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**Proteomic analysis reveals the role of Art2
 α -arrestin in endocytosis of thiamine
transporter Thi7 in *S. cerevisiae***

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ABSTRACT

The plasma membrane (PM) constitutes the first barrier separating the cell from its external medium. The PM protein and lipid composition must be tightly regulated for the adaptation of cells to their surrounding environment, ensuring a good nutritional status and proper responses to environmental cues. One essential mechanism underlying that regulation is endocytosis. In the yeast *Saccharomyces cerevisiae*, endocytosis of proteins is triggered by their ubiquitylation, which is catalyzed by a single ubiquitin ligase, Rsp5. However, 240 proteins are predicted to be localized at the PM. To ubiquitylate such a high number of targets, Rsp5 interacts with adaptor proteins, the α -arrestins. The α -arrestins bridge Rsp5 and its targets, thereby mediating the ubiquitylation of PM proteins. The genome of *S. cerevisiae* encodes thirteen adaptors but only a few PM proteins were shown to interact with them so far, leading to an incomplete understanding of their regulation.

To broaden our knowledge on α -arrestin involvement, the first part of this study focused on the identification of new targets of the α -arrestin system in an unbiased fashion. To that end, we performed a quantitative proteomic screening that led us to the discovery of 28 potential new targets. Among these, we decided to further investigate a poorly known subset of transporters, thiamine (vitamin B1) transporters. The second part of the study dealt with the characterization of the α -arrestin-mediated endocytosis of thiamine transporters. We unraveled the role of Art2 α -arrestin in the thiamine and stress-induced ubiquitylation and endocytosis of thiamine transporters. Art2 proves to be a new player in thiamine homeostasis by fine-tuning the abundance of thiamine transporters in response to various signals.

ABBREVIATIONS

(yE)GFP	(yeast enhanced) Green fluorescent protein
ABC	ATP-binding cassette
ACT	Amino acid/choline transporter family
ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
AP-1	Clathrin adaptor protein complex-1
AP-2	Clathrin adaptor protein complex-2
APC	Amino acid/polyamine/organocation superfamily
Arg	Arginine
ART	Arrestin-related trafficking adaptor
ATP	Adenosine triphosphate
B1	vitamin B1
BAR	Bin/Amphiphysin/Rvs
CCP	Clathrin-coated pit
CCV	Clathrin-coated vesicle
CHX	Cycloheximide
CIE	Clathrin-independent endocytosis
CME	Clathrin-mediated endocytosis
ctrl	Control
DHA1	Drug:H ⁺ antiporter family
DIC	Differential interference contrast
DNA	Deoxyribonucleic acid
DUB	Deubiquitinase enzyme
ENTH	Epsin N-terminal homology
ER	Endoplasmic reticulum
ERAD	Endoplasmic-reticulum-associated protein degradation
ESCRT	Endosomal sorting complexes required for transport
ESI	Electrospray ionization
GA	Golgi apparatus
HA	Hemagglutinin
HECT	Homologous to E6-associated protein C-terminus
HET	4-methyl-5-hydroxyethylthiazole
HMP	2-methyl-4-amino-5-hydroxymethylpyrimidine
IF	Inward facing
ILV	Intralumenal vesicle

iTRAQ	Isobaric tags for relative and absolute quantification
kDa	Kilodalton
LID	Loop-interaction domain
MDR	Multiple drug resistance family
MFS	Major facilitator superfamily
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
MVB	Multivesicular body
NAD	Nicotinamide adenine dinucleotide
NCS1	Nucleobase-Cation Symporter 1 family
OD _{600nm}	Optical density at 600 nm
OF	Outward facing
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
PDC	Pyruvate decarboxylase
PDH	Pyruvate dehydrogenase
PEST	Proline, glutamic acid, serine and threonine rich sequence
PI(4,5)P ₂	Phosphatidylinositol-4,5-bisphosphate
PM	Plasma membrane
PP-1	protein phosphatase 1 (Glc7/Reg1)
PY	(V/L/P)PxY sequence motif
RING	Really interesting new gene
SC	Synthetic complete
SDS	Sodium dodecyl sulfate
SPS	Ssy1-Ptr3-Ssy5
TBS	Tris-buffered saline
TBS-T	Tween 20-supplemented Tris-buffered saline
TE	Total protein extract
TGN	Trans-Golgi network
TMP	Thiamine monophosphate
TORC1	Target of rapamycin complex
TPP	Thiamine pyrophosphate
Ub	Ubiquitin
UBD	Ubiquitin-binding domain
UHPLC	Ultra-high-performance chromatography
WT	Wild-type
WW domain	two tryptophan residues separated by approximately 20 amino acids
YD	Yeast Dextrose medium

YFP	Yellow fluorescent protein
YNB	Yeast nitrogen base

INTRODUCTION

1 Endocytosis

1.1 General overview

Endocytosis is the process by which the plasma membrane (PM) invaginates and buds off vesicles toward the cytosol, internalizing integral membrane proteins (also called cargoes), lipids and molecules from the external medium.

Newly formed endocytic vesicles pinch off from the plasma membrane and are internalized. Several endocytic vesicles undergo fusion, giving rise to an endosome. Endosomes can themselves undergo budding and generate recycling endosomes, transporting proteins that will be recycled back to the PM, retrieving them from the degradation pathway. Otherwise, early endosomes will further be matured to become late endosomes / multivesicular bodies (MVBs), following the formation of intraluminal vesicles. These intraluminal vesicles are composed of membrane proteins that will be targeted to vacuolar/lysosomal degradation. After fusion of MVBs with the vacuolar membrane, intraluminal vesicles are discharged in the vacuolar lumen and PM proteins are degraded (Goode et al., 2015).

As more and more evidences suggest that endocytosis is well conserved from yeast to metazoans, the budding yeast, *Saccharomyces cerevisiae*, turns out to be a powerful tool for understanding its underlying molecular mechanisms. Therefore, this section of the Introduction will focus on the endocytic pathway of the budding yeast.

1.2 Function

Endocytosis is essential for plasma membrane homeostasis. It allows the recycling of PM lipids and proteins as well as the uptake of some molecules from the external medium. The removal of misfolded proteins or proteins that are no longer required in the current growth condition is essential for cellular metabolism and membrane integrity. Accumulation of various compounds, including nutrients, is toxic for the cell. Therefore, many transporters undergo substrate-induced endocytosis, as first shown for the inositol permease Itr1 (Lai et al., 1995). This process, associated with protein synthesis and exocytosis as well as regulation of the intrinsic activity of PM proteins is a major mechanism controlling the PM protein landscape and its interaction with the environment.

1.3 Induction

1.3.1 Ubiquitylation of plasma membrane proteins

In yeast, the endocytosis of proteins is mainly triggered by their covalent coupling with ubiquitin (Hicke and Riezman, 1996). Ubiquitylated proteins are further recognized by the endocytic machinery, thereby entering the endocytic pathway (see section 2). Prior phosphorylation of the cytosolic region of some proteins was shown to be required for their ubiquitylation. Such ubiquitylation-inducing phosphorylation takes place at a polypeptide sequence enriched in proline, glutamic acid, serine and threonine, called PEST-like sequence (Rechsteiner and Rogers, 1996; Roth and Davis, 2000). For example, phosphorylation of the PEST-like sequence of the pheromone receptors Ste2 and Ste3 as well as the uracil permease Fur4 by the PM-anchored casein kinase 1 (Yck1/2) is required for their ubiquitylation and degradation (Marchal et al., 1998, 2000; Roth and Davis, 2000).

1.3.2 Short-peptide motif

Although most of studied proteins undergo endocytosis following ubiquitylation, some proteins only require the exposure of a short peptide motif to be endocytosed. In yeast, the only known sorting motif is characterized by the sequence NPF(x)_{1,2}D (Tan et al., 1996). This sequence is recognized by a protein from the endocytic machinery, Sla1, via its SH3 domain (Src homology domain), incorporating the protein in an endocytic vesicle (Howard et al., 2002). The pheromone receptors Ste2 and Ste3, the cell wall sensor Wsc1 and the phospholipid flippases Drs2 and Dnf1 were shown to bind Sla1 via this motif (Howard et al., 2002; Piao et al., 2007; Liu et al., 2007).

1.4 Mechanism

In yeast, two types of endocytic pathways are co-existing according to whether clathrin is involved or not. Clathrin is a hexameric protein, which forms a “triskelion”, made up of three 192 kDa heavy chains, each bound to one light chain of 30 kDa (Dupré et al., 2004). Polymerisation of clathrin in vivo gives a polygonal lattice that coats endocytic vesicles, thereby called clathrin-coated vesicles (CCVs). The main function of the clathrin coat is the scaffolding of the endocytic vesicles containing the endocytic coat, the actin assembly machinery and ubiquitylated cargoes.

Clathrin-mediated endocytosis is the most studied endocytosis mechanism in yeast. All PM proteins studied so far follow this pathway (McMahon and Boucrot, 2011). However, recent studies have suggested a clathrin-independent endocytosis (CIE) pathway. No PM protein has been shown to use exclusively this pathway, but they are proposed to rather follow one pathway or another according to conditions (Prosser et al., 2011).

1.4.1 Clathrin-mediated endocytosis

The mechanism underlying CME consists of more than 50 proteins (reviewed in Goode et al., 2015 and Lu et al., 2016). The aim of this part is to summarize the major steps of endocytic vesicle formation and will not focus on all the proteins involved.

Endocytosis is composed of different steps, precisely controlled in space and time (Kaksonen et al., 2005). Ubiquitylated cargoes gather at one place, the membrane invaginates and forms a CCV, this vesicle severed from the membrane and then transported from the cell periphery to the inside of the cell thanks to actin cables. Each step is characterized by the arrival of different subsets of proteins: the early and late coat proteins, the actin nucleation promoting factors (NPFs) and the scission machinery. Of course, the whole endocytic machinery is subsequently recycled to ensure a new round of endocytosis (Figure 1).

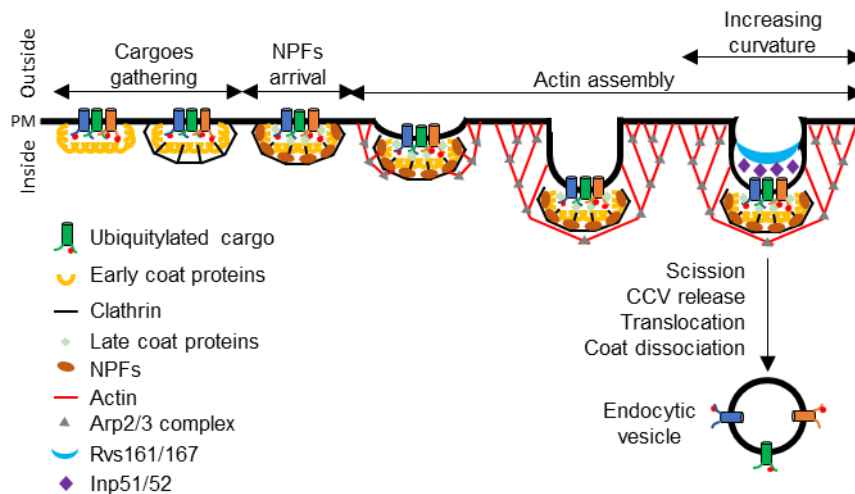


Figure 1 – Schematic view of the mechanism underlying clathrin-mediated endocytosis. (PM: Plasma Membrane)

Formation of the endocytic coat

Ubiquitylated proteins gather at the nascent endocytic site, which is selected and initiated by early coat proteins, Ede1 and Syp1. Their function is mostly to bind the cargoes, thanks to their ubiquitin-binding domain (UBD), and the PM. The recruitment of clathrin adaptor proteins (such as Yap1801, Yap1802 and the AP-2 complex) and clathrin takes place at this step. The function of these adaptor proteins is to link ubiquitylated cargoes, clathrin and PM together. Clathrin polymerization at the endocytic site contributes to the formation of a clathrin-coated pit (CCP). Additional proteins are recruited at the CCP to bridge membranes and the current endocytic coat to actin: Sla2, Ent1 and Ent2. Ent1/2 are also able to bind clathrin and further scaffold the CCP (McMahon and Boucrot, 2011; Boettner et al., 2011). Unlike other steps of CME, the duration of this step is not clearly defined. The accumulation of ubiquitylated cargoes was proposed as a checkpoint triggering the recruitment of the late endocytic coat proteins (Carroll et al., 2012).

The late arriving proteins consist of the first actin NPF (Pan1) and other scaffolding proteins (End3 and Sla1). End3 is known to link the clathrin adaptor complex-2 (AP-2) to ubiquitylated cargoes and coat proteins (Tang et al., 1997). Sla1 has a UBD and SH3-domain, allowing the binding to ubiquitylated and NPF(x)_{1,2}D motif containing proteins (Howard et al., 2002).

The last set of proteins recruited at the PM are the three-major actin NPFs: Las17 and Myo3/5. They promote actin polymerization by positively regulating the complex constituted of two actin-related proteins, Arp2 and Arp3, called the Arp2/3 complex (Boettner et al., 2011). At this step, the CCP is still flat. Actin is recruited, and polymerization takes place.

Invagination of the membrane

The invagination of the membrane is thought to be mostly due to the force generated by actin assembly and the recruitment of Bin-Amphiphysin-Rvs (BAR) domain-containing proteins (Weinberg and Drubin, 2012).

As mentioned in the previous part, actin polymerization is initiated by the Arp2/3 complex. When NPFs bind Arp2/3, the complex undergoes a conformational change and promotes the elongation of actin filaments as well as a branched actin network. Since the proteins of the coat are linked to NPFs and actin, the coat translates the actin assembly into mechanical force. The elongating actin filaments are suspected to push on the membrane at the initial curvature site, thereby promoting the membrane invagination (Mooren et al., 2012). The membrane invagination is made up of a tubule-shaped part (the neck) and a bud, the CCP, which is covered by the endocytic coat (Boettner et al., 2011).

BAR domain-containing proteins are known to sense curvature, to bind to curved membranes and to generate bending. The heterodimer composed of the BAR domain proteins, Rvs161 and Rvs167, is recruited to the neck by the rising curvature of the lipid bilayer. The more the membrane is bent, the more Rvs161/167 are recruited, and the more the curvature increases. Rvs161/167 are therefore essential to reduce the diameter of the neck, promoting fission of the CCP (Kaksonen et al., 2005).

Scission of the vesicle

Scission is thought to be the result of three factors: the force generated by the polymerization of actin, the constriction of the neck by BAR domain-containing proteins (Rvs161/167) and the creation of a line tension in the lipid bilayer. The line tension is due to the difference in lipid composition between the neck of the invagination and the CCP. Indeed, the phosphatidylinositol-4,5-bisphosphate (PI(4,5)P₂) phosphatases Inp51 and Inp52 dephosphorylate PI(4,5)P₂ from the bud, leading to its enrichment in phosphatidylinositol-4-phosphate. However, they fail to do so at the neck due to the BAR domain proteins covering it (Conibear, 2010).

Recycling of the endocytic machinery

Due to the change in lipid composition, many scaffolding proteins of the coat disassemble. Two kinases, Ark1 and Prk1, are also involved in coat dissociation by phosphorylating coat proteins and disrupting protein-protein interactions. This phosphorylation was shown to be counteracted by the action of the PP1-1 phosphatase, ensuring new endocytic events (Boettner et al., 2011).

1.4.2 Clathrin-Independent Endocytosis (CIE)

An endocytic pathway independent of clathrin and the actin assembly Arp2/3 complex was shown in *S. cerevisiae*. This pathway is based on the GTPase Rho1, the actin cable-assembling formin Bni1 and the major isoform of tropomyosin Tpm1 (Prosser et al., 2011). This pathway was emphasized by the rescue of the endocytosis defect in cells lacking clathrin-binding endocytic adaptor proteins (Ent1, Ent2, Yap1801, and Yap1802). Endocytosis of the α -pheromone receptor Ste3 was restored by overexpression of the GTPase *RHO1*, its guanine nucleotide exchange factors (GEF) *ROM1/2* or the cell wall sensor *MID2*. Moreover, in the absence of Bni1, overexpression of the latter genes fails to rescue endocytosis. Although the Arp2/3 complex is dispensable for CIE, actin is still required. Indeed, no Arp2/3-dependent actin patches are detected in CIE but rather actin cables generated by Bni1.

In addition to Ste3, the methionine transporter Mup1 was shown to use this pathway in that context (Prosser et al., 2015). Unfortunately, neither the underlying mechanism nor the role of this pathway in a WT background have been completely understood yet.

