differentiate slightly better than the cells expressing GFP-ATG8.1, as observed by both PPDK expression (Figure 32) as well as cytosolic localisation of PGK associated with procyclic cell morphology (Figure 31) and ASCT labelling in confocal immunomicroscopy (Figure 33).

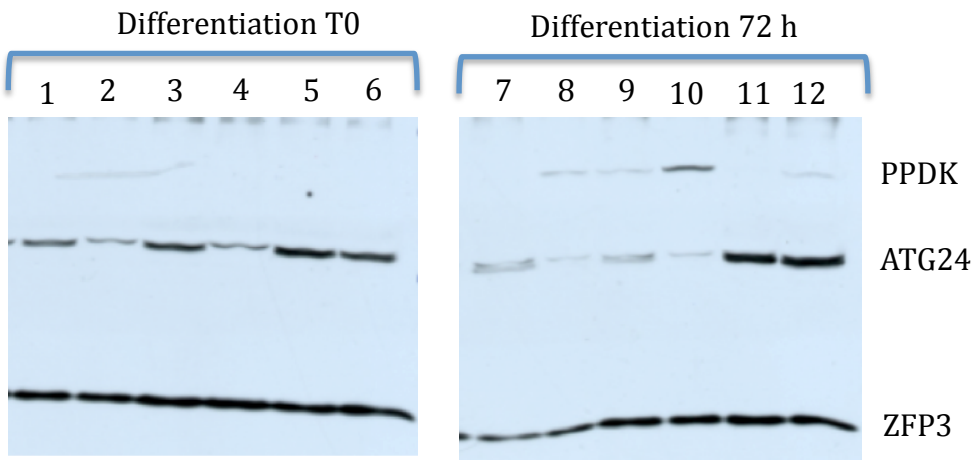

Figure 32: Knock-down of ATG24 expression affects *in vitro* differentiation of trypanosomes. RNAi against ATG24 decreases the levels of ATG24 and increases the rate of differentiation as seen in the levels of expression of the procyclic marker PPDK. Wells 1 and 7 – Bloodstream cells expressing GFP-ATG8.1 ATG24 RNAi Non-Induced, 2 and 8 – Bloodstream cells expressing GFP-ATG8.1 ATG24 RNAi Induced 48 h , 3 and 9 - Bloodstream cells expressing GFP-ATG8.2 ATG24 RNAi Non-Induced, 4 and 10 - Bloodstream cells expressing GFP-ATG8.2 ATG24 RNAi Induced 48 h , 5 and 11 – Bloodstream cells expressing GFP-ATG8.1, 6 and 12 – Bloodstream cells expressing GFP-ATG8.2. Samples 1 to 6 collected after 48 h RNAi induction, before induction of *in vitro* differentiation. Samples 7 to 12 collected after 72 h in differentiating medium. At all points, protein from a total lysate of approximately $8x10^6$ cells was loaded in the well. ZFP3 is a loading control.

Moreover, for both the wild-type and ATG24 RNAi bloodstream cells possessing the GFP-ATG8.2 construct, the GFP was detected in western blots sooner after induction of differentiation than in cells possessing the GFP-ATG8.1 construct. Since both constructs are under the control of the same procyclin promoter and bordered by the same UTRs, this indicates that GFP-ATG8.1 is probably participating in autophagy and being degraded. However cells with the GFP-ATG8.2 construct seem to differentiate at a faster rate than cells with GFP-ATG8.1, indicating that somehow ATG8.2 has a less detrimental effect for differentiation.

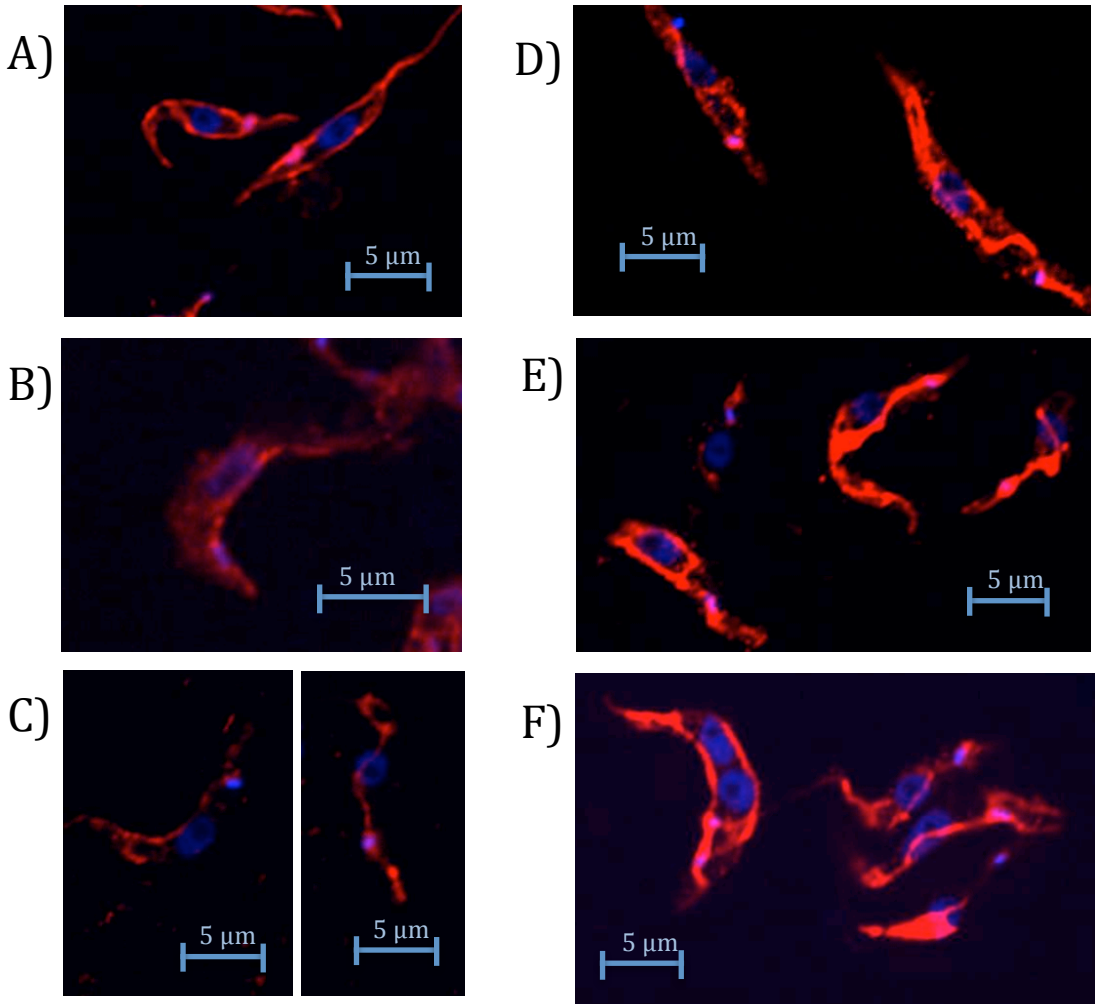

Figure 33: ATG24 RNAi cells display well-developed mitochondria sooner than wild-type cells upon *in vitro* differentiation. Confocal immunomicroscopy showing ASCT in red as a marker of mitochondrion development during differentiation. ASCT staining in procyclic and bloodstream wild-type cells are shown in A and B for comparison. Cells are in differentiation medium for 48 h. DNA is shown in blue. C) Cells possessing the GFP-ATG8.1 construct. D) Cells possessing GFP-ATG8.2 construct. E) ATG24 RNAi 48 h Induced, possessing the GFP-ATG8.1 F) ATG24 RNAi 48 h Induced, possessing GFP-ATG8.2.

4.8 GFP-ATG8 degradation upon autophagy induction

Since ATG8 participates in the formation of autophagosomes and remains attached to these organelles, it can be delivered into the lysosome. To follow the formation of autophagosomes labelled with GFP-ATG8.1 or GFP-ATG8.2, their

delivery to the lysosome and their degradation, procyclic cells expressing GFP-ATG8.1 or GFP-ATG8.2 were centrifuged and resuspended in pre-warmed PBS. This protocol is widely used to induce autophagy in trypanosomes and it has been shown to induce autophagy as early as 15 min after incubation. Samples were collected at several time points, up to 3 hours. Another approach was to resuspend the parasites in SDM80, a medium that contains no glucose but has a high concentration of amino acids. We considered that this would induce pexophagy since the glycosomal content would probably need to adapt to the changes in the carbon source. A third condition tested was to add 60 mM N-acetyl-D-glucosamine (GlcNac) to the procyclic medium SDM79, since it has been shown before that this compound blocks glucose uptake and induces a change in metabolism to the catabolism of proline and other amino acids (Ebikeme *et al*., 2008).

Since the two GFP-tagged isoforms of ATG8 are being expressed from the same genomically inserted vector, the sequences that control the levels of expression of the two proteins are identical and we expect the two to be expressed at the same levels. Figure 34 however show that at least for procyclic cells, ATG8.1 is expressed in lower levels than ATG8.2 (compare lines 1 to 3 and 5 to 8) in nutrient-rich conditions. Induction of autophagy by the nutritional change affected the levels of GFP-ATG8.1 in procyclic cells, irrespective whether the change involved transfer to SDM80 and or addition of GlcNac. Often the levels of GFP-ATG8.1 would decrease drastically at 24 h until the band was no longer detectable on western blots. The levels of GFP-ATG8.2 also seemed to decrease in most of these conditions, but not as dramatically as GFP-ATG8.1. These results seem to indicate that, despite having the same promoter and supposedly being expressed at same levels, GFP-ATG8.1 is degraded under most autophagy-inducing conditions in a rate that is different to GFP-ATG8.2.

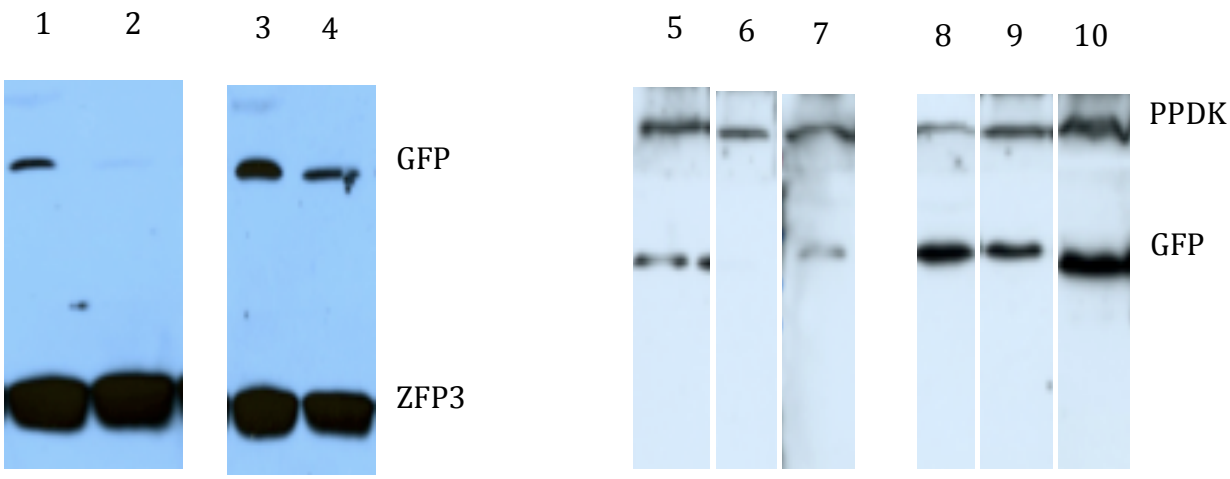

Figure 34: Autophagy induction causes decreased levels of autophagosomal marker ATG8. 1 – procyclic cells expressing GFP-ATG8.1 in nutrient-rich SDM79 medium. 2 - same cells incubated for 3 h in PBS. 3 – procyclic cells expressing GFP-ATG8.2 in SDM79 medium. 4 - same cells incubated for 3 h in PBS. ZFP3 is a loading control. 5 - procyclic trypanosomes expressing GFP-ATG8.1 in nutrient-rich SDM79 medium. 6 – same cells for 24 h in SDM80 medium. 7 - same cells for 24 h in presence of GlcNac. 8 - procyclic cells expressing GFP-ATG8.2 in SDM79 medium. 9 – same cells for 24 h in SDM80 medium. 10 – same cells for 24 h in the presence of GlcNac. PPDK is a loading control.

Chapter 5
Discussion, conclusions and perspectives

5.1 The identity and functions of *T. brucei* ATG24

A major objective of our project was to functionally characterise the *T. brucei* ATG24, a protein whose counterpart in different yeasts is not required for general autophagy, but has been shown to be essential for macro- and micropexophagy, the cytoplasm-to-vacuole pathway, PMN and mitophagy. Therefore, the trypanosome homologue might be a candidate for involvement in glycosome turnover. The *T. brucei* ATG24 is 22% similar to its *S. cerevisiae* homologue and also possesses the characteristic Phox-homology domain and BAR domain. The trypanosome expresses its ATG24 in both its bloodstream and procyclic forms, as observed by western blot analysis and immunomicroscopy using a polyclonal antiserum raised against the purified full-length protein expressed in *E. coli*. ATG24 was enriched in microsomal and cytosolic fractions obtained by differential centrifugation performed with lysates of wild-type procyclic cells in three independent experiments. Similar results were observed when the differential centrifugation experiments were performed with trypanosomes expressing myc-tagged ATG24 and a western blot of the fractions was subsequently analysed with a myc-specific antiserum (not shown). By fluorescence microscopy using either a C-terminal GFP-tag (only in bloodstream-form cells) or the anti-ATG24 antiserum (in both bloodstream- and procyclic trypanosomes) the protein appeared to be dispersed in the cytosol as well as concentrated in puncta between the nucleus and kinetoplast.

We demonstrated that the *T. brucei* homologue of yeast ATG24 indeed plays a role in autophagy, although it remains to be established what this role is and in which of the autophagy-related processes the role is exerted. In different autophagy-inducing conditions we observed a small but reproducible increase in the number of cells presenting autophagosomes in the trypanosomes in which ATG24 levels were decreased by RNAi compared to wild-type or RNAi non-induced cells exposed to similar conditions. An increase in the number of cells presenting autophagosomes could indicate an increase in autophagy levels or a

decreased fusion of autophagosomes with the lysosome. Because of the increase in the number of cells presenting autophagosomes we could speculate that ATG24 might be important for the fusion of autophagosomes (or other vesicles) to the lysosome. However in this latter case we would expect to see a similar effect for cells in which autophagosomes are marked with either of both GFP-tagged ATG8 isoforms, ATG8.1 or ATG8.2, in procyclics cells when they are starved in PBS. This was not the case; the increase of the number of cells presenting autophagosomes –thus the possible downregulation of their fusion with the lysosome– was only observed in cells with GFP-ATG8.1 marked autophagosomes. Also, the increase in the size of the lysosome in ATG24 RNAi cells points to the opposite direction (see below).

T. brucei ATG24 is required for the correct morphology of the lysosome. We observed that this organelle is enlarged in bloodstream ATG24 RNAi cells (seen in contrast phase and shown by concanavalin A localisation at 37°C for 10 to 30 min), a similar result as has been reported for *P. pastoris* where the absence of ATG24 had severe effects on the vacuolar morphology (Ano *et al*., 2005). The increase in the size of the lysosome could be explained by an increase in delivery of vesicles (autophagosomes or others) to it. Transmission electron microscopy images confirm the effect of ATG24 RNAi on the lysosome size, and the confluence of the lysosomal membrane around intracellular structures indicates that this increase in lysosome size is not caused by an osmotic effect. Other morphological effects of ATG24 RNAi seem to be present, especially in the ER and mitochondrion morphology. Despite not being essential, ATG24 seems to be important for the correct morphology of diverse organelles in bloodstream stage.

ATG24 could also have a role in regulating the fusion of vesicles of the endocytic system with the lysosome. Indeed, and initially surprisingly to us, several of our observations pointed to a possible role of ATG24 in the endocytic system. As mentioned above, by confocal fluorescence microscopy ATG24 was found dispersed in the cytosol but also concentrated in puncta between the nucleus and kinetoplast. This latter distribution is indicative of an endocytic localisation, as this region has been described as enriched in vesicles from endo- and exocytosis as well as the lysosome. An involvement of ATG24 in the *T. brucei* endocytic system was further supported by other results. First, the protein

partially colocalised with transferrin as well as concanavalin A when wild-type bloodstream cells were incubated with these proteins at 37° for up to 30 min, indicating an endocytic localisation. Previous observations made in various laboratories have led to the notion that proteins of the endocytic system tend to be upregulated or even that they are specific for the bloodstream stage and a recent analysis of mRNA levels in different stages of *T. brucei* found the corresponding mRNAs to be upregulated in the bloodstream stage (Jensen *et al*., 2009). However, we did not see a strong difference in the levels of expression of ATG24 when bloodstream and procyclic cells were compared.

Further support for a role of *T. brucei* ATG24 in endocytosis is provided by the observation that the bloodstream RNAi cell line has a defect in the receptor-mediated import of transferrin, showing very low levels of intracellular transferrin after 30 min at 37°C. Under these conditions the transferrin bound to its receptor should transit from the flagellar pocket into early endocytic vesicles, release the iron and be delivered to the lysosome, while the receptor is recycled back to the cell surface without reaching the lysosome. A decrease in the staining with transferrin strongly indicates a defect at the early stages of endocytosis. The alternative explanation of an increased lysosomal degradation can be excluded, because a transferrin uptake experiment performed in presence of the protease inhibitor leupeptin led to a similar result. A possible explanation for the decreased uptake of transferrin is a sequestration of the receptor away from the flagellar pocket in the ATG24 RNAi cells

Literature studies indicated that a role of ATG24 in endocytosis, although unexpected, was not entirely surprising. Our work is based on the apparent unambiguous identification of Tb09.211.4240 as the *T. brucei* putative orthologue of *S. cerevisiae* ATG24 in previous searches for autophagy-related genes (Herman *et al*., 2006; Rigden *et al*., 2009). Similarly, the corresponding proteins in *T. cruzi* and *Leishmania major* were identified as the most likely ATG24 candidates. However, this protein is a member of a family of homologous proteins with a considerable number of similar proteins in yeasts and mammalian cells, all with the presence of PX and BAR domains, named sorting nexins. The *S. cerevisiae* ATG24 was formerly called SNX4, homologous with SNX4 or ‘Sorting Nexin 4’ in mammals. Other former names of yeast ATG24 were

CVT13 and PAZ16. Indeed, the homologue in mammalian cells that is most related to TbATG24 is SNX4 whose major role is exerted in the endocytic system (see below). Also other proteins of this same family are involved in the mammalian endocytic system, such as SNX1, and in yeast the SNX1 orthologue VPS5.

The endocytic system of *T. brucei* has been analysed in great detail, at both the ultrastructural and molecular level by Field and collaborators (for a review see: Field and Carrington, 2009). This group reported recently the identification of the homologue of yeast VPS5, and its involvement in this process (Koumandou *et al*., 2011). When our experimental work was completed, and the manuscript of this thesis was in preparation, we became aware that Koumandou *et al*. (2011) had identified Tb09.211.4240 as the homologue of *S. cerevisiae* VPS5/SNX1, while our work was based on the identification of this same *T. brucei* protein as the counterpart of yeast ATG24/SNX4 (Herman *et al*., 2006; Rigden *et al*., 2009). Of course, ATG24/SNX4 and VPS5/SNX1 are homologous proteins, both possessing the PX and BAR domains.

To determine if Tb09.211.4240 is the most likely orthologue of either ATG24/SNX4 (as we assumed based on the analysis by Herman *et al*., 2006) or VPS5/SNX1 (as supposed by Koumandou *et al*., 2011), we performed first a thorough search for PX-BAR containing sequences in the genome database of *T. brucei*. Only two proteins were confidently identified, Tb09.211.4240 and Tb927.7.4500 (whose mRNA is also upregulated in the bloodstream form according to Jensen *et al*., 2009). Next, a phylogenetic analysis was performed to determine how similar these two *T. brucei* proteins are to all homologous proteins containing PX-BAR domains from human, yeast, *Drosophila melanogaster* and *Caenorhabditis elegans*. Three different algorithms were used for the analysis: Neighbour Joining, Maximum Parsimony and Minimum Evolution. One consensus tree, from the Minimum Evolution analysis, is shown in Figure 35, but essentially the same results were obtained with the other methods. In all analyses, Tb09.211.4240 was consistently, and with high confidence grouped with yeast ATG24/SNX4; in the tree shown in Figure 35 the bootstrap value is 78, based on 500 replicates. All three methods grouped seven other sequences from different organisms with Tb09.211.4240 and ScSNX4: the

human SNX4 (HsSNX4), ScSNX41, ScSNX42/ATG20 and HsSNX8, HsSNX30, HsSNX7 and ScMVP1. Surprisingly, no *D. melanogaster* or *C. elegans* sequences were present in this cluster. Very importantly, the cluster with Tb09.211.4240 and both human or yeast SNX4 was well separated from the cluster with ScVPS5/ScSNX1 and HsSNX1. The second *T. brucei* homologue with PX-BAR domains (although the latter domain being somewhat divergent), Tb927.7.4500, does not cluster with any of the counterparts from the other organisms. It is therefore not surprising that Tb09.211.4240 was identified by Koumandou *et al*. (2011) in a BLAST with ScVPS5/ScSNX1, but a reciprocal BLAST should have shown that it was not the orthologue.

The two domains found in SNX1 and SNX4 characterise the so-called sorting nexin (SNX) protein family: the N-terminal PX-domain is responsible for the interaction with PI3P in membranes, while the C-terminal BAR (Bin/Amphiphysin/Rvs) domain is involved in both the dimerization of the protein and the sensing of membrane curvature (Figure 36a). As mentioned above, not only SNX1 but also SNX4 is involved in the endosomal system, and apparently the yeast and trypanosome homologues of the latter too, despite also their role in autophagy, But the two SNXs have different roles and a different localisation in the endosomal system, as shown in studies with mammalian cells and yeasts. VPS5/SNX1 are members of the yeast and mammalian retromer, respectively (Nothwehr and Hindes, 1997; Horazdovsky *et al*., 1997). SNX4 has been shown in mammalian cells to participate in transferrin receptor recycling (Leprince *et al*., 2003; Traer *et al*., 2007) and in yeasts to participate in endosome-to-Golgi retrieval of the v-SNARE SNC1 independently of the retromer (Hettema *et al*., 2003).

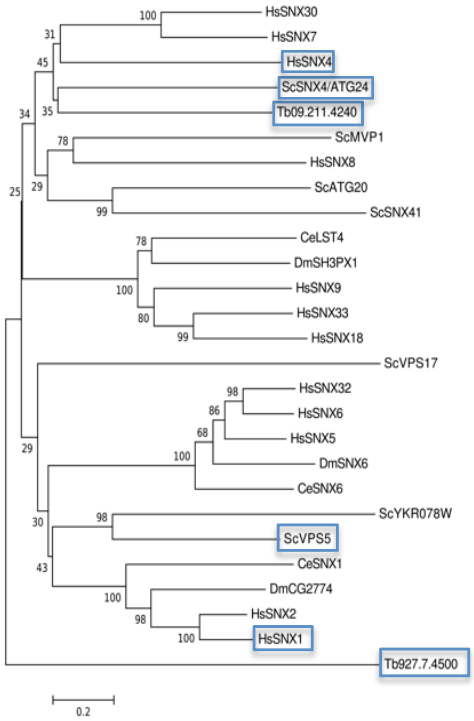

Figure 35: Phylogenetic tree of some PX-BAR containing proteins from different organisms. Sc - *Saccharomyces cerevisiae*, Hs – *Homo sapiens*, Ce – *Caenorhabditis. elegans*, Dm – *Drosophila melanogaster*, Tb – *Trypanosoma brucei*. The relationships were inferred by the Minimum Evolution method. The branches are drawn to scale, with lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed as described in section 3.17 (courtesy Dr Daniel J. Rigden, University of Liverpool, UK). The tree is bootstrapped; the numbers near the nodes indicate the percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates). Phylogenetic reconstructions using the Neighbour-Joining and Maximum Parsimony methods (not shown) gave essentially the same topology. Proteins considered important for the discussion are boxed.

The retromer is a protein complex that associates to the cytosolic face of endosomal membranes and participates in the retrieval of proteins from the endosomes to the Trans-Golgi Network (TGN) via tubulation of the membrane to form vesicles (Figure 36b). It is composed of two subcomplexes; in yeast cells one subcomplex that comprises a dimer of membrane-associated VPS5 and VPS17, while the other subcomplex contains VPS26, VPS29 and VPS35. The VPS5-VPS17 dimer interacts with PI3P-rich regions in endosomal membranes

and recruits the other components that are responsible for cargo recruitment. The TGN acts as a « sorting station » present at the trans face of the Golgi, that also receives extracellular material. The retromer functions in the endosome-to-Golgi retrieval of the vacuolar protein sorting receptor VPS10, as well as that of the Golgi-resident membrane protein A-ALP and the endopeptidases KEX2 and dipeptidyl amino peptidase (DPAP). The retromer, as unravelled in yeast, is conserved in mammals where homologues have been identified for all proteins except for VPS17, that is replaced by two functional equivalents, SNX5 and SNX6 (Wassmer *et al.*, 2007). Moreover two proteins, SNX1 and SNX2, have been identified as the mammalian homologues of VPS5 (Griffin *et al.*, 2005), with SNX1 having at least one splice variant. In *T. brucei*, homologues of all retromer components except VPS17 have been identified. A homologue is also lacking for the retromer cargo protein VPS10 (Koumandou *et al.*, 2011).

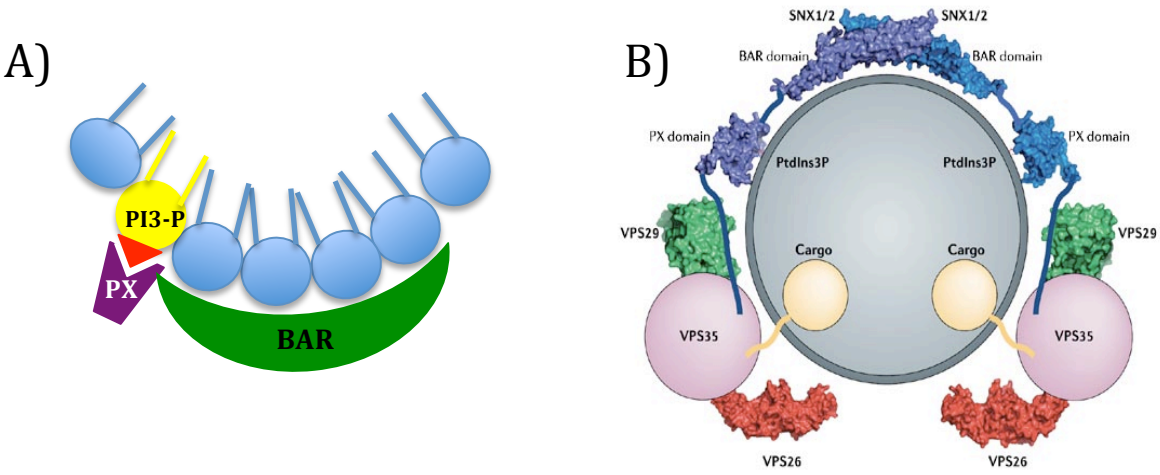

Figure 36: A) Scheme of the interaction of a PX-BAR protein with a curved lipid membrane. Phospholipids are shown in blue, the BAR domain in green, the PX domain in purple, PI3P in yellow and the 3-phosphate group in red. Adapted from: Lemmon. Nat. Rev. Mol. Cell Biol. 9 (2008) 99-111. B) Scheme of interactions between retromer components and cargo proteins at the membrane in mammalian cells. Taken from: Bonifacino and Rojas. Nat. Rev. Mol. Cell Biol. 7 (2006) 568-579.

The mammalian SNX4 resides on tubular profiles that are spatially and functionally distinct from retromer-labelled structures acting as endosome-to-TGN transport carriers (ETCs) and colocalises mainly with markers for early endosomes. It interacts with major proteins involved in endocytosis such as amphiphysin 2 (Leprince *et al*., 2003), clathrin and dynein (Skånland *et al*., 2007), and is involved in transferrin uptake. Leprince *et al*. (2003) showed decreased transferrin receptor endocytosis in SNX4 overexpressing cells, and attributed this result to a block in dynamin that was also observed when amphiphysin 2 was overexpressed. Moreover these authors observed colocalisation of SNX4 and transferrin. Another group found that the protein is required for recycling of transferrin receptors from endosomes to an endocytic recycling compartment (ERC) to be transported back to the cell surface. Decreased levels of SNX4 caused lysosomal degradation of the transferrin receptor, but it had no effect on retromer function (Traer *et al*., 2007). SNX4 was not found to directly associate with the transferrin receptor, and raised the possibility that the mammalian SNX4 regulates sorting through a hitherto not yet described protein complex. In yeasts the equivalent of SNX4 (ATG24) participates in a complex independent of the retromer, comprising also SNX41 and SNX42 (ATG20). The protein is involved in the recycling of the transmembrane protein SNC1 during exocytosis of vesicles derived from the Golgi into the plasma membrane. This SNC1, a v-SNARE, is then endocytosed again and SNX4 participates in its routing back to the Golgi (Hettema *et al*., 2003).

Importantly, among all SNX homologues in yeast, currently only ATG24 and ATG20 have been implicated in specific types of autophagy but not in general autophagy. ATG24 has been shown to participate in mitophagy, pexophagy, PMN and the Cvt pathway in different species. In yeast cells the protein is either required or important for different specific autophagy pathways, while we were surprised to see that in *T. brucei* the protein appears to be involved in general autophagy and particularly that it seems to play a negative regulatory role in it, as its depletion increased the number of autophagosomes in the cells under different autophagy-inducing conditions.

Koumandou *et al.* (2011) found that Tb09.211.4240, identified as TbVPS5/TbSNX1, but by us as TbATG24/TbSNX4, is upregulated in bloodstream-form cells together with other components of the retromer, as expected for proteins involved in endocytosis, and that RNAi cells present a severe growth defect. Moreover they observed that the protein colocalises with the *T. brucei* homologue of yeast VPS26. Upon knock-down of Tb09.211.4240, the lysosomal marker p67 showed an enlarged area with an increased immunofluorescence intensity, indicating an expanded lysosome, in agreement with our observations. The effect on the size of the lysosome is explained by the disruption of the retromer and subsequent increased traffic from intracellular vesicles to the lysosome. This group also detected decreased intracellular levels of ISG75, an invariant surface glycoprotein that is ubiquitinated and endocytosed *in vivo* and has a yet unknown endocytic route (Leung *et al*., 2011). This effect observed in their RNAi cells is partially explained by an increased delivery and faster rate of degradation of ISG75 in the lysosome upon depletion of Tb09.211.4240.

As mentioned previously, in our Tb09.211.4240 RNAi experiments we were able to detect an important decrease in transferrin-associated fluorescence in bloodstream-form cells, indicating that the protein is involved in receptor-mediated trafficking of transferrin. Uptake experiments in presence of leupeptin confirm that the compound is not taken up in the cells that are morphologically affected. This role of Tb09.211.4240 in transferrin uptake is reminiscent of what has been observed in mammalian cells for SNX4 but not for SNX1 or any other retromer component. Bryant *et al.* (2007) found no effect on transferrin receptor turnover during SNX1 depletion. Seaman *et al.* (2004) showed that transferrin uptake in mammalian cells is normal when VPS26 is knocked down. Gullapalli (2004) obtained similar results with SNX2. Moreover our experiments indicate colocalisation of GFP-tagged ATG24 and transferrin in wild-type bloodstream cells incubated at 37°C. Zhong *et al.* (2002) showed that mammalian SNX1 and transferrin receptor do not colocalise.

In conclusion, both our experiments and those by Koumandou *et al.* (2011) indicate that Tb09.211.4240 behaves as a retromer component, but also has other roles in endocytosis that are not expected to be performed by the

retromer, particularly in the uptake of transferrin in bloodstream cells. In addition, our data also show a role for Tb09.211.4240 in autophagy.

VPS34 is a conserved class III PI3-kinase that participates in two complexes in yeast, one that controls vacuolar protein sorting and the other one that controls induction of autophagy. The two complexes are fairly conserved in mammals (Itakura *et al*., 2008). The first complex comprises VPS15, VPS30 and ATG14, while in the second complex VPS38 replaces ATG14. No genes encoding homologues of either VPS38 or ATG14 (the two proteins responsible for the specificity of the complex) have been found in the trypanosomatid genome databases. *T. brucei* VPS34 has been characterised and is essential for the parasite's survival (Hall *et al*., 2006). Moreover, its role as an authentic PI3K was confirmed. Due to the fact that ATG24 has no predicted transmembrane domain whereas all homologues identified so far have a Phox-homology domain that interacts with PI3P, modifications often found on endosomal membranes, we used the PI3-kinase inhibitor wortmannin to see if this was also the case for *T. brucei* ATG24. In presence of increasing concentrations of wortmannin for 1 h, ATG24 was dispersed in the cytosol and no longer concentrated in the area between nucleus and the kinetoplast of procyclic cells. Some effect was already observed upon incubation in presence of 0.3 μM wortmannin, while in presence of 3 μM wortmannin the protein was almost completely dispersed in the cytosol. This seems to indicate that the *T. brucei* protein interacts with intracellular membranes via PI3P, as reported for all its homologues described to date. RNAi against VPS34, the only *T. brucei* PI3 kinase identified so far, also affected the subcellular localisation of ATG24 in bloodstream cells as shown by immunomicroscopy, indicating that the protein interacts with PI3P to bind to membranes and that it is affected by VPS34, as is the case in yeasts.

We created ATG24 RNAi cell lines to identify the role of this protein. Growth rate measurements indicated that *T. brucei* ATG24 was not essential in nutrient-rich conditions. However, this was not in agreement with the results by Koumandou *et al*. (2011). The difference between the two studies could be explained by an incomplete depletion of the protein by RNAi knock-down in our experiments that would maintain some ATG24 activity and allow cell survival. Despite the lack of a strong growth phenotype, we observed a clear effect on the

morphology of the lysosome that appeared enlarged. Moreover, transmission electron micrographs of bloodstream cells after 72 h of RNAi induction showed diverse morphological effects.

Next, we exposed these cells to different autophagy-inducing conditions to examine whether ATG24 is essential for autophagy. However, bloodstream-form trypanosomes are completely dependent on glucose for ATP production and other functions and do not survive for a long period when submitted to starvation conditions. Therefore we chose to observe autophagy and the role of ATG24 under differentiation conditions that have previously been shown to increase autophagy. Procyclic cells on the other hand are more resistant to starvation and can survive for hours in PBS. Moreover these cells can grow on glucose as well as on amino acids as energy source. In order to determine if the change from one carbon source to another would increase autophagy in procyclic cells, we used two approaches. The first involved changing the cells from glucose-rich SDM79 medium (that also contains amino acids) to SDM80 medium that lacks glucose but contains amino acids. A change of procyclic cells from SDM79 to SDM80 is usually carried out in a stepwise manner to allow the parasites to adapt metabolically to the new conditions; a decrease in cell growth is often observed during this adaptation. The other approach was to induce a switch in substrate utilization from glucose to amino acids is by adding 60 mM GlcNac to the SDM79 medium. This compound competes with glucose for its transporter but does not enter the cells.

When we imposed a change in the use of glucose as energy source to amino acids by transferring the procyclic cells from SDM79 to SDM80 medium, we failed to see an effect on the growth rate of ATG24 RNAi cells. This could indicate that either autophagy is not essential for these conditions or that the protein is not essential for autophagy under these conditions, similar to observations in starvation conditions in the methylotrophic yeasts *P. pastoris* and *H. polymorpha*. Another possibility is that the RNAi did not sufficiently deplete the levels of the protein and the remaining ATG24 was able to perform its role with no detrimental effects on the cells. A third possibility is that ATG24 acts in trypanosomatids as a negative regulator of autophagy, meaning that its decreased expression or depletion would stimulate autophagy. This is discussed

below. Yet another explanation is that changing the energy source from glucose to amino acids mainly requires mitochondrial enzymes to be upregulated and glycosomal turnover would not be a prerequisite for this adaptation.

Interestingly, monomorphic bloodstream-form cells induced for ATG24 depletion by RNAi showed an increased *in vitro* differentiation rate, triggered by changing the cells from bloodstream medium to differentiation medium in the presence of citrate and cis-aconitate and incubating the cells at a lower temperature, when compared to their parental wild-type cells. The ATG24 RNAi cells expressed marker proteins for the procyclic stage sooner and resumed growth again faster than the wild-type cells. We speculate that the negative effect of ATG24 on differentiation can be explained by its possible involvement in induction/control of autophagy. In yeasts ATG24 is part of a putative complex comprising ATG1, ATG13, ATG17, ATG20, ATG11 and VAC8 that controls the ATG1 (ULK1 in mammalians) kinase activity that, in turn, inhibits autophagy (Klionsky, 2005). In normal conditions ATG13 is hyperphosphorylated by TORC1 and does not interact with ATG1. When autophagy is induced, TORC1 is inactive and ATG13 becomes dephosphorylated and is able to interact with ATG1 as well as other ATG proteins, an autophagy-induction complex that comprises ATG17 in yeasts and ATG101 in mammals among other proteins. Half of the proteins present in this putative complex have no recognizable homologues in trypanosomatids, which hampers the prediction of the existence of a similar complex in *T. brucei*. These include ATG17 and ATG20 which were shown to directly interact with ATG24 in yeast species. Nonetheless there are homologues for ATG1, ATG13, ATG24 and VAC8 present in *T. brucei*, although for ATG1 is was not possible to point to one particular gene, as there are too many possible homologues in the genome. However, VAC8 is also expressed in procyclic and bloodstream cells and seen to be enriched in the microsomal fractions of differential centrifugation experiments (not shown, see Chapter 6), thus, raising the possibility that it interacts with ATG24.

Our experiments, in conjunction with the results published by Koumandou *et al*. (2011), show that Tb09.211.4240 functions in two different processes in trypanosomes: in the endocytic system and in autophagy. This is similar to observations made on the yeast orthologue, but the precise role in autophagy is

possibly not identical. However, the situation in mammalian cells as well as in *Drosophila* and *Caenorhabditis* seems quite different, and this raises the intriguing question about the scenario by which this may have evolved. Probably, the function of SNX proteins in the endocytic system is most ancient, because it has been observed in organisms belonging to different lineages such as yeasts, mammals and trypanosomes. Although autophagy itself is an ancient process, as it is present in representatives from all phylogenetic supergroups (Duszenko *et al.*, 2011), it seems likely that in yeast and trypanosomes the SNX4 isoform has, independently in both lineages, secondarily acquired an additional role in autophagy while retaining its endocytic functions. One can speculate that the SNX1/VPS5 isoform was lost from the trypanosome, with SNX4/ATG24 taking over its functions or that trypanosomatids never possessed it. Based on phylogenetic analysis (Figure 35), we cautiously consider the former explanation most likely. In this respect, it is important to note that mammals, *C. elegans*, *D. melanogaster* and yeasts all belong to one supergroup, the Opisthokonta, whereas trypanosomatids belong to a different group, the Excavata. In different lineages within the Opisthokonta, SNX proteins have expanded to a multi-member family of proteins, but apparently not in the Excavata. Currently no information is available on the function and origin of the second, rather aberrant SNX protein in the trypanosome, Tb927.7.4500.

5.2 Autophagy markers in *T. brucei*

To follow autophagy as well as differentiation of bloodstream cells we used two plasmids allowing the expression of GFP-ATG8.1 and GFP-ATG8.2 (kindly provided by Remo Schmidt and Dr Peter Bütikofer, Bern, Switzerland). These proteins are two of the three isoforms identified in *T. brucei* of the well characterised ATG8 (yeast)/LC3 (mammals). ATG8/LC3 participates in autophagosome formation and might remain attached to be delivered in the vacuole/lysosome where it is degraded together with the contents of the autophagosome. For this reason ATG8/LC3 is routinely used as the marker for autophagy and autophagosome formation. The GFP-ATG8 plasmids were also used to follow *in vitro* differentiation since they were designed to integrate into

the trypanosomatid genome and be expressed under the control of a procyclin promoter, that should only be active in procyclic cells. However we could also see very weak fluorescence in the bloodstream form transfected with these constructs by confocal microscopy, although western blot analysis did not detect the presence of GFP in these cells. The expression of N-terminally GFP-tagged ATG8 is widely used for autophagy studies in different species. We observed that the GFP-tagged ATG8.1 and ATG8.2 was associated to puncta in autophagy-inducing conditions, indicating that the GFP tag was not disturbing the interaction of the protein with the autophagosomal membrane.