2 Ubiquitylation of plasma membrane proteins

2.1 General overview

Ubiquitylation is a reversible post-translational modification that consists of the covalent coupling of proteins to ubiquitin. Ubiquitin is a small globular polypeptide of 76 amino-acids found in all eukaryotic organisms. The isopeptide linkage is performed between the ϵ -amino group of lysine residues of the target protein and the C-terminal glycine of ubiquitin. Ubiquitin has seven lysine residues: K6, K11, K27, K29, K33, K48 and K63. Ubiquitin can itself be coupled with ubiquitin moieties. Therefore, different ubiquitylation patterns are found on proteins: monoubiquitylation (one ubiquitin on one lysine residue), multi-monoubiquitylation (one ubiquitin on several lysine residues), diubiquitylation (a diubiquitin chain on one lysine) and polyubiquitination (extended polyubiquitin chain). The fate of ubiquitylated proteins is thought to differ according to the type of ubiquitylation. (Staub and Rotin, 2006).

2.2 Function

Ubiquitylation is a signal for protein degradation. In fact, ubiquitylation is already known to target cytosolic proteins to the 26S-proteasome (Dupré et al., 2004). K11-, K29- or more commonly K48-linked polyubiquitin chains of at least four residues are required to trigger proteasome-dependent degradation. However, some membrane proteins, including PM cargoes, are not accessible to the proteasome and are degraded in the vacuole (Glickman and Ciechanover, 2002).

Ubiquitin is therefore a sorting signal inducing the endocytosis of PM proteins and subsequent transfer to the vacuole (Hicke and Riezman, 1996). Monoubiquitylation as well as multi-

monoubiquitylation have been shown to be sufficient for internalization of yeast nutrient permeases (Dupré et al., 2004; Lauwers et al., 2010) . In addition to this, K63-linked di- or polyubiquitin chains are often observed on endocytosed PM cargoes but they seem to be only necessary for entry in the MVB pathway and not for the internalization itself (Lauwers et al., 2010).

2.3 General mechanism

Ubiquitylation is an ATP-dependent process that involves the sequential transfer of the ubiquitin moiety to a ubiquitin-activating enzyme (E1), a ubiquitin-conjugating enzyme (E2), and a ubiquitin ligase (E3). Substrate specificity is mainly controlled by the E3 ubiquitin ligase. There are one E1, eleven E2 and 53 E3 encoded by the yeast genome (Lauwers et al., 2010). The two main types of ubiquitin ligase are the HECT (homologous to E6-associated protein C-terminus) and RING (really interesting new gene)-domain containing ubiquitin ligases. They differ by their mechanism of the ubiquitin moiety transfer. The HECT domain are covalently linked to ubiquitin via a thioester bond before transferring it to the target protein. On the contrary, RING domain-containing ubiquitin ligase does not covalently bind ubiquitin but will directly catalyze ubiquitin transfer from the E2 ubiquitin conjugating enzyme to the substrate (Staub and Rotin, 2006).

2.4 The ubiquitin ligase Rsp5

Astonishingly, a single ubiquitin ligase was shown to be required for ubiquitylation of PM proteins, the HECT-type ubiquitin ligase Rsp5 (Springael J.Y., Galan J.M., Haguenauer-Tsapis R., 1999; Hicke and Dunn, 2003). Rsp5 is the sole yeast homolog of a metazoan family of ubiquitin ligases, called Nedd4. As a member of the Nedd4 family, Rsp5 has an N-terminal C2 domain, required for plasma membrane association, and a C-terminal HECT E3

ubiquitin ligase domain, which flanks three central WW domains (Wang et al., 1999). The WW domain is essential for Rsp5 interaction with its targets, via binding to their [V/P]PxY (PY) motif (Helliwell et al., 2001).

In addition to PM proteins, Rsp5 promotes ubiquitylation and degradation of many cytosolic and internal membrane proteins. Rsp5 is an essential protein since it is involved in the proteolytic activation of a transcription factor promoting the expression of oleate synthesis genes (Zhang et al., 1999; Hoppe et al., 2000). Moreover, many PM proteins do not have a PY motif. Therefore, the interaction of Rsp5 with its PM target is mediated by PY motif-containing adaptor proteins. These adaptors specifically bind proteins to be degraded, thereby bridging Rsp5 to its target (Sullivan et al., 2007).

2.5 Adaptors of Rsp5

Many Rsp5 adaptors are integral membrane proteins, like Bsd2p (Hettema et al., 2004), Tre1/2p (Stimpson et al., 2006), Ear1p, Ssh4p (Li et al., 2015), Sna3 (MacDonald et al., 2012) and Rcr1/2p (Kota et al., 2007).

They are mainly required for the proper targeting of proteins to the vacuole by ensuring their ubiquitylation and entry in MVBs as it was shown for the Bsd2 and Tre1/2-mediated ubiquitylation of the manganese permease Smf1 (Hettema et al., 2004; Sullivan et al., 2007). Ssh4 was recently shown to bridge vacuolar amino acid transporter Ypq1 to Rsp5, leading to its ubiquitylation and subsequent vacuolar degradation (Li et al., 2015; Zhu et al., 2017).

Another family of Rsp5 adaptor proteins was illustrated recently (Lin et al., 2008). Unlike the previous adaptors, they are cytosolic and are also acting at the level of the plasma membrane. These adaptors belong to the family of α -arrestins and are further developed in the next chapter of the introduction (see section 3).

2.6 Deubiquitylation

Ubiquitylation is antagonized by deubiquitylating enzymes, also called DUBs. They consist of ubiquitin isopeptidases that ensure the recycling of ubiquitin moieties within the cell. There are about twenty DUBs encoded by the genome of *S. cerevisiae*. One example is the ubiquitin carboxyl-terminal hydrolase, Doa4p, which acts at the late endosome/prevacuolar compartment to remove ubiquitin from ubiquitylated membrane proteins before their vacuolar degradation. Moreover, many DUBs and E3 ubiquitin ligases interact with each other and work in concert, such as Ubp2 and Rsp5 (Kee et al., 2005). The interaction between DUBs and E3 ubiquitin ligase fine-tunes substrate ubiquitination, as well as regulates their own stability or activity (Nijman et al., 2005).

3 *S. cerevisiae* α -arrestins

3.1 General overview

The *S. cerevisiae* α -arrestins, also called ART (Arrestin-Related Trafficking) adaptors are members of the arrestin superfamily, which includes the metazoan visual arrestins (arrestin 1 and 4) and β -arrestins (arrestin 2 and 3), also called “true arrestins” (Aubry and Klein, 2013). True arrestins were initially shown to “arrest” the signal from activated G-protein coupled receptors (GPCRs). Arrestin-1 and arrestin-2 were first shown to mediate the endocytosis of the photoactivated rhodopsin (Zuckerman and Cheasty, 1986) or the β 2-adrenergic receptor (Attramadal et al., 1992), respectively.

Up to now, thirteen ART proteins have been identified *S. cerevisiae* (Table 1). Ten of them have been renamed Art1 through Art10. The three remaining proteins, the ubiquitin ligase-binding proteins Bul1, Bul2 and Bul3, are more distantly related members of the family and are thought to be fungal specific distant variant of arrestin proteins (Nikko and Pelham, 2009; Aubry et al., 2009; Novoselova et al., 2012). Other yeast proteins are predicted to have structural similarity with arrestins, such as the protein of unknown function Spo23, the retromer subunit Pep8 and the subunit of the Golgi apparatus (GA) membrane nucleotide exchange factor Rgp1 (Aubry and Klein, 2013) (Table 1).

Table 1 – List of the arrestin domain-containing proteins in *Saccharomyces cerevisiae*. Two systematic names are available for Bul3 since Bul3 is the result of a translational readthrough (bypass of the STOP-codon). (N.A.: Not Applicable)

ART nomenclature	Name	Systematic name	Size (kDa)
Art1	Ldb19	Yor322c	90
Art2	Ecm21	Ybl101c	124
Art3	Aly2	Yjl084c	117
Art4	Rod1	Yor018w	92
Art5	N.A.	Ygr068c	65
Art6	Aly1	Ykr021w	103
Art7	Rog3	Yfr022w	80
Art8	Csr2	Ypr030w	125
Art9	Rim8	Ygl045w	61
Art10	N.A.	Ylr392c	60
N.A.	Bul1	Ymr275c	109
N.A.	Bul2	Yml111w	105
N.A.	Bul3	Ynr068/069c	31/55
N.A.	Pep8	Yjl053w	43
N.A.	Spo23	Ybr250w	60
N.A.	Rgp1	Ydr137w	75

3.2 Features

Although the sequence identity between the arrestin-domain of yeast α -arrestin and true arrestins is relatively low (about 10%), they are folded alike according to structural predictions of yeast arrestins. True arrestins are composed of two β -strand sandwich modules, the N- and C-arrestin fold domains, separated by a flexible region usually called the hinge (Aubry and Klein, 2013) (Figure 2).

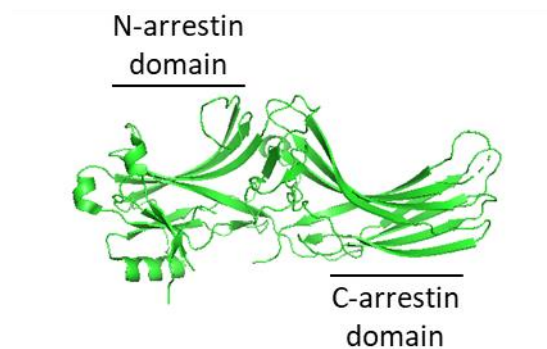


Figure 2 – Structure of bovine β -arrestin 1 by X-ray diffraction (Protein Data Bank ID: 1G4R). (Structure obtained by Han et al., 2001).

Yeast α -arrestins display either the N-arrestin, or the C-arrestin fold domain or both (Becuwe et al., 2012a). Unlike yeast α -arrestins containing both fold domains, the hinge of true arrestins is rich in charged amino acids, forming the polar core. The polar core has a regulatory function since it interacts with the C-terminal tail. Upon binding to its target, true arrestins undergo conformational change, releasing the C-terminal from the polar core. The C-terminal tail is therefore accessible for interaction with the endocytic machinery (clathrin and its adaptors). Such regulatory mechanism has not been illustrated for the yeast α -arrestins. In addition to their arrestin fold domain(s), all yeast α -arrestins except for the Bul adaptors also have a long C-terminal tail (C-tail) containing at least one PY motif ([V/L/P]PxY) (Figure 3).

Notably, in addition to true arrestins, α -arrestins are also found in metazoans (Alvarez, 2008). In addition to the five *arrestin domain-containing* (ArrDC) proteins, the thioredoxin-interacting protein TXNIP belongs to the human α -arrestin family. They are more similar to yeast α -arrestins in terms of sequence identity than β -arrestins. All the ArrDC proteins (except ArrDC5) and TXNIP have a PY motif on their C-terminal tail (Aubry and Klein, 2013).

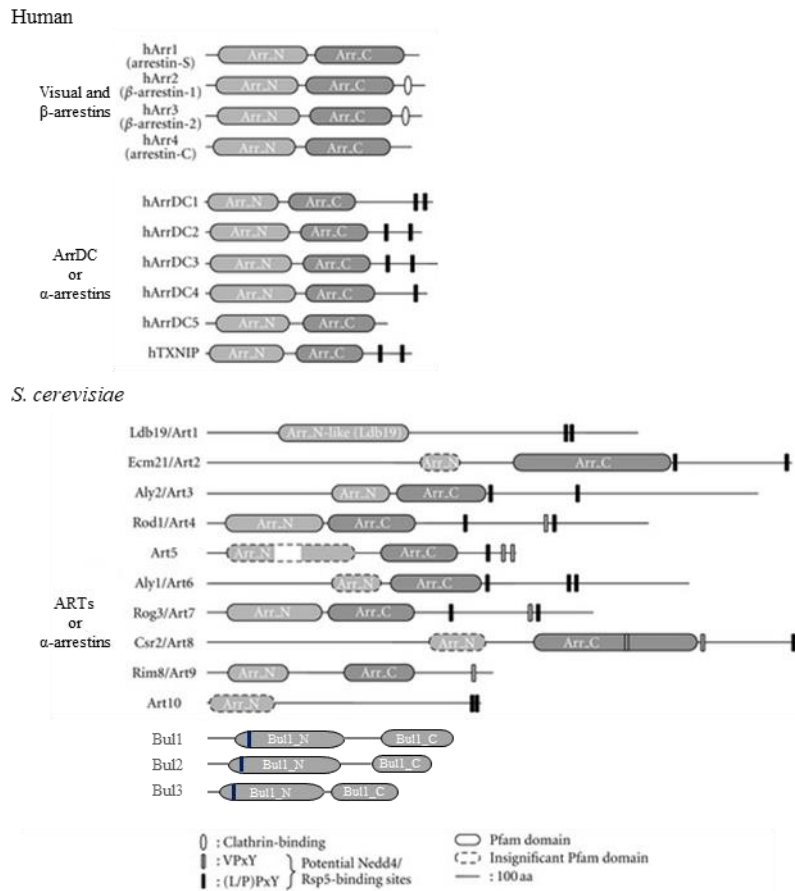


Figure 3 – List of the main arrestin domain-containing proteins in human and the yeast *Saccharomyces cerevisiae*. Ldb19, Bul1_N are related to N-arrestin fold domain (Arr_N), while Bul1_C is related to the C-arrestin fold domain (Arr_C). ArrDC stands for Arrestin domain-containing protein. Adapted from Becuwe et al., 2012a.

3.3 Function

The main α -arrestin function studied so far is their ability to recruit the ubiquitin ligase Rsp5 thanks to their C-terminal PY motif, therefore bridging Rsp5 to its PM target. The interaction with the target is likely to be dependent on the arrestin fold domain. Therefore, α -arrestins are mainly thought to be mediators of the Rsp5-dependent ubiquitylation of PM proteins, promoting their endocytosis. Most α -arrestins were shown to mediate the ubiquitylation of several PM targets according to the endocytosis-

triggering stimulus. For example, Art1 is required in the arginine and lysine induced endocytosis of the arginine permease Can1 and the lysine permease Lyp1, respectively (Lin et al., 2008; Nikko and Pelham, 2009; Merhi and Andre, 2012). Conversely, the ubiquitylation of PM proteins is often regulated by various α -arrestins according to conditions. The ubiquitylation of Lyp1 is mediated by Art1 in response to an excess of lysine but is Art2-dependent in response to cycloheximide (CHX), an inhibitor of protein synthesis known to induce endocytosis (Nikko and Pelham, 2009). The yeast α -arrestin system also presents some redundancy. Indeed, Bul1 and Bul2 are both sufficient to internalize Gap1 (Soetens et al., 2001; De Craene et al., 2001). In contrast with redundancy, several α -arrestins are required for the complete endocytosis of Fur4 after addition of uracil or cycloheximide (Nikko and Pelham, 2009), suggesting complementarity between adaptors. Table 2 lists all the PM proteins, whose endocytosis was shown to be mediated by α -arrestin, and the corresponding endocytosis-triggering stimulus.

Interestingly, the human α -arrestins (ArrDC) also appear to recruit ubiquitin ligase to their PM targets, mediating their ubiquitylation. For example, ArrDC3 recruits the Rsp5 homolog, Nedd4, to mediate ubiquitylation of β 2-adrenergic receptor and β 4-integrin (Nabhan et al., 2010; Draheim et al., 2010).

Table 2 – List of all targets whose endocytosis is regulated by α -arrestins. The stimulus promoting their endocytosis, the α -arrestins involved and the kinase(s) and phosphatase(s) known to regulate them are specified when they are known. Kinases that are proposed to act but without any rigorous evidences are marked with an asterisk (*). (N.B.: Not Determined; PKA: Protein Kinase A complex; PP1: Type 1 Protein Phosphatase (Glc7/Reg1))

Plasma membrane protein (agonist / substrate)	Endocytosis-triggering stimulus	Adaptor(s)	Kinase(s)	Phosphatase(s)	References
Fur4 (uracil)	Substrate excess	Art1/2/8, Bul1/2	Npr1*	N.D.	Galan and Haguenaier-Tasapis, 1997; Marchal et al., 2000; Nikko and Pelham, 2009; MacGurn et al., 2011; Keener and Babst, 2013; Crapeau et al., 2014
	Cycloheximide				
	Misfolding				
Gap1 (amino acids)	Ammonium	Bul1/2	Npr1	Sit4	Soetens et al., 2001; De Craene et al., 2001; Merhi and André, 2012, Crapeau et al. 2014
	Substrate excess				
	Various stresses (rapamycin, misfolding)				
Can1 (arginine)	Substrate excess	Art1, Bul1/2	Npr1	Sit4	Lin et al., 2008; MacGurn et al., 2011; Crapeau et al., 2014; Ghaddar et al., 2015; Gourmas et al., 2017
	Cycloheximide	Art1			
	Misfolding				
Mup1 (methionine)	Substrate excess	Art1	N.D.	N.D.	MacGurn et al., 2011; Zhao et al., 2013; Guiney et al., 2016
	Cycloheximide		N.D.	N.D.	
	Misfolding		N.D.	N.D.	
Lyp1 (lysine)	Substrate excess	Art1	Npr1*	N.D.	Nikko and Pelham, 2009; Zhao et al. 2013; Crapeau et al., 2014; Ghaddar et al., 2014
	Cycloheximide	Art2		N.D.	
	Misfolding	Art1/2		N.D.	
Tat1 (tryptophan)	High hydrostatic pressure	several Art	N.D.	N.D.	Suzuki et al., 2013
Tat2 (tryptophan)	Substrate excess	Art1/2/8, Bul1/2	N.D.	N.D.	Beck et al., 1999; Umehayashi and Nakano, 2003; Nikko and Pelham, 2009
	Cycloheximide	Art2/8, Bul1/2	N.D.	N.D.	
Dip5 (aspartate and glutamate)	Substrate excess	Art6/(3)	N.D.	Calcineurin	Hatakeyama et al. 2010, ; O'Donnell et al., 2013
Hxt1 (hexose)	2-deoxyglucose	Art4/7	Snf1	PP1	O'Donnell et al., 2015
Hxt2 (hexose)	Glucose depletion	Art8	N.D.	N.D.	Hovsepian et al., 2017
Hxt3 (hexose)	2-deoxyglucose	Art4/7	Snf1	PP1	O'Donnell et al., 2015
Hxt4 (hexose)	Substrate excess	Art8	N.D.	N.D.	Hovsepian et al., 2017
Hxt6 (hexose)	Substrate excess	Art4	N.D.	N.D.	Nikko and Pelham, 2009; Llopis-Terregrosa et al., 2016; Hovsepian et al., 2017
	Cycloheximide	Art8			
	Glucose depletion	Art8			
Hxt7 (hexose)	Glucose depletion	Art8	N.D.	N.D.	Hovsepian et al., 2017
	Glucose	Art4	Snf1	PP1	
Jen1 (lactate)	24h growth in lactate	Bul1/2	Npr1	Sit4	Paiva et al., 2003; Becuwe et al., 2012; Becuwe et al., 2014; Talaia et al., 2017
	cycloheximide	Bul1/2	Npr1	Sit4	
Itr1 (inositol)	Substrate excess	Art5	N.D.	N.D.	Nikko and Pelham, 2009
Stt1 (glycerol)	Glucose	Art4	N.D.	N.D.	Becuwe et al., 2014
Smf1 (manganese)	Cadmium	Art2/8	N.D.	N.D.	Nikko et al., 2008
Ctr1 (copper)	Substrate excess	Bul1/2	N.D.	N.D.	Liu et al., 2007
Ste2/3 (a and α -pheromone)	Stochastic misfolding	Art1	N.D.	N.D.	Alvaro et al., 2014; Alvaro et al., 2016
	Pheromone presence	Art4/7	Snf1, Ypk1 (Art4)	Calcineurin	
Rim21 (external pH sensor)	Medium alkalinisation	Art9	Yck1/2	N.D.	Herrador et al., 2010; Herrador et al., 2015
Pma1 (proton pump)	Inhibition of vacuolar ATPase	Art9	N.D.	N.D.	Smardon and Kane, 2014

3.4 Regulation of α -arrestins

Yeast α -arrestins were shown to be post-translationally modified. They are themselves substrate of the ubiquitin ligase Rsp5 and were often shown to be ubiquitylated in conditions triggering endocytosis of their target (Kee et al., 2006). In some other conditions, α -arrestins are phosphorylated, thereby regulating their ability to mediate Rsp5-dependent ubiquitylation of PM proteins (MacGurn et al., 2011).

3.4.1 Ubiquitylation

The consequences of the ubiquitylation are still matter of debate but ubiquitylation seems to be required for proper function of the α -arrestin adaptors. Generally, the PY motif-less version of adaptors, unable to interact with Rsp5 anymore, are not ubiquitylated and fail to mediate endocytosis of their corresponding PM target. Some studies propose that ubiquitylation is required for the correct localization of adaptors. For example, a non-ubiquitylable Art1 version (substitution of Rsp5-targeted lysine by arginine residues) does not localize anymore at the GA, unlike the WT version. Furthermore, it is mainly the ubiquitylated form of Art1 that interacts with the methionine transporter Mup1 in response to methionine addition to the medium. However, a high proportion of Art1 is ubiquitylated even in conditions where Mup1 is not endocytosed (Lin et al., 2008). On the contrary, the function of some α -arrestin adaptors does not always require their ubiquitylation. The ubiquitylation of Art8 is not required for its interaction with Hxt6 (Hovsepian et al., 2017). Moreover, the non-ubiquitylable mutants of Art1 and Art4 are both able to promote internalization of Ste2, while mutants defective for Rsp5 binding (PY-less mutant) are not (Alvaro et al., 2014).

Ubiquitylation could be necessary for other adaptor-protein interactions. For example, the ubiquitylation of Art9 is required for interaction with ESCRT-III subunit Vps23. Likewise, the

proper Rsp5-dependent ubiquitylation of Art9 needs its interaction with Vps23 (Herrador et al., 2010, 2013).

Two recent studies proposed that polyubiquitylation also affects the stability of α -arrestin adaptors by promoting their degradation by the 26S proteasome. This degradation has been studied for Art4 and Art8. It was artificially triggered by the deletion of two deubiquitinase (DUB), *UBP2* and *UBP15*, in the case of Art4 and induced by glucose addition for Art8. Besides, deletion of *UBP2* and *UBP15* affects the abundance of many α -arrestins. More specifically, Ubp2 and Ubp15 were shown to counteract the Rsp5-dependent degradation-promoting ubiquitylation of Art4 (Ho et al., 2017). On the contrary, degradation of Art8 in response to glucose is Rsp5-independent, suggesting that another ubiquitin ligase is involved in Art8 degradation. Ubp2 is again required to decrease the extent of Art8 polyubiquitylation but is probably associated to another DUB not yet identified (Hovsepian et al., 2017). The proteasomal degradation-promoting ubiquitylation of both Art4 and Art8 occurs at distinct sites from the one activating the adaptor. Moreover, although proteasomal degradation is usually triggered by K48-linked polyubiquitin chains, the ubiquitylation of Art4 and Art8 mainly involves K63-linked ubiquitin chains (Ho et al., 2017; Hovsepian et al., 2017).

3.4.2 Phosphorylation

In most of the cases, phosphorylation of the adaptors is inhibitory. Phosphorylation of the adaptors does not seem to affect their interaction with Rsp5 as it was shown for Art6 (O'Donnell et al., 2013). Moreover, phosphorylation does not prevent adaptor ubiquitylation. In a strain defective for the phosphatase Sit4, known to dephosphorylate Bul1 in response to ammonium, Bul1 is constitutively phosphorylated but still ubiquitylated. In that state, Bul1 is not able to mediate Gap1 ubiquitylation and endocytosis. Therefore, ubiquitylation of

phosphorylated Bul1 is not sufficient to elicit Gap1 endocytosis (Merhi and Andre, 2012).

For many α -arrestins, phosphorylation promotes the recruitment and interaction with two regulatory proteins, called 14-3-3 (MacGurn et al., 2011; Becuwe et al., 2012b; Merhi and Andre, 2012). The 14-3-3 proteins, Bmh1 and Bmh2, are known to interact with phosphoserine or threonine in the sequence Rxx[pS/T]xP (Bridges and Moorhead, 2005). Interestingly, a proteomic study revealed that almost all α -arrestins interact with 14-3-3 proteins (Kakiuchi et al., 2007). The exact role of this interaction has not been understood yet. Interaction with 14-3-3 proteins does not seem to impair the interaction between α -arrestins and Rsp5 (Becuwe et al., 2012b). However, they have been proposed to protect the adaptor from being ubiquitylated (Becuwe et al., 2012b; Merhi and Andre, 2012). Besides, upon glucose treatment, the protein kinase A (PKA)-dependent phosphorylation of the N-terminal part of Art8 was shown to promote its interaction with 14-3-3 and its subsequent deubiquitylation (Hovsepian et al., 2017). Concerning the interaction between α -arrestins and their target, the role of 14-3-3 proteins is yet to be determined. Over-expression of *BMH2* reduces the interaction of Art4 with its target, the hexose transporter Hxt6 (Llopis-Torregrosa et al., 2016). On the contrary, the rapamycin-induced endocytosis of the general amino acid permease Gap1 is still mediated by Bul1 and Bul2, while rapamycin does not trigger the dissociation of the Bul adaptors-14-3-3 complex (Crapeau et al., 2014)

Several kinases are involved in the phosphorylation of α -arrestins. The first kinase shown to be involved in their regulation is Npr1 (Soetens et al., 2001; De Craene et al., 2001). Npr1 has been shown to phosphorylate Bul1 and Art1, leading to their inactivation (Soetens et al., 2001; MacGurn et al., 2011). Npr1 is itself regulated in response to nutrient availability and turns out to be one of the link between α -arrestin-mediated ubiquitylation and the nutritional status of the cell (Merhi and Andre, 2012).

3.4.3 Nutrient availability-dependent regulation

Nitrogen source – dependent regulation

The TORC1 kinase complex is involved in α -arrestin regulation through the action of Npr1 in response to nutrients, especially amino acids (MacGurn et al., 2011; Merhi and Andre, 2012). The vacuolar membrane-localized complex is known to be activated when the nutritional status of the cell is good, thereby stimulating growth by acting on many players, i.e. transcription, protein synthesis, etc. On the contrary, TORC1 initiates the starvation response by promoting autophagy in response to a poor nutritional status (González and Hall, 2017).

As it was shown for the CHX-induced endocytosis of Can1 or the endocytosis of Gap1 when cells are switched from a poor-nitrogen source-containing medium (like urea or proline) to a rich one (ammonium or glutamine), TORC1 is activated and promotes the phosphorylation of Npr1 (Figure 3). The activation of TORC1 requires the metabolization of ammonium into amino-acids and is therefore thought to be linked to the increased amino-acid content in the cytosol. Concerning CHX, it was shown to stimulate TORC1 activity, which is likely the consequence of amino-acyl-tRNA accumulation in the cytosol following protein synthesis inhibition (Binda et al., 2009; MacGurn et al., 2011). Hyperphosphorylation of Npr1 by TORC1 is inhibitory. While adaptors cannot be inhibited by phosphorylation anymore, the dephosphorylation of α -arrestins by a phosphatase ensures the release of adaptors from 14-3-3 proteins (MacGurn et al., 2011; Merhi and Andre, 2012). Besides, this release is concomitant to their ubiquitylation. In the case of the ammonium-induced endocytosis of Gap1, the phosphatase involved is Sit4 (Merhi and Andre, 2012). The activity of Sit4 is itself regulated by TORC1 in response to ammonium supply. In conditions of low TORC1 activity, Sit4 is known to stimulate the activity of Npr1 by promoting its dephosphorylation (Schmidt et al., 1998). On the contrary, TORC1 inhibits Sit4-dependent dephosphorylation of Npr1 via its effector Tap42 upon addition of ammonium. Once phosphorylated by TORC1, Tap42 is thought to interact with Sit4.

This interaction would promote the dephosphorylation of Bul adaptors rather than the dephosphorylation of Npr1 (Merhi and Andre, 2012).

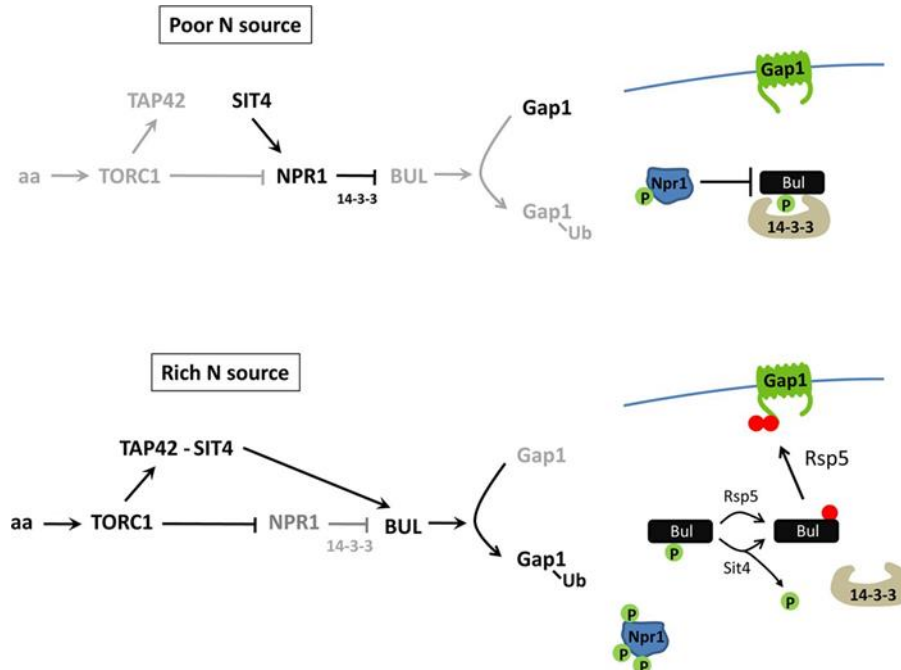


Figure 3 – TORC1-Npr1-Sit4-dependent regulation of Bul1/2-mediated endocytosis of the general amino acids permease Gap1 according to nitrogen source supply (Merhi and André, 2012). When cells are grown in poor nitrogen (N) source-containing medium (e.g. urea), the TORC1 kinase complex activity is low and does not phosphorylate its effectors Tap42 and Npr1. In these conditions, Tap42 does not inhibit the activity of the phosphatase Sit4, which is active. Sit4-dependent dephosphorylation stimulates the activity of Npr1. Npr1-dependent phosphorylation of Bul1/2 triggers their interaction with 14-3-3 proteins. Interaction with the 14-3-3 prevents the Bul adaptors to mediate ubiquitylation and endocytosis of Gap1. Upon addition of a rich N source (e.g. ammonium), TORC1 phosphorylates Npr1 and Tap42. Phosphorylated Tap42 would interact with Sit4 and inhibit the dephosphorylation of Npr1, which is therefore inhibited. Hyperphosphorylated Npr1 being inactive, Bul1/2 undergo dephosphorylation by Sit4 and are ubiquitylated in a Rsp5-dependent fashion, leading to the endocytosis of Gap1. (P: phosphate moiety, “red disk” and Ub: ubiquitin moiety).

Carbon source - dependent regulation

TORC1 is not the sole master regulator of α -arrestin activity. The regulation of the endocytosis of carbon-sources transporters in response to carbon source supply relies on another system of kinase and phosphatase. For example, the addition of glucose to cells grown on lactate was shown to trigger the endocytosis of the lactate transporter Jen1 (Paiva et al., 2002) (Figure 4). In lactate medium, the Art4 adaptor (Rod1) is phosphorylated by the AMP activated kinase (AMPK) homolog, Snf1. Once phosphorylated, Art4 interacts with 14-3-3 proteins. Like TORC1, which is activated by the presence of a rich N-source, the protein phosphatase 1 (PP-1) is activated in the presence of glucose and inhibits the activity of Snf1 by dephosphorylating the Snf1 activation loop (Hedbacker and Carlson, 2008). Concomitantly, PP-1 dephosphorylates Art4, promoting its release from the complex with 14-3-3 proteins. Then, the Rsp5-dependent ubiquitylation of Art4 takes place and leads to the endocytosis of Jen1 (Becuwe et al., 2012b).