The autophagosome marker ATG8/LC3 can be modified by the peptidase ATG4, allowing the protein to bind phosphatidylethanolamine (PE) at the expanding isolation membrane. Upon processing of mammalian LC3 by ATG4, the cleavage product is denominated as LC3I, and its lipid-bound form as LC3II. The two forms can be separated by SDS-PAGE. To separate the two forms in trypanosomatids, a protocol using 6 M urea gels was used. However we were unable to see a second band for ATG8 by western blotting under any conditions tested and for neither isoform (using anti-GFP antibody). As modification of the second band (caused by the association of ATG8 to PE) is expected to occur in cells, we can only explain our failure to detect it by the conditions in which the experiments were performed. The same difficulty in detecting the band representing ATG8 associated to PE in *T. brucei* has been reported even when anti-ATG8 was used (Herman *et al*., 2008; M. Duszenko and J. Mottram, personal communication). Our studies with GFP-ATG8.1 and GFP-ATG8.2 expressing bloodstream-form cell lines show that upon *in vitro* differentiation there is colocalisation of the glycosomal marker TPI and both autophagosomal markers, indicating that glycosomes are indeed targeted for degradation and both isoforms of ATG8 participate in their transport to the lysosome. Interestingly, we could see a difference in behaviour of ATG8.1 and ATG8.2 in procyclic cells. We observed that the band corresponding to GFP-ATG8.1 (but not GFP-ATG8.2) disappeared under certain autophagy-inducing conditions in procyclic cells, an indication that the tagged protein was degraded in the lysosome. Also the level of GFP-ATG8.2 seemed to decrease under these conditions but slower than GFP-ATG8.1. This isoform would decrease and eventually disappear upon prolonged

starvation (in PBS) as well as during incubations in presence of the glucose transport inhibitor GlcNac or when the cells were transferred from SDM79 to SDM80 without adaptation, while ATG8.2 levels would remain similar to levels seen under normal conditions or only decrease slightly. A difference in the behaviour of the two ATG8 isoforms would be expected since ATG8.1 has a C-terminal glycine residue already exposed and might not need processing by ATG4 to bind PE in autophagosome membranes. By contrast, ATG8.2 needs C-terminal cysteine cleavage by ATG4 to expose a C-terminal Gly residue to bind PE in the autophagosomal membrane during its formation. We imagine that ATG8.1 readily participates in autophagosome formation while ATG8.2, would be involved in autophagy in a more regulated fashion. Different roles of ATG8 isoforms have also been described for *L. major* (Williams *et al*., 2009) and *T. cruzi* (Alvarez *et al*., 2008). Interestingly, since we used a constitutively expressed construct, under the control of a procyclin promoter, we could see that the ATG24 RNAi cell lines expressed the tagged construct much sooner than wild-type or non-induced cells when they were exposed to *in vitro* differentiation conditions, in agreement with our observations that these cells differentiate into procyclics faster than wild-type cells. With regard to autophagy that takes place when the bloodstream forms are exposed to *in vitro* differentiation-inducing conditions, we observed that both ATG8 isoforms colocalised with glycosomal markers during differentiation, but cells expressing GFP-ATG8.2 always differentiated slightly faster than cells expressing GFP-ATG8.1 in both wild-type cells as in the ATG24 RNAi cell line. Interestingly we observed that the expression of the GFP-ATG8.1 or GFP-ATG8.2 construct in the wild type bloodstream cells severely affected the differentiation rate at different experiments. This indicates that the overexpression of either tagged proteins is deleterious for the cells during the *in vitro* differentiation conditions studied. A very recent article surprisingly shows that procyclic cells depleted for ATG8.1 or ATG8.2 survive longer than wild-type cells in starvation conditions (Li *et al*., 2012). If further experiments confirm that endogenous or tagged ATG8 is indeed deleterious to the cells, than autophagy will be another pathway in where these parasites are considered unique, as it is universally described as a response that allows cells to cope with different stressful conditions.

Interestingly for us, the differentiation of ATG24 RNAi cells did not seem to be affected by the GFP-ATG8.1 or GFP-ATG8.2 constructs. This could indicate some interaction between ATG24 and the two ATG8 proteins. In absence of ATG24, the negative effect of ATG8.1 and ATG8.2 is not observed in the differentiating cells. Other observation was that the deleterious effect on *in vitro* differentiation was higher for ATG8.1 than for ATG8.2

5.3 Perspectives

TbATG24 has been shown to participate in endocytosis, as its depletion affects the import of transferrin. It would be interesting to perform a more detailed study of the role of ATG24 in receptor-mediated endocytosis, and search for more target proteins besides the transferrin receptor that require ATG24 for their correct transit in the endosomal system in *T. brucei*. Moreover the fact that the protein is a functional homologue of SNX1 as well as SNX4 raises the question whether other proteins involved in endocytosis also exert a dual role in the parasites where distinct proteins are fulfilling the functions in yeasts and/or mammalian cells.

Cells induced for RNAi against ATG24 showed an increased rate of *in vitro* differentiation from long slender to procyclic form in monomorphic cells. A more authentic model, *i.e.* a pleomorphic cell line, should be used for *in vivo* differentiation in order to confirm a true physiological role of ATG24 in this process. In other organisms, ATG24 has been identified as one of the components of a crucial putative complex that is involved in the induction of autophagy. It will be important to identify in trypanosomes the network of ATGs and other proteins that interact with ATG24. This would probably give more detailed insight into how the parasite induces autophagy at the molecular level.

The effect of ATG24 RNAi on differentiation is possibly due to its role in induction of autophagy, as autophagy seems upregulated. Autophagy is vital for differentiation of the parasite (see Chapter 2). It would be important to identify the effect of ATG24 on differentiation also involves its role in endocytosis. For example, in that case the knockdown of the protein would result in the altered endocytic or exocytic targeting of one or more proteins that also might result in

an increased rate of differentiation. In addition to the similar effect on differentiation resulting from the protein's role as a negative controller of autophagy where knockdown would increase the rate of autophagy. To answer this question we would need to know not only the interacting partners of ATG24, but also which domains of the protein are important for its role in endocytosis and which ones are essential for autophagy-induction. With such information, one could try to block one role of the protein without interfering with the other one and distinguish the importance of the different protein domains on differentiation.

Moreover we were able to confirm the participation of ATG8.1 and, to a lesser extent, ATG8.2 in autophagy during starvation of procyclic cells in PBS. The first isoform is preferred under conditions where the energy source was changed from glucose to amino acids in the SDM80 medium or in the presence of GlcNac. Interestingly, in our experiments the second isoform was associated with faster *in vitro* differentiation in DTM medium containing 3 mM citrate and 3 mM cis-aconitate along with a drop in temperature to 28°C. However it is not known if there are conditions where only one of the two isoforms plays a role or if their functions overlap. Experiments performed with knock-out or knock-down cell lines for one or other isoform would help us to know whether these proteins are individually essential at each autophagy-inducing condition or if there exists redundancy so that one isoform can easily replace the other one without detriment for the cell.

5.4 Conclusions

Knowledge of autophagy in trypanosomatids was very limited until about a decade ago, when several groups started to focus their attention on the subject. Autophagy has since been associated with differentiation as well as virulence and as a mechanism to cope with effects of drugs in these parasites. This is also the case for other parasite protists such as *Plasmodium*, *Toxoplasma*, *Entamoeba* and *Acanthamoeba* species (see Chapter 2 and Duszenko *et al*., 2011). As a consequence, autophagy is today one of the fastest-growing fields of research in parasitology. Trypanosomatids have been shown to upregulate autophagy under

starvation, during differentiation and to cope with different sources of cellular stress. Importantly, blocking this response has been shown to be detrimental for the parasites, increasing the hope that targeting the autophagy machinery might offer a drug therapy. Despite the growing interest in the subject, the molecular mechanisms that control autophagy in trypanosomatids are still very much 'in the dark'. An increasing number of studies on autophagy in trypanosomatids seem to indicate that important differences exist when the pathway in these parasites is compared to autophagy in yeast or mammalian cells. Moreover, it has recently been argued that the autophagy process in yeast is highly specialized and in many respects not representative for that in other eukaryotes (King, 2012). Very little is known about how this pathway is induced in trypanosomatids. Our work has characterised ATG24 in *T. brucei* and associated this protein with different pathways, from receptor-mediated endocytosis to *in vitro* differentiation and autophagy. Our contribution that the protein is associated with this pathway represents a new step towards the elucidation of the network of ATGs that controls the induction of autophagy in trypanosomatids.

Chapter 6

Experiments on a candidate autophagy-related protein in *T. brucei*

Only a few of the proteins that participate in specific autophagy pathways have been identified in trypanosomatids (Herman *et al*., 2006; Rigden *et al*., 2009, Duszenko *et al*., 2011). Together with ATG24, that has been extensively described in Chapters 4 and 5, trypanosome homologues of the following proteins that participate in specific autophagy processes in yeasts have been found: GCN1 (previously known as PAZ10), GCN2 (PAZ11), GCN3 (PAZ5) and VAC8. GCN proteins have been described as translational regulators essential for micropexophagy in *P. pastoris* (Mukaiyama *et al*., 2002) and had, for that reason, not our highest priority.

VAC8 is a vacuolar protein that can be palmitoylated and myristoylated and participates in changes in the yeast vacuolar membrane that takes place during vacuolar inheritance and micropexophagy (Fry *et al*., 2006; Subramanian *et al*., 2006). VAC8 has been shown to interact with ATG13, making the protein a component of the putative ATG1 complex that participates in induction of autophagy. The *T. brucei* homologue for VAC8 has a predicted molecular mass of 121 kDa, almost twice the size of the *P. pastoris* protein, and possesses a conserved armadillo (ARM) repeat domain.

Using protocols similar to those we described for ATG24 in section 3.4, we have created a construct to express in *E. coli* BL21(DE3) cells a region of the TbVAC8 homologue containing the ARM repeat domain. We expressed and purified the protein and produced an antiserum in rabbit. A western blotting protocol was optimized by incubating the primary anti-VAC8 antiserum at a dilution of 1 :500 with 10% FCS in PBS in place of 1% milk.

The protein appeared to be expressed at very low concentrations in the parasite. It was found enriched in the microsomal and cytosolic fractions obtained by differential centrifugation and analysed by western blotting. In confocal immunomicroscopy the antiserum was not specific and stained

different cell compartments. It mainly appeared diffusely distributed throughout the cytosol. This may possibly not be the correct localisation of VAC8, which would be expected to interact with membranes. Our candidate VAC8 was also seen in puncta that did not colocalise with GFP-ATG8.1 nor GFP-ATG8.2 under starvation conditions.

A recently published study on the palmitoylated proteome of *T. brucei* did not include VAC8 (Emmer *et al*., 2011), although this modification has shown to be essential for the role of the *P. pastoris* protein. Our inability to see the subcellular localisation of VAC8 by confocal immunomicroscopy prevented further investigation to determine if the protein is palmitoylated in *T. brucei*. However during incubation with 2-bromopalmitate (2-BP), a palmitoylation inhibitor, the protein seemed to be enriched in the flagellum. Interestingly increasing the concentration of 2-BP caused a detachment of the flagellum from the cell body.

An RNAi construct targeting VAC8 was also made. However, repeated attempts to transfect both bloodstream and procyclic cells never resulted in the creation of a knock-down. This could indicate that, unlike in *P. pastoris*, the *T. brucei* protein is essential for the parasite.

Other unsuccessful experiments include different attempts to create a lysosomal marker. We have expressed in *E. coli* and purified recombinant p67, a *T. brucei* lysosomal membrane protein and used it for raising an antiserum. However this antiserum was not specific enough in western blotting nor in confocal microscopy, even following antibodies purification. The lack of specificity might be due to the fact that in trypanosomes, the protein is highly modified by protease processing and glycosylation en route to the lysosomal membrane. We also attempted to express a GFP-tagged fragment of p67 in trypanosomes for confocal microscopy experiments. The selected region of the protein had previously been identified by Bangs and coworkers as being necessary for the correct targeting of p67 to lysosomes in procyclic trypanosomes (Alexander *et al*., 2002). However the GFP-tagged construct was not directed to the lysosomal membrane in our experiments and fluorescence was found in the cytosol.

Finally we also used 1 mM 8-pCPT-cAMP, a cAMP analogue that has been described to induce slender-to-stumpy differentiation *in vitro* (Breidbach *et al*., 2002; Laxman *et al*., 2006). Despite several attempts, we did not see any effects on morphology or expression of the stumpy trypanosome-specific marker protein PAD1 (using an antiserum kindly donated by Prof. K. Matthews, Edinburgh; used at a 1 :1000 dilution), nor a change in the development of the mitochondrion, as seen in confocal immunomicroscopy using anti-ASCT. Moreover, when bloodstream cells treated with 1 mM 8-pCPT-cAMP for 48 h were placed in DTM medium, they failed to differentiate and the majority of the cells died before 72 h.

Chapter 7

Trypanosomes contain two highly different isoforms of peroxins PEX13 involved in glycosome biogenesis

This chapter has been published as a research paper:

Brennand A., Rigden D.J., Michels P.A.M.
Trypanosomes contain two highly different isoforms of peroxins PEX13 involved in glycosome biogenesis
FEBS Letters 586 (2012) 1765-1771.

Summary:

This chapter describes the identification and characterisation of TbPEX13.2, a peroxin involved in glycosome biogenesis. Like the previously identified and characterised PEX13.1 (Verplaetse *et al*., 2009), this protein does not possess all the characteristic features common to PEX13 in humans and different yeasts. We show that TbPEX13.2 is an essential protein for bloodstream trypanosomes, localised to glycosomes and involved in the import of PTS-1 and PTS-2 glycosomal matrix proteins. TbPEX13.2 is also involved in the association of PEX14 to the glycosomal membrane. This is the first case that two different forms of the peroxin PEX13 have been identified in one organism

FEBS Letters xxx (2012) xxx–xxx

FEBS Letters

journal homepage: www.FEBSLetters.org

Trypanosomes contain two highly different isoforms of peroxin PEX13 involved in glycosome biogenesis

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ARTICLE INFO

Article history:
Received 17 March 2012
Revised 3 May 2012
Accepted 10 May 2012
Available online xxxx

Edited by Wilhelm Just

Keywords:
Trypanosoma
Glycosome
Peroxisome
Biogenesis
Docking complex
Peroxin

ABSTRACT

We previously identified the peroxin PEX13 in *Trypanosoma brucei*. Although lacking some features considered typical of PEX13s, it appeared functional in the biogenesis of glycosomes, the peroxisome-like organelles of trypanosomatids. Here we report the identification of a very different trypanosomatid PEX13, not containing the commonly encountered PEX13 SH3 domain but having other typical features. It is readily detected with the jackhmmer database search program, but not with PSI-BLAST. This is the first time different PEX13 isoforms are reported in a single organism. We show that this PEX13.2, like the PEX13.1 previously described, is associated with glycosomes and that its depletion by RNA interference affects the biogenesis of the organelles and viability of trypanosomes. The features considered typical of PEX13s are discussed.

Structured summary of protein interactions:
Pex19 physically interacts with **Pex13.2** by two hybrid (View interaction)
Pex13.1 physically interacts with **Pex13.2** by two hybrid (View interaction)

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1. Introduction

Trypanosoma brucei is a parasitic protist responsible for sleeping sickness, a potentially fatal disease of humans in sub-Saharan Africa, and a similar disease called nagana of livestock animals. It lives in the bloodstream and other extracellular fluids of its mammalian host and is transmitted by the bite of tsetse flies. These parasites belong to the class Kinetoplastea, order Trypanosomatida [1] together with the other human pathogens *Trypanosoma cruzi*, responsible for Chagas' disease and *Leishmania* spp., the causative agents of a variety of disorders collectively called leishmaniases.

Trypanosomatids possess peroxisome-like organelles called glycosomes which, however, are unique in the sense that they contain the majority of the enzymes of the glycolytic pathway [2,3]. In other organisms glycolysis is a cytosolic process. It has been shown that this compartmentalization is essential for the viability of the bloodstream form (BF) of the parasite, for which glycolysis is the only source of ATP, and also for the procyclic form (PF), the life-cycle stage of the parasite present in the midgut of the tsetse fly, when in vitro cultured in the presence of glucose. Even a small degree of mislocalisation of glycolytic proteins from the glycosome into the cytosol can cause cell death (reviewed in [4,5]).

Peroxisomal and glycosomal matrix proteins are encoded in the nucleus and synthesized on free ribosomes in the cytosol. The post-translational import of these proteins into the organellar matrix is dependent on an amino-acid sequence motif, a so-called peroxisomal-targeting signal (PTS). More commonly this may be a PTS1, a specific C-terminal sequence such as SKL or three amino acids with similar physicochemical properties. The alternative PTS2 is a more complex nonapeptide sequence found in the N-terminal region of a number of other peroxisomal/glycosomal proteins. Targeting can also be mediated by a polypeptide internal sequence or I-PTS. However, the I-PTS motifs of different proteins do not share a consensus sequence and the means of import of proteins possessing such a PTS has not been unambiguously established: it may, at least in some cases, involve piggy-back transport on a PTS1 or PTS2 containing protein [6,7]. The PTS1 and PTS2 of newly synthesized proteins to be imported are recognized by the cytosolic receptors PEX5 and PEX7, respectively. The receptors, with their cargo-proteins bound, interact with an assembly of proteins associated with the peroxisomal/glycosomal membrane known as the docking complex. This complex comprises PEX13, PEX14 and in yeasts also PEX17. The docking leads to a number of successive conformational changes, protein–protein interactions (reviewed in [5]) and the

Abbreviations: PEX, peroxin; PTS, peroxisomal-targeting signal; RNAi, RNA interference; Tet, tetracycline; BF, bloodstream form; PF, procyclic form

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http://dx.doi.org/10.1016/j.febslet.2012.05.019

Please cite this article in press as: Brennand, A., et al. Trypanosomes contain two highly different isoforms of peroxin PEX13 involved in glycosome biogenesis. FEBS Lett. (2012), http://dx.doi.org/10.1016/j.febslet.2012.05.019

formation of a transient pore [8] resulting in the delivery of the cargo proteins into the matrix of peroxisomes/glycosomes. After releasing their cargos, the receptors PEX5 and PEX7 are retrieved from the organelle into the cytosol by a ubiquitination dependent process to become available for mediating new rounds of import. The energy required for the import seems to be mainly provided in the later steps of the cyclic process, by ATP that is hydrolyzed by the AAA^+-ATPases PEX1 and PEX6 for the retrieval of the ubiquitinated receptors from the organelles.

PEX13 is part of the docking complex and participates in the import of peroxisomal matrix proteins. It is an integral peroxisomal membrane protein that is only poorly conserved between different organisms. Its N- and C-termini are exposed to the cytosol. Common features in the N-terminal region are a proline-rich portion, often harbouring a KPWE motif, followed by a Gly-rich segment containing multiple YG motifs. The C-terminal region typically contains two membrane-spanning helices followed by a SH3 domain that interacts with PEX5 in yeast.

Peroxisome biogenesis has been studied in a variety of organisms, most notably in yeasts. A combined, not-redundant set of 33 peroxins (PEX) involved in the process have been identified in *Saccharomyces cerevisiae* and other organisms. Research on the biogenesis of *T. brucei* glycosomes has resulted in the identification and characterization of about 10 peroxins involved in matrix import, including PEX13 (reviewed in [4,5]). TbPEX13 shares very low overall sequence identity when compared to its counterparts from other organisms and is unusual in several respects: (1) it lacks the proline-rich motif usually found near the N-terminus; (2) unlike other PEX13s it has at its C-terminus a PTS1-like signal (TKL) that is conserved in all trypanosomatids [9]. The function for this signal is still unknown since the association of this PEX, being a peroxisomal membrane protein, with the glycosomes is supposed not to be dependent on a PTS1 or PTS2, but on another type of signal (a mPTS or membrane peroxisomal-targeting signal) that is recognized by another cytosolic receptor, PEX19. TbPEX13 has been confirmed to be a glycosomal membrane protein, to interact with TbPEX5 and TbPEX14 and to be involved in the import of glycosomal matrix proteins. By RNA interference (RNAi) it was shown to be essential for the survival of both bloodstream and procyclic cells [9].

In this paper we report the identification of another TbPEX13, called TbPEX13.2. It has a higher overall sequence identity to PEX13 from other organisms than does TbPEX13.1, also possesses a YG motif-containing, Gly-rich region and two predicted transmembrane regions, but lacks a SH3 domain and a PTS1. Its glycosomal localization and involvement in glycosome biogenesis were experimentally demonstrated. This is the first report from any species of two distinct, very different PEX13 isoforms being involved in the biogenesis of organelles of the peroxisome family.

2. Materials and methods

2.1. Bioinformatics

Iterative database searches were done with the jackhmmer program of the HMMER3 suite [10] or PSI-BLAST [11] in the UniProt database [12], varying inclusion thresholds – 1×10^{-3}, 1×10^{-6} or 1×10^{-9} – but leaving other parameters as default values. Motif searches were done with ps_scan [13], transmembrane helices predicted with TM-HMM [14], and alignments visualized and manipulated with Jalview 3 [15].

2.2. Trypanosome growth

Bloodstream-form (BF) *T. brucei* cells of strain Lister 427, cell line 90–13 [16] possessing a chromosomically integrated T7 polymerase gene and the tetracycline (Tet) repressor gene from *Escherichia coli* were cultured in HMI-9 medium containing 10% foetal calf serum (Gibco) and 2.5 μg/mL G418 (Gibco). The cells were incubated at 37 °C with 5% CO_2.

Procyclic-form (PF) cells of *T. brucei* Lister 427, cell line 449 [17] with the *E. coli* Tet repressor gene integrated in its genome were cultured in SDM-79 medium [18] containing 15% foetal calf serum and 0.5 μg/mL phleomycin (Cayla). PF trypanosomes of cell line 29–13 [16] were cultured in SDM-79 containing 15% foetal calf serum, 50 μg/mL hygromycin (Sigma–Aldrich) and 15 μg/mL G418. Cells were incubated at 28 °C with 5% CO_2.

2.3. Preparation, cloning and expression of DNA constructs

A construct was made with the trypanosome-specific pGC1 vector that allows the expression of an N-terminally GFP-tagged protein upon induction with Tet [19]. To that end the *PEX13.2* gene was amplified by PCR using the primers 5′-TTAAGCTTTT*ATG*TCGGCAGCATGG-3′ (forward) and 5′-AAGGATCC*CTA*ACGAGTAATCCTTTTT-3′ (reverse); the *Hind*III and *Bam*HI restriction sites are underlined and the start and stop codons are italicized in the primer sequences. The amplification conditions were: first a DNA denaturing step at 95 °C for two min, followed by 30 cycles of 95 °C for 30 s, 50 °C for 30 s and 72 °C for one min with a final extension step of 72 °C for five min. The amplified DNA was verified by sequencing (Macrogen, South-Korea). Clones of transfected PF cells having the construct incorporated in the genome were selected for blasticidin (Invitrogen) resistance. For fluorescence microscopy experiments GFP-tagged PEX13.2 was expressed by addition of 5 μg/mL Tet to the medium for 24 h.

Another trypanosome-specific construct was created using the vector p2T7–177 to generate RNAi against PEX13.2 in BF cells. The same amplified DNA was used to place the gene between two opposite Tet-inducible T7 promoters to generate two complementary RNA strands that will direct the degradation of mRNA matching the sequence and consequently the depletion of the protein from the cells. Positive clones were selected by resistance to phleomycin. RNAi was induced by the addition of Tet (1 μg/mL) to the medium.

2.4. Confocal fluorescence microscopy

For acquisition of images a Cell Observer Spinning Disk microscope was used. Poly-L-lysine was used to coat the slides. Trypanosomes were fixed with 4% formaldehyde and permeabilized with 1% Triton X-100. Anti-PEX11 (gift from F. Vonken), a glycosomal membrane protein, was used at a 1:5 dilution (in PBS containing 2% BSA) to assess for colocalization with GFP-tagged PEX13.2. Secondary antibody Alexa 568 anti-rabbit IgG (Molecular Probes) were used at a dilution of 1:800. Mowiol was used as the mounting medium.

2.5. Subcellular location of proteins by selective membrane permeabilization with digitonin

To study the effects of depleting PEX13.2 on the subcellular distribution of proteins, BF trypanosomes possessing an integrated RNAi p2T7-177-*PEX13.2* construct were grown in the presence or absence of Tet (1 μg/mL in DMSO). After 24–48 h samples were collected and the total protein concentration was determined. Samples of trypanosomes, each corresponding to 100–200 μg of total protein, were treated with the detergent digitonin. All samples were exposed for the same period, 4 min, to digitonin, each at a different concentration of the detergent. This allowed the permeabilization of the different membranes of the cells (plasma, glycosomal and mitochondrial membrane, successively) each to a different extent, according that membrane's sterol content. The incubation was then stopped by centrifugation (2 min, 13,000×g)

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and the supernatant collected for protein fractionation by SDS–PAGE and western blot analysis. Protein bands were revealed using an ECL system (Pierce) followed by film exposure. Films were scanned and all bands quantified with the ImageJ program. To assess if the RNAi targeting of PEX13.2 caused mislocalisation of glycosomal matrix proteins to the cytosol, the subcellular distribution of PTS1 and PTS2 proteins as well as cytosolic proteins was analyzed in wild-type, induced and non-induced trypanosomes.

2.6. Protein interaction studies with the yeast two-hybrid system

Possible interactions between TbPEX13.2 and other peroxins were tested using the yeast two-hybrid methodology. DNA segments corresponding to full-length proteins or fragments of proteins whose interactions were to be tested were expressed in *S. cerevisiae* as fusion proteins with $GAL4_{BD}$ or $GAL4_{AD}$, using the vectors pGAD424 and pGBT9 (Clontech). pGAD424 has also a targeting sequence that directs the two interacting proteins to the nucleus. After cotransformation, cells were kept at 30 °C for 72 h and then tested for a $lacZ^+$ phenotype in a X-gal assay. As negative controls, yeast cells were also cotransformed with each construct together with the empty opposite vector. For making a construct with the full-length *PEX13.2* gene amplification was performed with the forward primer 5'-AAGAATTC*ATG*TCGGCAGCAGT-3', where the *Eco*RI restriction site is underlined and the start codon italicized. The same oligonucleotide as was used to prepare the insert of the pGC1 construct (see section 2.3) served as reverse primer. For a construct with the N-terminal region of PEX13.2, the first 462 bp of the gene were amplified using the same forward primer as for the full-length construct and the oligonucleotide 5'-AAGGATCC*CTA*CATGAAAGTCATTTGAC-3' as reverse primer, where the *Bam*HI restriction site is underlined and the stop codon added to the sequence italicized. Yeast two-hybrid constructs with the same vectors have been prepared previously for the full-length PEX13.1 and its SH3 domain and for the full-length PEX5, PEX14 and PEX19 [9]. Yeast cell culturing, transformation and X-gal assays were performed according to the protocols provided by Clontech for its Matchmaker two-hybrid system.

3. Results and discussion

3.1. Identification of a second T. brucei PEX13 homologue and comparison of its sequence with other PEX13 sequences

Previously, the identification of trypanosomatid PEX13.1 could not be made by conventional (PSI-) BLAST database searches and instead resulted from a motif- and domain-based search strategy [9]. A likely contributor to the failure of the BLAST searches is the unusual sequence composition of PEX13, containing a long, Gly-rich region with multiple YG motifs: such regions of composition bias remain problematic for BLAST programs, leading to the contamination of search results with unrelated proteins, despite successive improvements in their treatment [20,21]. Comparison of a newer hidden Markov model-based program, jackhmmer, with PSI-BLAST showed that the former was significantly more resistant to search contamination (with obvious unrelated sequences such as ribonucleoproteins and ATP synthase) and it was employed to search for protist PEX13 sequences. Surprisingly, jackhmmer retrieved a second group of trypanosomatid sequences which, after their later characterization (below), were named PEX13.2. The *T. cruzi* PEX13.2 sequences, for example, could be retrieved using well-known PEX13 sequences, from human, *Arabidopsis* or yeast, after four, two or three iterations, respectively, using an inclusion threshold of 1×10^{-6}. Trypanosomatid PEX13.1 sequences, by contrast, failed to retrieve the new group. PSI-BLAST failed to retrieve the trypanosomatid PEX13.2 sequences using any of these query sequences before corruption of the searches occured.

An alignment of trypanosomatid PEX13.2 proteins is shown in Fig. 1A. In the present databases, the *Leishmania* sequences vary considerably at the N-terminus. The *L. infantum* sequence is much shorter than the *L. major* counterpart, suggestive of erroneous gene calling. On the other hand, other *Leishmania* sequence termini, especially that of *L. tarentolae*, are longer than that of *L. major* but bear little similarity to those of *Trypanosoma* species in the region whose translation starts are consistently similar. Experimental data may be required to resolve the issue. Fig. 1B shows a comparison of the features seen in different groups of PEX13 proteins. The SH3 domain was long considered a primary characteristic of PEX13 proteins [22], but it is absent from plant sequences [9] and also from the new trypanosomatid PEX13.2 proteins. Less commented, but well conserved and shared between fungal, animal and plant PEX13s is a KPWE motif, often preceded by several Pro residues, located near the N-terminus. This feature was absent from trypanosomatid PEX13.1 proteins and it is not conserved in all PEX13.2 sequences either, although the *T. cruzi* protein does contain a RPWE sequence starting at position 6. All known PEX13 proteins are integral membrane proteins, typically with two predicted transmembrane helices. Indeed TM-HMM predictions for PEX13.2 sequences pick out two regions (Fig. 1A). The PTS1 sequence found at the end of the PEX13.1 sequence, whose function remains cryptic, is not seen in the PEX13.2 proteins. Finally, like other PEX13s, the new group contain YG motif-containing, Gly-rich regions of 50–60 residues in length. As Fig. 1B makes clear, transmembrane regions aside, this region is the only obvious characteristic shared by all known PEX13 groups.

A comparison of trypanosomatid PEX13.1 and PEX13.2 sequences with PEX13 proteins from selected other organisms is shown in Supplementary Fig. S1. Sequence identity between the two trypanosomatid groups is insignificant at 11–16%. Indeed, the inclusion of PEX13.1 sequences in the alignment is for comparison only: homology between them and the other sequences cannot presently be demonstrated. While a trypanosomatid lineage-specific gene duplication followed by extreme divergence may still be considered the most likely explanation for the origin of the PEX13.1 and PEX13.2 groups, evidence is currently lacking. For example, although PEX13.1, yeast and animal sequences all contain an SH3 domain, that of PEX13.1 bears no particular evolutionary affinity for the others. The trypanosomatid PEX13.2 sequences, in contrast, are detectably similar to human, plant and yeast PEX13s by modern database search software (see above). PEX13.2 sequences share 10–22% sequence identity with the other proteins shown in Supplementary Fig. S1.

3.2. The putative T. brucei PEX13.2 is associated with glycosomes

To identify the subcellular localization of the putative *Tb*PEX13.2, a procyclic cell line was created that, upon induction by addition of Tet, expressed an N-terminally GFP-tagged version of the protein. The trypanosomes were collected 24 h after induction, and the localization of the fluorescent GFP-*Tb*PEX13.2 was determined by confocal microscopy. The glycosomal membrane protein PEX11 was used as a control for glycosomal localization. GFP-tagged TbPEX13.2 showed a punctate pattern characteristic of glycosomal localization and it colocalized with the glycosomal marker PEX11 (Fig. 2), indicating that the putative TbPEX13.2 is indeed associated with these organelles.

3.3. TbPEX13.2 is an essential protein, involved in the import of glycosomal matrix proteins

A *T. brucei* BF cell line was created to study the function of *Tb*PEX13 by decreasing its expression by RNAi. To that end, trypanosomes of the 90–13 cell line were transfected with the vector

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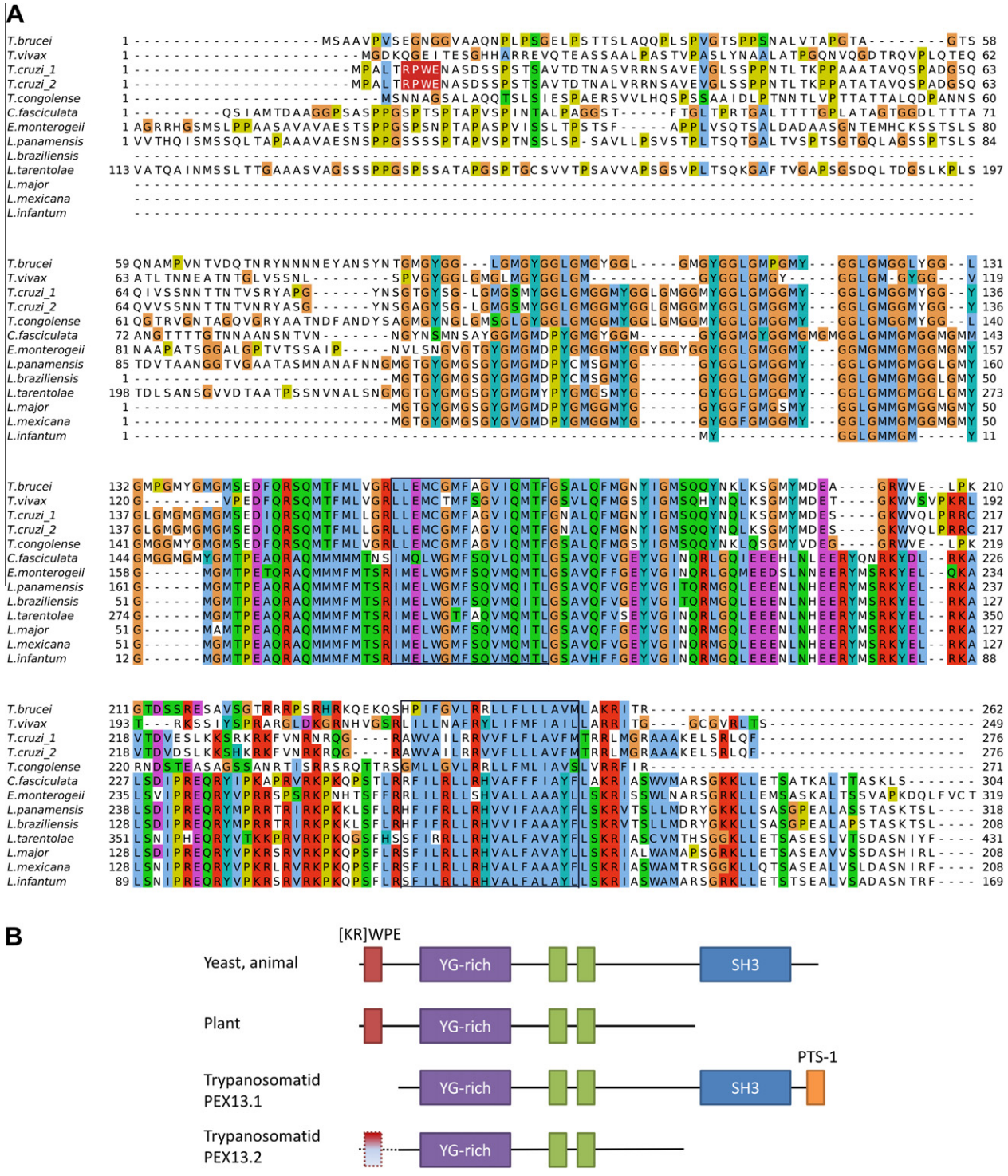

Fig. 1. (A) Sequence alignment of trypanosomatid PEX13.2 proteins in the TriTryp database release 4.0. Identifiers are as follows: *T. brucei*, Tb09.211.1050; *T. vivax*, TvY486_0904050; *T. cruzi*_1, Tc00.1047053506885.180; *T. cruzi*_2, Tc00.1047053510729.230; *T. congolense*, TcIL3000.0.23750; *Crithidia fasciculata*, Contig662-5-8715-7804; *Endotrypanum monterogeii*, EmtLcontig1-2-1808759-1809715; *L. panamensis*, LpnLscaffold72-6-131166-130213; *L. braziliensis*, LbrM.34.4910; *L. tarentolae*, LtaP35.4940; *L. major*, LmjF.35.4960; *L. mexicana*, LmxM.34.4960; *L. infantum*, LtaP35.4940. The N-terminal 112 residues of the *L. tarentolae* sequence are not shown. The RWPE motif in the *T. cruzi* sequences is picked out in white on red, boxes indicate predicted transmembrane helical regions. (B) Schematic figure, not to scale, of specific molecular features in PEX13 sequences from different organisms. Green boxes are predicted transmembrane helices. The dotted lines around the N-terminal motif in trypanosomatid PEX13.2 sequences indicate that the motif is only present in the *T. cruzi* sequences (see Fig. 1A).

p2T7–177 in which the *PEX13.2* gene had been placed between the two opposite, Tet-inducible, T7 promoters. Three clones were obtained of which the cells underwent important growth retardation upon induction of RNAi (Fig. 3). It was repeatedly observed that parasites of these clones started dying within 24 h after induction of RNAi, indicating the importance of PEX13.2 for the parasites. However, at 48 h after induction, the growth and death rates of cells balanced, as the number of cells remained the same, whereas after 48 h the population of parasites slowly recovered, eventually resulting in a growth rate similar to that of wild-type cells. This

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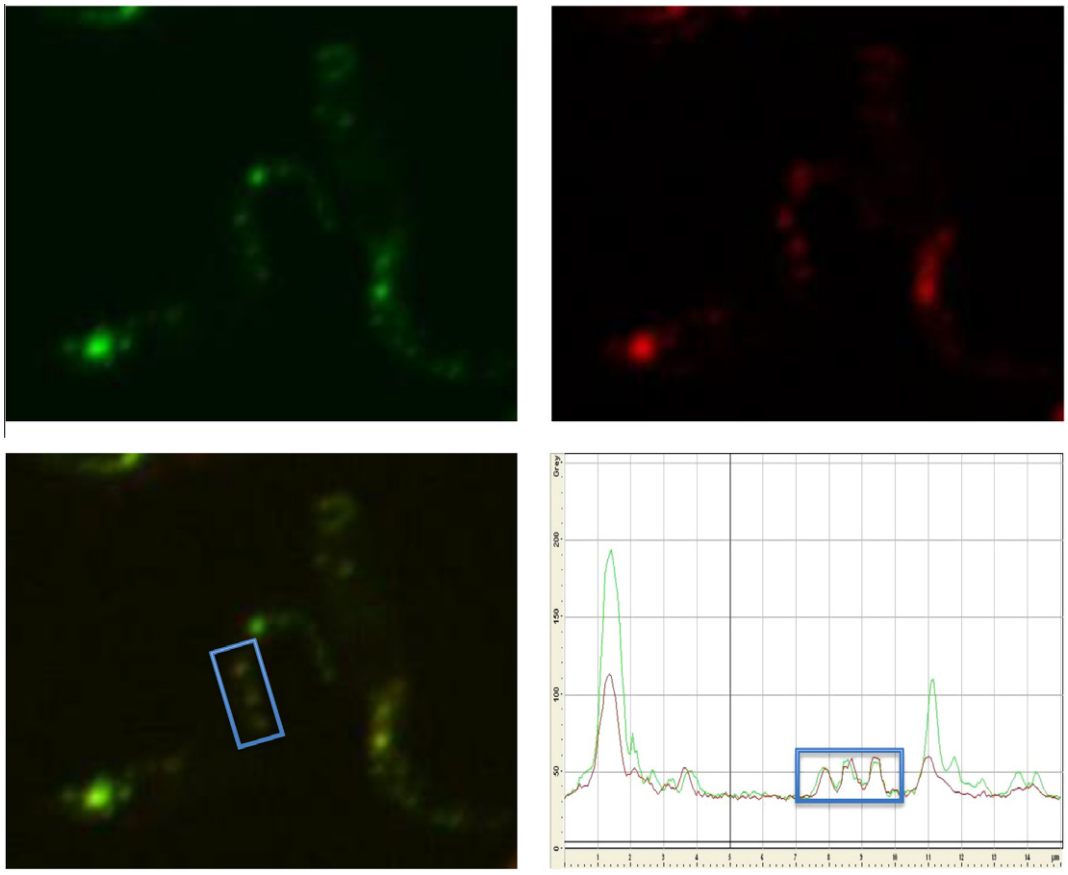

Fig. 2. Procyclic *T. brucei* expressing GFP-tagged PEX13.2 as visualised by confocal fluorescence microscopy. Two trypanosomes are present in the image. The green fluorescence of the tagged protein is shown in the top left panel. The glycosomal marker anti-TbPEX11, revealed in both trypanosomes by red fluorescence using anti-rabbit IgG conjugated Alexa-568, is shown in the top right panel. PEX13.2 and PEX11 colocalize, as shown by the yellow colour resulting from the merge of the signals (bottom left panel). The colocalisation of the two markers is further evidenced by a track analysis of the individual green and red fluorescence throughout the cell image with the merged signal (bottom right panel). The track analysis (giving intensity versus distance on track) was performed with AxioVision software (4.8.2; Zeiss, Germany).

recovery from an RNAi-dependent growth phenotype has been often reported for trypanosomes and was attributed to the arising of revertants which had undergone genomic changes affecting either the promoter region of the RNAi construct or the deletion of the deleterious RNAi cassette from the genome of the parasites [23,24]. Despite repeated attempts, no clones of procyclic trypanosomes with the RNAi construct were obtained.