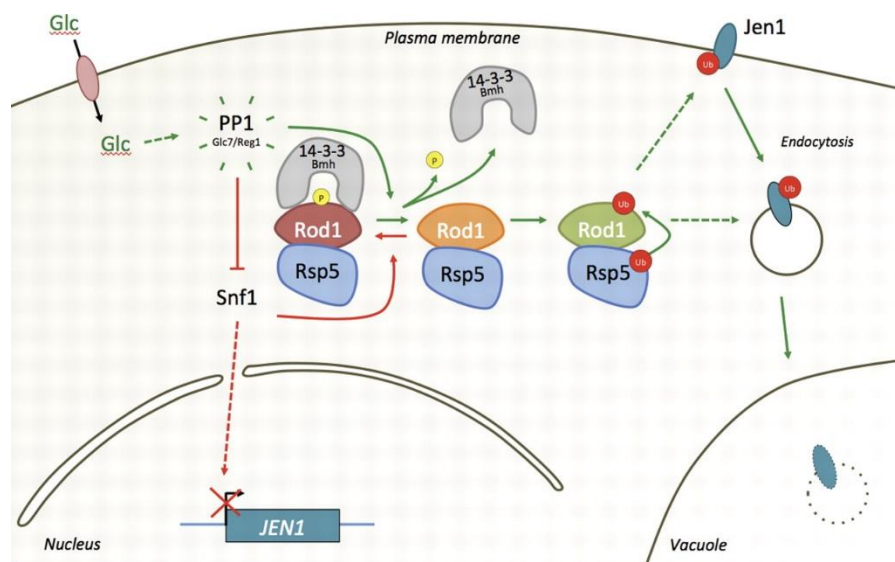


Figure 4 – PP1 and Snf1-dependent regulation of the Rod1/Art4-mediated endocytosis of the lactate transporter Jen1 upon glucose addition (Becuwe et al., 2012b). When cells are grown in lactate (a non-fermentable carbon source), the kinase Snf1 is activated, triggers the expression of *JEN1* and phosphorylates Rod1/Art4. Phosphorylated Art4 interacts with 14-3-3 proteins, Bmh1 and Bmh2, inhibiting Jen1 ubiquitylation that is therefore

stabilized at the PM. Upon addition of glucose, type 1 protein phosphatase (PP1), made of Glc7 and Reg1 subunits, is stimulated and inhibits Snf1, thereby preventing the expression of Jen1 (red arrows). In addition to that, PP1 dephosphorylates Art4, which is released from its complex with Bmh1/2. Simultaneously, dephosphorylated Art4 is ubiquitinated and mediates Jen1 ubiquitylation in a Rsp5-dependent fashion (green arrows).

This mechanism can be generalized to other transporters and other carbon sources. A study also demonstrated that the addition of the toxic analog of glucose, 2-deoxyglucose (2-DG), promotes the Rsp5-dependent Art4 and Art7-mediated endocytosis of hexose transporters, Hxt1 and Hxt3. Addition of 2-DG stimulates Snf1 activity that catalyzes the hyperphosphorylation of Art4 and Art7 (O'Donnell et al., 2015).

Snf1 regulatory function is broader than the phosphorylation-dependent inhibition of α -arrestins. When glucose-grown cells are switched to lactate, the high-affinity transporter Hxt6 and Hxt7 are endocytosed in an Art8-dependent fashion. In addition to its post-translational regulation, Art8 was shown to be transcriptionally regulated following glucose removal. Indeed, the Snf1-dependent glucose-induced repression of *ART8* expression is released after removal of glucose from the medium. In lactate medium, newly synthesized Art8 is then ubiquitinated and mediates Hxt6 and Hxt7 endocytosis (Hovsepian et al., 2017).

Notably, one α -arrestin can be the target of more than one kinase. A recent study showed that Art4 was not only phosphorylated by Snf1. The additional Ypk1-dependent phosphorylation of Art4 is regulating its ability to bind the receptor Ste2. Art4 is independently phosphorylated at Snf1 and Ypk1 sites. Likewise, PP-1 is not the only phosphatase involved in α -arrestin regulation. Calcineurin, the Ca^{2+} / calmodulin regulated protein phosphatase, dephosphorylates Art4, thereby promoting the endocytosis of Ste2 (Alvaro et al., 2016).

3.4.4 Substrate-induced endocytosis

Recent studies emphasized the fact that α -arrestins are at crossroads between many signals. As previously proposed, via the action of TORC1 kinase or the phosphatase PP-1, the adaptors are sensitive to the nutritional status of the cells. Above that regulation, there is another layer of specificity linked to the substrate-induced endocytosis of each transporter. Several yeast transporters were shown to be endocytosed after addition of an excess of their substrate in the extracellular medium (see Table 2).

The study on the uracil permease Fur4 brought many insights on the mechanism underlying this regulation (Figure 5). The N-terminal tail of Fur4 has a PEST-like sequence that is phosphorylated prior to the Rsp5-dependent ubiquitylation of nearby lysine residues, leading to Fur4 vacuolar degradation (Galan et al., 1996; Marchal et al., 1998, 2000). Moreover, uracil binding to Fur4 was proposed to be required for endocytosis-induction and direct routing of neo-synthesized permease from GA to the vacuole in response to high cytosolic concentration of uracil (Blondel et al., 2004). A recent study additionally pointed out that a domain localized in the N-terminal tail of Fur4, termed Loop-Interaction Domain (LID), acts as a conformational sensor. The LID sequence interacts with cytosolic loops via hydrogen bonds, thereby hiding the PEST-like sequence and lysine residues targeted by Rsp5, which are referred to as a degron. When uracil binds to Fur4, Fur4 undergoes a structural rearrangement from an outward facing (OF) open to an uracil-bound OF occluded conformation and subsequently to the inward facing (IF) conformation, releasing uracil in the cytosol. The conformational change partially disrupts the LID-loop interaction, leading to an increase availability of Fur4 degron and the subsequent Rsp5-dependent ubiquitylation of Fur4. The precise conformation (OF occluded or IF) that is recognized by the ubiquitylation machinery has not been precisely identified. Therefore, whether uracil release in the cytosol is required for Fur4 endocytosis remains elusive (Keener and Babst, 2013).

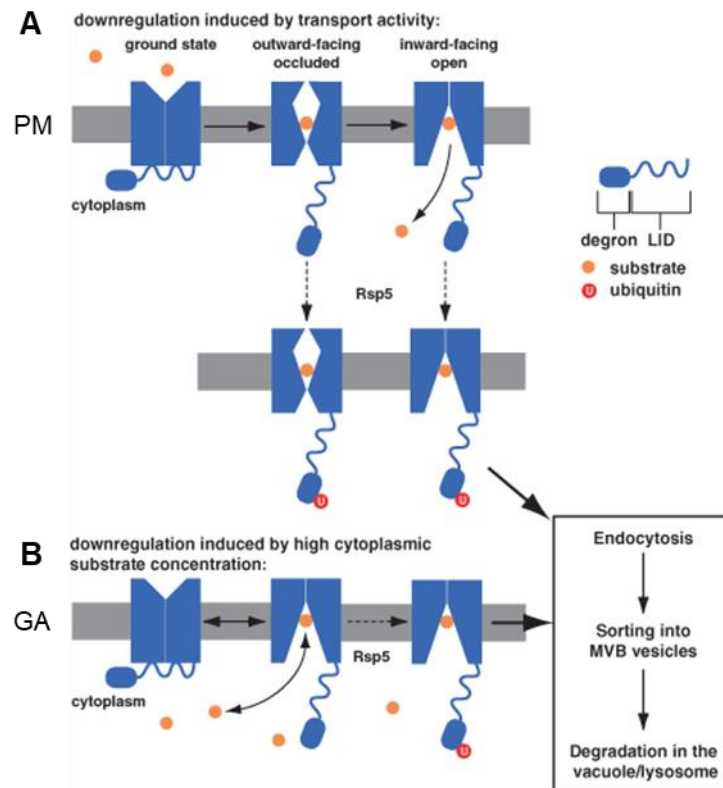


Figure 5 – Scheme of the mechanism underlying the uracil-induced endocytosis of the uracil permease Fur4. (A) Fur4 has a Loop Interaction Domain (LID) in its N-terminal part. The LID is localized downstream of a PEST-like sequence and Rsp5-targeted lysine residues, which are essential for its ubiquitylation and endocytosis, which constitutes a degron. In the absence of uracil, the LID interacts with cytosolic loops, hindering the interaction of the degron with the ubiquitylation machinery, i.e. α -arrestins and Rsp5. Upon external uracil binding, Fur4 (PM) undergoes a structural rearrangement from outward facing open to an outward facing (OF) occluded and subsequently an inward facing (IF) open conformation. The transition from the ground state to the OF occluded or the IF open conformation leads to the disruption of the interaction between the LID and cytosolic loops, exposing the degron and enabling ubiquitylation. (B) Newly synthesized Fur4 at the Golgi apparatus (GA) membrane is directly routed to the endosomes and ultimately the vacuole by the same conformational change-induced exposure of the degron sequence if the cytosolic uracil concentration is high enough. Adapted from Keener and Babst, 2013.

An additional clue strengthening the significance of the conformational regulation of permease endocytosis came from the evidence that constitutively active α -arrestin adaptors are necessary but not sufficient for mediating the internalization of their targets, as it was shown for the arginine permease Can1 (Ghaddar et al., 2014; Gournas et al., 2017). Indeed, a transport-inactive variant of Can1 is not endocytosed when its substrate (arginine) is transported by other permeases and metabolized, yet leading to high activity of TORC1 and α -arrestins (Ghaddar et al., 2014) (Figure 6). Indeed, catalysis of substrate transport, or at least substrate binding to the permease, is required to enable endocytosis. Arginine (Arg) binding triggers the transition from an outward-facing (OF) to an inward facing (IF) conformation, unveiling an Art1-binding site on the N-terminal tail of Can1 (Ghaddar et al., 2014; Gournas et al., 2017). Notably, Can1 also undergoes Arg-independent Bul1/2-mediated endocytosis in the absence of Art1 but fails to reach the vacuole and is recycled back to the PM. Bul1/2-targeted site is localised upstream of the one of Art1 and is proposed to be constitutively accessible (Gournas et al., 2017). Such Art1-targeting sequence was first shown to be required for the substrate-induced endocytosis of the methionine transporter Mup1. The positively-charged C-terminal region of Art1 is thought to interact with this acidic patch (Guiney et al., 2016). Therefore, accessibility of such acidic-patch is essential for α -arrestin-mediated endocytosis (Guiney et al., 2016; Gournas et al., 2017). It is proposed that a region of the N-terminal tail of Can1 localized between the acidic patch and the first transmembrane domain (TMD1) interacts with cytosolic loops, hiding the binding site in the absence of substrate. The conformational transition from the OF to the IF conformation upon substrate binding would partially disrupt this interaction through the repositioning of TMD1 and lead to the exposure of the α -arrestin-recognizing sequence. However, the IF conformation must be stable enough to be sufficiently populated, ensuring the proper recognition of the binding sequence by α -arrestins (Gournas et al., 2017). Therefore, the substrate-elicited conformational transition is likely to be the key

to ensure endocytosis specificity when several transporters are regulated by the same α -arrestin.

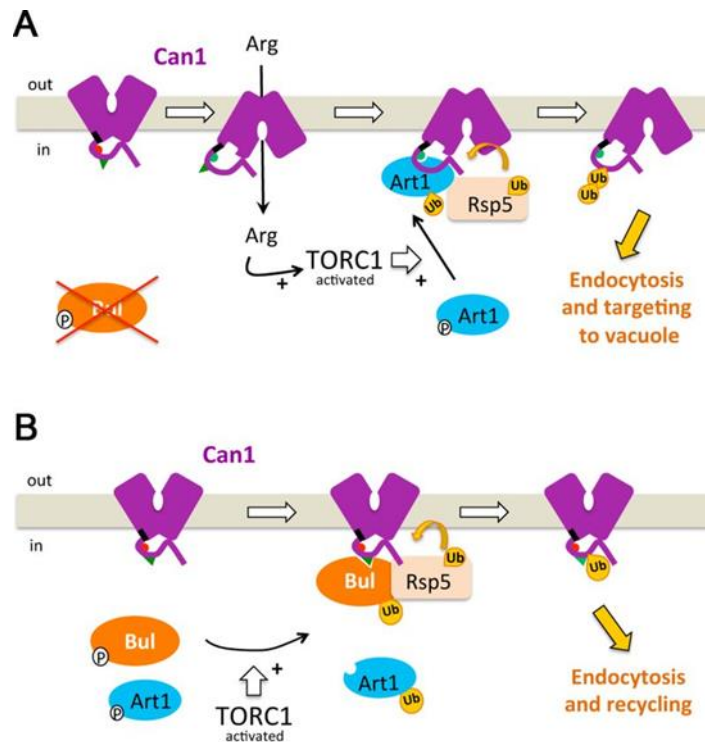


Figure 6 - Scheme of the substrate-induced endocytosis of Can1. (A) In the absence of its substrate (Arginine, Arg), Can1 is in its ground state and its Art1-binding site is not accessible (red disk). Upon binding to Arg, Can1 undergoes a structural rearrangement from an outward facing (OF) to an inward facing (IF) conformation. The conformational change triggers a partial disruption of the interaction between the N-terminal tail of Can1 and its cytosolic loops. This disruption leads to the exposure of a Art1-binding sequence, which is therefore accessible (green disk). In addition, Arg metabolization stimulates the activity of the TORC1 complex, stimulating the activity of Art1. Activated Art1 can therefore mediate the ubiquitylation (Ub) of Can1, leading to its endocytosis and vacuolar degradation. (B) Can1 is also ubiquitylated in a Bul1/2-dependent fashion. Without binding to Arg but in conditions activating TORC1, Bul1/2 as well as Art1 are activated. However, Can1 is blocked in the OF conformation, hiding the Art1-binding sequence (red disk). The Bul1/2 binding site being constitutively exposed (green triangle), Bul1/2 mediate Can1 ubiquitylation. Although Bul1/2-dependent ubiquitylation is enough to trigger its endocytosis, it fails to target it to the vacuole, Can1 is therefore recycled back to the plasma membrane (PM). Adapted from Gournas et al., 2017.

3.4.5 Stress-induced endocytosis

This type of endocytosis is not completely different from the two previous ones since many stresses tested in the context of α -arrestin mediated endocytosis are linked to either conformational changes due to natural or artificially-induced misfolding or signals mimicking either a good or a bad nutritional status.

Misfolding stress

PM proteins ensure their function during a definite time, which is characterized by their half time in a given condition. The basal turnover of PM proteins is likely to be due to their misfolding. Misfolding can also be artificially triggered after heat-shock, organic solvent exposure or oxidative stress. As developed in the previous paragraph, heat-shock, peroxide or uracil-induced endocytosis of Fur4 is thought to be mainly due to the disruption of the conformational sensor region (LID) and cytosolic loops, causing the exposure of the PEST-like sequence and Rsp5-targeted lysine (Keener and Babst, 2013). Although bringing a new insight to the question of PM protein quality control, the involvement of α -arrestin or the existence of a α -arrestin binding site in or nearby the LID sequence was not investigated in the latter study. Anyway, in a screening aiming at making the link between the endocytosis of several model PM proteins and α -arrestin adaptors, the CHX- and uracil-induced endocytosis of Fur4 was shown to be dependent of several adaptors, including Art1, Art8, Bul1 and Bul2 (Nikko and Pelham, 2009) (Table 2). Taking together, these results suggest that exposure of the PEST-like sequence following the structural rearrangement could trigger α -arrestin binding.

Notably, Rsp5 is the sole ubiquitin ligase that was shown to be involved in the heat-induced endocytosis of permeases, including the arginine permease Can1, the methionine permease Mup1 and the lysine permease Lyp1. In the same study, the endocytosis and degradation of misfolded proteins was always Art1-dependent, except for the one of Lyp1 that also required Art2. Besides, the deletion of ART1 was shown to increase

sensitivity upon growth at high temperature and the only two suppressors identified were *ART1* itself and *ART2*. The toxicity at high temperature was shown to be due to the accumulation of leaky misfolded transporters and is reduced by the over-expression of Art1 (Zhao et al., 2013). The stochastic misfolding of the pheromone receptor Ste2 is also Art1-dependent (Alvaro et al., 2016). These studies support the idea that Art1 is particularly important for recognition of misfolded proteins at PM.

Starvation

Starvation is a nutritional stress that can be mimicked by the addition of rapamycin to cells. Rapamycin is a well-known inhibitor of the TORC1 kinase complex that led to its discovery (González and Hall, 2017). Upon rapamycin addition, the activity of TORC1 is low. As previously stated, when cells are grown in a poor nitrogen source medium, TORC1 activity is low, Npr1 kinase is active and promotes the phosphorylation and inhibition of the two adaptors Bul1 and Bul2, stabilizing Gap1 (Merhi and Andre, 2012). Astonishingly, addition of rapamycin does not follow this path and leads to the endocytosis of Gap1. Accordingly, in a TORC1 defective mutant, *tco89Δ*, Gap1 is constitutively targeted to the vacuole. However, rapamycin and ammonium-induced endocytosis of Gap1 are slightly diverging. While ammonium-induced endocytosis of Gap1 is only Bul1/2-dependent, Art3 and Art6 are also required for the rapamycin-induced one. Although the four adaptors are phosphorylated and bound to 14-3-3 proteins, they are still able to mediate Gap1 ubiquitylation and endocytosis. Moreover, phosphorylated Bul1/2 are thought to interact with the C-terminal region of Gap1, while Bul1/2 are interacting with its N-terminal in response to ammonium. Two lysine residues undergo Bul1/2-dependent ubiquitylation in response to ammonium (K9 and K16) and rapamycin, while Art3/6 only target K16 residue after addition of rapamycin.

The rapamycin-induced pathway seems to converge with the stress induced one. Indeed, upon treatment of cells with a misfolding-inducing compound, Gap1 is internalized through the same pathway as after addition of rapamycin treatment (Shiga et

al., 2014; Crapeau et al., 2014) (Figure 7). The two permeases Can1 and Lyp1 are also endocytosed after addition of rapamycin. The deletion of *ART1* neither abolished the rapamycin-induced endocytosis of Can1 nor its constitutive targeting to the vacuole in *tco89Δ* strain, suggesting that other adaptors may be involved (Crapeau et al., 2014). Since there is no evident change of post-translational modification of Bul1/2 and Art3/6 upon addition of rapamycin, the mechanism inducing their binding to their target still needs to be investigated.

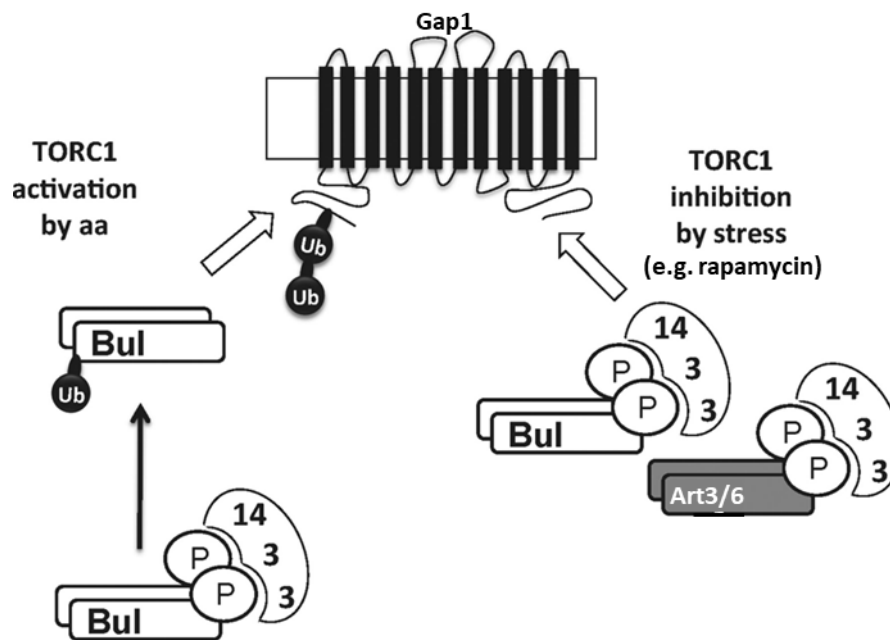


Figure 7 – Stress-induced endocytosis of the general amino-acids transporter Gap1. Addition of ammonium (converted into glutamate in the cell) or excess of amino acids in the medium triggers the activation of TORC1, leading to the activation of Bul1/2 (Bul) α-arrestins. Bul1/2 recruit Rsp5, are ubiquitylated and mediate the ubiquitylation of Gap1 by interacting with its N-terminal tail. Likewise, misfolding-inducing stresses or rapamycin, a TORC1 inhibitor, also trigger Gap1 endocytosis in a Bul and Art3/6-dependent way. In these conditions, TORC1 activity being low, adaptors are phosphorylated and interact with 14-3-3 proteins. Bul and Art3/6 adaptors, together with 14-3-3, also mediate the ubiquitylation of Gap1, but rather via their interaction with the C-terminal region of Gap1. Adapted from Crapeau et al., 2014

Cycloheximide

Addition of cycloheximide (CHX) can be categorized as a stress since it inhibits protein synthesis. However, CHX is also known to stimulate the activity of TORC1, mimicking a high cytosolic amino acid concentration (Binda et al., 2009). CHX triggers the endocytosis of many transporters like Can1, Lyp1, Hxt6, Fur4 and the tryptophan and tyrosine permease Tat2. Can1, Fur4 and Tat2 rely on the same α -arrestins for their substrate and CHX-induced downregulation (Lin et al., 2008; Nikko and Pelham, 2009). On the contrary, substrate- and CHX-induced endocytosis of Lyp1 and Hxt6 is mediated by different α -arrestins. The CHX-induced endocytosis of Lyp1 and Hxt6 is Art2-dependent and Art8-dependent, respectively. On the contrary, Art1 mediates Lyp1 endocytosis in response to an excess of lysine, while the endocytosis of Hxt6 is mediated by Art4 upon glucose addition. Therefore, there is no clear similarity in the stress-induced or more specifically CHX-induced endocytosis of all these transporters except that it often implies the action of at least one adaptor from the paralog pair Art2/Art8 (Nikko and Pelham, 2009).

Other stresses

The involvement of Art2/Art8 in stress-induced reveals to be true when studying toxicity to cadmium ions. Cadmium enters the cell via the manganese transporter Smf1 but is toxic for cells. In response to cadmium, Smf1 is endocytosed in an Art2 and Art8-dependent manner (Nikko et al., 2008).

On the contrary, Bul1 and Bul2 are required for Jen1 in response to prolonged growth in lactate (i.e. after 24h), while Art4 is required in response to glucose (Becuwe et al., 2012b; Talaia et al., 2017). The signal triggering this endocytosis of Jen1 is proposed to be the alkalinization of the medium. Interestingly, cycloheximide and rapamycin-induced endocytosis of Jen1 are also Bul1/2-dependent. The endocytosis of Jen1 appears therefore to be also controlled by the TORC1 complex, the Npr1 kinase and the Sit4 phosphatase. One big difference between the three stimuli is that transport activity of Jen1 is dispensable

for rapamycin-induced endocytosis but is required for endocytosis in response to CHX or prolonged growth in lactate (Talaia et al., 2017).

3.5 Post and non-endocytic functions

Yeast α -arrestins are not only involved in the recognition of PM proteins at the PM. Some of them have been shown to act at different organelles. Although they are mainly cytosolic, they seem to be tightly associated with GA membrane. Indeed, Art1 is able to translocate from GA to the PM after Can1 endocytosis induction with cycloheximide (CHX) or switch from a poor (yeast nitrogen base, YNB) to rich medium (YD) (Lin et al., 2008). Moreover, the adaptors were shown to mediate ubiquitylation of PM transporters at the GA. In Npr1-defective cells, the active Bul1 and Bul2 promote the ubiquitylation-dependent direct routing of Gap1 from the GA to the vacuole (De Craene et al., 2001; Soetens et al., 2001). In the same way, Art4 also acts at the Trans-Golgi network (TGN). As stated before, upon glucose addition to lactate grown cells, the lactate transporter Jen1-GFP is endocytosed. If glucose is removed quickly after, Jen1-GFP was shown to be recycled back to the PM via the TGN, its ubiquitylation being reset. If the presence of glucose is permanent, Jen1-GFP undergoes Rsp5-dependent Art4-mediated ubiquitylation at the TGN and is ultimately targeted to the vacuole. The post-endocytic localization of Jen1-GFP at TGN would act like a checkpoint. Art4 mediates Jen1 targeting to the vacuole when the endocytic signal is permanent. On the contrary, Jen1 is recycled to the PM if the stimulus is transient (Becuwe and Léon, 2014).

Another example of non-endocytic function of α -arrestin is illustrated by Art9. Art9 is involved in the external pH-sensing RIM pathway. When cells are switched from an acidic to a neutral pH, the C-terminal tail of the pH receptor Rim21 is exposed and interacts with Art9. Art9 turns out to be an essential link between the RIM and the ESCRT pathway. Indeed, Art9 interacts with

ESCRT-I subunit Vps23, thereby recruiting the ESCRT machinery. Interestingly, ESCRT-I recruitment could be directly localized at the PM and not endosomes, although this observation was based on over-expression of Art9 and Vps23. ESCRT recruitment is required for further processing of the Rim101 transcription factor, which upon proteolytic cleavage, will translocate to the nucleus, ensuring the response to a more alkaline pH (Herrador et al., 2010). As other α -arrestins, the activity of Art9 is regulated by its phosphorylation status. The PM-anchored casein kinase complex 1 (Yck1/2) phosphorylates Art9, counteracting its binding to the C-terminal tail of Rim21 (Herrador et al., 2015). Such function of Art9 in receptor signalling is reminiscent of true arrestins function in mammalian cells. As true arrestins, Art9 also performs endocytic function in addition to signalling. Art9 was shown to be required for the endocytosis of the H⁺-ATPase Pma1 in vacuolar V-ATPase deficient cells (Smardon and Kane, 2014). It seems also required for the PM localization of the sodium pump Ena1 in response to LiCl independently of *ENA1* expression (Marqués et al., 2015).

Finally, some α -arrestins were found to act at endosomes, like Art3 and Art6. When cells are switched from a rich nitrogen source-containing medium to a poor one, Art3 and Art6 are indeed necessary for retrograde transport of endocytosed Gap1 from the endosome to the GA. The N-terminal arrestin domain of both Art3/6 appears to interact with the clathrin-adaptor protein AP-1, suggesting that Art3/6 are mediating the sorting of Gap1 in recycling vesicles. However, since only the action of Art3 but not Art6 requires AP-1, Art6 may regulate other types of Gap1-containing vesicles. Moreover, regulation of Gap1 trafficking by Art3 but not Art6 is Npr1-dependent. Astonishingly, Rsp5 interaction to both Art3/6 is essential for their Gap1 sorting function although its role in the process is still unknown (O'Donnell et al., 2010). Again, the ability to interact with the sorting machinery evokes the ability of human β -arrestin to bind clathrin (Goodman et al., 1996).

3.6 Rsp5-independent function of α -arrestins

Some of the function of the α -arrestins do not require Rsp5 binding or their Rsp5-dependent ubiquitylation. It is the case of the involvement of Art4 and Art7 in the dampening of the pheromone-induced Ste2 or Ste3 signalling. More specifically, Art1 mediates the pheromone-independent basal turnover of Ste2 and Ste3 in a Rsp5-dependent fashion. When a pheromone binds to the receptor, Art4 and Art7 are both involved in their endocytic downregulation. However, the action of Art4 is Rsp5-dependent, whereas the one of Art7 is not (Alvaro et al., 2014). Recently, Art4 was also shown to reduce the signal of Ste2 in an Rsp5-independent fashion. The switch between the Rsp5-dependent and independent mechanism of Ste2 signalling desensitisation is likely to be controlled by the Art4 phosphorylation status (Alvaro et al., 2016).

Likewise, Art1, Art3 and Art6 are required independently of their recruitment of Rsp5 for the clathrin-independent endocytosis (CIE) of Ste2 and Ste3 (Prosser et al., 2015). In a CME-deficient strain, lacking the four clathrin-adaptors (Ent1, Ent2, Yap1801, and Yap1802), Ste2 and Ste3 endocytosis is impaired, but over expression of the CIE effector Rom1 is enough to initiate the internalization of the receptors. Over-expression of either wild-type or PY-less versions of the α -adaptors was also able to rescue the endocytosis of the receptor in the CME-deficient strain. Moreover, subunits of the CIE machinery, i.e. the GTPase Rho1 and its guanine nucleotide exchange factor (Rom1 and Rom2) interact with Art1, Art3 and Art6 (Prosser et al., 2015) (Figure 8).

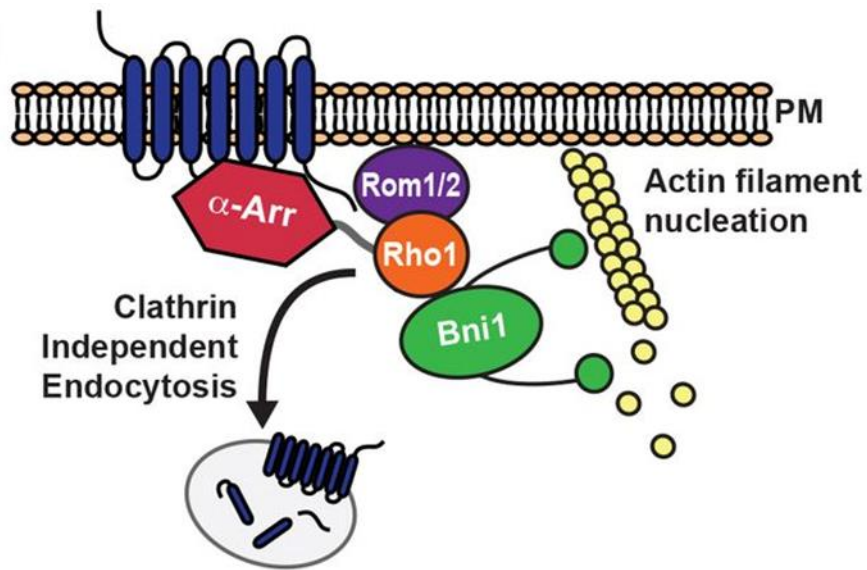


Figure 8 – Schematic representation of the involvement of α -arrestin in Clathrin-Independent Endocytosis (CIE). Involvement of α -arrestins (e.g. Art1/3/6) in CIE does not require their ability to recruit Rsp5 but rather the GTPase Rho1 and its guanine nucleotide exchange factors (GEF), Rom1 and Rom2. Rho1 is thought to locally stimulate the Bni1, leading to the nucleation of actin filaments, required for CIE. Adapted from Prosser et al., 2015

4 Thiamine homeostasis in yeast

Thiamine played an important role in our research. This chapter is therefore aiming at summarizing the current knowledge on thiamine homeostasis, especially in the yeast *S. cerevisiae*.

4.1 Thiamine

Thiamine (or thiamin), also known as vitamin B1, is a water-soluble molecule. Thiamine was discovered by Christiaan Eijkman and Gerrit Grijns in rice husk as able to prevent a neurological disorder, called the beriberi disease in 1912. It was then purified and its structure solved for the first time in 1926 by Jansen and Donath (Carpenter, 2012).

The active form of thiamine is thiamine pyrophosphate (or diphosphate), hereafter referred as TPP. Thiamine, and therefore TPP, is composed of a thiazolium ring and a 4-aminopyrimidine group (Figure 9). Thiamine carries a permanent positive charge coming from the thiazolium moiety. The pyrophosphate group and the pyrimidyl moiety are thought to be involved in the proper recognition and binding by the apoenzyme. The pyrimidyl and thiazolium moieties are both required for the catalysis process. The carbanion form (negatively charged carbon atom) of the acidic carbon C2 from the thiazole core reacts with the carbonyl group of its targets (Nemeria et al., 2009). This reaction allows the formation and transfer of an activated aldehyde to the substrate.

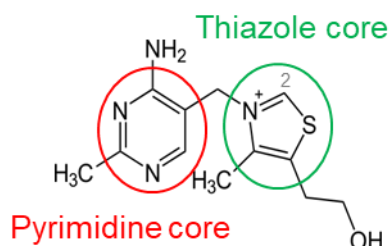


Figure 9 – Structure of thiamine (vitamin B1). The acidic carbon C2 involved in catalysis is identified by the number 2.

TPP is the coenzyme of many enzymes involved in the metabolism of sugars and amino acids.

TPP mainly contributes to the metabolism of carbohydrates via four enzymes: pyruvate decarboxylase (PDC), pyruvate dehydrogenase (PDH), α -ketoglutarate dehydrogenase and transketolase. The PDC enzymes (several isoforms in *S. cerevisiae*, Pdc1/5/6) are involved in the decarboxylation of pyruvate in acetaldehyde, which will be ultimately reduced in ethanol (alcoholic fermentation). The PDH complex is the essential link between the glycolysis and the Krebs cycle (tricarboxylic acid cycle). TPP is essential for the E1 subunit of the PDH complex (Pda1 and Pdb1) catalyzing the first step of acetyl-CoenzymeA (CoA) formation from pyruvate (Pronk et al., 1996). The Kgd1 subunit of the α -ketoglutarate dehydrogenase complex catalyzes the oxidative decarboxylation of α -ketoglutarate in succinyl-coA in the mitochondrion during the Krebs cycle (Repetto and Tzagoloff, 1989; Heublein et al., 2014). Finally, the transketolase (Tkl1/2) is a key enzyme in the non-oxidative phase of the pentose phosphate pathway (Lindqvist et al., 1992).