To assess the role of the protein in glycosome biogenesis, one BF RNAi clone was used for a digitonin permeabilization experiment in which aliquots of trypanosomes were incubated for the same period with increasing concentrations of the detergent, followed by centrifugation to separate the cells from the medium. Digitonin permeabilizes membranes as a function of their sterol levels, so that at low concentration of the detergent only the plasma membrane is affected allowing proteins present in the cytosol to be released from the cells. At higher concentrations the glycosomal and mitochondrial membranes are successively permeabilized, causing the release of glycosomal and eventually also mitochondrial matrix proteins. The RNAi induced effect on the sequestering of glycosomal matrix proteins was studied by comparing BF 90-13 wild-type trypanosomes, and cells of the PEX13.2 RNAi cell line that were either non-induced or induced for 24 h. The experiments were repeatedly performed and proteins in different cell fractions quantified as described in Section 2.5.

Differences were reproducibly observed in the release of glycosomal matrix proteins between BF trypanosomes induced for RNAi and wild-type trypanosomes, but not in the release of a cytosolic marker protein, the cytosolic isoenzyme of glyceraldehyde-3-phos-

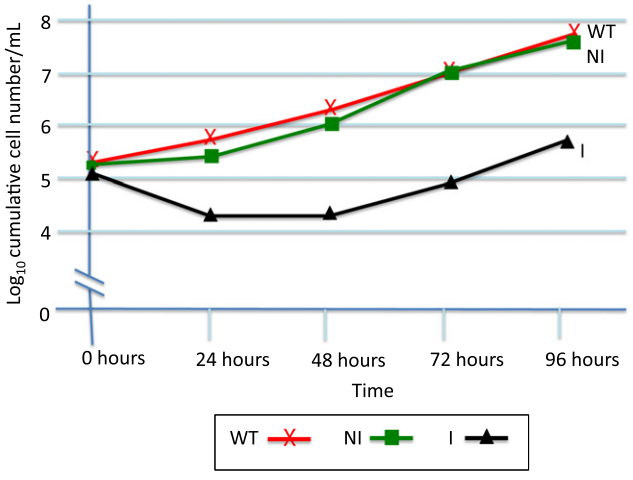

Fig. 3. Effect of RNAi-dependent depletion of PEX13.2 on the growth of bloodstream-form trypanosomes. The growth profiles of wild-type (WT) cells (X) or cells possessing the construct for production of double-stranded PEX13.2 RNA were determined for cells growing in the absence (■, NI) or the presence (▲, I) of the tetracycline, the inducer of double-stranded RNA expression. Tetracycline was added at $t = 0$. The increase in cell number in induced cultures after 48 h is attributed to revertants of the RNAi mutants. In different experiments, the timing of the appearance of RNAi revertants varied. Prolonged pre-culturing in the absence of tetracycline resulted in a more rapid appearance of revertants after the addition of inducer.

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phate dehydrogenase (cGAPDH). Essentially all cGAPDH was released, in wild-type and RNAi mutant trypanosomes, at digitonin concentrations of 0.05 mg/mg total cellular protein (Fig. 4). Low amounts of both the PTS1-protein glycerol kinase (GK) and the PTS2-protein aldolase (ALD) were already released below 0.25 mg digitonin/mg protein, a concentration at which usually, in wild-type cells, glycosomal proteins start to appear (Fig. 4). At this latter detergent concentration, almost all GK and ALD were released in the RNAi mutant, while the released levels in wild-type cells were consistently at least twofold lower. For the glycosomal isoenzyme of GAPDH (gGAPDH), release at low concentrations was never observed, but at 0.25 mg digitonin/mg protein all of this enzyme appeared, whereas in wild-type cells the release was only partial (about 20%) and higher detergent concentrations were required to free all GAPDH from the cells. No effect of RNAi was observed on the digitonin-dependent release of the I-PTS protein triosephosphate isomerase.

These data strongly suggest that TbPEX13.2 might be involved in the import of these glycosomal matrix proteins into the glycosomes. The RNAi-dependent decrease of the peroxin levels is considered to cause the import arrest of newly synthesized matrix proteins from the cytosol into the organelles. As explained previously, no drastic relocalization can be observed, because the presence of glycosomal enzymes outside the organelles, even at low activity, is highly detrimental for trypanosomes, particularly when they are dependent on glycolysis such as in the bloodstream stage [25–27].

We also observed an effect on the association of PEX14 with the glycosomal membrane when the RNAi mutant was induced. At 0.01 mg digitonin/mg protein, the RNAi cell line trypanosomes released almost four times more PEX14 into the supernatant than wild-type cells. PEX14 binds to the peroxisomal membrane in all cells analyzed, including *T. brucei* [24]. The correct association of PEX14 with the peroxisomal/glycosomal membrane has been shown to be dependent on PEX13 for yeast [28] and PEX13.1 in *T. brucei* (E. Verplaetse and PM, unpublished results). As shown in Fig. 4, upon PEX13.2 RNAi in bloodstream cells, PEX14 becomes partially mislocalized to the cytosol, indicating that PEX13.2, together with PEX13.1 and possibly other partners, plays a role in the correct localization of PEX14 to the glycosomal membrane in the parasite.

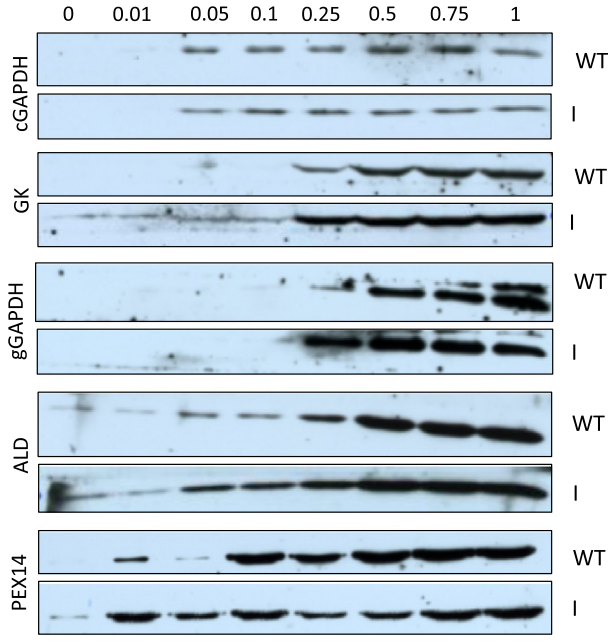

Fig. 4. Release of proteins from digitonin-treated trypanosomes. Bloodstream-form trypanosomes, wild-type (WT) cells and those of the PEX13.2 RNAi cell line induced (I) for RNAi by the presence of Tet, were treated with different concentrations of digitonin, indicated as mg digitonin added per mg of total cell protein. Measurements of induced cells were performed 24 h after addition of Tet. The appearance of glycosomal proteins in the supernatant, after centrifugation of the detergent treated trypanosomes, at lower digitonin concentration or to a larger extent in the induced clones than in the wild-type is an indication of mislocalisation of these proteins from the glycosome to the cytosol in the RNAi cell lines. The experiment was performed repeatedly. A representative result is shown in this figure. Quantification was done at least twice for each protein in independent experiments.

3.4. TbPEX13.2 interacts with other T. brucei PEX proteins

To verify the interaction of PEX13.2 and other peroxins of *T. brucei*, yeast two-hybrid assays were performed. Unfortunately, in most of the experiments an autoactivation by the N-terminal fragment of PEX13.2 expressed in the pGBT9 vector was observed. Moreover, there was often no cell growth when cells were transformed with the construct of the full-length PEX13.2 in the pGBT9 vector, preventing the reproducible use of this construct for protein–protein interaction tests in a X-gal assay. Results described here were observed, unless stated otherwise below, after co-transformations of constructs with the gene encoding the full-length or N-terminal PEX13.2 in the pGAD424 vector and the gene for a different peroxin in pGBT9, and where the negative controls yielded cells with a $lacZ^-$ phenotype in X-gal assays.

In four independent experiments PEX13.2 interacted with PEX13.1; one of these positive results was obtained in an experiment performed with just the SH3 domain of PEX13.1 and showed interactions between the peroxins in constructs made with both reciprocal combinations of the vectors. Twice an interaction of PEX13.2 with both PEX5 and PEX19 was observed. Interestingly, we observed only once a positive result for the interaction between the N-terminal domain of PEX13.2 and full-length PEX14, while several other attempts were negative. These latter results may be due to technical problems: for example these proteins, both expressed with the hydrophobic regions responsible for membrane association, are misfolded and cannot interact and/or one or both of them fail to reach the nucleus.

4. Conclusion

The data presented in this paper and previously [9] together show that trypanosomatids contain two functional PEX13 proteins. The reason for this is unclear, but it is interesting to note that most organisms only contain one: the only exceptions presently evident by database searching also containing two are the protist *Tetrahymena thermophila* and some plants, including wheat and rice but not *Arabidopsis*. Interestingly, these two very different *T. brucei* isoforms of PEX13 appear to interact with each other, as shown in the two-hybrid assays and are both important for the correct association of PEX14 with the glycosomal membrane. Moreover, they are not redundant; decreasing the cellular level of one of them, either PEX13.1 or PEX13.2, appears to affect glycosome biogenesis, as shown by relocation of glycosomal matrix enzymes to the cytosol, and is detrimental for growth of the trypanosomes.

Acknowledgements

PAMM gratefully acknowledges the 'Fonds de la Recherche Scientifique' and the Belgian Interuniversity Attraction Poles-Federal Office for Scientific, Technical and Cultural Affairs for financial support for the research performed. AB acknowledges a PhD scholarship from the 'Fonds pour la formation à la Recherche dans l'Industrie et dans l'Agriculture' (FRIA). We thank Eva Rico Vidal

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for assistance in some experiments and Dr Frank Voncken (University of Hull, UK) for the gift of the anti-PEX11 antiserum.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2012.05.019.

References

[1] Moreira, D., López-García, P. and Vickerman, K. (2004) An updated view of kinetoplastid phylogeny using environmental sequences and a closer outgroup: proposal for a new classification of the class Kinetoplastea. Int. J. Syst. Evol. Microbiol. 54, 1861–1875.
[2] Opperdoes, F.R. and Borst, P. (1977) Localization of nine glycolytic enzymes in a microbody-like organelle in *Trypanosoma brucei*: the glycosome. FEBS Lett. 80, 360–364.
[3] Michels, P.A.M., Bringaud, F., Herman, M. and Hannaert, V. (2006) Metabolic functions of glycosomes in trypanosomatids. Biochim. Biophys. Acta 1763, 1463–1477.
[4] Moyersoen, J., Choe, J., Fan, E., Hol, W.G.J. and Michels, P.A.M. (2004) Biogenesis of peroxisomes and glycosomes: trypanosomatid glycosome assembly is a promising new drug target. FEMS Microbiol. Rev. 28, 603–643.
[5] Galland, N. and Michels, P.A.M. (2010) Comparison of the peroxisomal matrix protein import system of different organisms. Exploration of possibilities for developing inhibitors of the import system of trypanosomatids for anti-parasite chemotherapy. Eur. J. Cell Biol. 89, 621–637.
[6] Van der Klei, I.J. and Veenhuis, M. (2006) PTS1-independent sorting of peroxisomal matrix proteins by Pex5p. Biochim. Biophys. Acta 1763, 1794–1800.
[7] Galland, N., de Walque, S., Voncken, F.G.J., Verlinde, C.L.M.J. and Michels, P.A.M. (2010) An internal sequence targets *Trypanosoma brucei* triosephosphate isomerase to glycosomes. Mol. Biochem. Parasitol. 171, 45–49.
[8] Meinecke, M., Cizmowski, C., Schliebs, W., Krüger, V., Beck, S., Wagner, R. and Erdmann, R. (2010) The peroxisomal importomer constitutes a large and highly dynamic pore. Nat. Cell Biol. 12, 273–277.
[9] Verplaetse, E., Rigden, D.J. and Michels, P.A.M. (2009) Identification, characterisation and essentiality of the unusual peroxin 13 from *Trypanosoma brucei*. Biochim. Biophys. Acta 1793, 516–527.
[10] Johnson, L.S., Eddy, S.R. and Portugaly, E. (2010) Hidden Markov model speed heuristic and iterative HMM search procedure. BMC Bioinformatics 11, 431.
[11] Altschul, S.F., Madden, T.L., Schaffer, A.A., Zhang, J., Zhang, Z., Miller, W. and Lipman, D.J. (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. 25, 3389–3402.
[12] The UniProt Consortium (2012) Reorganizing the protein space at the Universal Protein Resource (UniProt). Nucleic Acids Res. 40, D71–75.
[13] Gattiker, A., Gasteiger, E. and Bairoch, A. (2002) ScanProsite: a reference implementation of a PROSITE scanning tool. Appl. Bioinformatics 1, 107–108.
[14] Krogh, A., Larsson, B., von Heijne, G. and Sonnhammer, E.L. (2001) Predicting transmembrane protein topology with a hidden Markov model: application to complete genomes. J. Mol. Biol. 305, 567–580.
[15] Waterhouse, A.M., Procter, J.B., Martin, D.M., Clamp, M. and Barton, G.J. (2009) Jalview Version 2–a multiple sequence alignment editor and analysis workbench. Bioinformatics 25, 1189–1191.
[16] Wirtz, E., Leal, S., Ochatt, C. and Cross, G.A. (1999) A tightly regulated inducible expression system for conditional gene knock-outs and dominant-negative genetics in *Trypanosoma brucei*. Mol. Biochem. Parasitol. 99, 89–101.
[17] Biebinger, S., Wirtz, L.E., Lorenz, P. and Clayton, C. (1997) Vectors for inducible expression of toxic gene products in bloodstream and procyclic *Trypanosoma brucei*. Mol. Biochem. Parasitol. 85, 99–112.
[18] Brun, R. and Schönenberger, M. (1979) Cultivation and in vitro cloning or procyclic culture forms of *Trypanosoma brucei* in a semi-defined medium. Short communication. Acta Trop. 36, 289–292.
[19] Yernaux, C., Fransen, M., Brees, C., Lorenzen, S. and Michels, P.A.M. (2006) *Trypanosoma brucei* glycosomal ABC transporters: identification and membrane targeting. Mol. Membr. Biol. 23, 157–172.
[20] Schaffer, A.A., Aravind, L., Madden, T.L., Shavirin, S., Spouge, J.L., Wolf, Y.I., Koonin, E.V. and Altschul, S.F. (2001) Improving the accuracy of PSI-BLAST protein database searches with composition-based statistics and other refinements. Nucleic Acids Res. 29, 2994–3005.
[21] Altschul, S.F., Wootton, J.C., Gertz, E.M., Agarwala, R., Morgulis, A., Schaffer, A.A. and Yu, Y.K. (2005) Protein database searches using compositionally adjusted substitution matrices. FEBS J. 272, 5101–5109.
[22] Williams, C. and Distel, B. (2006) Pex13p: docking or cargo handling protein? Biochim. Biophys. Acta 1763, 1585–1591.
[23] Chen, Y., Hung, C.H., Burderer, T. and Lee, G.S. (2003) Development of RNA interference revertants in *Trypanosoma brucei* cell lines generated with a double stranded RNA expression construct driven by two opposing promoters. Mol. Biochem. Parasitol. 126, 275–279.
[24] Moyersoen, J., Choe, J., Kumar, A., Voncken, F.G.J., Hol, W.G.J. and Michels, P.A.M. (2003) Characterization of *Trypanosoma brucei* PEX14 and its role in the import of glycosomal matrix proteins. Eur. J. Biochem. 270, 2059–2067.
[25] Blattner, J., Helfert, S., Michels, P. and Clayton, C. (1998) Compartmentation of phosphoglycerate kinase in *Trypanosoma brucei* plays a critical role in parasite energy metabolism. Proc. Natl. Acad. Sci. USA 95, 11596–11600.
[26] Furuya, T., Kessler, P., Jardim, A., Schnaufer, A., Crudder, C. and Parsons, M. (2001) Glucose is toxic to glycosome-deficient trypanosomes. Proc. Natl. Acad. Sci. USA 99, 14177–14182.
[27] Haanstra, J.R., Van Tuijl, A., Kessler, P., Reijnders, W., Michels, P.A.M., Westerhoff, H.V., Parsons, M. and Bakker, B.M. (2008) Compartmentation prevents a lethal turbo-explosion of glycolysis in trypanosomes. Proc. Natl. Acad. Sci. USA 105, 17718–17723.
[28] Girzalsky, W., Rehling, P., Stein, K., Kipper, J., Blank, L., Kunau, W.H. and Erdmann, R. (1999) Involvement of Pex13p in Pex14p localization and peroxisomal targeting signal 2-dependent protein import into peroxisomes. J. Cell Biol. 144, 1151–1162.

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Supplementary data

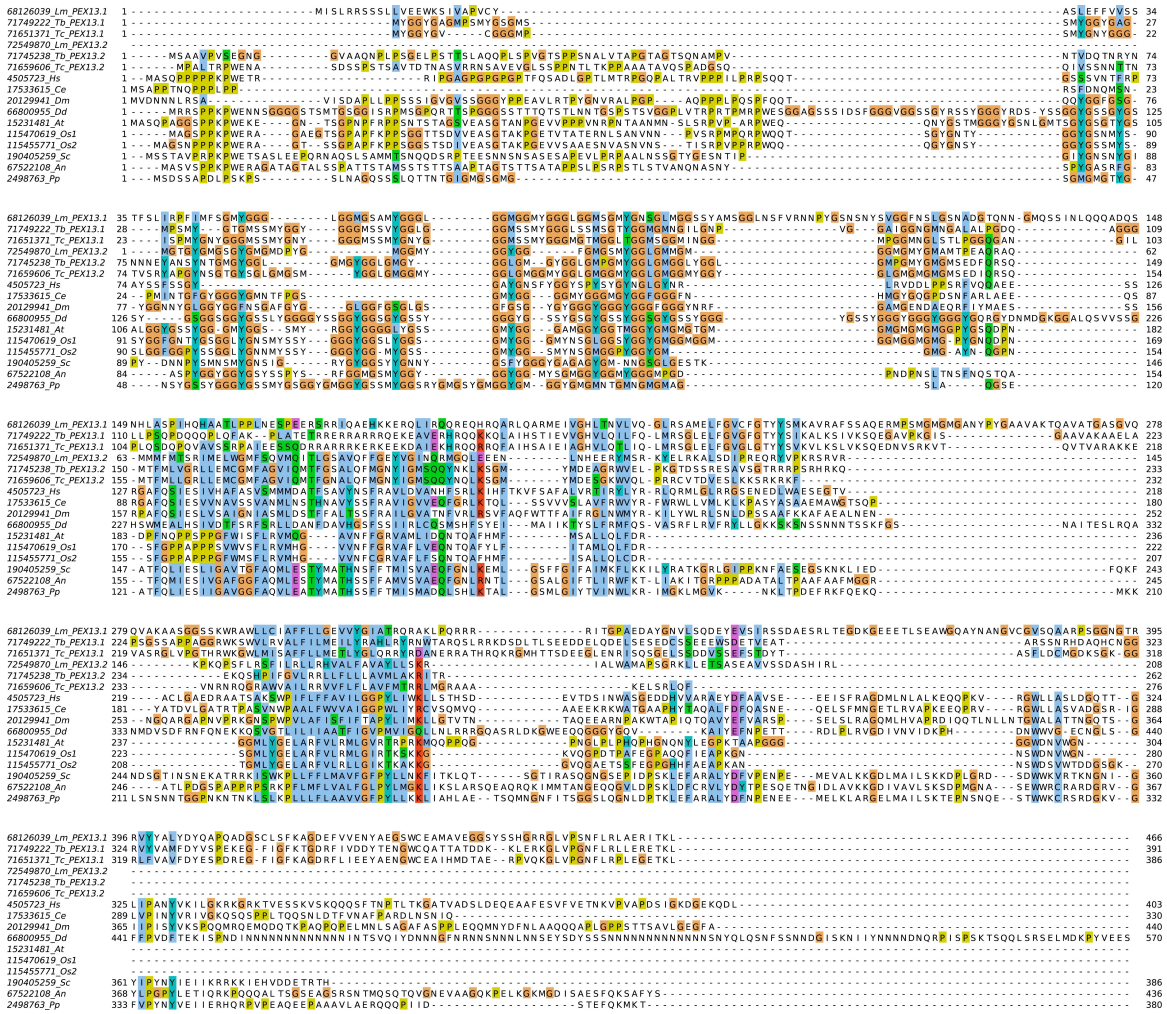

Fig. S1. Multiple sequence alignment of trypanosomatid PEX13.1 and PEX13.2 proteins with PEX13 sequences from other selected species. Each sequence is labelled with its gi code and a two letter abbreviation of the species name. Abbreviations are Lm, *L. major*; Tc, *T. cruzi*; Tb, *T. brucei*; Hs, *Homo sapiens*; Ce, *Caenorhabditis elegans*; Dm, *Drosophila melanogaster*; Dd, *Dictyostelium discoideum*; At, *Arabidopsis thaliana*; Os, *Oryza sativa*; Sc, *Saccharomyces cerevisiae*; An, *Aspergillus nidulans*; Pp, *Pichia pastoris*. Note that for *T. cruzi*, only one sequence from a similar pair is shown for each of PEX13.1 and PEX13.2. The uncharacterized pair of PEX13 homologues found in *Oryza sativa* are numbered 1 and 2. Note that inclusion in the alignment does not imply that all sequences are homologous throughout their entire lengths. In particular, the PEX13.1 sequences are not demonstrably homologous to the others with presently available database search software and sequence databases.

References

Alexander D.L., Schwartz K.J., Balber A.E., Bangs J.D. 2002. Developmentally regulated trafficking of the lysosomal membrane protein p67 in *Trypanosoma brucei*. J. Cell Sci. 115, 3253-3263.

Alvarez V.E., Kosec V., Sant'Anna C., Turk V., Cazzulo J.J., Turk B. 2008. Autophagy is involved in nutritional stress response and differentiation in *Trypanosoma cruzi*. J. Biol. Chem. 283, 3454-3464.

Aman, R.A., Kenyon, G.L., Wang, C.C. 1985. Cross-linking of the enzymes in the glycosome of *Trypanosoma brucei*. J. Biol. Chem. 260, 6966–6973.

Ano Y., Hattori T., Oku M., Mukaiyama H., Baba M., Ohsumi Y., Kato N., Sakai Y. 2005. A sorting nexin PpATG24 regulates vacuolar membrane dynamics during pexophagy via binding to phosphatidylinositol-3-phosphate. Mol. Biol. Cell 16, 446-457.

Bakker B.M., Michels P.A.M., Opperdoes F.R., Westerhoff H. 1999. What controls glycolysis in bloodstream form *Trypanosoma brucei*? J. Biol. Chem. 274, 14551-14559.

Barquilla A., Crespo J.L., Navarro M. 2008. Rapamycin inhibitis trypanosome cell growth by preventing TOR complex 2 formation. Proc. Natl. Acad. Sci. USA. 23, 14579-14584.

Bellu A.R., Salomons F.A., Kiel J.A.K.W., Veenhuis M., Van der Klei I. 2002. Removal of PEX3p is an important initial stage in selective peroxisome degradation in *Hansenula polymorpha*. J. Biol. Chem. 277, 42875-42880.

Besteiro S., Williams R.A.M., Morrison L.S., Coombs G.H., Mottram J.C. 2006. Endosome sorting and autophagy are essential for differentiation and virulence of *Leishmania major*. J. Cell Biol. 281, 11384-11396.

Biebinger S., Wirtz L.E., Lorenz P., Clayton C. 1997. Vectors for inducible expression of toxic gene products in bloodstream and procyclic *Trypanosoma brucei*. Mol. Biochem. Parasitol. 85, 99-112.

Blattner J., Clayton C. 1995. The 3'- untranslated regions from *Trypanosoma brucei* phosphoglycerate-encoding genes mediate developmental regulation. Gene 162, 153-156.

Bonifacino J.S., Rojas R. 2006. Retrograde transport from endosomes to the *trans*-Golgi network. Nat. Rev. Mol. Cell Biol. 7, 568-579.

Borst P., Sabatini R. 2008. Base J: discovery, biosynthesis, and possible functions. Ann. Rev. Microbiol. 62, 235-251.

Breidbach T., Ngazoa E., Sterveding V. 2002. *Trypanosoma brucei: in vitro* slender-to-stumpy differentiation of culture-adapted monomorphic bloodstream forms. Exp. Parasitol. 101, 223-230.

Brennand A., Gualdron-Lopez M., Coppens I., Rigden D.J., Ginger M.L., Michels P.A.M. 2011. Autophagy in parasitic protists: Unique features and drug targets. Mol. Biochem. Parasitol. 177, 83-99.

Brun R., Schönenberger M. 1979. Cultivation and *in vitro* cloning of procyclic culture forms of *Trypanosoma brucei* in a semi-defined medium. Short communication. Acta Trop. 36, 289-292.

Bryant D.M., Kerr M.C., Hammond L.A., Joseph S.R., Mostov K.E., Teasdale R.D., Stow J.L. 2007. EGF induces macropinocytosis and SNX1-modulated recycling of E-cadherin. J. Cell Sci. 120, 1818-1828.

Clayton C., Adams M., Almeida R., Baltz T., Barrett M., Bastien P., Belli S., Beverley S., Biteau N., Blackwell J., Blaineau C., Boshart M., Bringaud F., Cross G., Cruz A., Degrave W., Donelson J., El-Sayed N., Fu G., Ersfeld K., Gibson W., Gull K., Ivens A., Kelly J., Lawson D., Lebowitz J., Majiwa P., Matthews K., Melville S., Merlin G., Michels P., Myler P., Norrish A., Opperdoes F., Papadopoulou B., Parsons M., Seebeck T., Smith D., Stuart K., Turner M., Ullu E., Vanhamme L. 1998. Genetic nomenclature for *Trypanosoma* and *Leishmania*. Mol. Biochem. Parasitol. 97, 221-224.

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. Autophagy in protists. Autophagy 7, 1-32.

Ebikeme C., Peacock L., Coustou V., Riviere L., Bringaud F. 2008. N-acetyl-D-glucosamine stimulates growth of procyclic forms of *Trypanosoma brucei* by inducing a metabolic shift. Parasitology 135, 585-594.

Ellson C.D., Andrews S., Stephens L.R., Hawkins P.T. 2002. The PX domain: a new phosphoinositide-binding module. J. Cell Sci. 115, 1099-1105

Emmer B.T., Nakayasu E.S., Souther C., Choi H., Sobreira T.J.P., Epting C.L., Nesvizhskii A.I., Almeida I.C., Engman D.M. 2011. Global analysis of protein palmitoylation in African trypanosomes. Eukaryot. Cell 10, 455-463.

Engstler M., Boshart M. 2004. Cold shock and regulation of surface protein trafficking convey sensitization to inducers of stage differentiation in *Trypanosoma brucei*. Genes Dev. 18, 2798-2811.

Engstler M., Thllo L., Welse F., Grünfelder C.G., Schwarz H., Boshart M., Overath P. 2004. Kinetics of endocytosis and recycling of the GPI-anchored

variant surface glycoprotein in *Trypanosoma brucei*. J. Cell Sci. 117, 1105-1115.

Engstler M., Weise F., Bopp K., Grünfelder C.G., Günzel M., Heddergott N., Overath P. 2005. The membrane-bound histidine acid phosphatase TbMBAP is essential for endocytosis and membrane recycling in *Trypanosoma brucei*. J. Cell Sci. 118, 2105-2121.

Engstler M., Pfhol T., Herminghaus S., Boshart M., Wiegertjes G., Heddergott N., Overath P. 2007. Hydrodynamic flow-mediated protein sorting on the cell surface of trypanosomes. Cell 131, 505-515.

Erdmann R., Schliebs W. 2005. Peroxisomal matrix protein import: the transient pore model. Nat. Rev. Mol. Cell Biol. 6, 738-742.

Farré J.C., Manjithaya R., Mathewson R.D., Subramani S. 2008. PpAtg30 tags peroxisome for turnover by selective autophagy. Dev. Cell 14, 365-376.

Felsenstein J. 1985. Confidence limits on phylogenies: An approach using the bootstrap. Evolution 39, 783-791.

Fenn K., Matthews K.R. 2007. The cell biology of *Trypanosoma brucei* differentiation. Curr. Opin. Microbiol. 10, 539-546.

Field M.C., Carrington M. 2009. The trypanosome flagellar pocket. Nat. Rev. Microbiology 7, 775-786.

Fry M.R., Thomson J.M., Amasini A.J., Dunn Jr W.A. 2006. Early and late molecular events of glucose-induced pexophagy in *Pichia pastoris* require VAC8. Autophagy 2, 280-288.

Furuya T., Kessler P., Jardim A., Schnaufer A., Crudder C., Parsons M. 2001. Glucose is toxic to glycosome-deficient trypanosomes. Proc. Natl. Acad. Sci. USA. 99, 14177-14182.

Galland N., Michels P.A.M. 2010. Comparison of the peroxisomal matrix protein import system of different organisms. Exploitation of possibilities for developing inhibitors of the import system of trypanosomatids for anti-parasite chemotherapy. Eur. J. Cell Biol. 89, 621-637.

Grab D.J., Garcia-Garcia J.C., Nikolskaia O.V., Kim Y.V., Brown A., Pardo C.A., Zhang Y., Becker K.G., Wilson B.A., Lima A.P.C.A., Scharfstein J., Dumler J.S. 2009. Protease activated receptor signaling is required for African trypanosome traversal of human brain microvascular endothelial cells. PLoS Negl. Trop. Dis. 3 e479.

Griffin C.T., Trejo J., Magnuson T. 2005. Genetic evidence for a mammalian retromer complex containing sorting nexins 1 and 2. Proc. Natl. Acad. Sci. USA. 18, 15173-15177.

Gualdrón-López M., Brennand A., Hannaert V., Quiñones W., Cáceres A., Bringaud F., Concepción J.L., Michels P.A.M. 2012. When, how and why glycolysis became compartmentalised in the Kinetoplastea. A new look at an ancient organelle. Int. J. Parasitol. 42, 1-20.

Gullapalli A., Garrett T.A., Paing T.T., Griffin C.T., Yang Y., Trejo T. 2004. A role for sorting nexin 2 in epidermal growth factor downregulation: evidence for distinct functions of sorting nexins 1 and 2 in protein trafficking. Mol. Biol. Cell 15, 2143-2155.

Haanstra J.R., Van Tuijl A., Kessler P., Reijnders W., Michels P.A.M., Westerhoff H.V., Parsons M., Bakker B.M. 2008. Compartmentation prevents a lethal turbo-explosion of glycolysis in trypanosomes. Proc. Natl. Acad. Sci. USA. 105, 17718–17723.

Haile S., Papadopoulou B. 2007. Developmental regulation of gene expression in trypanosomatid parasitic protozoa. Curr. Opin. Microbiol. 10, 569-577.

Hall B.S., Gabernet-Castello C., Voak A., Goulding D., Natesan S.K., Field M.C. 2006. TbVPS34, the trypanosome orthologue of VPS34, is required for Golgi complex segregation. J. Biol. Chem. 281, 27600-27612.

Hart D.T., Misset O., Edwards S.W., Opperdoes F.R. 1984. A comparison of the glycosomes (microbodies) isolated from *Trypanosoma brucei* bloodstream form and cultured procyclic trypomastigotes. Mol. Biochem. Parasitol. 12, 25–35.

Herman M., Gillies S., Rigden D.J., Michels P.A.M. 2006. Autophagy and related processes in trypanosomatids. Insights from genomic and bioinformatic analyses. Autophagy 2,107-118.

Herman M., Pérez-Morga D., Schtickzelle N., Michels P.A.M. 2008. Turnover of glycosomes during life-cycle differentiation. Autophagy 4, 1-15.

Hertz-Fowler C., Figueiredo L.M., Quail M.A., Becker M., Jackson A., Bason N., Brooks K., Churcher C., Fahkro S., Goodhead I., Heath P., Kartvelishvili M., Mungall K., Harris D., Hauser H., Sanders M., Saunders D., Seeger K., Sharp S., Taylor J.E., Walker D., White B., Young R., Cross G.A.M., Rudenko G., Barry J.D., Louis E.J., Berriman M. 2008. Telomeric expression sites are highly conserved in *Trypanosoma brucei.* PLoS ONE 3, e3527.

Hettema E.H., Lewis M.J., Black M. W., Pelham H.L.B. 2003. Retromer and the sorting nexins SNX4p/41p/42p mediate distinct retrieval pathways from yeast endosomes. EMBO J. 22, 548-557.

Horazdovsky B.F., Davies B.A., Seaman M.N., McLaughlin S.A., Yoon S., Emr S.D. 2007. A sorting nexin-1 homologue, Vps5p, forms a complex with

Vps17p and is required for recycling the vacuolar protein-sorting receptor. Mol. Biol. Cell 8, 1529-1541.

Itakura E., Kishi C., Inoue K., Mizushima N. 2008. Beclin 1 forms two distinct phosphatidylinositol 3-kinase complexes with mammalian ATG14 and UVRAG. Mol. Biol. Cell 19, 5360-5372.

Jensen B.C., Sivam D., Kifer C.T., Myler P.J., Parsons M. 2009. Widespread variation in transcript abundance within and across developmental stages of *Trypanosoma brucei*. BMC Genomics 10: 482. Doi: 10.1186/1471-2164/10/482.

Kanki T., Klionsky D. 2008. Mitophagy in yeast occurs through a selective mechanism. J. Biol. Chem. 283, 32386-32393.

Kaushik S., Cuervo A.M. 2008. Chaperone-mediated autophagy. Methods Mol. Biol. 445, 227-244.

Kennedy P.G.E. 2008. The continuing problem of human African trypanosomiasis (Sleeping sickness). Ann. Neurol. 64, 116-127.

Kieft R., Capewell P., Turner C.M.R., Veitch N.J., MacLeod A., Hajduk S. 2010. Mechanisms of *Trypanosoma brucei gambiense* (group 1) resistance to human trypanosome lytic factor. Proc. Natl. Acad. Sci. USA. 107, 16137-16414.

King J.S. 2012. Autophagy across the eukaryotes: Is *S. cerevisiae* the odd one out? Autophagy 8, in press.

Klionsky D. 2005. The molecular machinery of autophagy : unanswered questions. J. Cell Sci. 118, 7-18.

Koumandou V.L., Klute M.J., Herman E.K., Nunez-Miguel R., Dacks J.B., Field M.C. 2011. Evolutionary reconstruction of the retromer complex and its function in *Trypanosoma brucei*. J. Cell Sci. 124, 1496-1509.

Krick R., Muehe Y., Prick T., Bremer S., Schlotterhose P., Eskelinen E.L., Mille, J., Goldfarb D.S., Thumm M. 2008. Piecemeal microautophagy of the nucleus requires the cora macroautophagy genes. Mol. Biol. Cell 19, 4492-4505.

Laxman S., Riechers A., Sadilek M., Schwede F., Beavo J.A. 2006. Hydrolysis products of cAMP analogs cause transformation of *Trypanosoma brucei* from slender to stumpy-like forms. Proc. Natl. Acad. Sci. USA. 103, 19194-19199.

Lemmon M.A. 2008. Membrane recognition by phospholipid-binding domains. Nat. Rev. Mol. Cell Biol. 9, 99-111.

Leprince C., Le Scolan E., Meunier B., Fraisier V., Brandon N., De Gunzburg J., Camonis J. 2003. Sorting nexin 4 and amphiphysin 2, a new partnership between endocytosis and intracellular trafficking. J. Cell Sci. 116, 1937-2948.

Leung K.F., Riley F.S., Carrington M., Field M.C. 2011. Ubiquitylation and developmental regulation of invariant surface protein expression in trypanosomes. Eukaryot. Cell 10, 916–931.

Li F.J., Shen Q., Wang C., Sun Y., Yuan A.Y., He C.Y. 2012. A role of autophagy in *Trypanosoma brucei* cell death. Cell. Microbiol., in press. Doi: 10.1111/j.1462-5822.2012.01795.x

McCulloch R., Vassella E., Burton P., Boshart M., Barry J.D. 2004. Transformation of monomorphic and pleomorphic *Trypanosoma brucei.* Meth. Mol. Biol. 262, 53-86.

Meijer W.H., Van der Klei I., Veenhuis M., Kiel J.A.K.W. 2007. ATG genes involved in non-selective autophagy are conserved from yeast to man, but the selective Cvt and pexophagy pathways also require organism-specific genes. Autophagy 3, 106-116.

Meinecke M., Cizmowski C., Schliebs W., Krüger V., Beck S., Wagner R., Erdmann R. 2010. The Peroxisomal importomer constitutes a large and highly dynamic pore. Nat. Cell Biol. 12, 273-277.

Mendl N., Occhipinti A., Müller M., Wild P., Dikic I., Reichert A.S. 2010. Mitophagy in yeast is independent of mitochondrial fission and requires the stress response gene WHI2. J. Cell Sci. 124, 1339-1350.

Moreira D., Lopez-Garcia P., Vickerman K. 2004. An updated view of kinetoplastid phylogeny using environmental sequence and a closer outgroup: proposal for a new classification of the class Kinetoplastea. Int. J. Syst. Evo. Microbiol. 54, 1861-1875.

Morgan G.W., Hall B.S., Denny P.W., Carrington M., Field M.C. 2002. The kinetoplastida endocytic apparatus. Part I: a dynamic system for nutrition and evasion of host defense. Trends Parasitol. 18, 491-496.

Motley A., Hettema E.W. 2007. Yeast peroxisomes multiply by growth and division. J. Biol. Chem. 178, 399-410.

Motley, A.M., Nuttall J.M., Hettema E.H. 2012. Pex3-achored Atg36 tags peroxisomes for degradation in *Saccharomyces cerevisiae*. EMBO J. , in press. Doi: 10.1038/emboj.2012.151

Moyersoen J., Choe J., Fan E., Hol W.G.J., Michels P.A.M. 2004. Biogenesis of peroxisomes and glycosomes: trypanosomatid glycosome assembly is a promising new drug target. FEMS Microbiol. Rev. 28, 603-643.

Mukaiyama H. Oku M., Baba M., Samizo T., Hammond A.T., Glick B.S. Kato N., Sakai Y. 2002. Paz2 and 13 other *PAZ* gene products regulate vacuolar engulfment of peroxisomes during micropexophagy. Genes Cells. 7, 75-90.

Mutomba M.C., Wang C.C. 1998. The role of proteolysis during differentiation of *Trypanosoma brucei* from the bloodstream to the procyclic form. Mol. Biochem. Parasitol. 93, 11-22.

Navarro M., Peñate X, Landeira D. 2007. Nuclear architecture underlying gene expression in *Trypanosoma brucei*. Trends Microbiol. 15, 263-270.

Nei M., Kumar S. 2000. Molecular evolution and phylogenetics. Oxford University Press, New York.

Nice D., Sato T., Stromhaug P., Emr S., Klionsky D. 2002. Cooperative binding of the cytoplasm to vacuole targeting pathway proteins CVT13 and CVT20 to phosphatidylinositol-3-phosphate at the pre-autophagosomal structure is required for selective autophagy. J. Biol. Chem. 277, 30198-30207.

Nothwehr S.F., Hindes A.E. 1997. The yeast VPS5/GRD2 gene encodes a sorting nexin-1-like protein required for localizing membrane proteins to the late Golgi. J. Cell Sci. 110, 1063-1072.

Overath P., Czichos J., Haas C. 1986. The effect of citrate/*cis*-aconitate on oxidative metabolism during transformation of *Trypanosoma brucei*. Eur. J. Biochem. 160, 175-182.

Pays E., Vanhollenbeke B. 2008. Mutual self-defense: the trypanolytic factor story. Microbes Infect. 10, 985-989.

Reuner B., Vassella E., Yutzi B., Boshart M. 1997. Cell density triggers slender to stumpy differentiation of *Trypanosoma brucei* in culture. Mol. Biochem. Parasitol. 1, 268-290.

Rigden D.J., Michels P.A.M., Ginger M.L. 2009. Autophagy in protistis. Examples of secondary loss, lineage-specific innovations and the conundrum of remodelling a single mitochondrion. Autophagy 5, 784-794.

Rzhetsky A., Nei M. 1992. A simple method for estimating and testing minimum evolution trees. Mol. Biol. Evol. 9, 945-967.

Saitou N., Nei M. 1987. The neighbor-joining method: A new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4, 406-425.

Sambrook J., Russell D.W. 2001. Molecular cloning. A laboratory manual. Cold Spring Harbor Laboratory Press. Cold Spring Harbor, N.Y. 3rd ed. Vol 1-3.

Santos C.C., Sant'Anna C., Terres A., Cunha e Silva N.L., Scharfstein J., Lima A.P.C.A. 2005. Chagasin, the endogenous cystein-protease inhibitor of

Trypanosoma cruzi modulates parasite differentiation and invasion of mammalian cells. J. Cell Sci. 118, 901-915.

Seaman M.N.J. 2004. Cargo-selective endosomal sorting for retrieval to the Golgi requires retromer. J. Cell Biol. 165, 111-122.