TPP is also required for the amino acid metabolism. TPP is used by the phenylpyruvate decarboxylase (Aro10) involved in the second step (decarboxylation) of tryptophan and tyrosine catabolism via the Ehrlich pathway (Hazelwood et al., 2008; Kneen et al., 2011). The PDC enzymes discussed earlier are also thought to contribute to the catabolism of other amino acids (such as leucine and valine) through the same pathway (Dickinson et

al., 2003). Moreover, the mitochondrial acetolactate synthase Ilv2 is a TPP-dependent enzyme catalyzing the conversion of pyruvate in acetolactate, which constitutes the first step of the leucine and valine synthesis pathway (Falco et al., 1985).

4.2 Thiamine biosynthesis

Unlike metazoans, the yeast *S. cerevisiae* is able to synthesize thiamine. Interestingly, the mechanism of thiamine biosynthesis in yeast (Figure 10) does not coincide with the well-described mechanism in prokaryotes, such as in *Bacillus subtilis*.

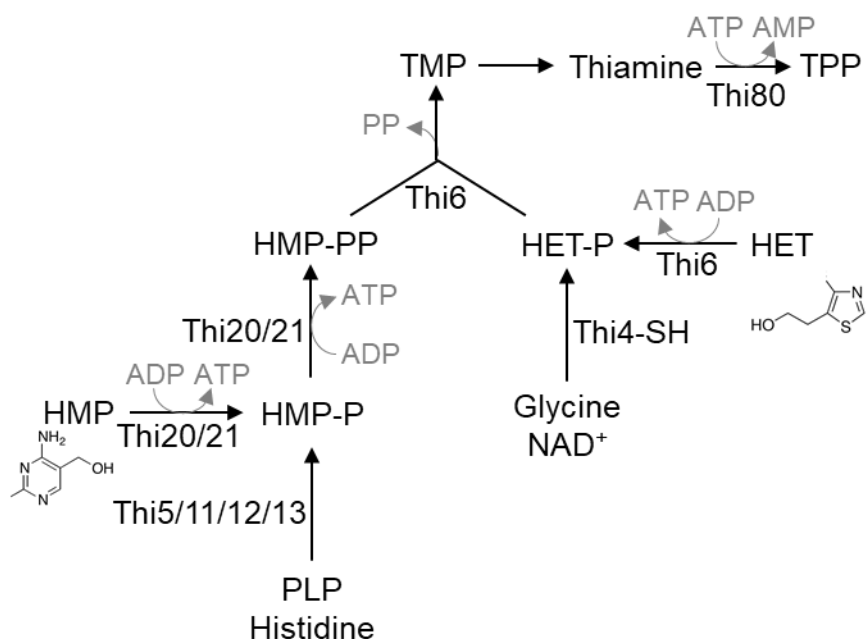


Figure 10 – Scheme of the thiamine biosynthesis pathway in *Saccharomyces cerevisiae*. (HMP: 2-methyl-4-amino-5-hydroxymethyl pyrimidine; HMP-P: HMP phosphate; HMP-PP: HMP pyrophosphate, PLP: pyridoxal phosphate, HET: 4-methyl-5-hydroxyethylthiazole, HET-P: HET phosphate, PP: pyrophosphate group, TMP: thiamine monophosphate, TPP: thiamine pyrophosphate, NAD⁺: nicotinamide adenine dinucleotide)

Precursors of the pyrimidine core of thiamine are histidine and pyridoxal phosphate (PLP), the active form of vitamin B6 (pyridoxine) (Tazuya et al., 1995; Begley et al., 2012). These precursors are thought to be the substrate of four very similar enzymes, known as members of the Thi5 family (including Thi5, Thi11, Thi12 and Thi13), which catalyze the synthesis of 2-methyl-4-amino-5-hydroxymethyl pyrimidine phosphate (HMP-P) in a redundant way (Wightman and Meacock, 2003). Two studies showed that an alternative pathway would be used by yeast in anaerobic conditions (Wightman and Meacock, 2003; Tanaka et al., 2000). Besides, some HMP can be also imported from the medium. HMP is phosphorylated in two steps by a HMP kinase and subsequently by a HMP phosphate (HMP-P) kinase, which are both encoded by the redundant *THI20* and *THI21* genes, producing HMP pyrophosphate (HMP-PP) (Kawasaki et al., 2005).

The synthesis of the thiazole moiety is catalyzed by a single enzyme, thiazole synthase Thi4. The precursors of the thiazole group are Nicotinamide Adenine Dinucleotide (NAD) (as a five-carbon source) and glycine. In addition to these two compounds, one cysteine residue (C205) from Thi4 is required as a sulphide donor during the process. Therefore, Thi4 is more a cosubstrate than an enzyme. The final product of Thi4-dependent pathway is 4-methyl-5-hydroxyethylthiazole phosphate (HET-P). Notably, Thi4 only undergoes a single turnover and harbours a 1:1 stoichiometry regarding thiamine produced (Chatterjee et al., 2011). Interestingly, it was shown that some HET can also be imported through an unknown protein. As a salvage pathway, transported HET would be subsequently phosphorylated in HET-P by Thi6 (Nosaka et al., 1994). Thi6 is a bifunctional protein forming a homooctameric complex with both HET kinase and a thiamine phosphate synthase activity (Kawasaki, 1993).

The condensation of HMP-PP and HET-P is catalyzed by the thiamine synthase Thi6 to form thiamine monophosphate (TMP) (Nosaka et al., 1994). Newly synthesized TMP is first dephosphorylated by a still unknown enzyme and then

pyrophosphorylated by the thiamine pyrophosphokinase Thi80 (Nosaka et al., 1993).

Thiamine biosynthesis is a highly energetically-demanding process, since five ATP-dependent phosphorylations, an equivalent of NAD⁺ as well as PLP are required (Hohmann and Meacock, 1998). Moreover, one equivalent of Thi4 enzyme is consumed during the process (Chatterjee et al., 2011).

Another enzyme also catalyzes the degradation of thiamine. Indeed, the HMP/HMP-P kinase Thi20 also has a thiaminase (type II) activity, catalysing the degradation of thiamine by producing its two precursors, HMP and HET (Haas et al., 2005; French et al., 2011). The rationale behind such degradation remains elusive.

4.3 Thiamine transport

Due to the high energetic cost of thiamine synthesis, the main source of thiamine in the cell is transported from the external medium to the cytosol. Thiamine is often found in the external medium in its phosphorylated forms, TMP or TPP. Phosphorylated thiamine is not transported *per se*. A periplasmic acid phosphatase, Pho3, is excreted in the periplasm, leading to the dephosphorylation of TPP in thiamine, that is further transported (Nosaka et al., 1989, 1988). In the cytosol, thiamine is pyrophosphorylated in TPP by the thiamine pyrophosphokinase, Thi80 (Nosaka et al., 1993).

Early studies have demonstrated in yeast the existence of an active transport of thiamine and one of its precursor, HMP. Transport was shown to be optimal at acidic pH, stimulated by glucose, inhibited by fluoride and nitride ion and to be characterized by an apparent K_m of 0.18 μM (Iwashima et al., 1973, 1990). Thiamine in the external medium is very quickly transported. Praekelt and co-workers showed that upon addition of thiamine at a final concentration of 1 μM in a thiamine-free medium, thiamine was totally sequestered from the medium by

the cells in about 30 min (Praekelt et al., 1994). Thiamine could be concentrated 10,000 times inside the cell (Iwashima et al., 1973). The maximum amount of thiamine reachable was shown to be equal to 1600 pmol per 10^7 cells before transport ceases to operate. A concentration of at least 40 μ M is therefore required to saturate a yeast culture in thiamine (Praekelt et al., 1994). The main actor of this active transport is the high-affinity thiamine plasma membrane transporter Thi7 (previously known as Thi10), a 598 amino acids protein (Singleton, 1997; Enjo et al., 1997) (Figure 11). Two other low affinity transporters, Thi71/Nrt1 and Thi72, were identified. Their contribution in thiamine transport is lower than the one of Thi7. For a same level of expression, Nrt1 and Thi72 accounts for 14% and 41% of the transport capacity of Thi7 (Enjo et al., 1997). Notably, Nrt1 was recently shown to be a high-affinity transporter of nicotinamide riboside ($K_m = 21 \mu$ M), a precursor of NAD^+ (Belenky et al., 2008). These three transporters share a high sequence identity: 87% and 85% of sequence identity between Thi7 and Nrt1 or Thi72, respectively.

Thi7 belongs to the Major Facilitator Superfamily (MFS), characterized by twelve transmembrane spans. From the same group, we find proteins that are relatively close to Thi7, the allantoin permease Dal4 (30% identity with Thi7) or the uracil permease Fur4 (28% identity with Thi7). Together with these two transporters, Thi7 belongs to the family of the Nucleobase-Cation Symporters (NCS1) of the amino acid/polyamine/organocation (APC) transporter superfamily. The structure of one NCS1 member, the benzyl-hydantoin transporter Mhp1 from *Mycobacterium liquefaciens* was solved and brought insights into the transport mechanism of yeast members (Weyand et al., 2008; Shimamura et al., 2010) (Figure 12). The modeled structure of Thi7 using Mhp1 as template suggests that the substrate binding site is formed by a four-helix bundle composed of the transmembrane helices 1,2,6 and 7 (Huang et al., 2012). Transport is the result of a structural rearrangement from an outside open to an inward open conformation. Moreover, the N-terminal tail of Thi7, Nrt1 and Thi72 presents a Loop-interaction Domain, which was found in Fur4. This LID sequence was shown

to interact with cytosolic loops, acting like a conformational sensor. Transport of the substrate triggers a conformational change and partially disrupts the LID-loops interaction (Keener and Babst, 2013). Unlike Fur4, no information is available concerning the endocytic downregulation of thiamine transporters. Nevertheless, the three thiamine transporters have a PY motif localized in a cytosolic loop, suggesting a direct interaction with an HECT-type ubiquitin ligase.

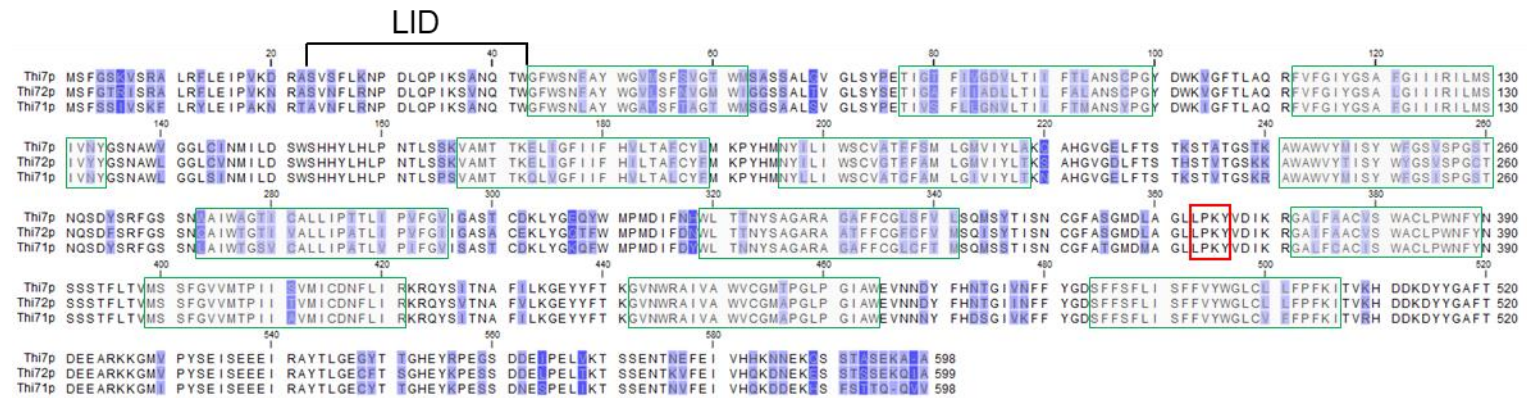


Figure 11 – Sequence alignment of Th7, Thi71/Nrt1 and Thi72. The less the amino acids are similar, the darker is the blue background. No background means that the amino acids are identical. Transmembrane spans are identified by green rectangles, while the PY motif is surrounded by a red one. (LID: loop-interaction domain)

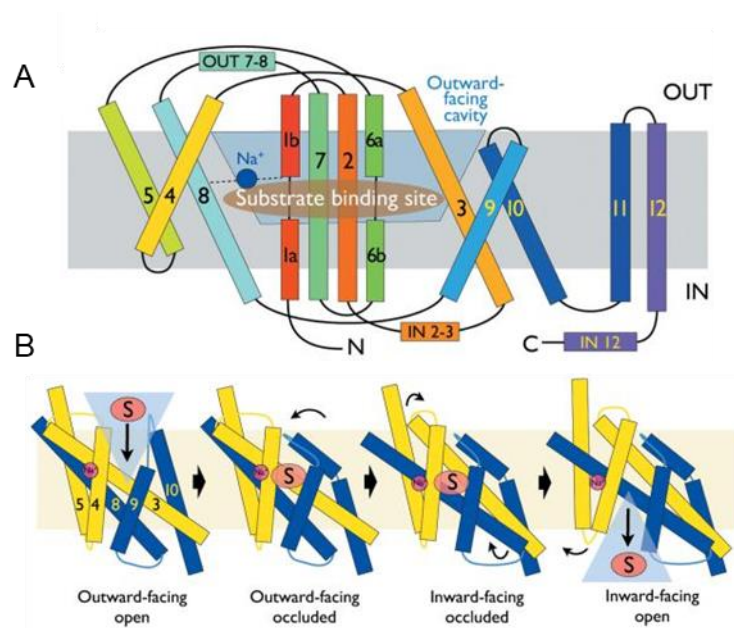


Figure 12 – Scheme of the structure (A) as well as the transport mechanism (B) of a NCS1 member, the sodium-hydantoin transporter Mhp1 from *Mycobacterium liquefaciens*. (S: substrate) Adapted from Weyand et al., 2008.

The *S. cerevisiae* genome also encodes a *THI73* gene, whose expression is controlled by thiamine concentration in the cytosol. Thi73 is predicted to be localized at the PM but its function is still unknown (Mojzita and Hohmann, 2006). Since some apoenzymes of TPP are localized in the mitochondrion, thiamine or its derivatives must be transported from the cytosol to the mitochondrion. The putative transporter Thi74 was proposed to transport thiamine across the mitochondrion membranes without clear evidence (Mojzita and Hohmann, 2006; Manzetti et al., 2014). On the contrary, a TPP transporter at the mitochondrion inner membrane, Tpc1, was identified and functionally characterized (Marobbio et al., 2002).

Eukaryotic organisms have evolved different mechanisms of thiamine transport, which are not necessarily similar to Thi7 from *S. cerevisiae*. In *Schizosaccharomyces pombe*, thiamine transport is mainly catalyzed by Thi9. Thi9 only shares about 13% of sequence identity with Thi7. Interestingly, while Thi7 is a member of the NCS1 family, Thi9 is rather a distant member of

the amino acid/choline transporter (ACT) family of the APC transporter superfamily. Thi9 closest *S. cerevisiae* transporters are the γ -aminobutyric acid (Uga4), choline (Hnm1), polyamine (Tpo5), and histidine (Hip1) transporters (Vogl et al., 2008). In a lesser extent, *S. pombe* vitamin B6 transporter Bsu1 is also able to transport thiamine across the PM. As Thi7, Bsu1 also belongs to the MFS family but is closer to transporters from the multiple drug resistance (MDR) subfamily (Stolz et al., 2005; Vogl et al., 2008). Bsu1 is therefore close to the *S. cerevisiae* family of Drug-H⁺ antiporter (DHA1) such as the putative drug transporter Yhk8 and the polyamine exporters Tpo3/4. In *Arabidopsis thaliana*, a transporter shown to facilitate polyamine transport, Put3, was also shown to be a thiamine transporter (Martinis et al., 2016).

Notably, two genes were shown to encode thiamine transporters in human, *SLC192A2* and *SLC192A3* (Dutta et al., 1999; Rajgopal et al., 2001). The corresponding proteins, named ThTR-1 and ThTR-2, share 6 and 13% of sequence identity with Thi7, respectively. However, expression of SLC192 in yeast cells defective for thiamine transport restored thiamine uptake (Huang et al., 2012). In the same way, expression of *THI7* was able to rescue thiamine transport in fibroblasts from patients suffering of thiamine-responsive megaloblastic anaemia, shown to be defective for high affinity thiamine uptake (Stagg et al., 1999).

4.4 *THI* regulatory system

In addition to its role of coenzyme, thiamine also regulates the expression of about 60 genes (Nosaka, 2006). Genes involved in the homeostasis of thiamine are referred to as *THI* genes. Generally, *THI* genes are highly expressed in the absence of thiamine and repressed upon addition of thiamine (Mojzita and Hohmann, 2006). A mutant in the thiamine pyrophosphokinase Thi80 led to the discovery that *THI* genes transcriptional regulation was dependent on the cytosolic concentration of TPP and not thiamine (Nishimura et al., 1992b). This mechanism is called the *THI* regulatory system or *THI* regulon.

Thi2, Pdc2 and Thi3 are positive regulators of the expression of *THI* genes. Thi2 is a Zn₂-Cys₆ zinc finger motif-containing transcription factor (Nosaka et al., 2005). Pdc2 is a transcription factor whose expression is stimulated by glucose. Pdc2 is known to be required for the expression of the pyruvate decarboxylase enzymes, Pdc1 and Pdc5 (Nosaka et al., 2012). Therefore, Pdc2 is the link between the expression of genes involved in thiamine synthesis and its use as coenzyme by Pdc1/5 (Mojzita and Hohmann, 2006). Thi3 is 50% identical to pyruvate decarboxylase. Thi3 has a TPP-binding motif and was shown to be the sensor of the cytosolic concentration of TPP (Hohmann and Meacock, 1998).

In thiamine-depleted conditions, Thi3 is thought to bridge Thi2 and Pdc2, initiating the transcription of *THI* genes. Indeed, interaction between Thi3 and the C-terminal part of Pdc2 is proposed to lead to the exposure of Pdc2 transactivation domain (Nosaka et al., 2008). When the intracellular concentration of TPP is high, TPP interacts with the TPP-binding site of Thi3 (Nosaka et al., 2005). TPP binding to Thi3 does not affect its nuclear localization but rather disrupts its ability to bind Thi2 and Pdc2, inhibiting *THI* gene transcription (Nosaka, 2006) (Figure 13).

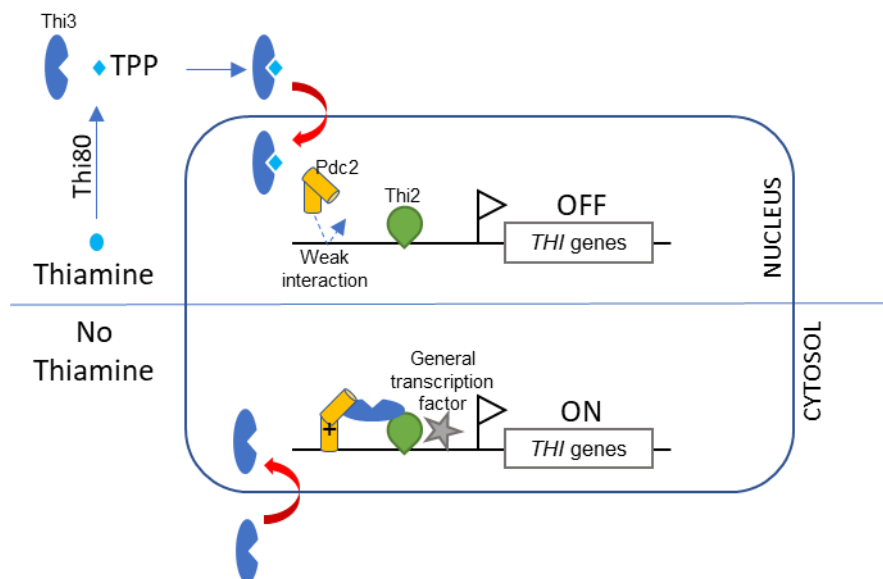


Figure 13 – Schematic view of the *THI* regulatory system. In the absence of thiamine, cytosolic thiamine pyrophosphate (TPP) concentration is low. In that condition, Thi3 interacts with the C-terminal part of Pdc2, triggering the exposure of its transactivation domain, and Thi2. The Pdc2 – Thi3 – Thi2 complex may also recruit other general transcription factors and initiates the transcription of *THI* genes. When TPP concentration is high, TPP binds to Thi3, hindering its interaction with Pdc2 and Thi2.

An activating sequence was found upstream of many thiamine repressible genes (like *PHO3*, *THI80* and *THI6*), characterized by GAAA(A/C)TCAA (Nosaka et al., 1992). By comparing many *THI* genes, a consensus sequence was later described as G*AACT*TTAGA. This sequence turns out to be a Thi2 binding sequence and is therefore required for sensitivity to thiamine (Harbison et al., 2004).

Although the reverse complement of this sequence is also found upstream to *THI7* (Enjo et al., 1997), *THI7* is still expressed in a strain defective for the transcription factor Thi2 (Nishimura et al., 1992a; Enjo et al., 1997). On the contrary, TPP was shown to reduce the uptake of thiamine in a Thi3-dependent way (Nishimura et al., 1992a). Indeed, *THI7* is not expressed in a strain defective for Thi3 in the presence or not of thiamine (Enjo et al., 1997). Therefore, the expression of *Thi7* is only dependent on Thi3 and Pdc2 but not Thi2. Like other *THI* genes, the expression of *THI7* is maximal when there is no thiamine in the

medium. In typical yeast growth medium, the concentration of thiamine (about 1.2 μM) triggers a strong decrease of *THI7* expression, reaching approximatively 4% of the maximal level (Singleton, 1997). Concerning *NRT1*, its expression seems to be linked to the cytosolic NAD^+ concentration via the NAD^+ -dependent histone deacetylase Hst1 (and its interactors Sum1 and Rfm1), which is thought to act as a global sensor of the intracellular concentration of NAD^+ (Bedalov et al., 2003). Recently, the expression of other *THI* genes was shown to be repressed by high cytosolic NAD^+ concentration via, among others, Hst1 (Li et al., 2010).

AIM OF THE THESIS

the plasma membrane (PM) composition must be constantly regulated to ensure its function of semipermeable barrier. Endocytosis is an essential mechanism involved in that regulation. The PM protein landscape is in constant adaptation to match the changing environmental conditions of the cell. Yeast cells can grow in various environments. Therefore, the need of rapid adaptation to the external medium is essential. In yeast, ubiquitylation of proteins is required to trigger endocytosis. Ubiquitylation of plasma membrane proteins is catalyzed by a single ubiquitin ligase, Rsp5, the *S. cerevisiae* homolog of the mammalian ubiquitin ligase Nedd4. Rsp5 is supported in its task by adaptor proteins, the arrestin-related trafficking (ART) adaptors also known as α -arrestins, mediating the ubiquitylation of its PM targets. The genome of *S. cerevisiae* encodes 13 α -arrestins that were shown to be required for the trafficking of many PM proteins.

About 240 plasma membrane proteins, including 125 transporters, are predicted to be localized at the PM. The α -arrestin mediated endocytosis of about 23 integral PM proteins has been investigated so far. These targets are mainly amino acids or carbon source transporters. Their study brought many insights on the mechanism underlying α -arrestin regulation in response to nutrient supply. However, the α -arrestin-mediated regulation of proteins that are not directly associated to carbohydrates or amino acids metabolism remains elusive.

In order to broaden our understanding of α -arrestins involvement, the first part of my thesis was aiming at finding new targets of the α -arrestin system in an unbiased fashion. To do so, we performed a PM quantitative proteomic screening. The proteomic screening led us to the study of the endocytosis of the PM thiamine (vitamin B1) transporter family. Thiamine is an essential coenzyme of many enzymes of the carbohydrate and amino acid metabolism, having a global impact on the cellular metabolism.

Therefore, the second part of my thesis focused on the characterization of the α -arrestin-mediated ubiquitylation of *S. cerevisiae* thiamine transporters.

RESULTS

This part will focus on the research I carried out during my four years of thesis. My first year was mainly dedicated to the proteomic screening that led to the study of thiamine transporters, which I performed during my two last years. During my second year of thesis, I took part in a proteomic screening aiming at completing the first one. This project was partially managed by another PhD student, Sylvain Nootens. This proteomic experiment was aborted for several reasons that will be further described in this part. Therefore, no result from that experiment will be developed here.

Some experiments of my thesis were performed in collaboration with master student and PhD student of the lab. The major part of the work was performed by myself and two master students I supervised, Xiaoqian Chen and Mathilde Bausart. During the writing of the thesis, I received kind help from Sylvain Nootens and the master student he supervised, Radia Adjadj. They participated to the interaction study of the three thiamine transporters.

1 Finding new targets of the alpha-arrestin adaptors

We applied a mass spectrometry-based quantitative proteomic experiment based on the iTRAQ labelling technology to yeast plasma membrane (PM) enriched fractions. We compared the PM proteome of twelve strains each lacking the gene of one specific alpha-arrestin (*artΔ* strains) and a WT strain after a 90-min incubation with cycloheximide (CHX), known to trigger the endocytosis of many transporters (Nikko and Pelham, 2009). We identified on average 110 peripheral and integral plasma membrane proteins. The relative abundance of proteins detected in the 13 strains was calculated and indicated by ratios, i.e. the abundance of each identified protein in a strain is divided by the abundance of the same protein in the CHX-treated WT (WT+CHX).

Among the identified proteins, the abundance of 28 proteins was affected by the CHX treatment, i.e. the ratio of their abundance in a mock-treated WT (WT ctrl) and a CHX-treated WT was different from one (Table 1, WT ctrl/WT+CHX), either significantly higher (in red) or lower (in blue). The abundance of these proteins was then compared between twelve strains lacking a given α -arrestin adaptor (*artΔ*) and a WT strain, both treated with cycloheximide. Again, red ratios, i.e. ratios significantly higher than one, indicate proteins that are more abundant in *artΔ*+CHX than in a WT+CHX strain, suggesting either the stabilization of the protein at PM or an increased expression in the absence of the adaptor. Alternatively, blue ratios, i.e. significantly lower than one, illustrate proteins whose abundance is lower in *artΔ*+CHX than in the WT+CHX condition. Ultimately, ratios that are not significantly different from one are represented in white. In Table 1, grey squares with the not determined (N.D.) mention represent proteins that were not identified in all the replicates by mass spectrometry, preventing the calculation of the corresponding fold change ratios.

1.1 The CHX treatment triggers a decrease of the abundance of 19 PM proteins

We identified 19 integral PM proteins whose abundance decreased following CHX treatment (ratio higher than one). The identified proteins are covering many functions. We identified a peptide transporter (Ptr2), the catalytic subunit of the 1,3- β -glucan synthase (Fks1), a thiamine transporter (Thi7), several hexose transporters (Hxt1, Hxt3 and Hxt7), a sodium pump (Ena5), membrane proteins involved in iron uptake (Fre1 and Fet3), ATP-binding cassette (ABC) transporters (Pdr5, Yor1), the H⁺-ATPase Pma1, a protein putatively involved in exocytosis (Rsn1), two β -1,3-glucanosyl transferases (Gas1, Gas3), two polyamine exporters (Tpo2 and Tpo4), and a protein of unknown function (Ina1) (Table 1).

The analysis of the *art* Δ +CHX/WT+CHX ratio of these 19 proteins reveals different profiles.

Some proteins are affected by a few α -arrestins, like the peptide transporter Ptr2. Ptr2 is the most affected protein in response to the CHX treatment. We observed that the *bul1* Δ +CHX on WT+CHX ratio of Ptr2 is equal to 8.1, suggesting a strong stabilization of Ptr2 at the PM in the absence of Bul1. This result suggests that Bul1 could be the adaptor bridging Rsp5 and Ptr2. Obviously, we lack data to compute ratios for the *art1* Δ to *art6* Δ strains. Therefore, we cannot rule out that the absence of these other α -arrestins does not affect Ptr2 abundance at the PM.

The abundance of other identified proteins seems to be affected by several α -arrestins. The abundance of Fks1 is increased at the PM in all *art* Δ except for the *bul2* Δ strain. This effect is intriguing since it suggests that many α -arrestins are required to fine-tune the abundance of Fks1. However, the different α -arrestins are not redundant since the lack of each α -arrestin results in an increase of Fks1 abundance.

Finally, some proteins are less abundant in the absence of a given α -arrestin. For Hxt4, Hxt7, Rsn1, Ina1, Gas1 and Gas3,

some or all *artΔ*+CHX/WT+CHX ratios are lower than one (blue ratios). That observation illustrates that their abundance is lower in the absence of specific α -arrestins although cells are treated with CHX. The lower abundance suggests either a decrease of the expression of the gene or an increased protein degradation. In the case of Hxt7, its expression was recently shown to be lower in the *art2Δ* strain (Hovsepian et al., 2017), supporting our results. Interestingly, Gas1 and Gas3 are glycosylphosphatidylinositol (GPI)-anchored proteins. They do not have any cytosolic part and are therefore unable to directly interact with α -arrestins. However, the abundance of Gas1 is lower in *art9Δ* than in the WT, while the abundance of Gas3 is lower in *art8/9Δ*, *bul1Δ* and *bul2Δ*. These results are the first examples of an effect of α -arrestin on the abundance of GPI-anchored proteins.

Notably, among the proteins we identified, some have already been studied in the context of α -arrestin-mediated endocytosis. Indeed, our screening partially supports previously described interactions between hexose transporters and some specific α -arrestins. Indeed, endocytosis of Hxt1 and Hxt3 is regulated by Art4 and Art7 (O'Donnell et al., 2015), while Hxt6 and Hxt7 endocytosis is mediated by Art8 (Hovsepian et al., 2017). The proteomic experiment confirmed that Art4 and Art7 are involved in Hxt1 endocytosis. Interestingly, we showed that several other adaptors could also be involved in Hxt1 endocytosis. Moreover, the screening illustrates the role of Art4 but not Art7 in Hxt3 endocytosis. It also points out potential new players in the endocytosis of the latter, namely Art1 and Art9. On the contrary, no red ratio is observed when looking at the relative abundance of Hxt7 between the *art8Δ* and WT strains. However, the proteomic experiment suggests a role of Art4 in the endocytosis of Hxt4 (Hxt7 paralog), a hexose transporter whose endocytosis upon glucose removal was shown to be Art2-dependent (Hovsepian et al., 2017).

Table 1 – Fold change of abundance of identified plasma membrane associated proteins after cycloheximide treatment by the proteomic experiment. The first and second column represent the accession number and name of proteins identified as affected by the CHX treatment. The first line corresponds to the strains being compared. The relative abundance is calculated as ratio. Red ratios stand for fold changes significantly higher than one (the protein being less abundant in the WT treated with CHX), while blue ratios stand for fold changes significantly lower than one. White ratios are not significantly different from one, meaning no difference could be statistically observed between strains. In some cases, ratios could not be calculated due to lack of identification or quantification data and are represented in grey with the “Not Determined” (N.D.) mention. Proteins are listed in order of effect of CHX (third column), i.e. ratios of the abundance in a mock-treated (WT ctrl) compared to a CHX-treated WT strain. The first protein being the most downregulated and the last, the most upregulated after treatment with CHX. The proteomic experiment has been performed three times, with six individual mock- and CHX-treated WT. Ratios observed are the geometrical average from three independent experiments.