Skånland S.S., Wälchli S., Utskarpen A., Wandinger-Ness A., Sandvig K. 2007. Phosphoinositide-regulated retrograde transport of ricin: crosstalk between hVPS34 and sorting nexins. Traffic 8, 297-309.

Skånland S.S., Wälchli S., Brech A., Sandvig K. 2009. SNX4 in complex with clathrin and dynein: implications for endosome movement. PLoS ONE 4:6 e5935.

Subramanian K., Dietrich L.E.P., Hou H., LaGrassa T.J., Meiringer C.T.A., Ungermann C. 2006. Palmitoylation determines the function of yeast VAC8 at the yeast vacuole. J. Cell Sci. 119, 2477-2485.

Tamura K., Peterson D., Peterson N., Stecher G., Nei M., Kumar S. 2011. MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol. Biol. Evol. 28, 2731-2739.

Till A., Lakhani R., Burnett S.F., Subramani S. 2012. Pexophagy: the selective degradation of peroxisomes. Int. J. Cell Biol., in press. Doi: 10.1155/2012/512721.

Traer C.J., Rutherford A.C., Palmer K.J., Wassmer T., Oakley J., Attar N., Carlton J.G., Kremerskothen J., Stephens D.J., Cullen P.J. 2007. SNX4 coordinates endosomal sorting of TfnR with dynein-mediated transport into the endocytic recycling compartment. Nat. Cell Biol. 9, 1370-1380.

Van den Abbeele J., Claes Y., van Bockstaele D., Le Ray D., Coosemans M. 1999. *Trypanosoma brucei* spp. Development in the tsetse fly: characterization of the post-mesocyclic stages in the foregut and proboscis. Parasitology 118, 469-478.

Van den Hoff M.J., Moorman A.F., Lamers W.H. 1992. Electroporation in 'intracellular' buffer increases cell survival. Nucleic Acids Res. 20, 2902.

Van Zutphen T., Veenhuis M., Van der Klei I.J. 2008. PEX14 is the sole component of the peroxisomal translocon that is required for pexophagy. Autophagy 4, 63-66.

Vanhaesenbroek B., Stephen L., Hawkins P. 2012. PIK3 signalling: the path to discovery and understanding. Nat. Rev. Mol. Cell Biol. 13, 195-203.

Vassella E., Van den Abbeele J., Bütikofer P. 2000. A major surface glycoprotein of *Trypanosoma brucei* is expressed transiently during

development and can be regulated post-transcriptionally by glycerol or hypoxia. Genes Dev. 14,615-626.

Walrad P.B., Capewell P., Fenn K., Matthews K. 2012. The post-transcriptional trans-acting regulator TbZFP3, co-ordinates transmission-stages enriched mRNAs in *Trypanosoma brucei.* Nucleic Acids Res. 40, 2869-2883.

Wassmer T., Attar N., Bujny M.B., Oakley J., Traer C.J., Cullen P.J. 2007. A loss-of-function screen reveals SNX5 and SNX6 as potential components of the mammalian retromer. J.Cell Sci. 120, 45-54.

Williams R.A.M., Tetley L., Mottram J.C., Coombs J.H. 2006. Cysteine peptidases PBA and CPB are vital for autophagy and differentiation in *Leishmania mexicana*. Mol. Microbiol. 61, 655-674.

Williams R.A.M., Woods K.L., Juliano L., Mottram J.C., Coombs J.H. 2009. Characterization of unusual families of ATG8-like proteins and ATG12 in the protozoan parasite *Leishmania major*. Autophagy 5, 159-172.

Zhong Q., Lazar C.S., Tronchère H., Sato T., Meerloo T., Yeo M., Songyang Z., Emr S.D., Gill G.N. 2002, Endosomal localisation and function of sorting nexin 1. Proc. Natl. Acad. Sci. USA. 99, 6767-6772.

List of full publications

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).
Autophagy in protists.
Autophagy. 7, 127-158 (solicited review).

Brennand, A.,* Gualdrón-López, M.,* Coppens, I., Rigden, D.J., Ginger, M.L., Michels, P.A.M. (2011).
Autophagy in parasitic protists: Unique features and drug targets. (solicited review)
Molecular and Biochemical Parasitology. 177, 83-99.
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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. (solicited review).
International Journal for Parasitology. 42, 1–20

Brennand, A., Rigden, D.J., Michels, P.A.M. (2012).
Trypanosomes contain two highly different isoforms of peroxin PEX13 involved in glycosome biogenesis.
FEBS Letters. 586, 1765-1771.

Gualdrón-López, M., Brennand, A., Avilán, L., Michels, P.A.M. (2012).
Translocation of solutes and proteins across the glycosomal membrane of trypanosomes; possibilities and limitations for targeting with trypanocidal drugs.
Parasitology. In press (solicited review).

Brennand, A.,* Rico Vidal, E.,* Michels, P.A.M. (2012).
Autophagy in trypanosomatids (Review)
Cells. Submitted.
(*joint first authors).

Annex

Autophagy in protists.

As annex is here presented a review paper.
I contributed to the preparation of this paper by writing parts of several sections, notably those concerning autophagy in trypanosomatids, by making comments on the entire manuscript at several stages of its preparation and by preparing some figures.

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).

Autophagy. 7, 127-158 (solicited review).

Summary:

In this section we discuss the occurrence of autophagy and the evolution of the pathway in different protist lineages, as inferred by genome-wide searches using yeast and mammalian ATGs as probes. The possible roles of autophagy in these organisms is discussed, by addressing the function of the process in both free-living and parasitic protists; how parasites use autophagy in their interactions with the hosts; how the host uses its autophagy to combat the parasites; and how the parasites utilize or manipulate the host autophagy machinery for their invasion. Moreover, the possibility to exploit autophagy for anti-parasite drug discovery is discussed.

Autophagy 7:2, 127-158; February 2011; © 2011 Landes Bioscience

Autophagy in protists

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Key words: autophagy, ubiquitination, pexophagy, evolution, free-living protist, parasitic protist, life-cycle differentiation, Trypanosomatidae, Apicomplexa, drug discovery

Abbreviations: ATG, autophagy-related; Cvt, cytoplasm-to-vacuole targeting; EC_{50}, effective concentration to cause 50% inhibition of growth; ER, endoplasmic reticulum; FR, flexible region; GFP, green fluorescent protein; HR, handle region; IFNγ, gamma interferon; IMC, inner membrane complex; LECA, last eukaryotic common ancestor; 3-MA, 3-methyladenine; MIPA, micropexophagic membrane apparatus; MVB, multivesicular body; MVT, multivesicular tube; PAS, phagophore assembly site; PCD, programmed cell death; PE, phosphatidylethanolamine; PGE2, prostaglandin E2; PtdIns3K, phosphatidylinositol 3-kinase; PtdIns(3)P, phosphatidylinositol 3-phosphate; PMN, piecemeal microautophagy of the nucleus; prApe1, precursor aminopeptidase; PV, parasitophorous vacuole; ROS, reactive oxygen species; TOR, target of rapamycin; TRAP, thrombospondin-related anonymous protein

Autophagy is the degradative process by which eukaryotic cells digest their own components using acid hydrolases within the lysosome. Originally thought to function almost exclusively in providing starving cells with nutrients taken from their own cellular constituents, autophagy is in fact involved in numerous cellular events including differentiation, turnover of macromolecules and organelles and defense against parasitic invaders. During the past 10–20 years, molecular components of the autophagic machinery have been discovered, revealing a complex interactome of proteins and lipids, which, in a concerted way, induce membrane formation to engulf cellular material and target it for lysosomal degradation. Here, our emphasis is autophagy in protists. We discuss experimental and genomic data indicating that the canonical autophagy machinery characterized in animals and fungi appeared prior to the radiation of major eukaryotic lineages. Moreover, we describe how comparative bioinformatics revealed that this canonical machinery has been subject to moderation, outright loss or elaboration on multiple occasions in protist lineages, most probably as a consequence of diverse lifestyle adaptations. We also review experimental studies illustrating how several pathogenic protists either utilize autophagy mechanisms or manipulate host-cell autophagy in order to establish or maintain infection within a host. The essentiality of autophagy for the pathogenicity of many parasites, and the unique features of some of the autophagy-related proteins involved, suggest possible new targets for drug discovery. Further studies of the molecular details of autophagy in protists will undoubtedly enhance our understanding of the diversity and complexity of this cellular phenomenon and the opportunities it offers as a drug target.

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Submitted: 06/08/10; Revised: 07/06/10; Accepted: 08/02/10
Previously published online:
www.landesbioscience.com/journals/autophagy/article/13310
DOI: 10.4161/auto.7.2.13310

Introduction

In the 1950s, Christian de Duve discovered lysosomes in mammalian cells as acidic organelles containing a large number of hydrolases responsible for intracellular degradation of proteins and other macromolecules, either taken up from outside the cell by endocytosis or hydrolysis of the cell's own constituents (reviewed in ref. 1). In 1963, de Duve coined the word autophagy for the process by which the cell's own proteins are degraded.[2,3] Subsequent research has identified lysosomes in many other eukaryotic organisms or similar organelles called vacuoles in yeasts, and has revealed various pathways by which cytosolic components as well as organelles are delivered to the lysosomes. Although autophagy was initially only detected by

electron microscopy, major advances in the last decade have enabled a detailed understanding of the process at the molecular level.[4] Many of the molecular components involved in autophagy have been identified in the budding yeast *Saccharomyces cerevisiae*, owing to its tractable genetics. Currently *ATG* (standing for AuTophaGy-related) genes encoding more than 30 proteins involved in these processes have been identified and characterized.[5] For many of them, orthologues have been identified in mammals, plants, other yeasts and fungi.[6] Moreover, detailed cellular and molecular biological studies have provided information about the steps of various autophagy pathways at which the different Atg proteins act, thereby revealing a defined molecular mechanism.[7]

Autophagy plays different roles in a cell (reviewed in ref. 8). First, it serves as a survival mechanism under conditions of "extracellular stress" such as nutrient starvation, hypoxia and high temperatures or as a housekeeping device under conditions of "intracellular stress" by removing damaged, redundant or otherwise unwanted cytoplasmic components which may include organelles. In yeasts, autophagy allows survival during nutrient deprivation by recycling the cytoplasmic constituents, as well as playing a major role in cellular remodeling. In particular, autophagy is important in adapting the cell's morphology and metabolic repertoire to changes of nutritional conditions. In addition, in mammals, autophagy plays a crucial role in health and disease.[9] It is not only involved in the removal of damaged or malfunctioning cell components, but it is important, too, for cell differentiation, tissue remodeling and defense against pathogenic organisms. As a result, links exist between, for example, autophagy and growth or the development of innate immunity. Autophagy has also been implicated in both promoting and preventing diseases such as cancer, neurodegenerative disorders, lysosomal storage diseases, viral, bacterial or parasitic infections and aging. Moreover, autophagy is one of three main cell death mechanisms: necrosis, apoptosis and cell death with autophagy.[10]

Different autophagic processes have been described (reviewed in ref. 6 and 11). The most common form is macroautophagy, a form of nonselective autophagy in which a bowl-shaped membrane is formed randomly around portions of the cytoplasm, bulk cytosol and other cytoplasmic components such as organelles, creating a structure called the phagophore or isolation membrane that develops into a double-membrane vesicle, the autophagosome. In yeast, a structure is observed called the phagophore-assembly site (PAS, also known as the preautophagosomal structure), which is important for different stages of the autophagy process including autophagosome formation. The PAS concentrates a large number of autophagy components, some Atg proteins and developing vesicles, probably to organize vesicle formation in a concerted manner. In yeast only a single, perivacuolar PAS is found, whereas mammalian cells contain multiple loci of Atg complexes. Once the autophagosome has been formed, its outer membrane fuses with the lysosomal/vacuolar membrane, forming the autophagic body or autolysosome. Due to the action of lysosomal hydrolases, the inner membrane of the autolysosome is ruptured before its content is degraded. The degradation products are recycled to the cytosol through the action of permeases within the lysosomal membrane. In addition, random uptake of cytoplasmic components may also occur by microautophagy, in which the uptake of a portion of the cytoplasm occurs by direct engulfment by the lysosomal membrane. The different mechanisms of macro- and microautophagy have distinct effects on the size of the lysosomal membrane. In the former process, its size increases as a result of fusion with the incoming autophagosomal membrane. In contrast, in microautophagy part of the lysosomal membrane is used to engulf cytoplasmic material resulting in a vesicle bounded by a single membrane of lysosomal origin. This vesicle, including both its membrane and its contents, is degraded. As a result, the size of the lysosomal membrane is decreased. Since newly digested material will be exported from the lysosome, until and unless new lysosomal membrane is synthesized, microautophagy may lead to a reduction in lysosomal volume and may play a homeostatic role in balancing membrane that is delivered to the lysosome via autophagy and other pathways that terminate at this organelle. A third form, a chaperone-mediated autophagy has been described.[12] Defined proteins, usually containing the KFERQ or a related pentapetide motif, bind to a cytosolic chaperone that targets the complex to bind to a lysosomal membrane receptor, where—with the help of additional chaperones—the respective protein is taken up and degraded.

Instead of directly fusing with the lysosome, the autophagosome may also be indirectly routed to the lysosome by first fusing with the endosomes resulting from endocytosis. The organelle formed by this process, designated the amphisome, will subsequently fuse with the lysosome.

Processes related to macroautophagy are also used for the selective routing of cytoplasmic macromolecules or organelles to the lysosome/vacuole. The selectivity of the system is in all cases endowed by the specific recognition of material to be degraded. In a few cases, proteins and cofactors responsible for this recognition have been identified, and some of these molecules undergo post-translational modification in order to provide the signal for degradation. However, the molecular basis of the recognition events remains rather elusive. The best-studied selective process is the cytoplasm-to-vacuole targeting (Cvt) pathway, which has only been identified in the yeasts *S. cerevisiae* and *Pichia pastoris*. This process does not serve to route proteins for degradation, but to deliver the resident vacuolar lumenal hydrolytic enzymes aminopeptidase I and α-mannosidase to their destination. These enzymes, after their synthesis in the cytosol, become incorporated into a double-membrane Cvt vesicle that fuses with the vacuole. The inactive precursor dodecameric aminopeptidase (prApe1) assembles into a large complex in the cytosol and is recruited by a receptor protein, Atg19 (originally called Cvt19). Atg19 also binds oligomeric α-mannosidase. The Atg19-prApe1-α-mannosidase complex is selectively targeted to the Cvt vesicle through the action of Atg11 and the subsequent interaction between Atg19 and Atg8. After delivery of the vesicle to the vacuole and the disruption of the inner membrane of the internalized Cvt vesicle, the complex is released into the vacuolar lumen, prApe1 processed into an active enzyme and Atg19 degraded. Other selective autophagy-like processes involve the degradation of redundant or damaged organelles. Among these processes

are (i) pexophagy, the selective degradation of peroxisomes, (ii) mitophagy, the selective degradation of (part) of mitochondria, (iii) ribophagy, the selective degradation of ribosomes and (iv) ER-phagy or reticulophagy, the degradation of part of the endoplasmic reticulum (ER). Moreover, part of the nucleus may be pinched off and degraded by microautophagy in a process called piecemeal microautophagy of the nucleus (PMN) or micronucleophagy. Selective pexophagy and mitophagy may also occur by either micro- or macroautophagic mechanisms.

As mentioned above, over 30 genes have been identified encoding proteins specifically involved in autophagy. About half of them participate in the core machinery, essential for formation of autophagosomes or Cvt vesicles, respectively, whereas the others complement this machinery according to the requirements of the specific process executed. Although autophagy occurs constitutively at a basal level, it is dramatically enhanced in response to stimuli from within the cell or from the environment. Such stimuli include starvation or other forms of stress, physiological signals such as hormones and growth factors and viral, bacterial or parasitic infection. Induction of autophagy involves common signaling cascades also involved in other cellular processes. For example, the serine/threonine protein kinase TOR (target of rapamycin) is crucial in the regulation of autophagy induction. The TOR protein occurs in two complexes, TORC1 and TORC2, each in control of a distinct signaling pathway. The rapamycin-sensitive TORC1 is involved in regulation of autophagy by responding to various sensors. On the one hand, it responds to the availability of nutrients, notably amino acids, by stimulating temporal cell growth. This is achieved, via a variety of downstream effectors, by orchestrating ribosome biosynthesis, amino acid uptake and translation initiation. On the other hand, nutrient or energy depletion causes TORC1 to maintain cellular viability via other effectors. This latter response includes use of the Atg proteins as effectors to activate autophagy and so increase the recycling of cellular components. Additionally some other enzymes are shared between autophagy pathways and other signaling cascades, such as the phosphatidylinositol 3-kinase (PtdIns3K) domain-containing Vps34, Vps15 and Vps30 (also termed Atg6 or Beclin 1 in mammals). These kinases are located at the PAS and are involved in autophagosome formation. A late step in the autophagy process involves fusion of autophagosomes or Cvt vesicles with the lysosome/vacuole. Other proteins involved in this fusion process are Vac8, SNAP and SNARE, the latter two of which are generally involved in membrane fusion.[13]

Autophagy processes are not restricted to mammalian cells and yeasts, but have been found in representatives of all eukaryotic phyla: vertebrates (mammals, birds, amphibians, fish), invertebrates such as the nematode *Caenorhabditis elegans* and the cnidarian polyp Hydra, insects (Drosophila, Lepidoptera and ticks), plants, fungi (different yeasts and filamentous fungi) and several protists.[6,14,15] This identification has been done experimentally, often by morphological studies of cells subjected to starvation or other forms of stress or by homology searches in sequence databases with yeast or human Atg sequences. Previously, we have presented genomic and experimental evidence for autophagy and pexophagy in parasitic protists belonging to the family Trypanosomatidae and genomic evidence that autophagy also occurs in a diverse variety of other protists, including some free-living taxa.[14,16-23] For some of the species examined so far, genomic signatures are complemented by experimental demonstrations of autophagy and/or analyses of candidate protein function. Significantly, however, bioinformatics surveys of protist genomes reveal some taxa in which autophagy, at least as defined by current paradigms, does not occur and others in which the canonical autophagy pathway has been subject to lineage-specific elaboration or moderation. Thus, even though only a limited spectrum of the many unicellular eukaryotes belonging to the 30–40 disparate phyla grouped in the Kingdom Protista can currently be readily subjected to genome or direct experimental analysis, one can anticipate considerable variation from the pathways described in yeast and animals during the past 10–20 years.

Human and animal pathogenic protists have received much more attention in biological research than either free-living protists or the parasites of plants. This is particularly true for parasites causing devastating human and animal diseases in tropical and subtropical parts of the world and for which no adequate treatment is available, such as the protists belonging to the family Trypanosomatidae that cause African sleeping sickness, Latin-American Chagas' disease and various manifestations of leishmaniasis and the order Apicomplexa responsible for malaria and theileriosis, but also for diseases occurring in parts of the world with a moderate climate such as toxoplasmosis. Some of these parasites (*Trypanosoma brucei*, responsible for sleeping sickness) live extracellularly in the blood of their human host. Others (like the Chagas' disease parasite *Trypanosoma cruzi*, Leishmania species and the apicomplexans), spend a major part of their pathogenic life inside host mammalian cells. The host's autophagy may play a major role in the infection process of some of these latter parasites. The process may either be used to eliminate live or dead pathogens or it may be exploited by the parasite to favor invasion. On the other hand, intracellular parasites may block host cell autophagy as a survival strategy, for example by reducing parasite-derived peptide presentation by host cell MHC to $CD4^+$ and $CD8^+$ T cells.

In this review, we will summarize the current knowledge about autophagy in protists, and present some new data. We will provide a rationalization for the differences found between protists and other phylogenetic groups and between different protist lineages with regard to the occurrence or variations in the process, the different pathways and the repertoire of ATGs. This will be done on the basis of evolutionary developments and habitats in which the different organisms live. We will also describe the details and relevance of the host autophagy process for invasion, survival or removal of pathogenic protists.

Molecular Analysis of Autophagy in Protists—Some Practical Considerations

Yeast as the model organism for autophagy studies. As mentioned above, autophagy is a process that has been found in representatives of all major eukaryotic phyla, including protists. Indications of autophagy in some unicellular eukaryotes were

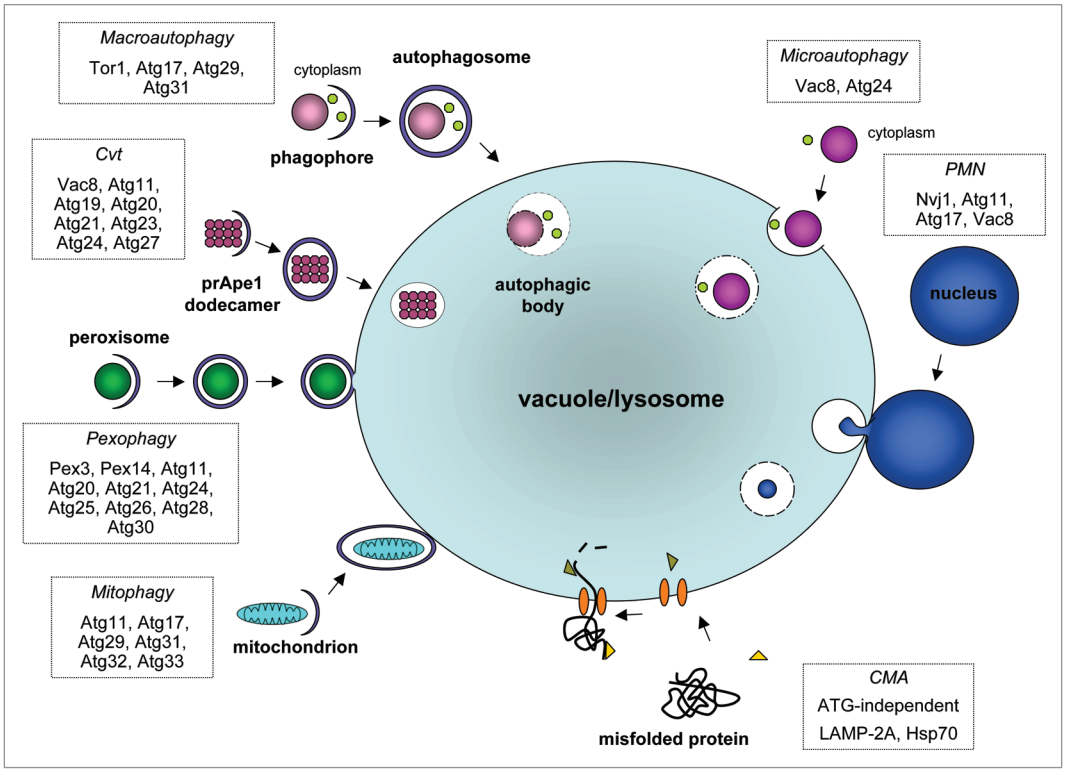

Figure 1. Schematic overview of autophagy-related processes. The main steps of the various pathways, as summarized in the main text of the paper, are indicated. Proteins shown to be specifically required for the different pathways are boxed.

initially obtained by electron microscopy. However, in the last decade most information about the distribution of autophagy in the highly diverse kingdom of protists has come from bioinformatic analyses based on homology searches in sequence databases using mammalian and particularly yeast Atg sequences as queries. Subsequently, the occurrence of the process in some of the better-known, often pathogenic protists has been experimentally confirmed. Since autophagy has been most intensively studied in *S. cerevisiae*, and this organism thus serves as a model to which other organisms are compared, we will first summarize the respective steps in yeast (reviewed in ref. 1, 24 and 25).

Macroautophagy, and the very similar Cvt pathway can conceptually be divided into a number of consecutive steps mediated by specific proteins (**Fig. 1**): (1) nutrient sensing and induction of autophagy; (2) cargo selection and packaging; (3) vesicle nucleation (formation of the phagophore); (4) vesicle expansion and completion (autophagosome or Cvt vesicle formation); (5) retrieval; (6) docking and fusion; (7) vesicle breakdown in the vacuole.

(1) Nutrient sensing and induction of autophagy. Under nutrient-rich conditions, when macroautophagy occurs at a basal level, the protein kinase TOR of the TORC1 complex maintains a hyperphosphorylated state of Atg13 that, together with Atg1, forms the core of the so-called Atg1 complex. When nutrients become limited, Atg13 is partially dephosphorylated causing an increased affinity for Atg1. The less phosphorylated Atg1-Atg13 complex induces, through the Ser/Thr kinase activity of Atg1, an increase of macroautophagy. This kinase activity appears to be also essential for the constitutive Cvt pathway. However, despite the similarity between the macroautophagy and Cvt pathways, and the fact that most components are shared between them, there are differences, too. Some Atgs have been shown to be essential only for the former pathway, whereas others only for the latter one. Atg1 interacts directly or indirectly via Atg13 or Atg11 with Atg20, Atg24 and Vac8 for the Cvt pathway, and with Atg17, Atg29 and Atg31 for macroautophagy. Atg11 is also essential for pexophagy, mitophagy and PMN, whereas Atg24 is additionally required for pexophagy in methylotrophic yeasts. These differences indicate a role of the Atg1 complex as a switch between nonselective autophagy and selective processes such as Cvt and pexophagy. A detailed discussion of the current knowledge of the elaborate regulation of autophagy by TOR and signaling pathways in *S. cerevisiae*, the nature of all above-mentioned Atgs involved in the induction step, and the temporal and spatial aspects of their interactions, can be found in reviews by Diaz et al.[26] and Jung et al.[27]

(2) Cargo selection and packaging. In contrast to nonselective macroautophagy, where bulk cytoplasm components are randomly sequestered, a defined cargo selection occurs in the cases of Cvt and selective autophagy processes. As mentioned in the introduction, the protein Atg19 or Cvt19 plays a crucial role in cargo selection for the Cvt pathway. The Cvt complex (consisting of prApe1 and α-mannosidase bound to Atg19) binds to Atg11 that brings the complex to the PAS where the Cvt vesicle will nucleate. All components of the core autophagy machinery (see below) are involved in the formation of the Cvt vesicle, notably

phosphatidylethanolamine (PE)-conjugated Atg8 that binds to Atg19 to ensure the correct sequestering of the complex within the vesicle.

For the selective process of pexophagy, several proteins are important in *S. cerevisiae* and/or the methylotrophic yeasts *P. pastoris* and *Hansenula polymorpha* (reviewed in ref. 28). Atg30 has been identified *in P. pastoris* as the peroxisome receptor for pexophagy. It is expressed and associates with peroxisomes already during proliferation of the organelles, but it becomes phosphorylated by an as yet unknown kinase only after induction of pexophagy. The interaction with peroxisomes occurs through two peroxins, Pex3 and Pex14, proteins with a major function in peroxisome biogenesis. In methylotrophic yeasts, Pex14 has been detected in both an unmodified and a phosphorylated form, and only the latter one appears to interact with Atg30. Atg30, after its association with peroxisomes, also interacts with the autophagy machinery at the PAS, and so targets the organelles for degradation to the vacuole.

A few other Atg proteins have been shown to be required for pexophagy: i.e., Atg25 in *H. polymorpha*, Atg26 in *P. pastoris* and the plant pathogenic fungus *Colletotrichum orbiculare* and Atg28 in *P. pastoris*. These proteins act at the later stages of pexophagy, but their precise role remains to be established.

Pexophagy in methylotrophic yeasts occurs not only by macropexophagy, but also by micropexophagy, depending on the carbon source and the ATP level.[29] Macropexophagy usually involves the sequestering of only a single peroxisome by the phagophore, whereas in micropexophagy often a cluster of organelles is engulfed by the vacuolar membrane. Micropexophagy is also a selective process, because the vacuolar membrane contains proteins that recognize the peroxisomes; the signaling is independent of Atg30 phosphorylation. In *P. pastoris*, the glycolytic enzyme phosphofructokinase plays a role in triggering micropexophagy. The morphological changes observed during micropexophagy are different from those in common microautophagy; in the latter, tubules invaginate into the vacuole lumen, whereas in the former, arm-like extensions of the vacuolar membrane are formed. To complete the sequestration by the arms, they fuse, in an Atg24-dependent manner, with a peculiar cup-shaped double-membrane structure called the micropexophagic membrane apparatus (MIPA). The origin of the MIPA is still unknown.

Autophagy of mitochondria in yeast occurs at a basic rate for regular turnover, but is induced upon change from a respiratory carbon source to a fermentative one. It may also be important upon organelle damage caused by reactive oxygen species (ROS) originating from a malfunctioning respiratory activity. Degradation of the organelle may occur by selective macro- or micromitophagy. Thus far, only two proteins specifically required for mitophagy have been identified in *S. cerevisiae*, Atg32, which appears to function as an outer membrane tag to mark the organelle,[30,31] and Atg33.[32] The selective nature of the organelle's degradation has been confirmed by the involvement of Atg11 (that binds Atg32, similar to its interaction with Atg19 in the Cvt pathway), Atg17 and Atg29.

Only limited information about induction, mechanism and the requirement of some Atg proteins involved in selective reticulophagy, ribophagy and piecemeal microautophagy of the nucleus is also available for yeast. However, it will not be discussed here because it is outside the scope of this review as no information about these processes in protists is as yet available.

(3) Vesicle nucleation. The nucleation of vesicles for cargo encapsulation involves the recruitment or formation of a membrane to generate a beginning for a vesicle (Cvt vesicle or autophagosome). The origin of the membrane for these vesicles has been debated for years. Several possibilities have been proposed, such as maturation from the ER or mitochondrion or de novo membrane formation by localized synthesis or transport. No unambiguous data are available for yeast, but recent studies on mammalian cells strongly suggest that both the ER and outer mitochondrial membrane can be the source of these membranes.[33-35] Cup-shaped ER protrusions enriched in phosphatidylinositol 3-phosphate [PtdIns(3)P] recruit autophagic effectors and expand for the phagophore to sequester cargo. Formation of PtdIns(3)P is dependent on the activity of a membrane-associated class III PtdIns3K complex comprising the Ser/Thr protein kinase Vps15, the PtdIns3K Vps34, as well as Atg6 and Atg14, which functions at the PAS. In yeast, Atg18, Atg20, Atg21 and Atg24, which all bind PtdIns(3)P, are recruited to the PAS. In *H. polymorpha*, but not in *S. cerevisiae*, Atg21 is essential for pexophagy.

(4) Vesicle expansion and completion. The expansion of the phagophore into an autophagosome or Cvt vesicle involves a process reminiscent of protein ubiquitination. Two sets of ubiquitination-like conjugation systems are implicated (reviewed in ref. 36). The first one, in which Atg8 as the ubiquitin-like modifier is conjugated to PE as the substrate, employs Atg7, Atg3 and the complex Atg12-Atg5 as E1, E2 and E3-like proteins, respectively; in the second system Atg12 plays the ubiquitin-like role, while Atg5 is the substrate and Atg7 and Atg10 exert the E1 and E2-like activities, respectively; here the E3 function is unknown. Atg16 can oligomerize and binds to the Atg12-Atg5 conjugate, usually resulting in the formation of a dimeric complex. Together, these activities lead to the following processes at the PAS: First, Atg8 is proteolytically processed at its C terminus by Atg4, exposing a glycine residue. This Atg8 as well as Atg12, also having a C-terminal Gly residue, are activated independently by the E1-like enzyme Atg7 by forming a thioester bond with it and subsequently transferred via E2-like Atg3 or Atg10, respectively, to membrane-associated PE or Atg5. As a result, Atg8 becomes covalently linked to PE and so anchored to both sides of the expanding, curved phagophore. The dimeric Atg12-Atg5-Atg16 complex covers mostly the outer surface of the curved organelle. After closure of the expanded phagophore, resulting in a double-membrane bounded vesicle, its outer surface loses its coating of Atg12-Atg5-Atg16 complex, and Atg4 acts once more on Atg8, releasing it from the outer surface by cleavage of the Atg8-PE linkage. The result is a cargo-filled vesicle with Atg8-PE at its inner membrane surface, now competent for fusion with the vacuole.

(5) Retrieval. Only Atg8-PE and Atg19 remain associated with the autophagosome or Cvt vesicle and are directed to the vacuole. Most other components involved in the autophagy and

Cvt pathways are soluble or peripheral membrane proteins and are easily retrieved from the phagophore/autophagosome/Cvt vesicle to the PAS to be reused in further cycles. Only retrieval of the integral membrane protein Atg9 requires PtdIns3K, the PtdIns3K-binding protein Atg18 and several other Atg proteins: 1, 2, 11, 13, 23 and 27. Atg23 is essential only for the Cvt pathway, but loss of this protein does result in a reduced number of autophagosomes.

(6) Docking and fusion. Vesicle targeting to the vacuole/lysosome, its docking to the degradative organelle and membrane fusion involve proteins that are generally used within the cell for other membrane docking/fusion processes and are not specific to autophagy and related processes. Therefore, they will not be further discussed in this review.

(7) Vesicle breakdown in the vacuole. Following fusion of the autophagosome with the vacuole and formation of the autophagic body, the membrane of this body (and that of organelles in it and the Cvt body) is ruptured to release and degrade its content. Vesicle lysis requires the vacuole's acidic pH and peptidase B (Prb1). Probably, this peptidase activates various vacuolar zymogens responsible for breakdown processes. In *S. cerevisiae* Atg15, an integral membrane protein with presumed lipase activity, is specifically involved in the degradation of this membrane. Another autophagy-related protein operating in the vacuole is Atg22, an integral protein of the organelle's boundary membrane which bears sequence similarity to permeases of the major facilitator superfamily. It is probably involved in export of regenerated amino acids to the cytosol.

Ubiquitination and selective autophagy. For many years, the ubiquitin-proteasome system has been considered as the main pathway responsible for the removal of misfolded proteins in all eukaryotic cells. Recently, however, it has been observed in mammalian cells that ubiquitination of cytosolic protein aggregates may also function as a specific signal to induce their selective degradation by autophagy (reviewed in ref. 37). By analogy with the proteasome system, where unfolded ubiquitinated proteins are recognized by ubiquitin-binding receptors that deliver them to the proteasome, ubiquitin-dependent selective autophagy involves a ubiquitin-binding receptor and its associated proteins, p62 and NBR1, respectively. Their simultaneous interaction with LC3 or GABARAP (mammalian proteins which belong to two ATG8 subfamilies), mediate the sequestering of ubiquitinated substrates (misfolded single proteins, inclusion bodies and aggresomes) in the autophagosome for their subsequent lysosomal degradation. The ability of p62 and NBR1 to bind ubiquitinated substrate depends on the presence of a C-terminal UBA domain, whereas the interaction with the ubiquitin-like family proteins LC3/GARABAP depends on a short linear motif called 'LC3-interacting region' (LIR) that is characterized by an acidic cluster and a conserved aromatic residue. A similar motif is present in Atg19 and is responsible for the interaction with Atg8 in the yeast's Cvt pathway. Nevertheless, functional yeast orthologues of p62/NBR1 have not been identified yet.

Regarding the regulatory mechanism that determines which pathway will be followed for the degradation of a specific ubiquitinated substrate, evidence has been reported that the specific architecture of the ubiquitin moieties (branched K48-linked polyubiquitin chains for proteasomal or mono-ubiquitin and linear K63-linked polyubiquitin chains for autophagy), together with the relative levels of co-chaperones BAG1 and BAG3 in the case of protein aggregates, are elements that select between the two pathways. However, p62 competes for ubiquitinated cargo with proteasomal receptors and is able to directly interact with the proteasome, which suggests the existence of additional regulatory mechanisms.

Ubiquitination is also involved in the selective autophagy of organelles like mitochondria, peroxisomes, ribosomes and intracellular bacteria. In the case of pexophagy, mono-ubiquitination of the integral membrane protein PMP34 is sufficient to induce autophagic degradation of mammalian peroxisomes in a p62-dependent manner.[38]

In general, most experimental evidence suggests that ubiquitination, too, might function as a signal for selective autophagic degradation of protein aggregates, organelles and pathogenic cells through a common mechanism involving autophagosome formation.

Terminology and methodology appropriate for the bioinformatics analysis of autophagy in protists. Comparative genomics provides a rapid way to exploit experimental data garnered for one species in order to elucidate the structure and operation of cellular pathways in other species. By compiling 'parts lists' for species for which experimental data may be sparse or hard to obtain, similarities and differences compared to model species can be visualized and their implications for physiology considered. Furthermore, mapping presences and absences of pathway components onto phylogenetic trees allows for a deeper understanding of the mechanisms by which the pathway evolved. Nevertheless, genome-mining exercises suffer from some fundamental and practical limitations and the risk of overinterpretation of data is ever present. A brief consideration of key terminology and common bioinformatic methodology illuminates some of the limitations of the area and explains some apparent inconsistencies in the literature.

Comparative genomics exercises cannot pinpoint genes or proteins with particular functions directly. Instead they seek statistically similar protein sequences to a query of known function: Where the similarity is strong enough to indicate homology (common evolutionary ancestry) then there is the possibility that the hit sequence has the same or similar function to the query. The improved statistics of the database search program BLAST[39,40] provide a reliable guide to significance, although certain kinds of sequences, such as those exhibiting strong compositional bias, e.g., coiled-coil regions, can still cause problems for the more sensitive, iterated search protocols of PSI-BLAST,[39] where search results can become 'contaminated' with unrelated sequences. The reliability of all searches is obviously dependent on the completeness of the protein sequence database and nucleotide databases should also be searched to allow for potential inaccuracies in gene calling. It is also prudent to employ query sequences from different species, where possible, since homologous queries may differ in their ability to retrieve divergent relatives. Even with all these efforts, however, it must be recognized that some divergent

homologues may resist detection with presently available queries and sequence databases.

Assuming homologues have been reliably found, the harder question emerges as to whether any of them have the same or similar function to the query. Here it is helpful to define two classes of homologue, orthologue and paralogue. Orthologues are defined as homologues that have arisen from a speciation event, i.e., their lineage traces back to a single gene in the last common ancestor of their respective species. Immediately after speciation these independent copies have the same function and, if selection pressure applies thereafter, will tend to retain that function. This consideration leads to the alternative definition of orthologues, as homologues in different species carrying out the same function. Very frequently, the two definitions are equivalent although there are exceptions where one of a pair of orthologues can alter or even lose its function.[41] Also, members of large families may have converged on the same function through more complicated evolutionary histories: These would be orthologues by the alternative definition but not by the original. Paralogues are those homologues deriving from gene duplication events within a single species. These are recognized as an important source of functional novelty during evolution and can be expected to differ, at least subtly, in their function(s).

The relationship between sequence identity and functional similarity of protein pairs is complicated and family-dependent.[42] However, there are several ways in which detected homologues can be justifiably reclassified as probable candidate orthologues. An accepted computational shortcut to prediction of orthology is the reciprocal BLAST search:[43] If two proteins, in different genomes, are each the top of the hit list of the other when queried by cross-genome BLAST, then they are likely to be orthologous and hence probably share the same function. Clearly, any experimental data regarding sequence characteristics key for activity should also be considered. These predictions are significantly enhanced by the availability of structural data but, as yet, rather few autophagy proteins have been fully structurally characterized.[18] Many autophagy proteins contain multiple domains[18] and these can cause problems, especially in the case of short domains and large inter-domain separations. Since BLAST will separate out individual domain matches in such cases, two proteins may pass the orthology prediction test yet differ in their domain composition. Nevertheless, domains may also be a useful filter in the search for likely orthologues. Some domains, such as those with catalytic activity, will be integral to a protein's molecular function, and less capable of substitution by alternative domains: Any putative orthologue should possess those core domains. Where candidate sequences require manual scrutiny, it is important to employ more sensitive domain annotation tools such as HHSEARCH,[44] since domain databases such as Pfam[45] and CDD[46] may fail to annotate divergent domain sequences, particularly short ones.

Therefore, different criteria may be used to consider a homologous sequence as a strong putative orthologue. For example, it has been initially suggested that the second Atg12-based conjugation system was absent in trypanosomatids.[18] However, later reports[14,15,23] identify Atg5 and Atg10 orthologues, recently experimentally characterized.[23] Interestingly, despite the identification in this last work of a Leishmania protein functionally resembling Atg12, and indeed now named ATG12, this is not likely to be an orthologue, strictly speaking, of the yeast protein. Instead, sequence comparisons suggest a closer, albeit still distant, resemblance to yeast Atg8.[14]

Finally, another challenge of genome mining is the possibility of gene displacement. Thus, the absence of a putative orthologue to a given protein in a certain organism need not necessarily mean that that function does not exist: an evolutionarily unrelated protein may have taken over the role. Autophagy provides a good example of this: The roles of the vacuolar hydrolases Pep4 (an aspartic peptidase) and Prb1 (a serine peptidase) in *S. cerevisiae* have been taken over by cysteine peptidases in trypanosomatids.[17] However, this resembles more the situation in humans, although the aspartic peptidase cathepsin D also plays an important role there.[47]

Genomic Predictions Regarding Autophagy and its Evolution in Protists

Independent secondary losses of, and variations on, 'canonical' autophagy. In current views of eukaryotic evolution most organisms are placed within one of six supergroups.[48] This classification is based upon results from large-scale phylogenetic analyses and careful comparisons of cell morphology, although some of the relationships between super-groups are uncertain and the identity of the deepest branch(es) that lie(s) closest to the root of the eukaryotic tree remains a hotly-debated topic.[49-52] The experimental characterization of autophagy in taxa widely accepted to have last shared a common ancestor at a very early stage in eukaryotic evolution—animals, yeast, trypanosomes and plants—strongly suggests that the basis for macroautophagy was already established prior to the divergence of the last common ancestor of known eukaryotes (or LECA). Using bioinformatics to map the distribution of known autophagy-related proteins in extant eukaryotes, however, indicates that among the protists canonical autophagy has been subjected to elaboration, moderation and even loss on multiple occasions (**Fig. 2**).[14] As exemplified by the experimental characterization of autophagy in malarial parasites (discussed under the heading 'Autophagy in Apicomplexa'), or the molecular characterization of ATG8 paralogues in *Leishmania major*[23] the cellular consequences of likely moderation to canonical autophagy, or the paralogue expansion of core *ATG*s are already under investigation in some species. The availability of reverse genetic tools for many of the other protists in which core ATGs have either been lost or subject to paralogue expansion, means that other variations in autophagy that are apparent at the genomic level should be amenable to experimental investigation, too.

Striking observations to emerge from a comparative genomic census of *ATG* genes are the loss of autophagy on at least three occasions during eukaryotic evolution and the moderation or divergence of autophagy in the Apicomplexa, such that, other than the ubiquitin-like conjugation systems, many readily recognizable ATGs are either absent or have diverged beyond obvious recognition. The absence of ATG8- or ATG12-related

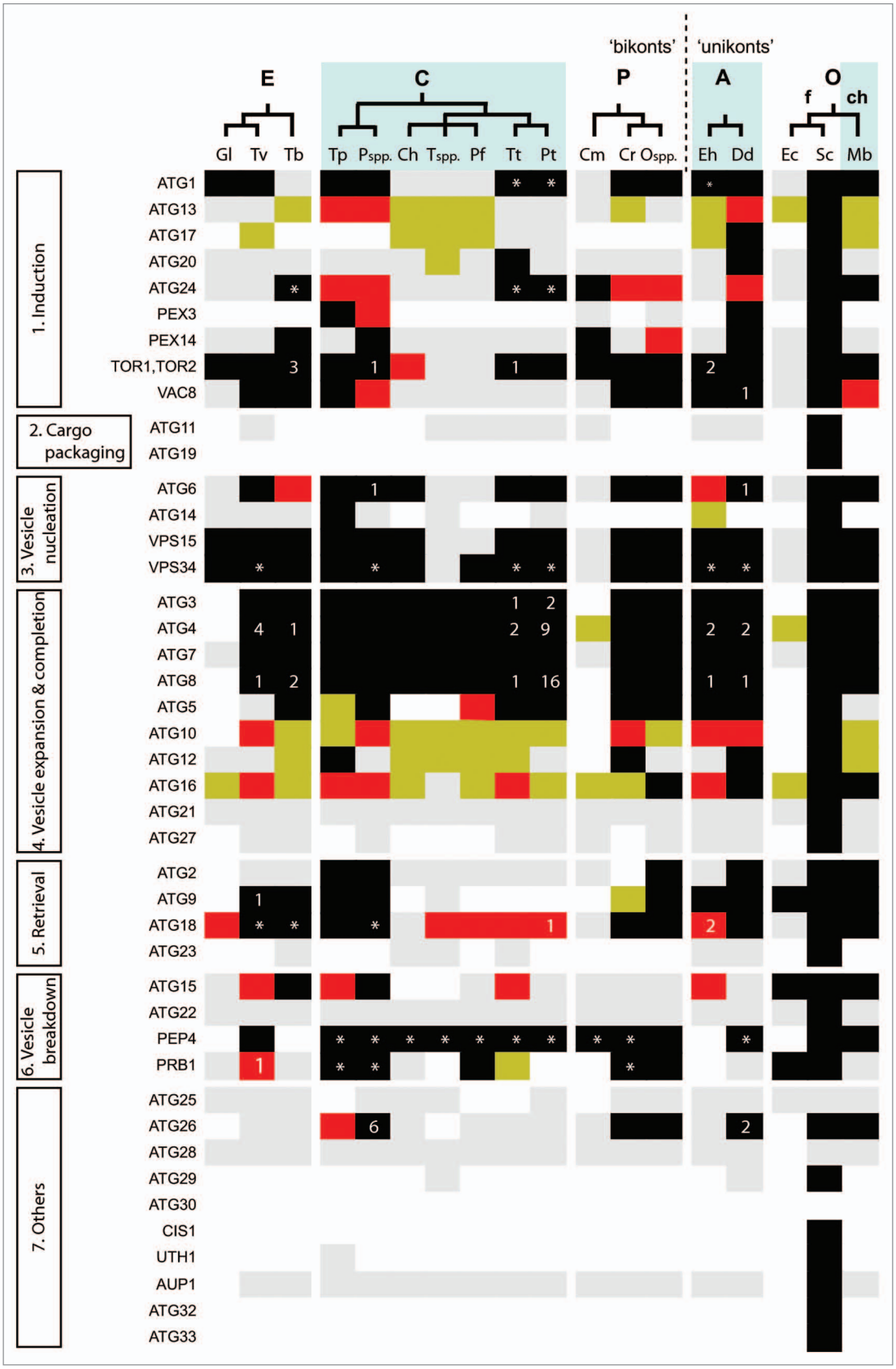

Figure 2. For figure legend, see page 9.

Figure 2 (See opposite page). Predicted distribution of ATG orthologues in protists. Modified from Rigden et al.[14] BLAST and PSI-BLAST were used to identify sequences in protists that were either homologous or putatively orthologous to known autophagy components. Proteins are grouped according to the stage of autophagy at which they principally function (see Fig. 1). RPS-BLAST and HHSEARCH were used to check the domain composition of identified hits. Putative orthologues were those corresponding to the query by being reciprocal top hits in BLASTs between complete sets of predicted genome-encoded proteins and by bearing similar domain composition. By this definition there can clearly be only a single orthologue per genome. Additional putative paralogues were defined as further protist hits in the same BLAST searches, which also retrieved the query in top place and had appropriate domain composition. Black cells indicate the presence of a putative orthologue identified using a yeast Atg query; red, a putative orthologue identified using a non-yeast ATG query; light grey, homologous sequences detected using a yeast Atg query (but without indications of orthology from reciprocal BLAST results and domain composition; reviewed in ref. 14 for details); yellow, homologous sequences detected using a non-yeast ATG query; blank, no homologues detected. Numbers denote additional predicted paralogues in an individual species. The figure, for consistency, comes from a uniform and purely computational analysis, and does not necessarily reflect experimental information. However, experimental data suggest that some of the putative paralogues in this figure have apparently very similar functions to the confirmed orthologues' functions. For example, *T. brucei*, by this methodological definition, contains one predicted orthologue and three paralogues of *S. cerevisiae* Tor1/Tor2. Nevertheless, experimental data show that two *T. brucei* proteins have TOR activity[116] and therefore, by some definitions based on functional analysis (see text), may both be considered as orthologues. Asterisks indicate the presence of homologous sequences that could not be reliably predicted as paralogous or not. Accepted relationships within eukaryotic 'supergroups' for the protists surveyed here are shown. There are competing hypotheses for how the divergence of these supergroups relates to the root of eukaryotic evolution. The last common ancestor of all eukaryotes has been suggested to have existed at the point of unikont-bikont divergence.[199] Alternatively, although molecular and morphological evidence for the monophyly of Excavata exist (e.g., reviewed in ref. 60), these data are equivocal and several excavate groups continue to be proposed as extant descendants from the earliest diverging eukaryotic lineage.[52,59] Abbreviations for eukaryotic 'supergroups': A, Amoebozoa; C, Chromalveolata; E, Excavata; O, Opisthokonta; P, Plantae. Abbreviations for other groupings: ch, choanoflagellates; f, fungi. The choanoflagellates represent the closest known unicellular relatives of animals. Species abbreviations: Ch, *Cryptosporidium hominis*; Cm, *Cyanidioschyzon merolae*; Cr, *Chlamydomonas reinhardtii*; Dd, *Dictyostelium discoideum*; Ec, *Encephalitozoon cuniculi*; Eh, *Entamoeba histolytica*; Gl, *Giardia lamblia*; Mb, *Monosiga brevicolis*; Ospp., *Ostreococcus* species; Pf, *Plasmodium falciparum*; Pspp., *Phytophthora* species; Pt, *Paramecium tetraurelia*; Sc, *Saccharomyces cerevisiae*; Tb, *Trypanosoma brucei*; Tp, *Thalassiosira pseudonana*; Tspp., Theileria species; Tt *Tetrahymena thermophila*; Tv, *Trichomonas vaginalis*.

conjugation systems from the complete genome sequences of the microsporidian parasite *Encephalitozoon cuniculi*,[53] the red alga *Cyanidioschyzon merolae*,[54] and the human pathogen *Giardia intestinalis*,[55] however, provides very strong evidence that the process of macroautophagy has been secondarily lost in a recent ancestor of these species. Significantly, the absence of macroautophagy is not the only illustration of where organellar cell biology has been subject to streamlining in these eukaryotes. For instance, neither microsporidia nor Giardia contain peroxisomes or related microbodies; molecular and cytological analyses indicate that the endomembrane networks are perhaps less complex than in other organisms. Mitochondrial function has also degenerated in both parasites and the relic organelles that remain (known as mitosomes) have no capacity for ATP production.[56] In the case of Giardia, the absence of autophagy also correlates with the absence of lysosomes. Instead, one contiguous tubular vesicular network appears to combine ER, endosome and lysosome functions.[57] Yet, that is not to suggest that differentiation and intracellular remodelling do not occur. For instance, Giardia and microsporidians form cysts and spores, respectively. In Giardia the appearance of the secretory network is induced to facilitate the deposition of cyst wall components.[58] It is not clear whether any turnover of macromolecular structures during the final stages of cyst formation or excystation is dependent upon a process resembling microautophagy.

In contrast to Giardia and the microsporidia, the extremophile red alga *C. merolae* contains a peroxisome, an aerobic mitochondrion and a photosynthetic chloroplast. Thus, secondary loss of macroautophagy does not always correlate with the extreme loss of metabolic functions that often accompanies the adaptation to parasitism, although niche adaptation to acidic, volcanic hot springs nonetheless likely facilitated the loss of ATGs in *C. merolae*. A wider comparison with the green algae reveals an extensive genomic footprint for canonical autophagy in the freshwater alga *Chlamydomonas reinhardtii* and in various oceanic phytoplankton (e.g., the diatom *Thalassiosira pseudonana* and the picoeukaryote Ostreococcus) (**Fig. 2**). This clearly indicates that in contrast to *C. merolae*, fluctuations in nutrient supply, competition for nutrients, other ecological pressures or developmental programs (e.g., sexual reproduction) necessitate the retention of autophagy pathways.

It is worthwhile to note that some might view the absence of macroautophagy from Giardia not as the product of secondary loss, but as a reflection of Giardia's last common ancestor with other eukaryotes possibly diverging prior to the appearance of the ATGs in eukaryotic evolution. Similar arguments have been put forward to explain the minimalism that pervades other aspects of Giardia's molecular cell biology.[55] For many years there was a longstanding belief that Giardia was an amitochondriate descendent of a primitive eukaryote that diverged before the α-proteobacterial endosymbiosis that gave rise to the proto-mitochondrion occurred. This now seems not to be the case: relic mitochondria are present in Giardia,[56] and although it is as yet difficult to refute entirely the suggestion that Giardia belongs to the earliest diverging lineage,[59] much of the divergent cell biology that characterizes this important human parasite is more often considered to be a product of reductive evolution. In molecular phylogenetic analyses Giardia is often recovered in a clade containing another human parasite Trichomonas (e.g., reviewed in ref. 60) which, in contrast to Giardia, contains obvious ATG homologues. Published thin section electron micrographs of Trichomonas are also suggestive of autophagosome formation following serum deprivation, hydroxyurea treatment or berberine sulphate treatment.[61,62] Like Giardia, Trichomonas has a streamlined metabolism:[63] In Trichomonas, cellular energy generation is dependent upon carbohydrate metabolism and, to a lesser extent, the catabolism of some amino acids, but peroxisomes and many biosynthetic pathways are absent and its anaerobic

mitochondria (known as hydrogenosomes) have lost the capacity for cytochrome-dependent respiration and oxidative phosphorylation. Thus, the example of *Trichomonas vaginalis* indicates that a streamlined metabolism is not necessarily a prelude to moderation or loss of macroautophagy.

In other protists the genomic survey points towards either moderation or divergence from the autophagy pathway that is broadly conserved in animals, plants, Dictyostelium (see below), and fungi. Experimental studies are required to distinguish between these possibilities. In the malarial parasite *Plasmodium falciparum*, and other apicomplexans, the differences with other eukaryotes are likely to be more extreme. Here, there are likely to be lineage-specific adaptations to the mechanisms by which autophagy is induced (e.g., a typical TOR-ATG1-ATG13 complex is not predicted) and the expansion of the phagophore membrane occurs (e.g., homologues of neither Atg9, which is an otherwise well-conserved integral membrane protein, nor Atg2, required for retrieval and recycling of Atg9 during phagophore expansion, are readily predicted by bioinformatics searches, although likely Atg18 orthologues were readily identified in *P. falciparum* and Theileria). Intriguingly, Atg9 orthologues are also not detected in ciliates, which are a sister group to the Apicomplexa.

Of course, an absence of readily detectable Atg orthologues in evolutionarily diverse protists is as likely, if not more likely, to reflect gene divergence, as it is to be due to gene displacement or gene loss. Yet, comparative genomics also throws up curious examples of ATG gene conservation. An unexpected example of conservation is the unambiguous example of an Atg17 orthologue in the amoeba *Dictyostelium discoideum*. Although proteins with low amino-acid identity to either yeast or the putative Dictyostelium ATG17 can be detected in a range of other protists, including *Entamoeba histolytica* and the choanoflagellates *Monosiga brevicolis* and Protereospongia, none is confidently predicted by the reciprocal BLAST to be a likely orthologue. The possible evolutionary significance of these observations was discussed previously.[14] *ATG17* remains one of the rare examples of an autophagy gene that potentially appeared early in evolution (before the divergence of the Amoebozoa and fungi), but which was subsequently lost or diverged beyond easy recognition during the evolution of lineages, including metazoans, where autophagy is an essential feature of development and normal cell physiology.

Paralogue expansions of some ATGs are evident in evolutionarily related and unrelated protists. For instance, there is a species-specific expansion of *ATG8* paralogues into four distinct families totaling 25 genes in Leishmania, but not Trypanosoma species as was noted previously.[23] Preliminary localization of some of these paralogues using GFP-tagging approaches suggests that some ATG8-family members have cellular functions in addition to or other than autophagy (reviewed in ref. 23; see also below, '*Autophagy in Trypanosomatidae as inferred from bioinformatics studies of genome sequences*'). From the wider comparative genomic survey of autophagy summarized in **Figure 2**, the most notable examples of paralogue expansion are *ATG4* and *ATG8*, duplicated several times in *Paramecium tetraurelia*, but to a much lesser extent in another ciliate, *Tetrahymena thermophila*. Phylogenetic analyses suggest that the expansions in these two species occurred largely independently of each other (data not shown). The Paramecium ATG4 and ATG8 homologues are not identical. Paralogue expansion of other Paramecium gene families has been reported previously, and it appears that genome architecture and gene content in Paramecium have been shaped by multiple rounds of whole genome duplicaton.[64] In instances where examples of unexpected paralogue expansion in Paramecium have been subject to experimental examination (e.g., actin-related proteins[65] and vacuolar ATPase[66]), the different gene products have often been observed to have distinct cellular functions. This suggests that future study of Paramecium *ATG4* and *ATG8* paralogues will be of merit.

In protists, selective autophagy of organelles—such as glycosomes, the peroxisome equivalents of trypanosomes (see section below 'Autophagy in Trypanosomatidae'; *Trypanosoma brucei*)—has been experimentally observed. Some proteins involved in these pathways in yeasts also participate in other autophagy-related processes or entirely different processes. These include Atg24, the peroxin Pex14 and the glycolytic enzyme phosphofructokinase, all involved in pexophagy in methylotrophic yeasts and Atg11, Atg17 and Atg29 involved in mitophagy in *S. cerevisiae*. Putative orthologues of these have been identified in protists but their role in organelle degradation in protists remains to be proven. Importantly, however, no genes have yet been detected in protists encoding proteins homologous to those found in yeasts that are specifically devoted to recognition/initiation of pexophagy (such as Atg25 and Atg30) or mitophagy (Atg32, Atg33). Thus, Atg25 and Atg30 are probably fungi-specific additions to the core autophagy pathways and their functions may be exerted by different proteins in other organisms.

Ubiquitination and autophagy in protists. Since the connection between these two processes has only recently been clarified, the proteins involved have not been included in genomic surveys. We initially used the same methodology as previously[14] to look for putative orthologues of p62 and NBR1 in a set of complete protist genome sequences. Using human sequences as queries, no putative orthologues were determined by the pipeline. However, p62 and NBR1 both contain relatively short domains separated by regions of intrinsic disorder, compositional bias and some coiled-coil regions (NBR1 alone). As mentioned above, these characteristics complicate automated genome mining. We therefore took a different approach and searched for proteins in the same protist genomes that contained the same PB1-ZZ-UBA domain combination shared by p62 and NBR1. The hits were also analyzed for the presence of coiled-coil and possible LIR motifs for interaction with small ubiquitin-like modifiers represented by [WY]-x-x-[LI] (as in Atg32).[67,68]

By this approach we identified proteins in five protists that each contain PB1, ZZ and UBA domains including two in *Naegleria gruberi* (**Fig. 3**). Clearly, the presence of these three domains in the protist sequences is no guarantee that they share a p62/NBR1-like function, and not all contain a consensus LIR between ZZ and UBA domains. However, recent data for the *D. discoideum* protein are at least consistent with its sharing a similar function to its human counterpart.[69] Thus, *Vmp1*$^{-}$ *D. discoideum* cells accumulated large ubiquitinated protein aggregates,

including the p62-like and ATG8 proteins, in a way that resembled the results of mutating *Atg5* or *Atg7* genes in mouse models of neurodegenerative diseases.[70,71] Furthermore, this domain combination seems relatively rare: No other human proteins appear to contain all three characteristic domains, for example. It is also interesting to note that the domain order is retained (albeit with some additional domains) and, with the exception of the *Thalassiosira pseudonana* protein, the PB1 domain maintains its N-terminal location, while the UBA domain is invariably positioned at the very C terminus, as in p62 and NBR1. Interestingly, we found two p62/NBR1-like proteins in the amoeboflagellate *N. gruberi*, which belongs to the supergroup Excavata. Although Naegleria was not included in our larger scale census of ATGs, we were mindful of the identification of both Atg17 and p62/NBR1-like homologues in Dictyostelium and wished to ask whether homologues might have been retained in evolutionarily distant amoebae (in the case of Naegleria, not likely to have shared an ancestor with Dictyostelium since LECA). No clear candidate for an Atg17 orthologue in Naegleria emerged from this analysis, although the p62/NBR1-like proteins described here all represent interesting candidates for further study.

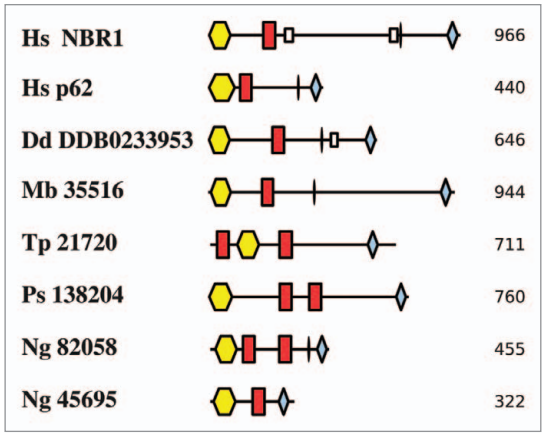

Figure 3. Domain architectures of human NBR1 and p62 compared to protist proteins with similar domain compositions. See text for a discussion of these proteins. Each protein is labeled with its species of origin (Hs, *Homo sapiens*; Dd, *Dictyostelium discoideum*; Mb, *Monosiga brevis*; Tp, *Thalassiosira pseudonana*; Ps, Phytophthora spp.; Ng, *Naegleria gruberi*) and a genome identifier. Domains are PB1 (yellow hexagons), ZZ (red rectangles) and UBA (blue diamonds), small boxes represent coiled-coil regions and ellipses mark LIR motifs. Numbers to the right are sequence lengths, and the diagram is approximately to scale.

Functional Analyses of Autophagy in Free-Living Protists

Amoebae. Amoebae are a polyphyletic group of protists that have radiated into many terrestrial and aquatic habitats, and also include some medical pathogens[72]—see below 'Functional analyses of autophagy in parasitic protists'. Some species are known for their ability to undergo dramatic morphological changes in response to appropriate environmental cues. Perhaps the most extreme of these developmental programs are found among the social amoebas which aggregate together to form multicellular fruiting bodies when starved.[73] (For a scheme of the Dictyostelium life cycle, see **Fig. 4**). This developmental process is best described in the soil-dwelling *D. discoideum*, which normally hunts bacteria and yeast within aerobic leaf litter. Significantly, *D. discoideum* also serves as an experimentally tractable model phagocyte for the study of bacterial pathogenesis and host-cell dependent killing mechanisms.[74] Autophagy is important in both multicellular development and in the phagocytosis and digestion of bacterial prey. Intriguingly, the molecular analysis of autophagy in *D. discoideum* suggests that in this protist the core features of macroautophagy retain some interactions that are otherwise thought to occur only in yeast (Atg17-dependency,[14] see the previous section), to occur only in yeast and plants (Atg13-dependency) or to occur in mammals but not yeast (VMP1-dependency[75,76]). In addition, some Dictyostelium ATG proteins exhibit structural architectures that are more reminiscent of animal homologues than they are of yeast homologues (ATG1, ATG16).[77,78] Finally, membrane whorls similar to the autophagosomes seen in mammalian cells are evident following cytological examination of *ATG5*$^-$ and *ATG7*$^-$ Dictyostelium mutants, but not wild-type amoebae, thereby providing further evidence that the cell biology of autophagy in social amoebae is more similar to mammals than it is to yeast.[79]

Thus, genomic analyses have identified most of the core ATG components that have been experimentally characterized in yeast or animals, including candidate orthologues of ATG13 and ATG17 that were missed in the earliest studies of autophagy in Dictyostelium.[14,78,79] In response to starvation, cAMP-dependent signaling leads to the aggregation of many thousands of adhering cells that transform into a motile slug, which undergoes morphogenesis to produce a 1 to 2 mm high fruiting body containing a spore mass at the tip of a thin stalk. Within the stalk, cells are highly vacuolated; under normal circumstances the induction of starvation-induced autophagy is followed by extensive vacuolization and DIF-1-dependent autophagic cell death.[80] (DIF-1 is a chlorinated small molecule morphogen synthesized by Dictyostelium). Autophagic cell death is also dependent upon inositol 1,4,5-triphosphate signaling and Ca^{2+} release from the ER, and for vacuolization and the subsequent autophagy-dependent cell death to proceed normally a UDP-glucose derivative is required.[81,82] Phenotypic analysis of the various *ATG* gene knock-outs that have been made in *D. discoideum* reveals that the absence of various ATG components either compromises or results in failure of fruiting body formation, but that failure of the developmental program occurs at different points depending upon the *ATG* gene that is inactivated.[78,79,83,84] Following *ATG* gene inactivation, the morphological defect that occurs during development can vary depending upon the experimental protocol used to induce multicellular development. Thus, amoebae can be grown axenically and then starved on nitrocellulose filters, induced for development within plaques on bacterial lawns or grown on bacterial lawns and then transferred to nitrocellulose for starvation-induced development. Autophagy is also induced, and readily scored using microscopy, when amoebae are maintained in amino acid-deficient media. Vegetative growth of Dictyostelium appears to be largely unaffected by *ATG* gene

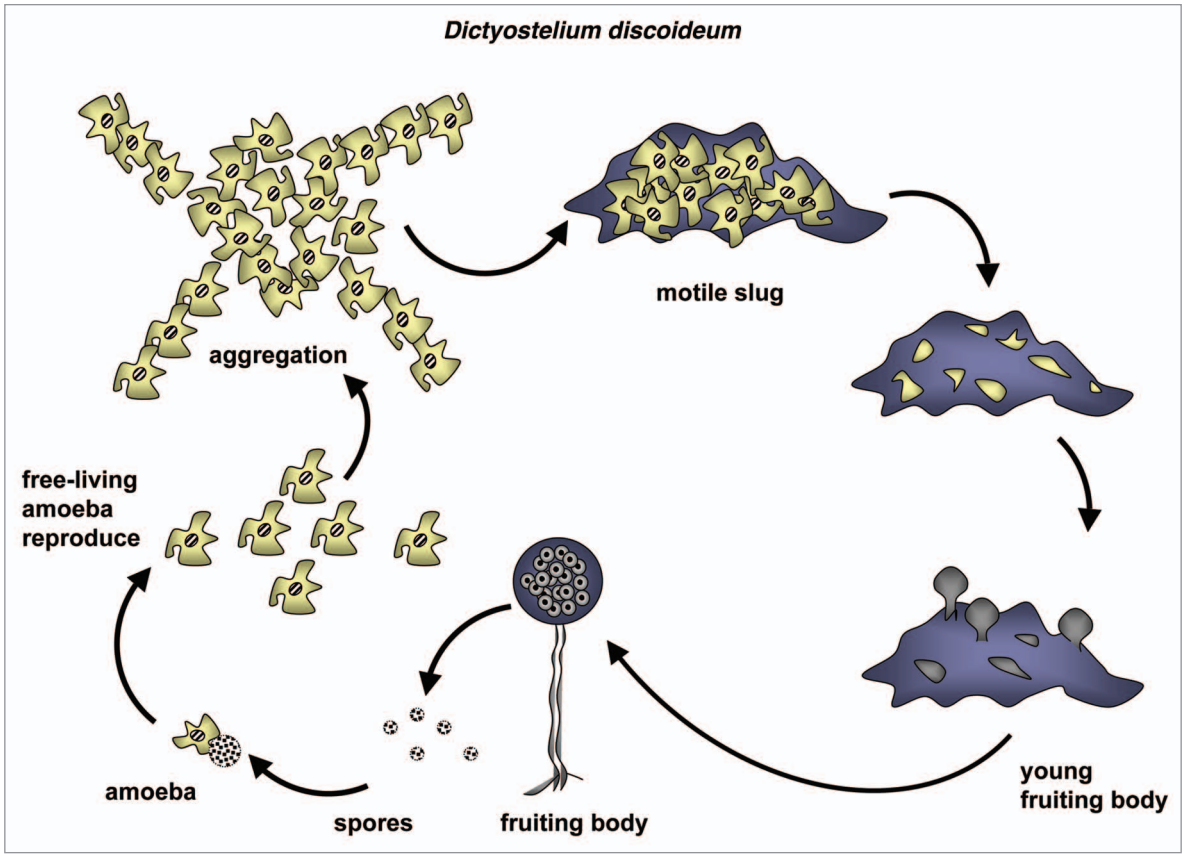

Figure 4. Diagram of the life cycle of *Dictyostelium discoideum*. For a detailed discussion of the life cycles of this and each of the organisms in Figures 6–8 and 10, see the text.

inactivation, at least for the genes examined thus far;[78,79,83,84] a result which mirrors the phenotypes seen in parasitic protists where autophagy is also important during cellular differentiation—see next main section.

Following induction of autophagy, transcription of the Dictyostelium *ATG1* homologue (*DdATG1*) is upregulated (as are the *ATG8* and *ATG9* homologues discussed below, reviewed in ref. 79); the ATG1 protein subsequently colocalizes with the 'classical' autophagosome marker ATG8,[78] and presumably interacts with a hypophosphorylated form of the Dictyostelium ATG13 homologue (DdATG13). *ATG1* null mutants die rapidly in amino acid-free media, are unable to form mature autophagosomes, and fail to aggregate properly when induced for multicellular development on nitrocellulose filters.[78] Use of a temperature-sensitive *ATG1* mutant reveals that the requirement for ATG1 in autophagy is retained throughout development.[78] Moreover, additional mutagenesis studies revealed that the kinase activity of DdATG1 and the C-terminal region of DdATG1 that contains a 122 amino-acid domain conserved in animal, but not yeast, homologues are both required for autophagy to occur in Dictyostelium.[77] However, no homologue of the *C. elegans* protein UNC-14,[85] which interacts with the *C. elegans* ATG1 orthologue UNC-51 through a C-terminal domain conserved in animal and amoebal ATG1, is evident in the *D. discoideum* genome.

DdATG5- and *DdATG7*-deficient amoebae are unable to undergo ATG12-ATG5 dependent conjugation and, when developed for multicellularity on nitrocellulose filters, aggregate to form slugs that form multiple, small, abnormal fruiting bodies with thick stalks and empty sori (**Fig. 5**).[79] However, in contrast to the phenotypes seen in *DdATG5*$^-$ and *DdATG7*$^-$ amoebae, the requirements for ATG8 conjugation to PE and for PtdIns3K signaling during autophagosome expansion are less critical. Thus, while autophagosomes are formed and development proceeds to fruiting body formation in Dictyostelium mutants that lack either the DdATG8 or the DdATG6 isoforms characterized by Kessin and co-workers, these mutants form fruiting bodies with atypical morphologies (depending upon whether mutants were developed on nitrocellulose filters or bacterial plaques) and produce fewer spores than wild-type amoebae.[83] Interestingly, there are paralogues of DdATG6 and DdATG8 encoded in the nuclear genome,[14] perhaps pointing to the possibility that redundancy provides an explanation for the less severe developmental phenotypes seen in *ATG6*- and *ATG8*-deletion mutants compared to the other *ATG* gene deletions examined in Dictyostelium thus far. However, the lack of cytoplasmic turnover or recognizable autophagosomes in amino acid-starved *DdATG6*$^-$ or *DdATG8*$^-$ amoebae[83] indicates that these paralogues cannot fully compensate for the absence of their experimentally characterized counterparts.

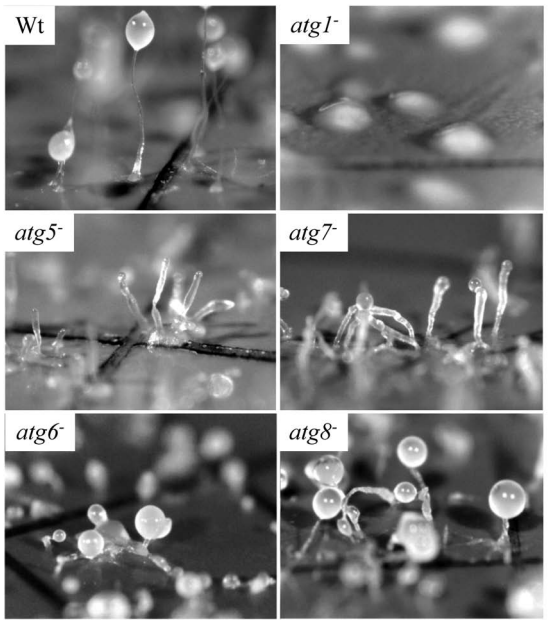

Figure 5. Dictyostelium autophagy mutants vary in the severity of their developmental phenotype. Amoebae were photographed following multicellular development on nitrocellulose filters. Wild-type (Wt) Dictyostelium elaborate fruiting bodies. Amoebae lacking *ATG1* form loose mounds that rarely develop further. Amoebae lacking either *ATG5* or *ATG7* form multi-tipped aggregates, which form abnormal, small fruiting bodies at the end of thickened stalks; viable, detergent-resistant spores are not produced. Mutants lacking either *ATG6* or *ATG8* form multi-tipped aggregates with small, but otherwise normal fruiting bodies; some viable, detergent-resistant spores are formed by these mutants. This research was originally published in the Journal of Biological Chemistry: parts Wt, *atg5*$^-$ and *atg7*$^-$ as Figure 3A, C and E, respectively, in Otto et al. Macroautophagy is required for multicellular development of the social amoeba *Dictyostelium discoideum*. J Biol Chem 2003; 278:17636–45 and parts *atg1*$^-$, *atg6*$^-$ and *atg8*$^-$ as Figure 3B, G and H, respectively, in Otto, et al. Dictyostelium macroautophagy mutants vary in the severity of their developmental defects. J Biol Chem 2004; 279:15621–9. ©The American Society for Biochemistry and Molecular Biology.

The most recent *ATG* gene to be experimentally characterized in Dictyostelium is *DdATG*9.[84] The protein encoded by this gene contains the conserved 'ATG9' domain, six putative transmembrane helices, and when fused to green fluorescent protein (GFP) localizes in vesicles that plausibly track along microtubules to the site of autophagosome formation, as in mammalian cells. As with other eukaryotes, ATG9 is a candidate molecule involved in the delivery of lipids to the expanding autophagosome and, akin to other Dictyostelium *ATG* gene mutants, amoebae lacking DdATG9 are attenuated in development. The ability of *DdATG9*$^-$ mutants to form slugs and occasionally form abnormal-looking fruiting bodies when developed on nitrocellulose filters suggests a phenotype more severe than DdATG6 or DdATG8 deficiency, but less pronounced than loss of DdATG1, DdATG5 or DdATG7.[84] Retrieval of Atg9 from the phagophore membrane in yeast requires Atg1 activity.[86] Assuming this dependency is evolutionarily conserved, the striking differences in phenotype severity between *DdATG1*- and *DdATG9*-deficient amoebae suggest DdATG1 is likely to most critically function at an early, as opposed to late (ATG9-interacting) stage in the autophagy process. Another intriguing phenotype arising from DdATG9 deletion is the impairment of phagocytosis, providing unexpected evidence of a link between the function of an *ATG* gene product and microbial engulfment.[84] The role of autophagy in microbial digestion by Dictyostelium has been studied using bacteria that are either generally readily cleared by the amoebae (*Salmonella typhimurium*[87]) or which are able to replicate intracellularly (*Legionella pneumophila*[84]). The data from these experiments echo the results from studies of autophagy in mammalian cells and underscore the importance to some intracellular microbes of manipulating the autophagy process in order to replicate.

In line with work on mammalian cells, recent work has also revealed that the orthologue of vacuole membrane protein 1 or VMP1, is required for autophagy in Dictyostelium.[69,75,76] As in mammalian cells, this protein, which is absent from yeast, is multifunctional and found at different intracellular locations making it difficult to assess whether the effect of *VMP1* deletion on autophagy is direct or a pleiotropic consequence of losing a protein involved in numerous cellular processes. There is, however, some evidence that VMP1 in Dictyostelium is present in autophagosomes.[69] Following the resuspension of amoebae in minimal salts solution, the detection of p62-related protein in *VMP1*$^-$, *DdATG1*$^-$, *DdATG5*$^-$ and *DdATG7*$^-$ mutants within ubiquitin-containing protein aggregates that colocalize with GFP-ATG8 indicates conservation of ubiquitination-dependent delivery of cytosolic protein aggregates to autophagosomes, as observed in mammalian cells.[69] The limited data regarding the function and mechanisms of autophagy in other amoebae are cited in the section on 'Experimental analyses of autophagy in parasites.'

Other heterotrophs and phototrophs. Although reverse genetic approaches necessary to facilitate molecular studies of autophagy are available for a diverse assortment of free-living heterotrophic and phototrophic protists, it is only Dictyostelium that has been subject to extensive molecular analysis. However, in addition to genomic signatures that suggest intriguing variations on canonical macroautophagy (e.g., the puzzling expansion of ubiquitin-like protein conjugation components in *Paramecium tetraurelia*—see above 'Genomic predictions regarding autophagy and its evolution in protists'), there are various ultrastructural descriptions consistent with roles for autophagy or similar processes in a variety of species. Here, cell cytology not only points towards the importance of autophagy in development (e.g., in ciliates where autophagy is implicated in degradation of the macronucleus during conjugation[88]), differentiation (e.g., during encystment of Tetrahymena;[89] long-term 'gametic' survival of plate-grown *Chlamydomonas reinhardtii*, a ubiquitous green alga[90,91]), and programmed cell death (PCD) (reviewed in ref. 92 and 93), but also towards the upregulation of basal levels of autophagy in response to the spatially and temporally heterogeneous environments in which many protists are found (reviewed in ref. 94). Given the paucity of relevant molecular data we consider experimental studies relating to autophagy in

free-living protists other than Dictyostelium and related amoebae in one combined section.

An involvement of autophagic-like processes in possible turnover or remodeling of metabolic organelles (mitochondria, chloroplasts) was revealed by studies with *C. reinhardtii* mutants deficient for chlorophyll production (due to the absence of phytoene synthase),[95] and *Tetrahymena thermophila* mutants in which mitochondrial function and fission were compromised by either overexpression or deletion of septin.[96] In the Tetrahymena mutants, far higher numbers of mitochondria were seen in association with autophagosome-like vesicles than in wild-type or starved ciliates. Following treatment with trans-resveratrol, inclusion of mitochondria within autophagosome-like structures was also seen in another ciliate species, *Philasterides dicentrarchi*.[97]

In the Chlamydomonas phytoene synthase mutant,[95] an increased frequency of cytosolic autophagosome-like vesicles correlated with aberrant plastid structure where thylakoid stacks and starch-containing pyrenoid bodies were missing. This increase in the number of autophagic vesicles could have been due to either the pleiotrophic consequence of a defect in chloroplast function and the general poor health of a mutant that could only grow heterotrophically in the dark,[95] or because of autophagy during attempted remodeling of the abnormally functioning chloroplast present in these cells. The dramatic remodeling of organellar architecture that follows N-limitation and the entry of Chlamydomonas cultures into stationary phase (reviewed in refs. 90 and 98) provides a more likely example of where autophagy is important in *C. reinhardtii*. However, it is also relevant to remember that we speculated previously on the possible importance of intra-organellar protein turnover rather than autophagy for remodeling of the single mitochondrion and/or plastids found in many protists (the mitochondrion and chloroplast are single copy organelles in *C. reinhardtii*).[14] The conservation of many *ATG* homologues in algal and ciliate genomes[14,99] suggests that organelle and protein turnover is more likely to occur by 'classical' macroautophagy, than it is by the substantially moderated process implicated in the turnover of organelles in malarial parasites and perhaps other apicomplexan parasites, too (see below, 'Autophagy in Apicomplexa').

In the green alga *Micrasterias denticulata*, *s*tress-induced autophagy can be accompanied by a PCD-like process in which the presence of autophagosomes is readily observed by transmission electron microscopy.[93] On the one hand, for some free-living unicellular opportunists the benefits conferred by altruistic cell death of an individual are not necessarily obvious. Yet, in other instances, such as in the seasonal cycling of phytoplankton blooms, the spread of viral infection through an algal population or following excessive ROS-induced damage,[100] the benefits of (autophagy-dependent) PCD on a large-scale appear plausible (and even provide a rational explanation for ill-understood examples of phytoplankton cultures that crash in the laboratory).[101] The possible ecological significance of PCD, and its link with autophagy, therefore provides interesting parallels with parasitic protists where occurrence of PCD in vector-borne parasites has been proposed as an adaptation that aids life-cycle progression (reviewed in ref. 102). A need for altruistic PCD, coupled with an accommodation of seasonal variation in nutrient availability, provides obvious selective pressures for the retention of 'classical' *ATG* genes in the genomes of ubiquitous pico-phytoplankton, such as the Ostreococcus species. In contrast, autophagy-dependent remodeling of cellular metabolism has either been lost or severely moderated in the extremophile *Cyanidioschyzon merolae* (**Fig. 2**), which is found only within acidic warm springs. We speculated previously that limited variation in the supply of or competition for, core nutrients may have, at least in part, facilitated loss of ATG homologues and a capacity for macroautophagy.[14] More obvious, clear-cut examples of autophagic cell death in algae are perhaps likely to be found during the development of species such as *Volvox carteri*, a multicellular green alga and a close evolutionary relative of *C. reinhardtii*.[103]

Molecular characterization of the Chlamydomonas ATG8 homologue has recently been reported.[104] Here, stress- and rapamycin-induced processing and relocalization of CrATG8 were described, consistent with morphological and genomic studies suggesting the occurrence of a typical autophagy pathway. Interestingly, the rapamycin-sensitive TOR-LST8 complex implicated in the regulation of autophagy initiation in Chlamydomonas[104,105] localizes to the peri-basal body region of the alga.[106] The overlapping localization of TOR and LST8 homologues in *C. reinhardtii* within the vicinity of the flagellar basal bodies not only strengthens existing data linking flagellum function and intracellular cell signalling in this alga (reviewed in ref. 107), but also draws to mind the increase in mTOR activity in renal epithelial cells from human patients with polycystic kidney disease or mouse models of polycystic kidney disease where intracellular signalling is perturbed due to defects or loss in function of immotile primary (or sensory) cilia.[108,109]

Functional Analyses of Autophagy in Parasitic Protists

Parasitic protists such as the organisms responsible for African sleeping sickness (*Trypanosoma brucei*), Chagas' disease (*Trypanosoma cruzi*), leishmaniasis (Leishmania), malaria (Plasmodium), toxoplasmosis (Toxoplasma), East Coast Fever and Tropical Theileriosis (Theileria) and various kinds of diarrheal diseases (e.g., Entamoeba), have received considerable interest from the scientific community, notably biochemists and molecular and cell biologists, because of the serious diseases they cause, often to people and domestic animals in developing countries. Since safe and efficient drugs are still not available or are undermined by drug resistance, there is considerable pressure to identify new drug targets and develop lead compounds for therapeutic intervention. Autophagy could represent a valuable drug target, as it may lead to autophagy-induced cell death. Beside this, protists may serve as model organisms, because in many respects they represent early exponents of evolution and may be easier to analyze than higher eukaryotes. Roles for autophagy during exponential growth and encystment in *Entamoeba histolytica* and the encystment of another amoebal pathogen, the opportunist *Acanthamoeba castellanii*, have been reported previously.[110,111] However, most of the experimental analysis regarding

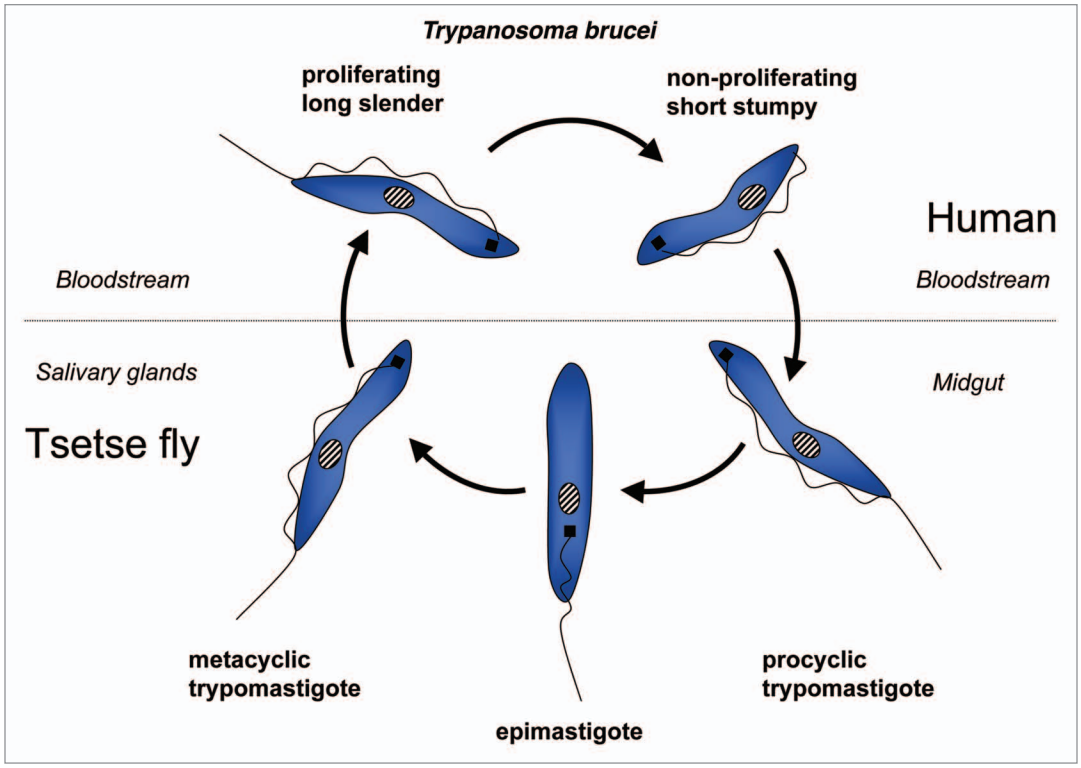

Figure 6. Diagram of the life cycle of *Trypanosoma brucei.*

autophagy and parasitic protists has been performed with trypanosomatid and apicomplexan parasites. This work is discussed below.

Autophagy in Trypanosomatidae. *Trypanosomatids: life cycle, morphology and metamorphosis.* Trypanosomatidae belong to the order Kinetoplastida that comprises flagellated protists characterized by the presence of a DNA-containing compartment, the kinetoplast, at the base of the flagellum. This compartment is actually part of the single mitochondrion present in these organisms, and the kinetoplast DNA is a complex of standard mitochondrial DNA with other DNA, having a unique structure and function (reviewed in ref. 112). All known species of the Trypanosomatidae are pathogenic for human, animals or crops. Best studied are the human pathogens *T. brucei*, responsible for sleeping sickness in sub-Saharan Africa, *T. cruzi*, the causative agent of Chagas' disease in Latin America, and various Leishmania species causing a variety of disorders in tropical and subtropical areas worldwide. These are all so-called neglected diseases (because of the little attention they receive from pharmaceutical companies due to the limited market potential in the countries of affected people) that often are fatal without adequate treatment, and for which efficacious, nontoxic drugs are not generally available. Each of these parasites has a complicated life cycle (**Figs. 6–8**). They are transmitted between human (or other mammals) by insects and undergo in each of their hosts sequential developmental changes resulting in alterations in their morphology which may be considerable, i.e., major changes in cell size and form; repression or derepression of the mitochondrion; expression of a flagellum providing high motility or immotile forms without flagellum; position of organelles, etc.

T. brucei is an extracellular parasite that is introduced as a metacyclic form in the mammalian bloodstream, from the tsetse fly's saliva when the insect takes a blood meal (**Fig. 6**). Once in the blood, parasites proliferate as a form that has a slender morphology. During the course of infection, the parasites differentiate into nonproliferating stumpy trypanosomes, which are pre-adapted to life in the tsetse fly's midgut once taken up during a blood meal. Stumpy trypanosomes differentiate into procyclic forms before they migrate to the salivary glands, while undergoing several additional developmental changes to form new metacyclic forms that are infective to mammals.

T. cruzi infections are caused by reduviid bugs when they defecate during feeding. Infective metacyclic trypomastigotes from the feces enter the host through skin lesions and mucous tissues (**Fig. 7**). The trypomastigote stages do not divide but spread through the blood infecting any tissue, but especially muscle tissue of the heart and alimentary tract, where they develop into proliferating amastigotes that are responsible for the pathogenic symptoms and, after several rounds of multiplication, develop into new trypomastigotes that are released in the blood and will invade new cells. The trypomastigotes can also be taken up again by an insect and develop in its gut into epimastigote forms. In the rectum, where the insect's urine is discharged, the epimastigotes differentiate into human infective metacyclic trypomastigotes, induced by the locally very low nutritional content.

Leishmania species are transmitted by sandflies, which infect the host with metacyclic promastigotes from the gut, by

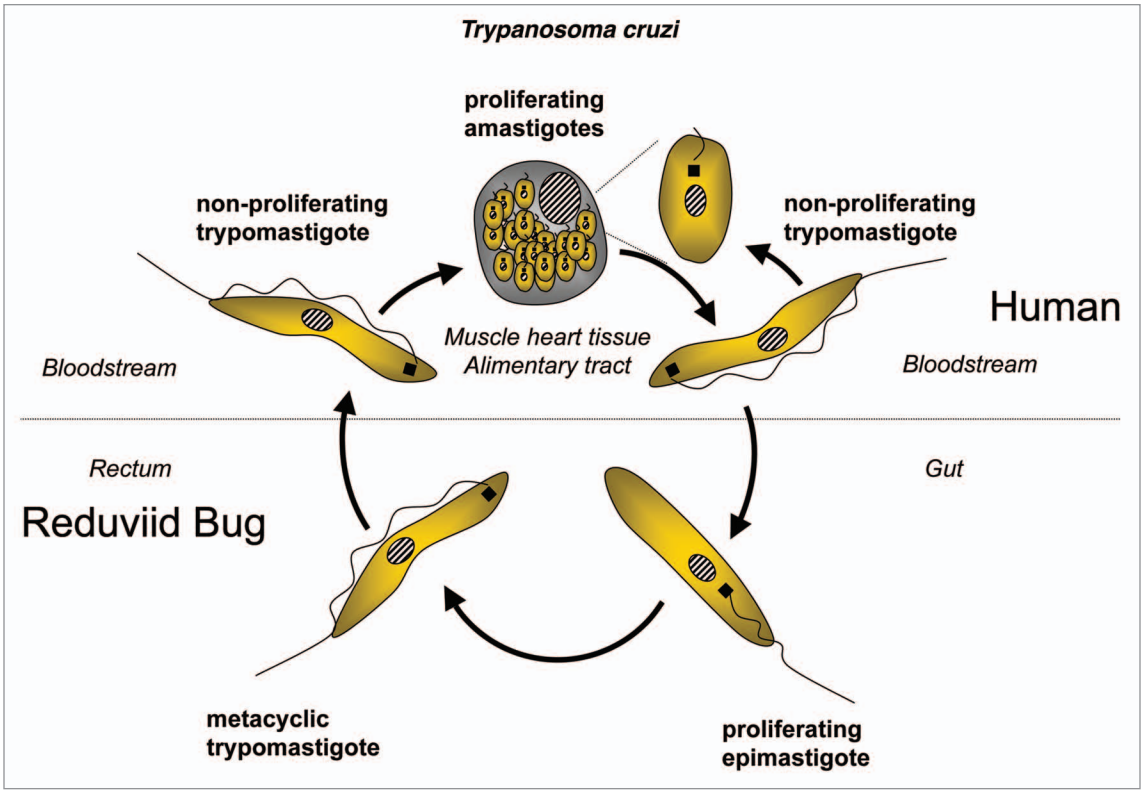

Figure 7. Diagram of the life cycle of *Trypanosoma cruzi.*

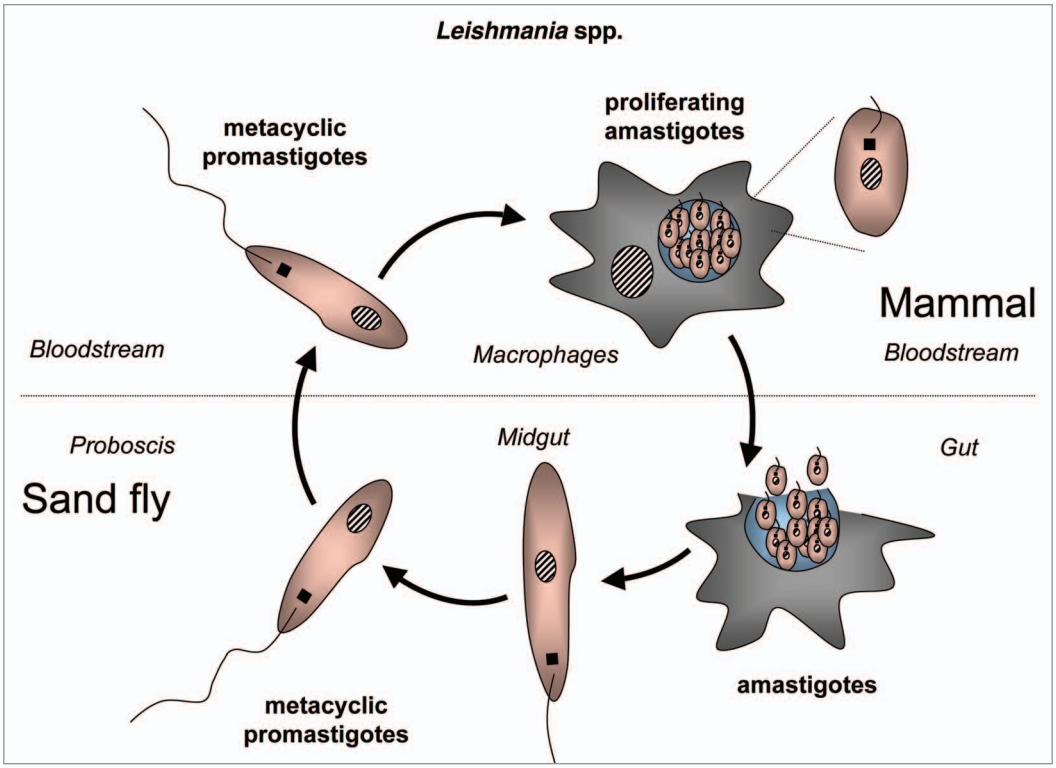

Figure 8. Diagram of the life cycle of Leishmania spp.

regurgitation during feeding (**Fig. 8**). In the mammalian host, these infected forms enter macrophages where they multiply as amastigotes in the phagolysosomes.

During the developmental cycles these parasites encounter highly different environments with different nutrients, different levels of oxygen supply and different temperatures. For example, *T. brucei* when living in blood encounters a rather constant, abundant supply of glucose. It generates ATP only by glycolysis because the mitochondrion is nearly completely repressed. In contrast, glucose is rarely present in the tsetse midgut. Therefore, amino acids, notably proline, become the major energy source, and they are oxidized in the then well-developed mitochondrion. Similarly, nutrients available to intracellular amastigotes of *T. cruzi* and Leishmania spp., present in the cytosol and phagolysosomes, respectively, are different from those in the blood and insect gut. Consequently, the metabolic profiles may differ in the different life-cycle stages of the various parasites, and it is expected that, at least where important metabolic reprogramming occurs, autophagy plays a major role in achieving this during transitions from one stage to the next.

Autophagy in Trypanosomatidae as inferred from bioinformatics studies of genome sequences. Genome database searches supplemented with more advanced analyses have revealed that only half of the autophagy-associated components characterized in *S. cerevisiae* are present in trypanosomatids.[14,15,17,18,20] However, comparisons with other organisms show less dramatic differences since data suggest that a number of autophagy-related proteins e.g., Atg19, Atg21, Atg22, Atg23, Atg25, Atg28, Atg29, Atg30 and Cis1/Atg31 arose as innovations in the fungal lineage: There is no good evidence that they have orthologues outside fungi.[6,14] Nevertheless, it remains likely that further lineage-specific innovations, as well as expansions outlined elsewhere, remain to be discovered. Until they are unraveled, it seems impossible to assess the true degree of similarity between the trypanosomatid autophagy machinery and the equipment of other species.

ATG8 has undergone an unusual evolution within the genus Leishmania.[23] Bioinformatics analysis reveals that the *L. major* genome has 25 ORFs encoding ATG8-like proteins. They could be classified into four families, designated ATG8, ATG8A, ATG8B and ATG8C. The four families are also present in other Leishmania species, but with different copy numbers. The ATG8 protein is in all cases encoded by a single-copy gene. The members of the other three families are encoded by gene arrays. Experimental studies by Williams et al.[23] discussed below, indicated different functions for the different families, although many questions remain to be answered about these functions and the reason for expansion of the ATG8 repertoire in this trypanosomatid genus. In contrast to the situation in Leishmania, the number of ATG8 genes found in *T. brucei* and *T. cruzi* is much more limited. *T. brucei* contains three isoforms of ATG8, designated ATG8.1, ATG8.2 and ATG8.3, while two isoforms, ATG8.1 and ATG8.2, have been detected in *T. cruzi* (discussed further below). The finding of multiple ATG8 homologues may reflect either functional redundancy and/or the ability of the proteins to carry out additional function(s).

Results of experimental studies. Trypanosoma brucei. The morphology of the different life-cycle stages of African trypanosomes has been studied in great detail by electron microscopy in the 1970s by Vickerman and colleagues.[113,114] These studies provide the first indications that autophagy plays an important role during the transition from one differentiation stage to another. However, experimental evidence of autophagy in these organisms was only obtained in the last few years, after ATG homologues had been discovered and their function studied using specific antisera, inhibitors and reverse genetics. Rapamycin, the classical inhibitor of TOR, induces growth inhibition leading to cell death of bloodstream-form *T. brucei* with the formation of vacuoles stained with monodansylcadaverine that were proposed at the time to be autophagosomes.[22] However, this effect was only seen at a high concentration (EC_{50} 6.5 μM) of rapamycin, at least 10-fold higher than that required to induce autophagy in yeast. No apoptotis or necrosis was observed. In addition, electron microscopy suggested the formation of phagophores, autophagosomes and autolysosomes. Rapamycin also caused formation of densely packed membrane clusters associated with the kinetoplast near the flagellar pocket, which was also observed upon starvation, but not under several other stress conditions. These clusters were not seen in differentiating cells.

TOR is a kinase that regulates the balance between protein synthesis and degradation via autophagy. There are two different TOR-containing complexes with distinct functions: TOR complex 1 (TORC1) controls processes such as ribosome biogenesis, transcription, translation and induction of autophagy, while TORC2 controls spatial aspects of cell growth by actin cytoskeleton remodeling (review in ref. 115). Metazoans coordinate cell growth via a single TOR kinase (mTOR), while some single-cell organisms such as yeast have two TOR proteins, Tor1 and Tor2. Nonetheless, the different TOR complexes and their distinct functions are conserved in all eukaryotes studied. Like some other unicellular eukaryotes, trypanosomes contain two TOR kinases, TOR1 and TOR2; however, two additional TOR-related proteins (TOR-like 1 and TOR-like 2) have also been described.[116] TOR1 and TOR2 participate in the complexes TORC1 and TORC2, respectively, which differ in function, subcellular localization and rapamycin sensitivity in trypanosomes.[116] As in yeast, TORC1 and TORC2 are associated with the orthologues KOG1/raptor and AVO3/rictor. TOR1 is predominantly nuclear, whereas TOR2 is excluded from the nucleus and is associated with the ER and the mitochondrion. TORC1 is involved in regulating cell polarization and cytokinesis. In particular, rapamycin, with an EC_{50} of 152 nM severely reduces proliferation by inhibiting TORC2 formation. However, contrary to the situation in other eukaryotes, rapamycin, even at concentrations up to 1 μM, appears to have no effect on TORC1, since no cell cycle arrest in G_1 or protein synthesis inhibition are detected. Moreover, a rapamycin-FKBP12 complex does not bind to the recombinant TOR1 rapamycin-binding domain, while it does to TOR2 in vitro and in vivo. Although trypanosome TORC1 is insensitive to rapamycin, it was shown by RNA interference that TOR1 knockdown triggers the appearance of autophagic-like vesicles. Its depletion also causes morphological changes such

as abnormal appearance of the ER and formation of membranous whorls similar to those that occur in other eukaryotes upon TOR1 inhibition.[117]

Recently, punctate structures that were proposed to be autophagosomes, detected by expression of a fusion construct of yellow-fluorescent protein and ATG8.2, have been described for procyclic *T. brucei* cells exposed to ER stress.[118] Furthermore, both ATG8.1 and ATG8.2 have been validated as autophagosome markers in both bloodstream and procyclic form *T. brucei* (Proto W and Mottram JC, unpublished).

Since TORC1 is entirely insensitive to rapamycin, it remains to be determined how the morphological effects observed after treatment with high concentrations of this compound are caused and if the observed structures are indicative of death by autophagy.

Another molecule that induces autophagy in African trypanosomes is dihydroxyacetone (DHA).[19,119] Many cells can use DHA as an energy substrate because, after its phosphorylation by DHA kinase, it may enter the glycolytic pathway. Trypanosomes, however, which take up DHA via aquaglyceroporins, lack this kinase. As a consequence, DHA causes growth inhibition (at an EC_{50} of 1 mM) as a result of cell cycle arrest in the G_2/M phase and morphological alterations (multivesicular bodies, vacuoles, characteristic changes of mitochondrial structure) typical of autophagy.

Also, some neuropeptides kill the mammal-infective form of *T. brucei*. Surprisingly parasite death does not occur by the classical antimicrobial peptide mechanisms, i.e., by forming pores or inducing membrane damage. Instead the neuropeptides are endocytosed and appear to induce autophagic cell death of the trypanosomes.[120,121] The authors show that endocytosed peptides reach the lysosome whose integrity is subsequently disrupted. In addition, the peptides can be released from the late endosomes or lysosome into the cytosol, where they accumulate in various intracellular compartments. Interaction of peptides with glycosomes causes partial relocalization of the glycosomal enzymes, with the consequence that the cellular ATP is depleted. The failure of their energy metabolism results in cell death with involvement of autophagy, as suggested by ultrastructural morphological analysis, whereas no signs of necrosis or apoptosis are observed. These studies, which show the progressive formation and fusion of multiple lysosome-like vesicles and autophagic-like vacuoles, are corroborated by the results of immunostaining using mammalian anti-LC3 (ATG8) antibodies that are suggestive of the appearance of autophagosomes.

As mentioned above, *T. brucei* possesses three isoforms of ATG8 called ATG8.1, ATG8.2 and ATG8.3. ATG8.3 contains a unique insertion that is positioned near the interface in such a way that the binding properties of this protein might be different from those of other ATG8-like proteins. Moreover, some analyses suggest it is syntenic with an ATG8-like protein identified in *L. major* that has functionally been shown to act as an ATG12. ATG8.1 ends with a Gly residue and is therefore expected to be active without a need for processing by ATG4. The crystal structure of ATG8.2, with a C-terminal extension that should be processed, has been determined. It has the typical ubiquitin fold and its overall structure is very similar to that of its human homologue ATG8/LC3.[122] *T. brucei* contains two ATG4 homologues, one of which (the closer relative of human ATG4) was modeled using available ATG4 crystal structures from other organisms. The model was docked onto the TbATG8 structure, and the complex compared with the known structure of the mammalian LC3-ATG4 complex. Despite the low sequence identity (30% between human and trypanosome ATG4) the overall structure is very similar and the catalytic C-D-H triad is also present in TbATG4, strongly suggesting conservation of function.[122]

Among the various unique features of Kinetoplastida is the possession of glycosomes.[123] These organelles, which may be present in 10–100 copies per cell, dependent on the species and the respective developmental stage, belong to the peroxisome family but, unlike typical peroxisomes, contain the majority of the glycolytic enzymes, hence their name. In some of the Trypanosomatidae, such as the bloodstream form of *T. brucei*, glycolytic enzymes may contribute over 90% of the protein content of the organelles. Enzymes of some other important catabolic and anabolic pathways may also be present in glycosomes, such as those involved in gluconeogenesis, the pentose-phosphate pathway, β-oxidation of fatty acids, purine salvage and the biosynthesis of pyrimidines, ether lipids and squalenes, as well as typical peroxisomal enzymes responsible for peroxide metabolism. Dependent on the species, the importance of these different processes may vary with the environmental conditions encountered during the life cycle of the parasites. Indeed, glycosomes of African trypanosomes exhibit considerably different enzyme content among successive life-cycle stages. Consequently, an enhanced turnover of glycosomes during differentiation from one stage to another is expected. Pexophagy is invoked to be responsible for this turnover.[18,21] Indeed, immunofluorescence microscopy studies reveal a small tendency of glycosomes to associate with the lysosome during differentiation from the slender to the stumpy bloodstream form: Colocalization is observed in 40% of the trypanosomes, whereas less than 10% of nondifferentiating cells display such colocalization. A fast and drastic increase in the association of the organelles is observed in all trypanosomes during their transition from stumpy to procyclic forms. Moreover, the lysosome shows a transient but dramatic increase in size during this latter differentiation process. Immunoelectron microscopy confirms the autophagic degradation of glycosomes and the appearance of glycosomal enzymes within lysosomes during the differentiation steps.[21] Interestingly, the observed surrounding of glycosomes by the lysosome is suggestive of a process similar to micropexophagy, whereas the size increase of the lysosome may rather suggest macropexophagy involving the formation of autophagosomes. Indeed, bioinformatics analysis reveals putative orthologues of most of the Atg proteins specifically involved in micropexophagy in *S. cerevisiae*.[18] In contrast, in Leishmania species, only macroautophagy has been experimentally observed so far (see below).[16,17] Whether African trypanosomes and Leishmania spp. use different degradation pathways or if both pathways can function in all trypanosomatids but the induction of either depends on the conditions encountered, as observed for *H. polymorpha* and *P. pastoris*,[124] remains to be determined.

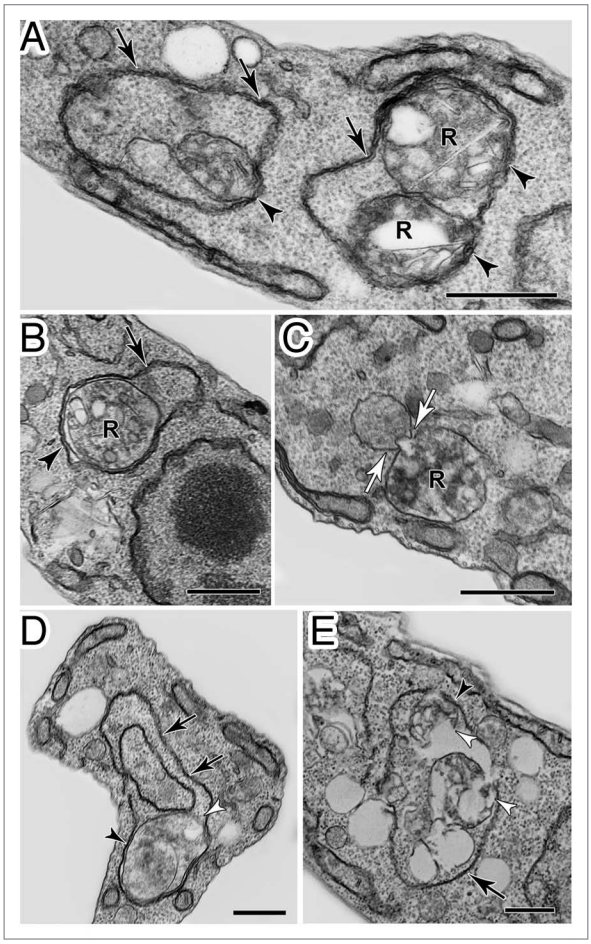

Figure 9. Transmission electron microscopy analysis of *Trypanosoma cruzi* epimastigotes treated with naphthoimidazole. (A, B, D and E) The parasites showed well-developed endoplasmic reticulum profiles (black arrows) surrounding organelles, especially reservosomes (R), suggesting a close contact between both membranes (black arrowheads), and commonly loss of the integrity of these organelle structures (white arrowheads). (C) A cytoplasmic vesicle near to a reservosome was also observed, suggesting fusion with the organelle (white arrows). Scale bars = 0.5 μm.

Table 1. Autophagic events in *T. cruzi* treated with naphthoimidazoles

	Epimastigotes	Trypomastigotes
RE profiles close to organelles	+	+
Concentric membrane structures	+	+
ATG RNA detection	+	nd
Increase in number of autophagic vacuoles	+	+
Autophagy inhibition	+	+

nd, not determined.

Trypanosoma cruzi. Several papers have reported ultrastructural changes indicative of autophagy after treatment of *T. cruzi* parasites with toxic compounds or their exposure to adverse conditions. Protein kinase and PtdIns3K inhibitors like wortmannin, staurosporin and genistein led to growth inhibition of epimastigotes and caused various changes including the formation of autophagosome- and myelin-like structures. No effect was found on division of intracellular amastigotes or their differentiation to trypomastigotes.[125] In epimastigotes, the lysophospholipid analogue edelfosine, alone or combined with the steroid inhibitor ketoconazole, induces morphological alterations, with changes in plasma membrane and lysosomes (also called reservosomes in this parasite), and promotes the appearance of autophagic structures and multinucleation.[126] Other trypanocidal agents such as the dinitroanilines trifluralin and chloralin lead to the appearance of vacuoles containing damaged membranes.[127] Also some natural products such as geranylgeraniol, propolis and naphthoquinones lead, in epimastigote and trypomastigote forms, to the appearance of concentric membrane structures in the cytosol, which are apparently associated with an autophagic process.[127,128]

Ultrastructural studies in epimastigotes and trypomastigotes treated with either one of three naphthoimidazoles N1, N2 and N3 derived from β-lapachone demonstrate that the mitochondrion, Golgi and reservosomes are the main targets.[128,129] Frequently, treatment with these three compounds induces morphological autophagic characteristics in the parasites such as the occurrence of concentric membrane structures in the cytosol and an increase in the number of autophagosome-like bodies, observations reinforced by the strong increase of monodansylcadaverine labeling.[130] The effect of the naphthoimidazoles on both forms of *T. cruzi* is blocked by E64 and calpain I inhibitor,[129] suggesting the participation of calpain in the autophagic process as in mammalian cells.[131] Pre-incubation with the autophagic inhibitors wortmannin and 3-methyladenine (3-MA) completely abolish the effects of N1, N2 and N3, pointing to autophagic cell death as a consequence of this treatment.[130] In treated epimastigote forms, extensive ER profiles surround structures, especially reservosomes, proving a close contact between the membranes of both organelles (**Fig. 9 and Table 1**). This morphological finding supports the hypothesis that the ER provides membranes for PAS formation in *T. cruzi*, as recently shown for mammalian cells.[33,34] The participation of reservosomes in *T. cruzi* autophagy deserves further investigation, as this organelle, a pre-lysosomal compartment present only in epimastigotes,[132] may participate in autolysosome formation as lysosomes do in mammalian cells.

Subsequent studies unambiguously confirmed the occurrence of autophagy in *T. cruzi*. As mentioned above, homologues of about 20 yeast Atg proteins were identified. One of the steps where the bioinformatics analysis[18] was confirmed by experimental data is vesicle expansion and completion.[20] Although sequence length varies between the *T. cruzi* homologues and the corresponding yeast Atg protein, the protein domain composition remains conserved, supporting their proposed function. As has also been seen in some other trypanosomatids, *T. cruzi* may contain more than one protein where only a single one is found in yeast. Cases in point are ATG4-like and the ATG8-like proteins.[20] *T. cruzi* contains two ATG4 and two ATG8 homologues, in contrast to the single-copy genes in yeasts. Like in *T. brucei*, one of these ATG8 homologues is possibly syntenic with *L. major* ATG12, but biochemical support for an ATG12 activity remains to be provided. Each of the *ATG4* gene copies (*TcATG4.1* and *TcATG4.2*) could,

with similar efficiency, complement a yeast deletion strain and restore the Cvt and autophagy pathways, whereas only one of the *ATG8* genes (*TcATG8.1*) is able to functionally reconstitute autophagy in the yeast deletion mutant, and the corresponding protein concentrates in large vesicles in epimastigotes after the cells are subjected to starvation.[20] Mutation of the conserved Gly residue abolishes the localization of ATG8 into the vesicles, indicative of the need of ATG8-PE conjugation for association with the membrane. Only TcATG4.1 is able to efficiently process both ATG8.1 and ATG8.2 in vitro, suggesting that this protein is involved in autophagy, whereas the role of ATG4.2 remains to be established. Together, these results suggest that the ATG8 conjugation system in *T. cruzi* functions very similarly to that in yeasts and mammalian cells.

RT-PCR data indicate the participation of ATG3, ATG4, ATG7 and ATG8 in the autophagic process in epimastigotes treated with naphthoimidazoles,[130] although the detailed pathway and proteins involved in the mechanisms of cell death require further investigation.

A major differentiation step in the life cycle of *T. cruzi* is the transition of epimastigotes into metacyclics triggered by nutritional stress within the rectum of the insect vector. This can be reconstituted in vitro, by starvation of cultured epimastigotes. Initially, the epimastigotes have access to sufficient nutrients due to the reservosome, an organelle unique to this stage. It is part of the lysosomal system and harbors endocytosed proteins and lipids, as well as secretory proteins synthesized by the parasite.[133] Upon starvation, the proteins are degraded by cruzipain,[20,134] the most abundant cysteine peptidase of the lysosome and reservosome and whose activity is critical for differentiation. The organelle then shrinks and disappears. Starved epimastigotes as well as cultured epimastigotes undergoing spontaneous differentiation to metacyclic trypomastigotes display structures related to autophagosomes that are highlighted by TcATG8.1 staining.[20] The staining decreases considerably in metacyclics where, during differentiation, the ATG8 signal colocalizes with that of serine carboxypeptidase and decreases considerably in metacyclics, thus indicative of delivery of the autophagosome content to the reservosome/lysosome system.

Leishmania spp. The importance of protein turnover for differentiation of Leishmania has been known for several decades. Different life-cycle stages vary considerably in morphology, and the same holds true for its unusual lysosome. In promastigotes, the lysosome is present as a large single vesicular structure also known as 'multivesicular tubule' (MVT). It has, however, a low lytic capacity and a relatively high lumenal pH value. Upon differentiation of promastigotes into intracellular amastigotes the shape of the organelle changes into a large compartment with aspect, size and number dependent on the species. Moreover, its lytic capacity increases considerably. Initially this organelle was called a 'megasome' because of its large size. Megasomes may represent 5–15% of the total cell volume.[135,136] Furthermore, Leishmania is known to have high peptidase activity, particularly of cysteine peptidases, that is upregulated during metacyclogenesis, the transformation of metacyclic promastigotes to amastigotes. The peptidases are involved in cellular reshaping and metabolic reprogramming during differentiation and also nutrient acquisition by the different life-cycle stages. Genome sequencing reveals a large arsenal of peptidases in *L. major*—at least 154 peptidases representing 1.8% of the genome—and a very similar repertoire of such enzymes in other Leishmania species (reviewed in ref. 137). Two main peptidases are the lysosomal cysteine peptidases CPA and CPB; the expression of these increases considerably during differentiation in parallel with the appearance of megasome-like structures. Leishmania, like all trypanosomatids, lack orthologues of the yeast major vacuolar aspartic peptidase Pep4 and serine peptidase Pbr1. There is evidence that their function is replaced by CPA and CPB, as inhibition of these enzymes or deletion of both genes results in promastigote cells that are more susceptible to nutrient withdrawal, fail to differentiate and do not degrade GFP-ATG8 labeled autophagosomes within the lysosome.[17]

Besteiro and coworkers also analyzed the phenotype of a *L. major* dominant-negative mutant of the yeast Vps4 orthologue, an ATPase known to be involved in the endosomal system and notably in the formation of late endosomes with a multivesicular aspect called multivesicular bodies (MVB). Indeed, as predicted, the mutant form of the VPS4 accumulated in vesicles of the endocytic pathway, and those cells are deficient in endosome sorting.[16] Interestingly, autophagy and differentiation are also affected in these mutants. In wild-type cells, the GFP-ATG8 staining is most intense, and autophagosomal structures are most abundant, during differentiation of procyclic promastigotes to infective metacyclic promastigotes. In wild-type metacyclic parasites, the signal moves to the MVT-lysosome. However, in the mutant cells the number of autophagosomal structures that are stained remains high throughout. Furthermore, the level of ATG8–PE conjugate is consistently higher than in wild-type cells. These data suggest an inability of the autophagosomes to fuse with the endosomal/MVT-lysosomal compartment. They also underscore the importance of late endosomal function, and more specifically autophagy, for the transformation to the infective metacyclics. *L. major* contains two *ATG4* genes, *LmATG4.1* and *LmATG4.2*. A null mutant of the cysteine peptidase ATG4.2 is defective in the ability to differentiate, corroborating the earlier notion based on the results obtained with the VPS34 mutant that autophagy plays a role in this process.

As mentioned above, Leishmania species contain a large number of ATG8-like proteins, which can be classified into four families. The 25 *'ATG8'* genes in *L. major* code for one ATG8, three ATG8A, eight ATG8B and 13 ATG8C proteins. Despite the low sequence identities with ATG8 homologues of other organisms, LmATG8 and representatives of each of the three other families are able to complement a *S. cerevisiae atg8* null mutant, indicating that they can fulfill the Atg8 function in the Cvt pathway. However, a complete ATG8 physiological function can probably only be attributed to LmATG8, as it is involved in autophagosome formation during both differentiation and starvation. In contrast, the role of LmATG8A is probably only in starvation-induced autophagy as suggested by the observation that it forms puncta upon starvation. LmATG8B and LmATG8C are both located close to the flagellar pocket, but from the pattern of labeling of GFP-fusion products seem not to be involved in autophagy.

In addition to the four *ATG8* gene families, *L. major* has another *ATG8*-like gene that codes for a protein with a C-terminal extension beyond a scissile glycine that becomes exposed by ATG4 activity prior to conjugation to ATG5. Intriguingly, it has a 58-residue insertion somewhat similar to that seen in the yeast ATG12, raising the possibility of its having an ATG12-like function (see below).

L. major has two ATG4 genes, *LmATG4.1* and *LmATG4.2*. LmATG4.1 exerts proteolytic activity towards ATG8, ATG8B and ATG8C, whereas LmATG4.2 shows only activity towards ATG8A. Searching the *L. major* genome, possible candidates are also found for ATG5, ATG10 and ATG12, although with low similarity with their orthologues in other organisms. These parasite genes are able to complement the respective yeast mutants, confirming their functional identity. However, the *ATG12* gene complemented the respective mutant only when expressed without residues beyond the scissile glycine. In this heterologous expression system it is not processed by ATG4.1 or ATG4.2, suggesting that processing naturally in Leishmania requires an as yet to be identified peptidase. Furthermore, ATG12 localizes in part to ATG8-containing puncta in *L. major* promastigotes, suggesting the functionality of the ATG12-ATG5 conjugation pathway in this parasite. Using an in vitro assay, biochemical evidence has been obtained that ATG12 can be conjugated with ATG5 (Williams R, Mottram JC and Coombs GH, unpublished results). Interestingly, *L. major* ATG12 fails to cluster reliably with yeast and mammalian ATG12 sequences by phylogenetic analyses (neighbor-joining, minimum evolution and maximum parsimony trees calculated with MEGA-4;[138] Daniel J. Rigden, data not shown) and bears a closer resemblance to ATG8 sequences, particularly in the N-terminal region. Thus, it appears possible that ATG12-like activity may have evolved more than once. The same phylogenetic analysis fails to consistently cluster any trypanosomal sequences with *L. major* ATG12, including those syntenic with it (see above), again highlighting the need to assess the roles of the latter experimentally.

Although Leishmania, like *T. brucei,* encounters different nutritional conditions during its life cycle, there are no conclusive data showing that the differentiation of promastigotes to amastigotes is accompanied with metabolic reprogramming involving an increased turnover of glycosomes by pexophagy, as is observed during differentiation of *T. brucei*. The size of amastigotes is reduced significantly, however, so it is likely that some glycosomes are digested.[135,139] Pexophagy thus may be involved in adjusting the glycosome number, consistent with other observations on the role of autophagy in cellular remodeling. Gluconeogenesis rather than glycolysis is more important in amastigotes than in promastigotes and indeed proteomic analysis shows a two-fold decrease of the last three enzymes of the glycolytic pathway—phosphoglycerate mutase, enolase and pyruvate kinase, which are all present in the cytosol—during the late stage of differentiation. In contrast, the levels of glycolytic enzymes within the glycosomes are on average 1.4-fold increased.[140] It seems probable that the altered metabolic fluxes result largely from the different nutrient availability and/or post-translational regulation of enzyme activity, rather than from a different enzyme network.[141]

Autophagy in Apicomplexa. *Apicomplexa: life cycle, morphology and metamorphosis.* Apicomplexa form a large group of protists comprising approximately 5,000 known parasitic species, but this number is probably a gross underestimation. This group is characterized by an apical complex, a collection of organelles at the anterior end of the cell that serves to penetrate host cells. Apicomplexa are exclusively parasites of animals, both vertebrates—including humans, livestock and other mammals—and invertebrates. Apicomplexa are responsible for very serious human diseases like malaria and toxoplasmosis, as well as diseases of economically important livestock like theileriosis, babesiosis and coccidiosis. They are caused by species of the genera Plasmodium, Toxoplasma, Theileria, Babesia and Coccidia, respectively. In particular, malaria is a worldwide public health problem with 3.2 billion people at risk, an estimated 350–500 million new cases each year and over one million deaths annually. Apicomplexa have complex life cycles (**Fig. 10**), involving both asexual and sexual reproduction, but with considerable variation among the different subgroups. They can be transmitted to new hosts by insects, via feces or when a predator eats an infected prey. Typically, motile forms called sporozoites enter host cells within which the parasite divides to produce numerous merozoites. When the host cells burst, merozoites are released and infect new and sometimes different host cells, a process that can occur repeatedly. Some haploid asexual forms transform into sexually reproductive cells or gamonts. When haploid gamonts join in pairs to form gamontocysts, division occurs to produce multiple gametes. Pairs of gametes fuse to form zygotes, which by meiosis result in new sporozoites, so completing the life cycle.

In the next section, the role of autophagy in Plasmodium species will be discussed. Much less is known as yet about autophagy in other Apicomplexa genera. However, the role of host autophagy in controlling infections by Toxoplasma and Theileria is a topic of intense study and will be discussed in a later section of this review.

Plasmodium. Malaria sporozoites are transmitted from the mosquito vector to the mammalian host and first take up residence in the liver before initiating red blood cell infection (**Fig. 10**). Following penetration into hepatocytes, the parasite converts from an invasion-competent, motile, elongated sporozoite to a metabolically active, round trophozoite. Subsequent to this conversion, a mitotic phase begins, characterized by multiple fissions of nuclei, transforming the parasite into a giant syncytium (up to 10,000 nuclei). The end of the schizogony marks a phase of organelle biogenesis that is critical for erythrocyte invasion. Mature merozoites enclosed in a membrane, called a merosome, are released from hepatocytes and invade red blood cells: This erythrocyte infection is the cause of the pathology associated with malaria.[142-144]

The sporozoite is adeptly equipped for migration through tissues and penetration into hepatocytes. It possesses a robust membrane cytoskeleton essential for the maintenance of its shape and motility. The parasite body is delimited by a specialized cortical structure that is composed of the plasma membrane tightly associated with flattened membrane cisternae forming the inner membrane complex (IMC).[145] The IMC is continuous along the entire

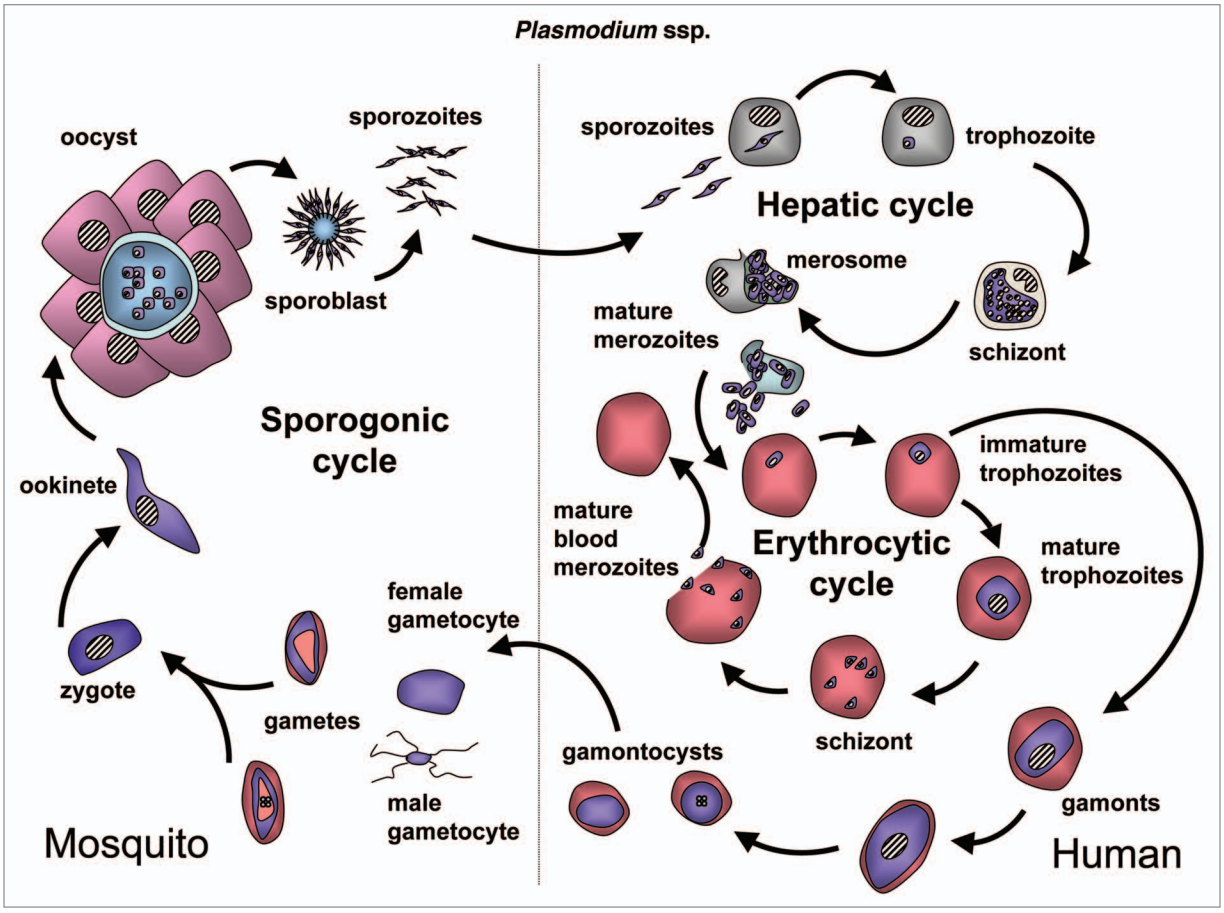

Figure 10. Diagram of the life cycle of Plasmodium spp.

length of the sporozoite. The process of sporozoite-to-trophozoite conversion is characterized by a spectacular change of the parasite shape and a major interior remodeling that is accompanied by the elimination of organelles unnecessary for the replication of the liver forms.[146] The beginning of the metamorphosis is marked by the dismantling of the IMC, inducing the repositioning of the parasite nucleus to the middle of the parasite. As this median bulbous region expands, the two distal ends of the sporozoite gradually retract and disappear, leading to parasite sphericalization. The IMC is then reorganized into dense lamellar arrays within the cytoplasm and is expulsed by converting sporozoites in the parasitophorous vacuole (PV) wherein the parasite resides.

During metamorphosis, other organelles such as micronemes are also cleared from the sporozoite. Micronemes are abundant secretory vesicles that contain proteins such as the thrombospondin-related anonymous protein (TRAP), which facilitate parasite adhesion to hepatocytes.[147,148] In converting parasites, micronemes are packaged into large compartments and partially discharged into the PV.[146] Sporozoites maintained under axenic conditions undergo similar transformations as intravacuolar parasites, demonstrating that the signals and triggers of sporozoite differentiation are not host cell-dependent.[146,149] At the completion of metamorphosis, mature trophozoites only retain organelles involved in biosyntheses, e.g., the ER, mitochondria and the three genomes (nuclear, mitochondria and apicoplast—i.e., the relic of a secondary endosymbiontic plastid; reviewed in ref. 150), that are necessary for the successive rounds of parasite divisions.

A critical role of autophagy for organelle turnover during the differentiation of parasitic protists that shuttle between an insect vector and mammalian host is clearly emerging.[21] Scrutiny of the cytoplasmic content of converting sporozoites reveals the presence of novel double-membrane structures containing organelles such as micronemes (**Fig. 11**). These double-membrane compartments are morphologically similar to mammalian autophagosomes and, interestingly, the peak of their biogenesis temporally coincides with the packaging of micronemes within the parasite.[146] These autophagosome-like profiles could then be indicative of functional autophagy in the malaria parasite. An autophagic activity is furthermore substantiated by treatment of parasites with the inhibitor 3-MA that induces a significant delay in the conversion process of sporozoites (Jayabalasingham B and Coppens I, unpublished data). 3-MA interferes with autophagy by blocking class III PtdIns3K activity.[151,152] The Plasmodium orthologue of the main enzymatic component of the PtdIns3K complex and the target of 3-MA, VPS34, has been recently identified and characterized in blood-stage malaria parasites.[152,153]

In addition to VPS34, Plasmodium genomes contain autophagy-related proteins. Of the 33 Atg proteins present in yeast,

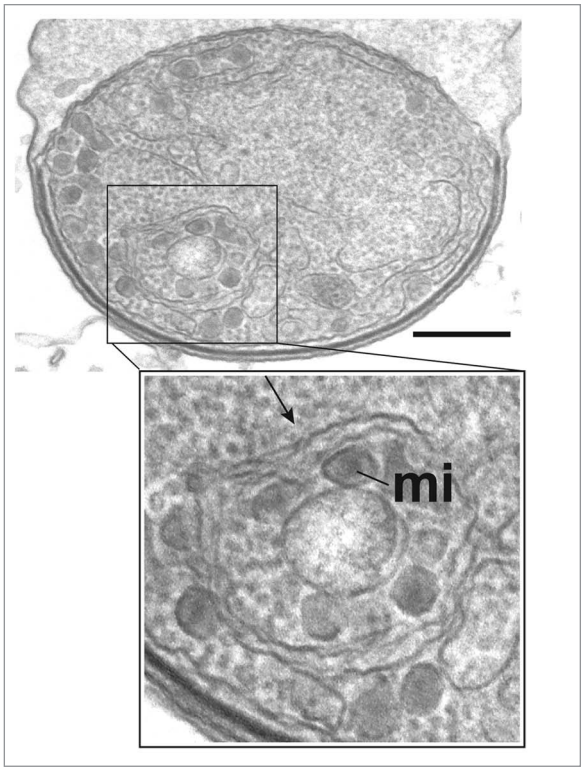

Figure 11. Presence of an autophagosomal structure in converting Plasmodium sporozoites. Electron microscopy of *P. berghei* sporozoites maintained in axenic condition for 3 h. The cytoplasm contains a double-membrane structure (arrow) sequestering micronemes (mi). Scale bar = 300 nm.

eight ATG putative orthologues have been clearly identified in the malaria parasites. These include: ATG1, ATG17 and ATG18 of the ATG9 cycling system, ATG12 of the ATG12 conjugation system, and all components of the ATG8 conjugation system comprising ATG3, ATG4, ATG7 and ATG8.[14] The malaria parasite is therefore another example of a protist that apparently harbors a limited repertoire of autophagy-related genes. As reported for other protists, it remains also plausible that the set of ATG proteins expressed by Plasmodium define the minimum conserved cohort required for functional autophagy in this parasite.

The putative ATG orthologues of the human malaria parasite *P. falciparum* and the rodent malaria parasite *P. berghei* are 50–87% identical to each other. Among the components of the ATG8 conjugation system, ATG4 of *P. falciparum* (or PfATG4) shares 33% identity with yeast Atg4. An ATG4 homologue in *P. berghei* cannot be accurately analyzed because of incomplete annotation of this region of the genome. PfATG7 and ATG7 of *P. berghei* (PbATG7) show 38% and 36% identity to yeast Atg7, and PfATG3 and PbATG3 are 26% and 29% identical to yeast Atg3. PfATG8 and PbATG8 are the most similar to their yeast counterparts, sharing 40% and 45% identity, respectively. A putative orthologue of Atg12 is present in the *P. falciparum* genome, but like ATG4 cannot be identified in *P. berghei*. PfATG12 shares 32% identity with yeast Atg12.

The single *P. berghei* orthologues of ATG8 and ATG3 share close structural similarity with their yeast and mammalian counterparts (Jayabalasingham B and Coppens I, unpublished data). Cloned PbATG8 is 124 amino acids long and its sequence predicts two N-terminal α-helices tethered to an ubiquitin fold core. The prediction of the PbATG8 structure is supported by the conservation of amino acids involved in maintaining the tertiary structure of the protein. PbATG8 is predicted to have two hydrophobic patches as well as a number of basic residues on one face of the protein that form the scaffold for binding to ATG3, ATG4 or ATG7. While the surface-exposed residues located on one side of all ATG8 homologues are highly conserved, many of the surface-exposed residues located on the opposite face of the protein exhibit low conservation, even among homologues expressed in humans. It is possible that the reduced conservation of residues on one face reflects a reduction in evolutionary constraints, and it is tempting to hypothesize that this face mediates the interactions that are unique to different ATG8 homologues. Such potential binding partners remain to be identified for the ATG8 homologues expressed in Plasmodium species. To this point, PbATG8 and its orthologues in other Plasmodium species all share a unique inserted loop consisting of nine amino acids between α-helix 3 and β-sheet 3, suggesting that such binding partners may exist. A similar insert is also present in ATG8 homologues expressed in other protist species such as *T. cruzi*, *T. gondii* and Entamoeba species, though no function has yet been ascribed to these species-specific insertions. Finally, a functionally important residue conserved among all ATG8 homologues is a C-terminal Gly. In most homologues of ATG8, this Gly is followed by additional amino acids that are cleaved from the protein by ATG4. In this respect, the Plasmodium ATG8 sequences contain a C-terminal glycine that is not followed by additional residues, similarly to the *T. gondii* and one of the *C. elegans* ATG8 (CeLGG-2) homologues.[154] Whether this implies that processing of PfATG8 by PfATG4 is not necessary is an interesting and open question.

Cloned PbATG3 is 286 residues long and the predicted sequence contains the catalytic cysteine as found in other ATG3 proteins. Two regions of interest have been identified in the yeast Atg3 homologue: the 'handle region' (HR), which mediates the binding between ATG3 and ATG8, and the 'flexible region' (FR), which is implicated in the enzyme's conjugation activity and binding to ATG7.[155] Interestingly, the *P. berghei* orthologue shares greater structural similarity with the human and *Arabidopsis thaliana* orthologues, as all three either have a truncated HR or lack this region completely, and share an extension of the FR, which is absent in yeast Atg3. It is possible that the smaller HR in PbATG3 is functionally compensated by inserted residues present in the FR. Yeast Atg3 interacts with Atg8 via its N-terminus to form an intermediate that appears to be essential for the formation of the Atg8-PE moiety.[156] The C-terminal HR of Atg3 mediates the interaction between Atg3 and Atg8.[155] PbATG8 and PbATG3 also physically interact, as demonstrated in yeast two-hybrid assays (Jayabalasingham B and Coppens I, unpublished data). However, unlike the yeast Atg3-Atg8 interaction, the interaction between the *P. berghei* proteins is not

dependent on any single region of ATG3. Indeed, PbATG8 is able to interact with the FR region of PbATG3, as well as with PbATG3 lacking either the FR region or the C-terminal region including the catalytic cysteine residue. Compared to yeast Atg3 in which the C terminus forms a long α-helix, the C terminus of PbATG3 is shorter and is not predicted to form such secondary structure.[155] Unsurprisingly, PbATG8 cannot interact with yeast Atg3 and is unable to complement *atg8*Δ yeast probably due to these structural disparities between Plasmodium ATG3 and yeast Atg3. Thus, PbATG3 interacts with PbATG8 via several unique interfaces and this interaction more closely resembles that of the mammalian and plant homologues, which also have comparatively short C-terminal regions.

PbATG8 is expressed in Plasmodium blood and liver forms, and located in the peripheral membrane of large vesicles. This implies specific interaction of PbATG8 with membrane components of these organelles. In yeast and mammalian cells, nutrient deprivation stimulates Atg8 conjugation to a phospholipid of the phagophore to initiate the autophagy process. Lipidation has been visualized for many homologues of Atg8 by the presence of a second lower band in western blots after separation by SDS-PAGE in the presence of urea. However, only a single band corresponding to PbATG8 is detectable upon urea-SDS-PAGE separation (Jayabalasingham B and Coppens I, unpublished data). One possible explanation is that Plasmodium homologues of Atg8 are predominantly present in the parasite in either a conjugated or unconjugated state. Alternatively, PbATG8 may not be conjugated to a lipid moiety as reported for one of the two isoforms of ATG8 in *T. cruzi*, and GATE-16 and GABARAP, two mammalian homologues of Atg8.[157] No other parasite-specific protein bands are detected in immunoblots against PbATG8, indicating that PbATG8 is not covalently linked to a protein like other ubiquitin-like molecules. When expressed in mammalian cells upon starvation conditions, Plasmodium ATG8 never associates with autophagosomes, but remains cytosolic. It is possible that the specific conformation of PbATG8 is responsible for the lack of interaction of this protein with the mammalian autophagy machinery, and that in general the interaction of PbATG8 and partners have evolved in a species-specific manner.

PbATG8-containing structures are prominently present in converting parasites, and some of them colocalize with TRAP-labeled structures (Jayabalasingham B and Coppens I, unpublished data). This suggests physical interactions between PbATG8-labeled compartments and micronemes and potentially implicates PbATG8 in facilitating microneme disposal. Interestingly, after sporozoite metamorphosis, PbATG8-labeled structures are still visible within the parasite and persist during the replication phase of development. In yeast, *ATG8* is transcriptionally upregulated by starvation induction.[158] By contrast, the *PbATG8* transcripts are detected in sporozoites, hepatic stage parasites and mixed blood stage parasites, suggestive of constitutive expression of ATG8 by Plasmodium throughout the life cycle (Jayabalasingham B and Coppens I, unpublished data). *PbATG3* transcription, however, is limited to sporozoites and mixed blood stages, and is below the detection level in intrahepatic parasites one day post-infection. This suggests that *PbATG3* transcription is downregulated relative to *PbATG8* during the hepatic phase of infection. Such a lack of PbATG3 expression suggests that autophagic activity is decreased as early as one day post-hepatocyte infection, and that PbATG8 may take on a second function during the anabolic phase of parasite growth in the liver.

Genetic ablation of Plasmodium *ATG8* results in a lethal phenotype, indicating that ATG8 is essential for blood stage development. Other organisms including parasitic protists have several ATG8 homologues, permitting the deletion of one gene without deleterious consequences for the organism. For example, the human genome encodes three isotypes of the Atg8 homologue, LC3. By contrast, only a single Plasmodium ATG8 homologue has been identified. Such a duplication of ATG8 isotypes may reflect specific functions of each ATG8. Functional divergence of ATG8 homologues has been observed in mammals, in which LC3 and GATE-16/GABARAP subfamilies are both essential yet act differently in autophagosome biogenesis.[159] However, even the function of LC3 does not seem to be limited to autophagy, as it is also able to bind fibronectin RNA enhancing its translation.[160,161] Given the identification of only one ATG8 homologue in Plasmodium species it is possible that this single protein performs multiple functions within the parasite making its expression essential for parasite survival. Thus, in addition to a role in organelle degradation via autophagy, we hypothesize additional functions that could include a role in vesicular trafficking between organelles, as supported by the persistence of PbATG8-labeled vesicles during the anabolic phase of intrahepatic development.

Autophagy in Host-Parasitic Protist Interactions

Autophagy of host mammalian cells may play an important or even crucial role in the interaction with pathogenic protists, as it does with pathogenic viruses and bacteria. Autophagy may act to control the infection by microorganisms or the microorganisms may exploit the process to their advantage. The different roles of autophagy in host-pathogen interactions, and aspects of the mechanism of the interactions, have been studied in great detail for a variety of bacteria. A detailed description is beyond the scope of this paper about autophagy in protists, but can be found in a variety of authoritative reviews.[162-166]

Autophagy used by host cells to direct live parasites to lysosomes. Macrophages play an important role in controlling infections by ingesting pathogens and trapping them in compartments such as phagosomes. These then fuse with a lysosome resulting into a phagolysosome, where the pathogen is digested. However, several pathogens, both bacteria (e.g., *Mycobacterium tuberculosis*) and protists (e.g., Leishmania, Toxoplasma, Theilera), are able to survive in macrophages. They achieve this by different strategies, such as inhibiting phagosome acidification, manipulating vesicular trafficking of the host cells and so avoiding fusion with lysosomes, egressing from phagosomes or creating a specialized compartment that remains isolated from the host endocytic network. *T. gondii* relies on the latter mechanism; the membrane of its PV is nonfusogenic due to its unique composition lacking host proteins. Nonetheless, macrophages infected with Toxoplasma can reroute the pathogen-containing compartment to lysosomes.

Autophagy plays an important role in such an innate, cell-mediated immune defense mechanism.[167,168] When CD40 of human or mouse macrophages infected with *T. gondii* is stimulated with (CD154$^+$)CD4$^+$ T cells or exposed to anti-CD4 antibodies, the nonfusogenic nature of the PV is reversed and the vacuole fuses with late endosomes and lysosomes. This fusion is dependent on autophagy, as indicated by observations that it is impaired by knockdown of Beclin 1 and treatment with 3-MA, an inhibitor of phagophore formation, whereas it is stimulated by rapamycin. Moreover, CD40 activation stimulates expression of LC3/Atg8 that localizes to the PV.[168] Furthermore, bafilomycin A_1 and LY294002, drugs that inhibit autophagosome formation and degradation of the content of autolysosomes, inhibit the fusion of the PV with lysosomes.

Autophagy used by host cells to eliminate dead parasites. *T. gondii* not only infects macrophages, but also a variety of other cells such as fibroblasts, epithelial cells, endothelial cells, astrocytes and other microglial cells. Whereas the role of autophagy in parasite killing and elimination has been well established for macrophages, no evidence for the engulfment of the parasite or the PV in autophagosomes or autolysosomes has been found in other cells, such as astrocytes, where it has been sought. However, in these latter cells it has been observed that, after gamma interferon (IFNγ) stimulation, the parasites are degraded by another route that is also present in macrophages.[169-173] This route involves the delivery of at least one of the p47 GTPases to the PV, followed by the disruption of first the PV membrane and subsequently the parasite's plasma membrane. Autophagy is then activated, resulting in the elimination of the damaged parasites. Treatment of infected cells with autophagy inhibitors leads to the accumulation of Toxoplasma debris in the cytosol. In this case, autophagy does not play a role in PV disruption or parasite killing, and indeed 3-MA and wortmannin have no effect on PV disposal.[169] The process of clearance of dead parasites by autophagy seems necessary for host cell survival, since large amounts of parasite products accumulated in cells induce necrosis.[169,172] In macrophages, the killing of parasites by the autophagic route, as described in the previous section, probably enables the cells to present pathogen antigens via the MHC class II pathway and thus to stimulate an adaptive immune response. It remains to be determined if autophagy of degraded parasite material in astrocytes also leads to antigen presentation and so would allow an intracerebral immune response to *T. gondii*.[173] Intriguingly, in primary mouse macrophages IFNγ-induced recruitment of p47 GTPase to the PV is Atg5 dependent;[171] no localization of the GTPase is observed in cells lacking this Atg protein, nor clearance of parasites. Yet, the IFNγ-dependent nitric oxide production, necessary for parasite killing, is not affected. Since no classical hallmarks of autophagy are detected, it remains unknown how Atg5 is involved in causing this IFNγ-dependent cellular immunity against *T. gondii*.

Host cell autophagy exploited by parasites to stimulate their invasion or growth. There are several examples where parasitic protists exploit the autophagy process of the host cell to their advantage. A first one is also from *T. gondii*, as observed when it grows in nonphagocytic cells, such as HeLa cells and primary fibroblasts.[174] Upon infection of host cells and formation of the PV, the parasite undergoes a phase of rapid proliferation. This rapid growth is dependent on the host cell for the supply of nutrients, such as amino acids, lipids and purines. Interestingly, the PV is surrounded by host cell organelles such as endolysosomes, ER and mitochondria. Host lysosomes can even be in close contact with the PV and encroached in invaginations of the PV membrane, thereby allowing the parasite access to nutrients supplied by the host endocytic circuit.[175] In general, autophagy contributes to increase the pool of nutrients derived from autolysosomes via the recycling of autophagy degradation products. Wang et al.[174] showed that the host cell autophagy machinery is stimulated by Toxoplasma in order to exploit the nutritive function of autophagy and therefore boost its multiplication. Infection results in lipidation of LC3 and the accumulation of PtdIns(3)P or LC3-containing vesicles near the PV. Also Beclin 1 concentrates in the vicinity of the PV. All these effects are abolished in Atg5-deficient host cells, resulting in parasite growth arrest under conditions of low amino acid levels. The autophagic-dependent parasite growth correlates with an autophagy-dependent decrease of host cell mass. Interestingly, the stimulation of host autophagy by the parasite is independent of mTOR signaling, as suggested by the fact that no alteration in the phosphorylation state of the downstream effectors 4E-BP1 and S6K1 is observed. However, this process seems to be dependent on calcium signaling and abscisic acid, an inducer of calcium release from intracellular stores. In contrast, *T. gondii* stimulates mTOR-dependent host cell growth, as determined by rapamycin-sensitive phosphorylation of the ribosomal protein S6 and cell cycle progression.[176]

A second example of the exploitation of host autophagy by parasitic protists has been provided by studies of nonphagocytic cells infected with *T. cruzi* trypomastigotes.[177] This parasite can enter its host cell via two routes: Lysosome-dependent and -independent entry pathways. The first route involves the calcium-dependent recruitment of host lysosomes to the site of parasite entry via the activation of PtdIns3Ks and the exploitation of lysosomal membranes by the parasite as sources of lipids to form its PV.[178,179] Although nonlysosomal entry is quantitatively more frequent,[180] the lysosome-dependent pathway is essential for establishment of a productive infection and intracellular proliferation.[181] The alternate nonlysosomal and actin-independent entry pathway involves the formation of a tightly associated host cell plasma membrane-derived vacuole enriched in the lipid products of class I PtdIns3Ks.[180] Using CHO cells transfected with GFP-LC3, Romano and coworkers showed that autophagosomes are also recruited to the entry site during *T. cruzi* invasion and are thus involved in formation of the PV as heterolysosomes.[177] The low pH inside the PV may initiate the differentiation of trypomastigotes into amastigotes and activates the production of a pore-forming toxin, Tc-Tox, by trypomastigotes or intermediate forms allowing these forms to escape the PV.[182] The completion of the differentiation process and proliferation of amastigotes occurs subsequently in the cytosol, followed by release from the cell. Induction of host cell autophagy by starvation or addition of rapamycin appears to favor the invasion process, but not the differentiation and proliferation of the parasites.[177] The level of

parasite infection in host cells starved for nutrients is decreased by classical inhibitors of autophagy such as wortmannin, 3-MA and vinblastine. Furthermore, in Atg5-deficient cells or in cells where Beclin 1 expression has been reduced by RNA interference, autophagosome formation, parasite entry and the association of the PV with the autolysosomal marker are reduced. Together, these results provide strong indications that *T. cruzi* exploits the autophagy process of its host cells to establish a productive infection.

A third example is illustrated by BALB/c macrophages infected with *Leishmania amazonensis*. Exogenously added IFNγ increases the intramacrophagic replication of *L. amazonensis* and this affect is attenuated by co-treating the cultures with either 3-MA or wortmannin.[183] IFNγ treatment of infected cells stimulates the biogenesis of autophagosomal profiles, however, the amastigotes do not colocalize with these double-membrane vacuoles. Correlation between Leishmania replication and host autophagy activity is confirmed by induction of nutrient deprivation resulting in a boost of parasite growth. Starvation significantly increases the number of lipid bodies in infected BALB/c macrophages. Lipid bodies are involved in eicosanoid synthesis,[184] and the prostanoid Prostaglandin E2 (PGE2) increases the intracellular load of Leishmania in macrophages.[185] The levels of PGE2 are significantly augmented in infected BALB/c macrophages[183] compared to uninfected macrophages, and starvation increases two-fold the PGE2 amounts produced by infected macrophages. Addition of the cyclooxygenase blocker indomethacin prevents the increase in parasite load induced by starvation. This suggests a role of PGE2 in macrophage responses to starvation-induced autophagy. Of interest, induction of autophagy that correlates with increased Leishmania load is selective for BALB/c and J774 macrophages but not C57BL/6 macrophages, suggesting the involvement of intrinsic host cell factors in the regulation of the outcome of infection following autophagy stimulation.

Suppression of host cell autophagy induction by parasites to avoid degradation. Many intracellular pathogens can block their recognition by autophagosomes and/or the maturation of pathogen-containing autophagosomes into acidified autolysosomes. For example, viruses actively suppress initiation of the autophagy pathway, whereas certain bacterial virulence factors are necessary for bacterial evasion of autophagy. However, to date, there is no published evidence indicating that parasites block the induction of autophagy in infected cells. Nevertheless, unpublished work from the Langsley laboratory indicates that Theileria may be able to do so. Tropical Theileriosis and East Coast Fever are tick-born diseases of cattle caused by the parasitic protists *Theileria annulata* and *T. parva,* respectively. Both theilerioses display an enlargement of the lymph node draining the tick bite followed by a generalized lymphadenopathy associated with fever, anorexia and respiratory distress. Theileria sporozoites are injected with the tick saliva during a blood meal and they infect mononuclear cells. Their rapid development occurs in the absence of a PV membrane that characterizes the related Plasmodium and Toxoplasma parasites. Consequently, intracellular Theileria reside free in the host cell cytosol, closely associated with an array of host cell microtubules. In the case of *T. parva*, intracellular development to the multinucleated macroschizont only takes place in bovine T and B lymphocytes, whereas *T. annulata* transforms monocytes/macrophages and B cells (recently reviewed in ref. 186). The macroschizont stage is largely responsible for the pathology of Theileria infections, and multinucleated parasites induce leukocyte proliferation leading to clonal expansion of the parasitized cell population. Environmental signals trigger differentiation to the microschizont stage and upon merogony the host cell stops dividing. Released merozoites infect erythrocytes and differentiate into piroplasms that will eventually be taken up during a tick's blood meal. An efficacious immune response to East Coast fever involves cytotoxic $CD8^+$ T cells, and additional CD4 responses are also thought to be necessary for protection against Tropical Theileriosis (reviewed in ref. 187). Very recently, a potent cysteine peptidase inhibitor of *P. berghei* PbICP, which is involved in sporozoite invasion and prevents hepatocyte cell death was identified. The inhibitor can potentially control parasites, as well as host cell-derived peptidases, which sheds some additional light on parasite-host cell interaction.[188]

Since autophagy plays a role in antigen presentation (reviewed in ref. 189), the hypothesis that intracellular Theileria parasites modify the autophagic response of infected leukocytes was tested. Theileria-infected lines that express Cherry-tagged LC3/Atg8 were made. Autophagy was induced by nutrient starvation of infected leukocytes and followed by the formation of large Cherry-LC3 aggregates. Confirmation that these corresponded to macroautophagosomes was provided by electron microscopy. Surprisingly, in the absence of nutrient starvation live Theileria parasites do not provoke Cherry-LC3 aggregates; i.e., the presence of intracellular Theileria appears to inhibit an autophagic response despite it being exposed (not enclosed with a PV) in the leukocyte cytosol. However, when parasites are killed with a specific drug called buparvacone they are readily recognized and digested by the autophagosome. As good CD4 and CD8 T cell responses to infection eliminate parasites, this suggests that Theileria might be deliberately inhibiting host cell autophagosome function to reduce peptide presentation to specific CD4 (by cross-presentation) and CD8 T cells, as a way of dampening the host's immune response and increasing its chances of survival. This exciting possibility is currently being tested in collaboration with I. Morrison (University of Edinburgh, UK).

Concluding remarks about autophagy in host-parasite interactions. On the one hand, the original role of autophagy as an efficient antimicrobial defense mechanism has expanded to immunological processes that participate in both innate and adaptive immunity to neutralize infections. On the other hand, intracellular protist parasites have developed specialized anti-autophagy adaptations allowing them to prevent or block autophagic elimination. Many parasites have also built up multipronged strategies to utilize functions or components of autophagy to facilitate their own invasion of, and promote their replication rate in, mammalian cells. This concept of autophagy helping to 'feed' intracellular intruders is particularly relevant for parasites that reside in sequestered PV with limited access to host nutrients. However, observations on enhanced development of parasites upon autophagy induction are based upon in vitro studies and a "microbe-friendly" role of autophagy in parasite

replication needs to be established in vivo. Returning to the point of origin, autophagy as a cell-autonomous defense of the host cell has met its adversarial counterpart in autophagy as a pro-microbial process. Manifestly, autophagy works without respite to protect the master eukaryotic cell, often on both sides of the host-parasite relationship.

Discussion and Conclusions

Evolutionary aspects. In this review, we addressed the occurrence, variability and evolution of autophagy in protists. However, the term 'protists' covers species of unicellular eukaryotes, which not only are very large in number, but also very broadly in diversity. In classical taxonomy they form one of the four kingdoms of eukaryotes, the other kingdoms being formed by the animals, plants and fungi, respectively. However, the increased availability of genome sequences for a large variety of organisms, in the last decade, and advances in molecular phylogenetics have allowed a reconsideration of this classical division. Today, most eukaryotes have been classified within one of six 'suprakingdom-level'- or 'super'-groups, each containing protists.[48,49,60] One supergroup, the Opisthokonta, contains animals and fungi as well as certain protists, while another, the Archaeplastida, harbors plants and some groups of photosynthetic protists (e.g., Chlamydomonas). In the other supergroups (called Amoebozoa, Chromalveolata, Rhizaria and Excavata) only protist organisms are found. The presence of a nucleus, ER, Golgi, the occurrence of mitosis, and the possession of mitochondria or relic mitochondrial organelles in each of these major groups, indicates that the common ancestor of all extant eukaryotes was already a 'typical' eukaryotic cell. Also ATG genes have been found in protist representatives of the supergroups, examined thus far (there are no data available for the Rhizaria) as well as experimental indications that autophagy occurs in protists belonging to Amoebozoa, Chromalveolata, Archaeplastida and Excavata. For example, the trypanosomatids belong to the Excavata, the apicomplexans and ciliates to the Chromoalveolata, and Dictyostelium to the Amoebozoa (**Fig. 2**). Therefore, the process of macroautophagy must have been part of the repertoire of the common ancestor. Based on the distribution of ATGs in a taxonomically diverse range of protists, it may be concluded that this ancestor contained a core autophagy machinery that has been retained in all extant organisms in which autophagy can be found. The machinery has further developed with the acquisition of additional Atgs in opisthokonts (yeasts, mammals) and has been entirely lost in some protists, probably as a result of niche adaptation. In other protists the basic process has been simplified, by the probable loss of specific ATGs in some lineages or elaborated by paralogue expansion of other ATGs. It is equally possible that expansion of the core repertoire has occurred in other groups, but by recruitment of proteins unrelated to those developed in the opisthokont supergroup, and therefore not identified in the genomic searches performed with sequences of Atgs from experimentally better-characterized organisms like yeast. Only by future experimental cell biological and genetic research in more diverse unicellular taxa, similar to that performed in yeast, will the full autophagic process and the machinery involved be unraveled.

As mentioned, both ubiquitin-like proteins (Atg8, Atg12) and ubiquitin itself (with the involvement of p62 or NBR1) play key roles in the autophagy of ubiquitinated proteins. Following the recent discovery of ubiquitin-like proteins in bacteria[190] it is tempting to speculate that they, too, may be involved in internal degradation mechanisms, but the differences in cell biology between prokaryotes and eukaryotes are so great that any such mechanisms would necessarily be very different relative to eukaryote autophagy.

Translational aspects. A large number of the currently known protists are free-living organisms, but also a considerable number are pathogens of other eukaryotes, including human and domestic animals. Because of their importance for health and the economy, these pathogens have received attention in scientific research with an objective to explore the possibility of developing drugs against them. This interest for research of pathogenic protists also involved the study of the role of autophagy in these organisms and the identification of the autophagic machinery. Autophagy is essential for cellular differentiation of each of the three genera of human-infective trypanosomatid parasites and thus for the progress of the parasites though their life cycle. In addition, interference with the activity of individual proteins acting in different stages of autophagy pathways, either by reverse genetics of the use of inhibitors, affects in some cases not only the differentiation, but also the morphology, virulence or viability of the parasites. These observations, together with the generally low level of sequence conservation of ATGs and other proteins involved in autophagy in parasites, offer prospects for the development of new parasite-specific drugs.

Particularly, in the case of *T. cruzi* and Leishmania species, interference with the activity of proteins that are essential for differentiation may offer a possible chemotherapeutic strategy.[191-195] After their introduction in the human host, these parasites have to undergo the transition from extracellular promastigotes or trypomastigotes to intracellular amastigotes, which are the stage responsible for the major pathogenic symptoms. The intracellular *T. cruzi* amastigotes, after several rounds of multiplication, differentiate again into trypomastigotes that burst out of the host cells to infect additional ones. In contrast, in the case of Leishmania, the parasites are released as amastigotes that initiate new macrophage infections.

Blocking of autophagy may prevent these differentiation steps and thus pathogenicity. For *T. cruzi* this has not yet been proven, contrary to Leishmania, where inactivation of autophagy-related gene products indeed prevents the differentiation of promastigotes.[16] On the other hand, in *T. brucei*, the major morphological and metabolic changes, associated with upregulated autophagy, occur upon the transition from the human bloodstream form to the insect procyclic form and not when the parasite resides within the human host.[21] Therefore, the effectiveness of autophagy inhibitors in treatment of sleeping sickness may be questionable. One could imagine, however, that African trypanosomes may try to overcome the deleterious effects caused by drugs by an

autophagic response, and in that case combined administration with autophagy inhibitors may be effective.

One may wonder if treatment with autophagy inhibitors will be feasible for the initial phase of Chagas' disease and leishmaniasis because the period during which the insect-stage parasites will differentiate into the intracellular amastigotes, after the infection of human, is relatively short. However, at least in the case of *T. cruzi*, maintenance of infection involves a cyclic differentiation of amastigotes and trypomastigotes, suggesting that it could be targeted using inhibitors of autophagy.

In addition to interfering with the ATG proteins, it is possible to block autophagy also at a later stage, i.e., at the level of the autolysosome, the terminal step of the process. Using gene ablation or inhibition by chemical inhibitors it was demonstrated that the *Leishmania mexicana* cysteine peptidases CPA and CPB, which are orthologues of the mammalian lysosomal cysteine cathepsins, are important for both the autophagy and differentiation of the parasite. In addition, *L. mexicana* CPA/CPB-deficient mutants lack virulence in macrophages and mice.[17] Similarly, chemically blocking the peptidases prevents amastigote replication and infectivity, although the effect on autophagy was not followed at that time.[196] Moreover, similar results were obtained not only in Leishmania, but also in *T. cruzi* and other trypanosomatids,[196-198] thereby setting the ground for possible therapeutic intervention.

An alternative chemotherapeutic strategy could be the induction of autophagic cell death. The feasibility of this approach has been shown for each of the two Trypanosoma species as discussed in the previous sections. For *T. brucei*, the example is the killing of parasites as a result of administration of DHA or neuropeptides,[120,121] while for *T. cruzi* the same was achieved with lysophospholipid analogues and naphtoimidazoles.[127,130] However, similar data regarding the induction of autophagic cell death of Leishmania parasites by administration of compounds are not available.

Apicomplexan parasites also undergo major morphological changes during the differentiation between the successive life-cycle stages. For Plasmodium, it has been discussed above that, during the sporozoite-to-trophozoite conversion, important interior remodeling occurs involving the elimination of many organelles. Morphological analysis, in conjunction with immunolabeling with anti-ATG8, indicates that this conversion involves autophagy. Although the essentiality of autophagy for this differentiation has not yet been demonstrated by genetic means, the observed delay in stage conversion caused by the administration of the autophagy inhibitor 3-MA renders it very likely and suggest that specifically interfering with components of the parasite autophagy machinery might combat infection.

The development of inhibitors that interfere only with ATGs of parasitic protists and not with those of the host cells may be feasible because of the low level of sequence conservation of these proteins. This will require further studies on the structure of these ATGs, permitting the design and synthesis of selective inhibitors, or the production of recombinant ATGs and the development of sensitive binding assays to be used in high-throughput screening of compound libraries for the selection of inhibitors.

Alternative strategies may be attempted to interfere with specific host-parasite interactions. For example, stimulation of autophagy in macrophages infected with Toxoplasma parasites has been shown to promote the fusion of PVs with lysosomes.[173] On the contrary, in cases where parasites (*T. gondii*, *T. cruzi*, *L. amazonensis*) exploit host autophagy to stimulate their invasion or growth, administration of inhibitors of the human autophagy process may be a means to control the infection. Equally, reversing this suppression may combat parasites such as Theileria, which appear to ablate host cell autophagy to avoid degradation. Of course, activation or inhibition of host autophagy is not selective and will also affect uninfected cells or tissues and thus risk causing side effects.

Autophagy has already received major attention in therapeutic intervention in human medicine—targeting autophagy may be beneficial as monotherapy or to sensitize cells to other conventional therapies (e.g., cancer). Research to explore the possibility of targeting autophagy in parasites is still at a very preliminary stage, but as reasoned above, the approach may be valuable for treatment of some of the parasite-associated diseases.

Acknowledgements

The authors gratefully acknowledge the following organizations: A.B., M.G.L. and P.A.M.M. the 'Fonds de la Recherche Scientifique' (FRS-FNRS) and associated foundations (FRSM and FRIA) and the Belgian Interuniversity Attraction Poles-Federal Office for Scientific, Technical and Cultural Affairs for support of their research on autophagy in trypanosomes; M.L.G. the Royal Society for an University Research Fellowship; G.L. the Wellcome Trust for a Special Initiative grant (075820/A/04/Z) for support for 'An integrated approach for the development of sustainable methods to control tropical theileriosis'; J.C.M. and G.H.C. the Medical Research Council for support (G0700127); M.N. the MICINN for support (SAF2009-07587); B.T. and V.S. the Slovenian Research Agency for research grants (P1-0140, J1-9520, J3-2258); S.L.d.C. and R.M.B. the Brazilian agencies CNPq and Faperj; M.I.C. and P.R. the ANPCYT (PICT 2005 # 38420) and Comisión Nacional Salud Investiga in Argentina for research grants; B.T. and V.S. would like to thank Vito Turk for valuable discussions.

Note

Slightly different systems are being used for abbreviations of proteins and their genes in mammals, yeasts and protists. Throughout this paper, we used for proteins and genes from protists the consensus nomenclature system for trypanosomatid protists: upper case for proteins, italicized upper case for genes.[200]

References

1. de Duve C. The lysosome turns fifty. Nat Cell Biol 2005; 7:847-9.
2. de Reuck AVS, Cameron MP. Ciba Foundation Symposium on Lysosomes. London: J.A. Chrurchill Ltd 1963.
3. Klionsky DJ. Autophagy revisited: a conversation with Christian de Duve. Autophagy 2008; 4:740-3.
4. Klionsky DJ. Autophagy: from phenomenology to molecular understanding in less than a decade. Nat Rev Mol Cell Biol 2007; 8:931-7.
5. Klionsky DJ, Cregg JM, Dunn WA Jr, Emr SD, Sakai Y, Sandoval IV, et al. A unified nomenclature for yeast autophagy-related genes. Dev Cell 2003; 5:539-45.
6. Meijer WH, van der Klei IJ, Veenhuis M, Kiel JAKW. ATG genes involved in non-selective autophagy are conserved from yeast to man, but the selective Cvt and pexophagy pathways also require organism-specific genes. Autophagy 2007; 3:106-16.
7. Yang Z, Klionsky DJ. An overview of the molecular mechanism of autophagy. Curr Top Microbiol Immunol 2009; 335:1-32.
8. He C, Klionsky DJ. Regulation mechanisms and signaling pathways of autophagy. Annu Rev Genet 2009; 43:67-93.
9. Mehrpour M, Esclatine A, Beau I, Codogno P. Autophagy in health and disease. 1. Regulation and significance of autophagy: an overview. Am J Physiol Cell Physiol 2010; 298:776-85.
10. Kroemer G, Galluzzi L, Vandenabeele P, Abrams J, Alnemri ES, Baehrecke EH, et al. Nomenclature Committee on Cell Death 2009. Classification of cell death: recommendations of the Nomenclature Committee on Cell Death 2009. Cell Death Differ 2009; 16:3-11.
11. Yorimitsu T, Klionsky DJ. Autophagy: molecular machinery for self-eating. Cell Death Differ 2005; 12:1542-52.
12. Bejarano E, Cuervo AM. Chaperone-mediated autophagy. Proc Am Thorac Soc 2010; 7:29-39.
13. Fader CM, Sánchez DG, Mestre MB, Colombo MI. TI-VAMP/VAMP7 and VAMP3/cellubrevin: two v-SNARE proteins involved in specific steps of the autophagy/multivesicular body pathways. Biochim Biophys Acta 2009; 1793:1901-16.
14. Rigden DJ, Michels PAM, Ginger ML. Autophagy in protists: Examples of secondary loss, lineage-specific innovations and the conundrum of remodeling a single mitochondrion. Autophagy 2009; 5:784-94.
15. Kiel JA KW. Autophagy in unicellular eukaryotes. Philos Trans R Soc Lond B Biol Sci 2010; 365:819-30.
16. Besteiro S, Williams RA, Morrison LS, Coombs GH, Mottram JC. Endosome sorting and autophagy are essential for differentiation and virulence of *Leishmania major*. J Biol Chem 2006; 281:11384-96.
17. Williams RA, Tetley L, Mottram JC, Coombs GH. Cysteine peptidases CPA and CPB are vital for autophagy and differentiation in *Leishmania mexicana*. Mol Microbiol 2006; 61:655-74.
18. Herman M, Gillies S, Michels PA, Rigden DJ. Autophagy and related processes in trypanosomatids: insights from genomic and bioinformatic analyses. Autophagy 2006; 2:107-18.
19. Uzcátegui NL, Denninger V, Merkel P, Schoenfeld C, Figarella K, Duszenko M. Dihydroxyacetone induced autophagy in African trypanosomes. Autophagy 2007; 3:626-9.
20. Alvarez VE, Kosec G, Sant'Anna C, Turk V, Cazzulo JJ, Turk B. Autophagy is involved in nutritional stress response and differentiation in *Trypanosoma cruzi*. J Biol Chem 2008; 283:3454-64.
21. Herman M, Pérez-Morga D, Schtickzelle N, Michels PA. Turnover of glycosomes during life-cycle differentiation of *Trypanosoma brucei*. Autophagy 2008; 4:294-308.
22. Denninger V, Koopmann R, Muhammad K, Barth T, Bassarak B, Schönfeld C, et al. Kinetoplastida: model organisms for simple autophagic pathways? Methods Enzymol 2008; 451:373-408.
23. Williams RA, Woods KL, Juliano L, Mottram JC, Coombs GH. Characterization of unusual families of ATG8-like proteins and ATG12 in the protozoan parasite *Leishmania major*. Autophagy 2009; 5:159-72.
24. Klionsky DJ. The molecular machinery of autophagy: unanswered questions. J Cell Sci 2005; 118:7-18.
25. Suzuki K, Ohsumi Y. Molecular machinery of autophagosome formation in yeast, *Saccharomyces cerevisiae*. FEBS Lett 2007; 581:2156-61.
26. Díaz-Troya S, Pérez-Pérez ME, Florencio FJ, Crespo JL. The role of TOR in autophagy regulation from yeast to plants and mammals. Autophagy 2008; 4:851-65.
27. Jung CH, Ro SH, Cao J, Otto NM, Kim DH. mTOR regulation of autophagy. FEBS Lett 2010; 584:1287-95.
28. Manjithaya R, Nazarko TY, Farré JC, Subramani S. Molecular mechanism and physiological role of pexophagy. FEBS Lett 2010; 584:1367-73.
29. Ano Y, Hattori T, Kato N, Sakai Y. Intracellular ATP correlates with mode of pexophagy in *Pichia pastoris*. Biosci Biotechnol Biochem 2005; 69:1527-33.
30. Okamoto K, Kondo-Okamoto N, Ohsumi Y. Mitochondria-anchored receptor Atg32 mediates degradation of mitochondria via selective autophagy. Dev Cell 2009; 17:87-97.
31. Kanki T, Wang K, Cao Y, Baba M, Klionsky DJ. Atg32 is a mitochondrial protein that confers selectivity during mitophagy. Dev Cell 2009; 17:98-109.
32. Kanki T, Wang K, Baba M, Bartholomew CR, Lynch-Day MA, Du Z, et al. A genomic screen for yeast mutants defective in selective mitochondria autophagy. Mol Biol Cell 2009; 20:4730-8.
33. Hayashi-Nishino M, Fujita N, Noda T, Yamaguchi A, Yoshimori T, Yamamoto A. A subdomain of the endoplasmic reticulum forms a cradle for autophagosome formation. Nat Cell Biol 2009; 11:1433-7.
34. Ylä-Anttila P, Vihinen H, Jokitalo E, Eskelinen EL. 3D tomography reveals connections between the phagophore and endoplasmic reticulum. Autophagy 2009; 5:1180-5.
35. Hailey DW, Rambold AS, Satpute-Krishnan PP, Mitra K, Sougrat R, Kim PK, Lippincott-Schwartz J. Mitochondria supply membranes for autophagosome biogenesis during starvation. Cell 2010; 141:656-67.
36. Geng J, Klionsky DJ. The Atg8 and Atg12 ubiquitin-like conjugation systems in macroautophagy. 'Protein modifications: beyond the usual suspects' review series. EMBO Rep 2008; 9:859-64.
37. Kirkin V, McEwan DG, Novak I, Dikic I. A role for ubiquitin in selective autophagy. Mol Cell 2009; 34:259-69.
38. Kim PK, Hailey DW, Mullen RT, Lippincott-Schwartz J. Ubiquitin signals autophagic degradation of cytosolic proteins and peroxisomes. Proc Natl Acad Sci USA 2008; 105:20567-74.
39. Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ. Gapped BLAST and PSI-BLAST: A new generation of protein database search programs. Nucleic Acids Res 1997; 25:3389-402.
40. Altschul SF, Wootton JC, Gertz EM, Agarwala R, Morgulis A, Schaffer AA, Yu YK. Protein database searches using compositionally adjusted substitution matrices. FEBS J 2005; 272:5101-9.
41. Patthy L. Protein Evolution. Oxford, UK: Blackwell 2008.
42. Addou S, Rentzsch R, Lee D, Orengo CA. Domain-based and family-specific sequence identity thresholds increase the levels of reliable protein function transfer. J Mol Biol 2009; 387:416-30.
43. Tatusov RL, Fedorova ND, Jackson JD, Jacobs AR, Kiryutin B, Koonin EV, et al. The COG database: An updated version includes eukaryotes. BMC Bioinformatics 2003; 4:41.
44. Soding J. Protein homology detection by HMM-HMM comparison. Bioinformatics 2005; 21:951-60.
45. Finn RD, Mistry J, Tate J, Coggill P, Heger A, Pollington JE, et al. The pfam protein families database. Nucleic Acids Res 2010; 38:211-22.
46. Marchler-Bauer A, Anderson JB, Chitsaz F, Derbyshire MK, DeWeese-Scott C, Fong JH, et al. CDD: Specific functional annotation with the conserved domain database. Nucleic Acids Res 2009; 37:205-10.
47. Turk B, Turk V. Lysosomes as 'suicide bags' in cell death: myth or reality? J Biol Chem 2009; 284:21783-87.
48. Simpson AG, Roger AJ. The real 'kingdoms' of eukaryotes. Curr Biol 2004; 14:693-6.
49. Roger AJ, Simpson AG. Evolution: revisiting the root of the eukaryote tree. Curr Biol 2009; 19:165-7.
50. Embley TM, Martin W. Eukaryotic evolution, changes and challenges. Nature 2006; 440:623-30.
51. Rogozin IB, Basu MK, Csürös M, Koonin EV. Analysis of rare genomic changes does not support the unikont-bikont phylogeny and suggests cyanobacterial symbiosis as the point of primary radiation of eukaryotes. Genome Biol Evol 2009; 2009:99-113.
52. Cavalier-Smith T. Kingdoms Protozoa and Chromista and the eozoan root of the eukaryotic tree. Biol Lett 2010; 6:342-5.
53. Katinka MD, Duprat S, Cornillot E, Méténier G, Thomarat F, Prensier G, et al. Genome sequence and gene compaction of the eukaryote parasite *Encephalitozoon cuniculi*. Nature 2001; 414:450-3.
54. Matsuzaki M, Misumi O, Shin-I T, Maruyama S, Takahara M, Miyagishima SY, et al. Genome sequence of the ultrasmall unicellular red alga *Cyanidioschyzon merolae* 10D. Nature 2004; 428:653-7.
55. Morrison HG, McArthur AG, Gillin FD, Aley SB, Adam RD, Olsen GJ, et al. Genomic minimalism in the early diverging intestinal parasite *Giardia lamblia*. Science 2007; 317:1921-6.
56. Tovar J, León-Avila G, Sánchez LB, Sutak R, Tachezy J, van der Giezen M, et al. Mitochondrial remnant organelles of Giardia function in iron-sulphur protein maturation. Nature 2003; 426:172-6.
57. Abodeely M, DuBois KN, Hehl A, Stefanic S, Sajid M, DeSouza W, et al. A contiguous compartment functions as endoplasmic reticulum and endosome/lysosome in *Giardia lamblia*. Eukaryot Cell 2009; 8:1665-76.
58. Marti M, Regös A, Li Y, Schraner EM, Wild P, Müller N, et al. An ancestral secretory apparatus in the protozoan parasite *Giardia intestinalis*. J Biol Chem 2003; 278:24837-48.
59. Roger AJ, Hug LA. The origin and diversification of eukaryotes: problems with molecular phylogenetics and molecular clock estimation. Philos Trans R Soc Lond B Biol Sci 2006; 361:1039-54.
60. Hampl V, Hug L, Leigh JW, Dacks JB, Lang BF, Simpson AG, Roger AJ. Phylogenomic analyses support the monophyly of Excavata and resolve relationships among eukaryotic "supergroups". Proc Natl Acad Sci USA 2009; 106:3859-64.
61. Benchimol M. Hydrogenosome autophagy: an ultrastructural and cytochemical study. Biol Cell 1999; 91:165-74.
62. Kaneda Y, Torii M, Tanaka T, Aikawa M. In vitro effects of berberine sulphate on the growth and structure of *Entamoeba histolytica*, *Giardia lamblia* and *Trichomonas vaginalis*. Ann Trop Med Parasitol 1991; 85:417-25.
63. Carlton JM, Hirt RP, Silva JC, Delcher AL, Schatz M, Zhao Q, et al. Draft genome sequence of the sexually transmitted pathogen *Trichomonas vaginalis*. Science 2007; 315:207-12.
64. Aury JM, Jaillon O, Duret L, Noel B, Jubin C, Porcel BM, et al. Global trends of whole-genome duplications revealed by the ciliate *Paramecium tetraurelia*. Nature 2006; 444:171-8.
65. Sehring IM, Reiner C, Mansfeld J, Plattner H, Kissmehl R. A broad spectrum of actin paralogs in *Paramecium tetraurelia* cells display differential localization and function. J Cell Sci 2007; 120:177-90.

66. Wassmer T, Kissmehl R, Cohen J, Plattner H. Seventeen a-subunit isoforms of Paramecium V-ATPase provide high specialization in localization and function. Mol Biol Cell 2006; 17:917-30.
67. Kirkin V, Lamark T, Sou YS, Bjorkoy G, Nunn JL, Bruun JA, et al. A role for NBR1 in autophagosomal degradation of ubiquitinated substrates. Mol Cell 2009; 33:505-16.
68. Okamoto K, Kondo-Okamoto N, Ohsumi Y. Mitochondria-anchored receptor Atg32 mediates degradation of mitochondria via selective autophagy. Dev Cell 2009; 17:87-97.
69. Calvo-Garrido J, Escalante R. Autophagy dysfunction and ubiquitin-positive protein aggregates in Dictyostelium cells lacking Vmp1. Autophagy 2010; 6:100-9.
70. Hara T, Nakamura K, Matsui M, Yamamoto A, Nakahara Y, Suzuki-Migishima R, et al. Suppression of basal autophagy in neural cells causes neurodegenerative disease in mice. Nature 2006; 441:885-9.
71. Komatsu M, Waguri S, Chiba T, Murata S, Iwata J, Tanida I, et al. Loss of autophagy in the central nervous system causes neurodegeneration in mice. Nature 2006; 441:880-4.
72. Pawlowski J, Burki F. Untangling the phylogeny of amoeboid protists. J Eukaryot Microbiol 2009; 56:16-25.
73. Schaap P, Winckler T, Nelson M, Alvarez-Curto E, Elgie B, Hagiwara H, et al. Molecular phylogeny and evolution of morphology in the social amoebas. Science 2006; 314:661-3.
74. Hilbi H, Weber SS, Ragaz C, Nyfeler Y, Urwyler S. Environmental predators as models for bacterial pathogenesis. Environ Microbiol 2007; 9:563-75.
75. Calvo-Garrido J, Carilla-Latorre S, Escalante R. Vacuole membrane protein 1, autophagy and much more. Autophagy 2008; 4:835-7.
76. Calvo-Garrido J, Carilla-Latorre S, Lazaro-Dieguez F, Egea G, Escalante R. Vacuole membrane protein 1 is an endoplasmic reticulum protein required for organelle biogenesis, protein secretion and development. Mol Biol Cell 2008; 19:3442-53.
77. Mizushima N, Kuma A, Kobayashi Y, Yamamoto A, Matsubae M, Takao T, et al. Mouse Apg16L, a novel WD-repeat protein, targets to the autophagic isolation membrane with the Apg12-Apg5 conjugate. J Cell Sci 2003; 116:1679-88.
78. Tekinay T, Wu MY, Otto GP, Anderson OR, Kessin RH. Function of the *Dictyostelium discoideum* Atg1 kinase during autophagy and development. Eukaryot Cell 2006; 5:1797-806.
79. Otto GP, Wu MY, Kazgan N, Anderson OR, Kessin RH. Macroautophagy is required for multicellular development of the social amoeba *Dictyostelium discoideum*. J Biol Chem 2003; 278:17636-45.
80. Giusti C, Tresse E, Luciani MF, Golstein P. Autophagic cell death: analysis in Dictyostelium. Biochim Biophys Acta 2009; 1793:1422-31.
81. Lam D, Kosta A, Luciani MF, Golstein P. The inositol 1,4,5-trisphosphate receptor is required to signal autophagic cell death. Mol Biol Cell 2008; 19:691-700.
82. Tresse E, Kosta A, Giusti C, Luciani MF, Golstein P. A UDP-glucose derivative is required for vacuolar autophagic cell death. Autophagy 2008; 4:680-91.
83. Otto GP, Wu MY, Kazgan N, Anderson OR, Kessin RH. Dictyostelium macroautophagy mutants vary in the severity of their developmental defects. J Biol Chem 2004; 279:15621-9.
84. Tung SM, Unal C, Ley A, Pena C, Tunggal B, Noegel AA, et al. Loss of Dictyostelium ATG9 results in a pleiotropic phenotype affecting growth, development, phagocytosis and clearance and replication of *Legionella pneumophila.* Cell Microbiol 2010; 12:765-80.
85. Ogura K, Shirakawa M, Barnes TM, Hekimi S, Ohshima Y. The UNC-14 protein required for axonal elongation and guidance in *Caenorhabditis elegans* interacts with the serine/threonine kinase UNC-51. Genes Dev 1997; 11:1801-11.
86. Reggiori F, Tucker KA, Stromhaug PE, Klionsky DJ. The Atg1-Atg13 complex regulates Atg9 and Atg23 retrieval transport from the pre-autophagosomal structure. Dev Cell 2004; 6:79-90.
87. Jia K, Thomas C, Akbar M, Sun Q, Adams-Huet B, Gilpin C, Levine B. Autophagy genes protect against *Salmonella typhimurium* infection and mediate insulin signaling-regulated pathogen resistance. Proc Natl Acad Sci USA 2009; 106:14564-9.
88. Kobayashi T, Endoh H. Caspase-like activity in programmed nuclear death during conjugation of *Tetrahymena thermophila*. Cell Death Differ 2003; 10:634-40.
89. McArdle EW, Bergquist BL, Ehret CF. Structural changes in *Tetrahymena rostrata* during induced encystment. J Protozool 1980; 27:388-97.
90. Martin NC, Goodenough UW. Gametic differentiation in *Chlamydomonas reinhardtii*. I. Production of gametes and their fine structure. J Cell Biol 1975; 67:587-605.
91. Wang ZT, Ullrich N, Joo S, Waffenschmidt S, Goodenough U. Algal lipid bodies: stress induction, purification and biochemical characterization in wild-type and starchless *Chlamydomonas reinhardtii*. Eukaryot Cell 2009; 8:1856-68.
92. Affenzeller MJ, Darehshouri A, Andosch A, Lutz C, Lutz-Meindl U. PCD and autophagy in the unicellular green alga *Micrasterias denticulata*. Autophagy 2009; 5:854-5.
93. Affenzeller MJ, Darehshouri A, Andosch A, Lutz C, Lutz-Meindl U. Salt stress-induced cell death in the unicellular green alga *Micrasterias denticulata*. J Exp Bot 2009; 60:939-54.
94. Fenchel T. The life history of *Flabellula baltica* Smirnov (Gymnamoebae, Rhizopoda): adaptations to a spatially and temporally heterogeneous environment. Protist 2010; 161:279-87.
95. Inwood W, Yoshihara C, Zalpuri R, Kim KS, Kustu S. The ultrastructure of a *Chlamydomonas reinhardtii* mutant strain lacking phytoene synthase resembles that of a colorless alga. Mol Plant 2008; 1:925-37.
96. Wloga D, Strzyzewska-Jowko I, Gaertig J, Jerka-Dziadosz M. Septins stabilize mitochondria in *Tetrahymena thermophila.* Eukaryot Cell 2008; 7:1373-86.
97. Morais P, Lamas J, Sanmartín ML, Orallo F, Leiro J. Resveratrol induces mitochondrial alterations, autophagy and a cryptobiosis-like state in scuticociliates. Protist 2009; 160:552-64.
98. Martin NC, Chiang KS, Goodenough UW. Turnover of chloroplast and cytoplasmic ribosomes during gametogenesis in *Chlamydomonas reinhardi*. Dev Biol 1976; 5:190-201.
99. Diaz-Troya S, Perez-Perez ME, Florencio FJ, Crespo JL. The role of TOR in autophagy regulation from yeast to plants and mammals. Autophagy 2008; 4:851-65.
100. Parker MS, Mock T, Armbrust EV. Genomic insights into marine microalgae. Annu Rev Genet 2008; 42:619-45.
101. Franklin DJ, Brussaard CPD, Berges JA. What is the role and nature of programmed cell death in phytoplankton ecology? Eur J Phycol 2006; 41:1-14.
102. Duszenko M, Figarella K, Macleod ET, Welburn SC. Death of a trypanosome: a selfish altruism. Trends Parasitol 2006; 22:536-42.
103. Golstein P, Aubry L, Levraud JP. Cell-death alternative model organisms: why and which? Nat Rev Mol Cell Biol 2003; 4:798-807.
104. Pérez-Pérez ME, Florencio FJ, Crespo JL. Inhibition of target of rapamycin signaling and stress activate autophagy in *Chlamydomonas reinhardtii*. Plant Physiol 2010; 152:1874-88.
105. Crespo JL, Diaz-Troya S, Florencio FJ. Inhibition of target of rapamycin signaling by rapamycin in the unicellular green alga *Chlamydomonas reinhardtii*. Plant Physiol 2005; 139:1736-49.
106. Diaz-Troya S, Florencio FJ, Crespo JL. Target of rapamycin and LST8 proteins associate with membranes from the endoplasmic reticulum in the unicellular green alga *Chlamydomonas reinhardtii*. Eukaryot Cell 2008; 7:212-22.
107. Huang K, Diener DR, Mitchell A, Pazour GJ, Witman GB, Rosenbaum JL. Function and dynamics of PKD2 in *Chlamydomonas reinhardtii* flagella. J Cell Biol 2007; 179:501-14.
108. Mostov KE. mTOR is out of control in polycystic kidney disease. Proc Natl Acad Sci USA 2006; 103:5247-8.
109. Shillingford JM, Murcia NS, Larson CH, Low SH, Hedgepeth R, Brown N, et al. The mTOR pathway is regulated by polycystin-1, and its inhibition reverses renal cystogenesis in polycystic kidney disease. Proc Natl Acad Sci USA 2006; 103:5466-71.
110. Picazarri K, Nakada-Tsukui K, Nozaki T. Autophagy during proliferation and encystation in the protozoan parasite *Entamoeba invadens*. Infect Immun 2008; 76:278-88.
111. Moon EK, Chung DI, Hong YC, Kong HH. Autophagy protein 8 mediating autophagosome in encysting Acanthamoeba. Mol Biochem Parasitol 2009; 168:43-8.
112. Lukes J, Hashimi H, Zíková A. Unexplained complexity of the mitochondrial genome and transcriptome in kinetoplastid flagellates. Curr Genet 2005; 48:277-99.
113. Brown RC, Evans DA, Vickerman K. Developmental changes in ultrastructure and physiology of *Trypanosoma brucei*. Trans R Soc Trop Med Hyg 1972; 66:336-7.
114. Vickerman K, Tetley L. Recent ultrastructural studies on trypanosomes. Ann Soc Belg Med Trop 1977; 57:441-57.
115. Wullschleger S, Loewith R, Hall MN. TOR signaling in growth and metabolism. Cell 2006; 124:471-84.
116. Barquilla A, Crespo JL, Navarro M. Rapamycin inhibits trypanosome cell growth by preventing TOR complex 2 formation. Proc Natl Acad Sci USA 2008; 105:14579-84.
117. Barquilla A, Navarro M. Trypanosome TOR as a major regulator of cell growth and autophagy. Autophagy 2009; 5:256-8.
118. Goldshmidt H, Matas D, Kabi A, Carmi S, Hope R, Michaeli S. Persistent ER stress induces the spliced leader RNA silencing pathway (SLS), leading to programmed cell death in *Trypanosoma brucei*. PLoS Pathog 2010; 6:1000731.
119. Uzcátegui NL, Carmona-Gutiérrez D, Denninger V, Schoenfeld C, Lang F, Figarella K, Duszenko M. Antiproliferative effect of dihydroxyacetone on *Trypanosoma brucei* bloodstream forms: cell cycle progression, subcellular alterations and cell death. Antimicrob Agents Chemother 2007; 51:3960-8.
120. Delgado M, Anderson P, Garcia-Salcedo JA, Caro M, Gonzalez-Rey E. Neuropeptides kill African trypanosomes by targeting intracellular compartments and inducing autophagic-like cell death. Cell Death Differ 2009; 16:406-16.
121. Campos-Salinas J, Gonzalez-Rey E. Autophagy and neuropeptides at the crossroad for parasites: to survive or to die? Autophagy 2009; 5:551-4.
122. Koopmann R, Muhammad K, Perbandt M, Betzel C, Duszenko M. *Trypanosoma brucei* ATG8: structural insights into autophagic-like mechanisms in protozoa. Autophagy 2009; 5:1085-91.
123. Michels PA, Bringaud F, Herman M, Hannaert V. Metabolic functions of glycosomes in trypanosomatids. Biochim Biophys Acta 2006; 1763:1463-77.
124. Bellu AR, Kiel JAKW. Selective degradation of peroxisomes in yeasts. Microsc Res Tech 2003; 61:161-70.
125. Braga MV, de Souza W. Effects of protein kinase and phosphatidylinositol-3 kinase inhibitors on growth and ultrastructure of *Trypanosoma cruzi*. FEMS Microbiol Lett 2006; 256:209-16.

126. Santa-Rita RM, Lira R, Barbosa HS, Urbina JA, de Castro SL. Anti-proliferative synergy of lysophospholipid analogues and ketoconazole against *Trypanosoma cruzi* (Kinetoplastida: Trypanosomatidae): cellular and ultrastructural analysis. J Antimicrob Chemother 2005; 55:780-4.
127. Menna-Barreto RFS, Salomão K, Dantas AP, Santa-Rita RM, Soares MJ, Barbosa HS, De Castro SL. Different cell death pathways induced by drugs in *Trypanosoma cruzi*: an ultrastructural study. Micron 2009; 40:157-68.
128. Menna-Barreto RFS, Henriques-Pons A, Pinto AV, Morgado-Diaz JA, Soares MJ, De Castro SL. Effect of a β-lapachone-derived naphthoimidazole on *Trypanosoma cruzi*: identification of target organelles. J Antimicrob Chemother 2005; 56:1034-41.
129. Menna-Barreto RF, Corrêa JR, Pinto AV, Soares MJ, de Castro SL. Mitochondrial disruption and DNA fragmentation in *Trypanosoma cruzi* induced by naphthoimidazoles synthesized from β-lapachone. Parasitol Res 2007; 101:895-905.
130. Menna-Barreto RFS, Corrêa JR, Cascabulho CM, Fernandes MC, Pinto AV, Soares MJ, De Castro SL. Naphthoimidazoles promote different death phenotypes in *Trypanosoma cruzi*. Parasitology 2009; 136:499-510.
131. Codogno P, Meijer AJ. Atg5: more than an autophagy factor. Nat Cell Biol 2006; 8:1045-7.
132. Soares MJ. The reservosome of *Trypanosoma cruzi* epimastigotes: an organelle of the endocytic pathway with a role on metacyclogenesis. Mem Inst. Oswaldo Cruz 1999; 94:139-41.
133. Sant'Anna C, Nakayasu ES, Pereira MG, Lourenço D, de Souza W, Almeida IC, Cunha-E-Silva NL. Subcellular proteomics of *Trypanosoma cruzi* reservosomes. Proteomics 2009; 9:1782-94.
134. Cazzulo JJ, Stoka V, Turk V. Cruzipain, the major cysteine proteinase from the protozoan parasite *Trypanosoma cruzi*. Biol Chem 1997; 378:1-10.
135. Coombs GH, Tetley L, Moss VA, Vickerman K. Three-dimensional structure of the Leishmania amastigote as revealed by computer-aided reconstruction from serial sections. Parasitology 1986; 92:13-23.
136. Waller RF, McConville MJ. Developmental changes in lysosome morphology and function Leishmania parasites. Int J Parasitol 2002; 32:1435-45.
137. Besteiro S, Williams RA, Coombs GH, Mottram JC. Protein turnover and differentiation in Leishmania. Int J Parasitol 2007; 37:1063-75.
138. Kumar S, Nei M, Dudley J, Tamura K. MEGA: A biologist-centric software for evolutionary analysis of DNA and protein sequences. Brief Bioinform 2008; 9:299-306.
139. Hart DT, Opperdoes FR. The occurrence of glycosomes (microbodies) in the promastigote stage of four major Leishmania species. Mol Biochem Parasitol 1984; 13:159-72.
140. Rosenzweig D, Smith D, Opperdoes F, Stern S, Olafson RW, Zilberstein D. Retooling Leishmania metabolism: from sand fly gut to human macrophage. FASEB J 2008; 22:590-602.
141. Saunders EC, Souza DP, Naderer T, Sernee MF, Ralton JE, Doyle MA, et al. Central carbon metabolism of Leishmania parasites. Parasitology 2010; 137:1303-13.
142. Frevert U, Engelmann S, Zougbede S, Stange J, Ng B, Matuschewski K, et al. Intravital observation of *Plasmodium berghei* sporozoite infection of the liver. PLoS Biol 2005; 3:192.
143. Amino R, Thiberge S, Martin B, Celli S, Shorte S, Frischknecht F, Ménard R. Quantitative imaging of Plasmodium transmission from mosquito to mammal. Nat Med 2006; 12:220-4.
144. Sturm A, Amino R, van de Sand C, Regen T, Retzlaff S, Rennenberg A, et al. Manipulation of host hepatocytes by the malaria parasite for delivery into liver sinusoids. Science 2006; 313:1287-90.
145. Morrissette NS, Sibley LD. Cytoskeleton of apicomplexan parasites. Microbiol Mol Biol Rev 2002; 66:21-38.
146. Jayabalasingham B, Bano N, Coppens I. Metamorphosis of the malaria parasite in the liver is associated with organelle clearance. Cell Res 2010; 20:1043-59.
147. Robson KJ, Frevert U, Reckmann I, Cowan G, Beier J, Scragg IG, et al. Thrombospondin-related adhesive protein (TRAP) of *Plasmodium falciparum*: expression during sporozoite ontogeny and binding to human hepatocytes. EMBO J 1995; 14:3883-94.
148. Sinnis P, Sim BK. Cell invasion by the vertebrate stages of Plasmodium. Trends Microbiol 1997; 5:52-8.
149. Kaiser K, Camargo, Kappe SH. Transformation of sporozoites into early exoerythrocytic malaria parasites does not require host cells. J Exp Med 2003; 197:1045-50.
150. Lim L, McFadden GI. The evolution, metabolism and functions of the apicoplast. Philos Trans R Soc Lond B Biol Sci 2010; 365:749-63.
151. Seglen PO, Gordon PB. 3-Methyladenine: specific inhibitor of autophagic/lysosomal protein degradation in isolated rat hepatocytes. Proc Natl Acad Sci USA 1982; 79:1889-92.
152. Petiot A, Ogier-Denis E, Blommaart EF, Meijer AJ, Codogno P. Distinct classes of phosphatidylinositol 3'-kinases are involved in signaling pathways that control macroautophagy in HT-29 cells. J Biol Chem 2000; 275:992-8.
153. Vaid A, Ranjan R, Smythe WA, Hoppe HC, Sharma P. PfPI3K, a phosphatidylinositol-3 kinase from *Plasmodium falciparum*, is exported to the host erythrocyte and is involved in hemoglobin trafficking. Blood 2010; 115:2500-7.
154. Alberti A, Michelet X, Djeddi A, Legouis R. The autophagosomal protein LGG-2 acts synergistically with LGG-1 in dauer formation and longevity in *C. elegans*. Autophagy 2010; 6:622-33.
155. Yamada Y, Suzuki NN, Hanada T, Ichimura Y, Kumeta H, Fujioka Y, et al. The crystal structure of Atg3, an autophagy-related ubiquitin carrier protein (E2) enzyme that mediates Atg8 lipidation. J Biol Chem 2007; 282:8036-43.
156. Hanada T, Satomi Y, Takao T, Ohsumi Y. The amino-terminal region of Atg3 is essential for association with phosphatidylethanolamine in Atg8 lipidation. FEBS Lett 2009; 583:1078-83.
157. Kabeya Y, Mizushima N, Yamamoto A, Oshitani-Okamoto S, Ohsumi Y, Yoshimori T. LC3, GABARAP and GATE16 localize to autophagosomal membrane depending on form-II formation. J Cell Sci 2004; 117:2805-12.
158. Kirisako T, Baba M, Ishihara N, Miyazawa K, Ohsumi M, Yoshimori T. Formation process of autophagosome is traced with Apg8/Aut7p in yeast. J Cell Biol 1999; 147:435-46.
159. Weidberg H, Shvets E, Shpilka T, Shimron F, Shinder V, Elazar Z. LC3 and GATE-16/GABARAP subfamilies are both essential yet act differently in autophagosome biogenesis. EMBO J 2010; 29:1792-802.
160. Zhou B, Boudreau N, Coulber C, Hammarback J, Rabinovitch M. Microtubule-associated protein 1 light chain 3 is a fibronectin mRNA-binding protein linked to mRNA translation in lamb vascular smooth muscle cells. J Clin Invest 1997; 100:3070-82.
161. Cann GM, Guignabert C, Ying L, Deshpande N, Bekker JM, Wang L, et al. Developmental expression of LC3α and β: absence of fibronectin or autophagy phenotype in LC3β knockout mice. Dev Dyn 2008; 237:187-95.
162. Swanson MS. Autophagy: eating for good health. J Immunol 2006; 177:4945-51.
163. Colombo MI. Autophagy: a pathogen driven process. IUBMB Life 2007; 59:238-42.
164. Huang J, Klionsky DJ. Autophagy and human disease. Cell Cycle 2007; 6:1837-49.
165. Deretic V. Autophagy in infection. Curr Opin Cell Biol 2009; 22:252-62.
166. Deretic V, Levine B. Autophagy, immunity and microbial adaptations. Cell Host Microbe 2009; 5:527-49.
167. Andrade RM, Wessendarp M, Gubbels MJ, Striepen B, Subauste CS. CD40 induces macrophage anti-*Toxoplasma gondii* activity by triggering autophagy-dependent fusion of pathogen-containing vacuoles and lysosomes. J Clin Invest 2006; 116:2366-77.
168. Subauste CS. CD40, autophagy and *Toxoplasma gondii*. Mem Inst Oswaldo Cruz 2009; 104:267-72.
169. Melzer T, Duffy A, Weiss LM, Halonen SK. The gamma interferon (IFNγ)-inducible GTP-binding protein IGTP is necessary for Toxoplasma vacuolar disruption and induces parasite egression in IFNγ-stimulated astrocytes. Infect Immun 2008; 76:4883-94.
170. Ling YM, Shaw MH, Ayala C, Coppens I, Taylor GA, Ferguson DJ, Yap GS. Vacuolar and plasma membrane stripping and autophagic elimination of *Toxoplasma gondii* in primed effector macrophages. J Exp Med 2006; 203:2063-71.
171. Zhao Z, Fux B, Goodwin M, Dunay IR, Strong D, Miller BC, et al. Autophagosome-independent essential function for the autophagy protein Atg5 in cellular immunity to intracellular pathogens. Cell Host Microbe 2008; 4:458-69.
172. Zhao YO, Khaminets A, Hunn JP, Howard JC. Disruption of the *Toxoplasma gondii* parasitophorous vacuole by IFNgamma-inducible immunity-related GTPases (IRG proteins) triggers necrotic cell death. PLoS Pathog 2009; 5:1000288.
173. Halonen SK. Role of autophagy in the host defense against *Toxoplasma gondii* in astrocytes. Autophagy 2009; 5:268-9.
174. Wang Y, Weiss LM, Orlofsky A. Host cell autophagy is induced by *Toxoplasma gondii* and contributes to parasite growth. J Biol Chem 2009; 284:1694-701.
175. Coppens I, Dunn JD, Romano JD, Pypaert M, Zhang H, Boothroyd JC, Joiner KA. *Toxoplasma gondii* sequesters lysosomes from mammalian hosts in the vacuolar space. Cell 2006; 125:261-74.
176. Wang Y, Weiss LM, Orlofsky A. Intracellular parasitism with *Toxoplasma gondii* stimulates mammalian-target-of-rapamycin-dependent host cell growth despite impaired signalling to S6K1 and 4E-BP1. Cell Microbiol 2009; 11:983-1000.
177. Romano PS, Arboit MA, Vázquez CL, Colombo MI. The autophagic pathway is a key component in the lysosomal dependent entry of *Trypanosoma cruzi* into the host cell. Autophagy 2009; 5:6-18.
178. Tardieux I, Webster P, Ravesloot J, Boron W, Lunn JA, Heuser JE, Andrews NW. Lysosome recruitment and fusion are early events required for trypanosome invasion of mammalian cells. Cell 1992; 71:1117-30.
179. Rodríguez A, Samoff E, Rioult MG, Chung A, Andrews NW. Host cell invasion by trypanosomes requires lysosomes and microtubule/kinesin-mediated transport. J Cell Biol 1996; 134:349-62.
180. Woolsey AM, Sunwoo L, Petersen CA, Brachmann SM, Cantley LC, Burleigh BA. Novel PI 3-kinase-dependent mechanisms of trypanosome invasion and vacuole maturation. J Cell Sci 2003; 116:3611-22.
181. Andrade LO, Andrews NW. The *Trypanosoma cruzi*-host-cell interplay: location, invasion, retention. Nat Rev Microbiol 2005; 3:819-23.
182. Ley V, Robbins ES, Nussenzweig V, Andrews NW. The exit of *Trypanosoma cruzi* from the phagosome is inhibited by raising the pH of acidic compartments. J Exp Med 1990; 171:401-13.
183. Pinheiro RO, Nunes MP, Pinheiro CS, D'Avila H, Bozza PT, Takiya CM, et al. Induction of autophagy correlates with increased parasite load of *Leishmania amazonensis* in BALB/c but not C57BL/6 macrophages. Microbes Infect 2009; 11:181-90.
184. Bozza PT, Melo RC, Bandeira-Melo C. Leukocyte lipid bodies regulation and function: contribution to allergy and host defense. Pharmacol Ther 2007; 113:30-49.
185. Guimarães ET, Santos LA, Ribeiro dos Santos R, Teixeira MM, dos Santos WL, Soares MB. Role of interleukin-4 and prostaglandin E2 in *Leishmania amazonensis* infection of BALB/c mice. Microbes Infect 2006; 8:1219-26.

186. Chaussepied M, Langsley G. Theileria-induced leukocyte transformation: an example of oncogene addiction? In: Kaufmann SHE, Rouse BT, Sacks DL, Eds., The Immune Response to Infection. Washington DC: ASM Press 2010; In press.
187. Morrison WI. Progress towards understanding the immunobiology of Theileria parasites. Parasitology 2009; 136:1415-26.
188. Rennenberg A, Lehmann C, Heitmann A, Witt T, Hansen G, Nagarajan K, et al. Exoerythrocytic Plasmodium parasites secrete a cysteine protease inhibitor involved in sporozoite invasion and capable of blocking cell death of host hepatocytes. PLoS Pathog 2010; 6:1000825.
189. Münz C. Antigen processing via autophagy—not only for MHC class II presentation anymore? Curr Opin Immunol 2010; 22:89-93.
190. Pearce MJ, Mintseris J, Ferreyra J, Gygi SP, Darwin KH. Ubiquitin-like protein involved in the proteasome pathway of *Mycobacterium tuberculosis*. Science 2008; 322:1104-7.
191. Cazzulo JJ, Stoka V, Turk V. The major cysteine proteinase of *Trypanosoma cruzi*: a valid target for chemotherapy of Chagas disease. Curr Pharm Des 2001; 7:1143-56.
192. Docampo R, Moreno SN. Current chemotherapy of human African trypanosomiasis. Parasitol Res 2003; 90:10-3.
193. Steverding D, Tyler KM. Novel antitrypanosomal agents. Expert Opin Investig Drugs 2005; 14:939-55.
194. Alvarez VE, Kosec G, Sant Anna C, Turk V, Cazzulo JJ, Turk B. Blocking autophagy to prevent parasite differentiation: a possible new strategy for fighting parasitic infections? Autophagy 2008; 4:361-3.
195. Duschak VG, Couto AS. Cruzipain, the major cysteine protease of *Trypanosoma cruzi*: a sulfated glycoprotein antigen as relevant candidate for vaccine development and drug target. A review. Curr Med Chem 2009; 16:3174-202.
196. Selzer PM, Pingel S, Hsieh I, Ugele B, Chan VJ, Engel JC, et al. Cysteine protease inhibitors as chemotherapy: lessons from a parasite target. Proc Natl Acad Sci USA 1999; 96:11015-22.
197. Franke de Cazzulo BM, Martínez J, North MJ, Coombs GH, Cazzulo JJ. Effects of proteinase inhibitors on the growth and differentiation of *Trypanosoma cruzi*. FEMS Microbiol Lett 1994; 124:81-6.
198. McKerrow JH, Engel JC, Caffrey CR. Cysteine protease inhibitors as chemotherapy for parasitic infections. Bioorg Med Chem 1999; 7:639-44.
199. Stechmann A, Cavalier-Smith T. The root of the eukaryote tree pinpointed. Curr Biol 2003; 13:665-6.
200. Clayton C, Adams M, Almeida R, Baltz T, Barrett M, Bastien P, et al. Genetic nomenclature for Trypanosoma and Leishmania. Mol Biochem Parasitol 1998; 97:221-4.