Accession number	Name	WT ctrl / WT+CHX	<i>art1Δ</i> + CHX / WT+CHX	<i>art2Δ</i> + CHX / WT+CHX	<i>art3Δ</i> + CHX / WT+CHX	<i>art4Δ</i> + CHX / WT+CHX	<i>art5Δ</i> + CHX / WT+CHX	<i>art6Δ</i> + CHX / WT+CHX	<i>art7Δ</i> + CHX / WT+CHX	<i>art8Δ</i> + CHX / WT+CHX	<i>art9Δ</i> + CHX / WT+CHX	<i>art10Δ</i> + CHX / WT+CHX	<i>bul1Δ</i> + CHX / WT+CHX	<i>bul2Δ</i> + CHX / WT+CHX
P32901	PTR2	16.3	ND	ND	ND	ND	ND	ND	1.3	1.2	1.4	1.8	8.1	0.6
P38631	FKS1	7.4	6.7	4.0	6.8	2.5	4.6	2.2	2.1	1.5	4.2	1.8	3.8	0.9
Q05998	THI7	6.6	1.2	3.0	0.7	0.4	0.5	0.2	0.5	0.6	11.7	0.4	0.6	0.2
P32465	HXT1	5.8	7.6	4.3	6.4	15.3	4.9	2.2	3.9	3.2	2.4	3.6	2.5	3.3
Q12691	ENA5	3.7	1.8	2.3	4.2	3.2	1.7	2.2	1.2	1.9	1.9	1.2	0.8	1.5
P32791	FRE1	3.0	ND	ND	ND	ND	ND	ND	1.8	1.7	2.0	2.0	2.3	1.8
P32466	HXT3	2.7	2.5	1.3	1.7	8.0	1.3	0.6	1.6	0.8	3.2	1.1	1.3	0.6
P33302	PDR5	2.4	ND	ND	ND	ND	ND	ND	2.3	1.3	1.9	2.0	0.5	1.5
P53049	YOR1	2.3	ND	ND	ND	ND	ND	ND	1.2	1.5	1.6	1.4	1.2	1.0
P39004	HXT7	2.3	0.6	0.3	0.4	1.2	0.6	0.3	0.8	0.5	0.1	0.6	0.1	0.4
P05030	PMA1	2.2	ND	ND	ND	ND	ND	ND	1.4	0.7	0.3	0.8	0.6	0.4
P32467	HXT4	2.1	0.6	0.5	0.6	2.9	0.5	0.5	0.7	0.5	0.4	0.5	0.3	0.4
P38993	FET3	2.0	ND	ND	ND	ND	ND	ND	1.0	0.8	1.8	0.8	1.3	1.1
Q03516	RSN1	2.0	ND	ND	ND	ND	ND	ND	0.9	1.0	1.6	1.3	0.5	0.8
P53283	TPO2	1.9	ND	ND	ND	ND	ND	ND	1.6	1.1	0.5	1.0	1.1	1.3
Q06689	INA1	1.8	ND	ND	ND	ND	ND	ND	0.9	0.6	0.6	0.8	0.4	0.6
P22146	GAS1	1.7	ND	ND	ND	ND	ND	ND	1.3	0.9	0.2	1.3	1.0	0.7
Q03655	GAS3	1.7	ND	ND	ND	ND	ND	ND	0.9	0.6	0.4	0.7	0.2	0.3
Q12256	TPO4	1.6	ND	ND	ND	ND	ND	ND	1.4	1.2	0.2	1.1	0.8	1.1
P01120	RAS2	0.4	1.0	0.6	1.3	1.0	1.0	0.6	ND	ND	ND	ND	ND	ND
Q03640	TCB3	0.4	0.3	0.5	0.5	0.6	0.5	0.3	0.7	0.8	0.9	0.8	0.5	0.5
Q12207	NCE102	0.4	0.8	0.6	0.9	0.3	0.6	0.4	ND	ND	ND	ND	ND	ND
Q12466	TCB1	0.3	ND	ND	ND	ND	ND	ND	0.7	0.8	0.7	0.7	0.5	0.3
P38250	IST2	0.3	ND	ND	ND	ND	ND	ND	0.6	0.6	1.1	0.8	0.8	0.5
P06780	RHO1	0.2	1.0	0.7	0.9	0.6	0.9	0.4	ND	ND	ND	ND	ND	ND
P53252	PIL1	0.2	0.5	0.3	0.6	0.5	0.5	0.3	0.4	0.4	0.1	0.5	0.3	0.3
Q12230	LSP1	0.1	0.6	0.3	0.5	0.4	0.6	0.4	0.6	0.6	0.1	0.6	0.4	0.6
Q08245	ZEO1	0.1	0.7	0.9	1.3	0.5	0.9	0.5	1.1	0.9	0.3	0.9	0.5	0.4

1.2 The CHX treatment triggers an increase of the abundance of nine proteins

Some proteins were more abundant in response to the CHX treatment (WT ctrl/WT+CHX ratio lower than one). All these proteins are peripheral or tightly associated to the PM.

The less affected protein in response to the CHX treatment is Ras2. Ras2 is a GTP-binding protein known to be palmitoylated and farnesylated, which ensures its anchorage to the PM. Ras2 is involved in the regulation of sporulation, filamentous growth and nitrogen starvation response by stimulating the adenylate cyclase (Cyr1). Recently, Ras2 was also shown to be activated by the glucose metabolism (Peeters et al., 2017). Ras2 physically interacts with Art1, Art4 and Art7 (Prosser et al., 2015) and with two other hits of the screening, the ABC transporters Yor1 and Pdr5 (Snider et al., 2013). In our results, the absence of Art1 to Art6 α -arrestins did not result in a change of Ras2 abundance. Unfortunately, we could not conclude for the remaining strains (*art7* Δ to *bul2* Δ).

Three proteins involved in the ER-PM membrane tethering are also more abundant after the addition of CHX: the tricalbins Tcb1 and Tcb3, as well as Ist2 (Manford et al., 2012). ER-PM tethering is required for the regulation of the PM lipid composition via the action at the PM of the integral ER phosphatidylinositol 4-phosphate (PI4P) phosphatase Sac1. At membrane contact sites Sac1 dephosphorylates PI4P from the PM in phosphatidylinositol (PI) (Stefan et al., 2011). The abundance of Tcb3 is lower than in a WT in strains lacking Art1 to Art6 α -arrestins after the CHX treatment. Ratios for the remaining strains are not available. Concerning Tcb1 and Ist2, they both are less abundant at the PM in *art7* Δ , *bul1* Δ and *bul2* Δ . Additionally, the abundance of Tcb1 is decreased in *art9* Δ , and the abundance of Ist2 is lower in *art8* Δ and *art10* Δ .

Three components of the eisosomes, Nce102, Pil1 and Lsp1 were identified as more abundant at the PM after the CHX treatment. Eisosomes are protein complexes laterally organizing

the PM protein and lipid composition by forming sphingolipids and ergosterol-enriched membrane compartment, called membrane compartments containing Can1 (MCC). The MCC were shown to gather other transmembrane proteins in addition to the arginine permease Can1, like Nce102, Sur7 and many other permeases (Grossmann et al., 2008). Among the identified proteins of the screening, Nce102 is a transmembrane protein proposed to be a sphingolipid sensor (Fröhlich et al., 2009). Lsp1 and Pil1 are peripheral BAR domain-containing proteins localized on the cytosolic leaflet of the PM. Pil1 seems to be essential for the assembly of eisosomes, while the function of Lsp1 is still unclear. Although the physiological role of eisosomes in endocytosis is still debated, Gournas and co-workers recently showed that the clustering of Can1 in the MCC prevents its ubiquitylation and endocytosis upon arginine addition (Gournas et al., 2018).

The abundance of the GTP-binding protein Rho1 is increased in response to the CHX treatment. Rho1 is involved in the cell integrity pathway, cell polarity and is known to regulate the activity of Fks1, which is downregulated in response to CHX. Rho1 and Fks1 were shown to be required for the efficient internalization of the pheromone receptor Ste2 (deHart et al., 2003). Moreover, Rho1 and one of its effector, the formin Bni1, is involved in the CIE endocytosis of Ste2 and Mup1. The Rho1-dependent CIE of Ste2 was also shown to depend on Art3 and Art6 (Prosser et al., 2011, 2015). However, no effect of the deletion of *ART1* to *ART6* α -arrestin genes was observed in the screening and we did not obtain the results for the remaining α -arrestin genes.

The identified protein whose abundance is the most increased following the CHX treatment is Zeo1. Zeo1 is a peripheral protein regulating the cell integrity pathway (Green et al., 2003). Its exact function is still unclear. The abundance of Zeo1 is decreased in *art4 Δ* , *art9 Δ* , *bul1 Δ* and *bul2 Δ* .

In order to complete this global analysis, we carried out a second proteomic screening (data not shown). This work was performed in collaboration with another PhD student of the lab, Sylvain Nootens. We used a strain lacking all α -arrestin genes (except for ART9), named EN67, which we transformed with various plasmids coding for α -arrestin adaptors (Nikko and Pelham, 2009). Our aim was to compare the PM proteome of the EN67 strain with and without expression of an additional α -arrestin adaptor. To study the involvement of Bul1 and Bul2, we used the EN63 and EN60 strain, in which *BUL1* and *BUL1/2* are still expressed in addition to *ART9*, respectively. As EN67 and the α -arrestin-coding plasmids, the latter strains were provided by Professor H. Pelham (MRC, UK). Many proteins were stabilized at PM in the EN67 strain due to the lack of α -arrestin to mediate their ubiquitylation. We expected to see specific endocytosis following addition of a single α -arrestin in the EN67 background.

Using this approach, we identified almost 60 new potential targets of the α -arrestin network. This second screening pointed out an interesting regulation pattern of the Ssy1-Ptr3-Ssy5 (SPS)-regulated amino acid transporter family, including the high-affinity glutamine permease Gnp1, the low-affinity amino acid permease Agp1 and high-affinity leucine permease Bap2. The abundance of these three transporters was significantly lower in the EN67 strain expressing *ART4* or *ART7* than in EN67, suggesting the involvement of both adaptors in their ubiquitylation. Unfortunately, we were not able to confirm these results using other approaches (data not shown). In addition to that, some clues suggested that the multiple gene deletion in the EN67 strain resulted in the accumulation of many indirect effects, impacting the abundance of PM proteins in an unspecific way. This part of the project was therefore aborted.

2 Thiamine transporters as new targets of the α -arrestin system

2.1 The abundance of Thi7 is higher in *art2Δ* or *art9Δ* strains than in a WT after the addition of CHX

Apart from proteins previously stated, a protein caught our attention, the high affinity vitamin B1 (thiamine) transporter Thi7. Indeed, the regulation of vitamin transporters is still poorly understood. Thiamin is essential and is involved in many processes, raising the question of how the α -arrestin system copes with this complexity. Therefore, we decided to focus our work on this Thi7 transporter.

The addition of cycloheximide triggers a strong decrease of Thi7 at the PM. Indeed, Thi7 is on average seven times less abundant in a WT treated with cycloheximide than in a mock-treated WT. Moreover, when Art2 is absent (*art2Δ*), Thi7 is three times more abundant at PM than in a WT. Thi7 abundance is 12 times higher in an *art9Δ* strain than in a WT (Table 1). The results of the screening suggest that Art2 and Art9 are involved in the regulation of the abundance of Thi7 at PM. They could not be fully redundant since the presence of *ART9* in *art2Δ* seems to not rescue the downregulation of Thi7 and *vice versa*.

2.2 Deletion of *ART2* or *ART9* does not increase *THI7* expression

We checked if the higher abundance of Thi7 at the PM of *art2Δ* and *art9Δ* strains was due to an impairment of its degradation or to an increased expression. We examined the abundance of genomically-tagged Thi7-EYFP (enhanced Yeast Fluorescent Protein) in YD grown strains after CHX treatment. The advantage

of the genomic insertion was to monitor the abundance of Thi7 in the growth medium used in the screening, i.e. in YD rich medium without any selection pressure. We compared by western blotting the abundance of Thi7-EYFP in a WT, *art2Δ* and *art9Δ* after 90 min incubation of ethanol (mock-treatment) or CHX (Figure 1). The calculation of the relative abundance was performed through quantification of bands intensity, from which the background signal was subtracted, normalized by the intensity of the band corresponding to the loading control, Pma1. The theoretical size of Thi7 and Thi7-EYFP is 67 kDa and 94 kDa, respectively. We noticed that Thi7-EYFP appears as a doublet in all conditions we tested in our study. The upper band is slightly higher and the lower one is lower than the 100 kDa-corresponding band of the ladder.

After mock-treatment, the protein level of Thi7-EYFP is lower in *artΔ* strains compared to the WT. Indeed, Thi7-EYFP intensity of *art2Δ* and *art9Δ* is 1.6 and 2.1 times lower than the one in the WT. Moreover, we observed by immunoblotting that the abundance of Thi7-EYFP is 45% lower in the CHX-treated than in the mock-treated WT. On the contrary, the intensity of Thi7-EYFP corresponding band after CHX incubation is only 30% decreased in *art2Δ*, suggesting that the protein is less degraded compared to a WT. However, the ratio of Thi7-GFP amount between mock-treatment and CHX-treatment is similar in *art9Δ* and in a WT.

Although the experiment was performed once and should be repeated, the results suggest that CHX is decreasing Thi7-EYFP abundance as it did in the proteomic screening. The difference observed in the screening for *art2Δ* and *art9Δ* is not due to an increase of Thi7 expression since we did not see any significant increase of Thi7-EYFP level when comparing these strains to a WT. However, our results support that the CHX-induced decrease of Thi7 abundance is impaired in *art2Δ*, but not in *art9Δ*.

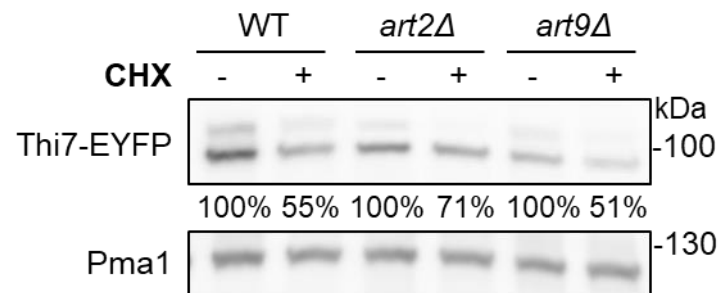


Figure 1 – Cycloheximide triggers a greater decrease of Thi7-EYFP abundance in a WT than in *art2Δ* strain. We genomically-fused THI7 to EYFP in a WT, *art2Δ* and *art9Δ* strains that were grown in YD rich medium to mid-log phase, treated with 50 μ g/ml CHX (in ethanol) or 0.5% ethanol as a control for 90 min, and harvested for total protein extraction. Samples were immunoblotted using anti-GFP and anti-Pma1 (as a loading control). No free YFP signal was shown because EYFP is not stable in the vacuole. Quantification is based on the intensity of the Thi7-EYFP bands, from which background is subtracted, normalized by the corresponding Pma1 band intensity. Experiment was performed once.

2.3 GFP fusion of thiamine transporters does not impair transport

To further study the role of the alpha-arrestin adaptors in thiamine transporter regulation, we engineered N-terminal and C-terminal yeast enhanced Green Fluorescent Protein (GFP) fusion of Thi7 and its two homologs, the low affinity thiamine transporters Nrt1 and Thi72, expressed under the control of the *THI7* promoter. A phenotypic growth test of a thiamine transport-deficient strain expressing the different GFP-fused versions was performed on a toxic analog of thiamine, oxythiamine, to check whether their fusion to the GFP affects their ability to transport. A *thi7Δnrt1Δthi72Δ* strain (called CVY3), impaired for thiamine transport but not for thiamine synthesis, was transformed with the GFP fusion constructs and the non-fused version of the transporters, and grown in thiamine-free medium to early exponential phase. Ten-fold serial dilutions of cell cultures were spotted on a selective thiamine-free medium (SC-U-B1) supplemented with oxythiamine (Figure 2A). Oxythiamine cannot sustain the coenzyme function of thiamine and therefore impairs carbohydrate and amino acid metabolism, leading to a growth defect. We used this growth defect as readout of the transport activity. As expected, the CVY3 strain transformed with an empty plasmid turns out to be resistant to oxythiamine. However, a growth defect was detected for all the strains expressing Thi7 and Thi72, fused or not to GFP. The GFP-Nrt1-transformed strain showed a growth defect in response to oxythiamine, while the non-fused and carboxy (C)-terminal version were not sensitive. Globally, only some slight differences were detected between the native and C-terminal GFP-fusions. Therefore, we performed the next experiments with the C-terminally tagged version, their ability to transport oxythiamine being the most similar to the native version.

In order to confirm the previous results, we also directly tested the activity of the C-terminally fused transporters for thiamine uptake using the same methodology. We transformed a *thi7Δnrt1Δthi72Δthi4Δ* (called CVY4) strain lacking thiamine

transport and synthesis with the native version of the transporters and their GFP-fused counterparts. Cultures were spotted on medium containing different concentrations of thiamine. This time, the empty vector-transformed strain did not grow, except on high concentration of thiamine, i.e. 100 μ M (Figure 2B). The same trends as in the oxythiamine test were observed. All the strains expressing a transporter were able to grow. Growth of strains expressing the GFP fusions was similar to that of the strain expressing the native transporters.

These results also suggest some differences in the transport activity of the three transporters. We observed that *NRT1*-expressing strains were less affected by oxythiamine than the two other transporters. The growth defect was higher for *THI72*-expressing strains than for those expressing *THI7*. In the same way, the expression of *NRT1* in the CVY4 strain rescued growth in a lesser extent than *THI7* and *THI72*. To have a more rigorous information about transport activity, these experiments should be complemented with a fluorescent thiamine analog or radioactive thiamine uptake assay.

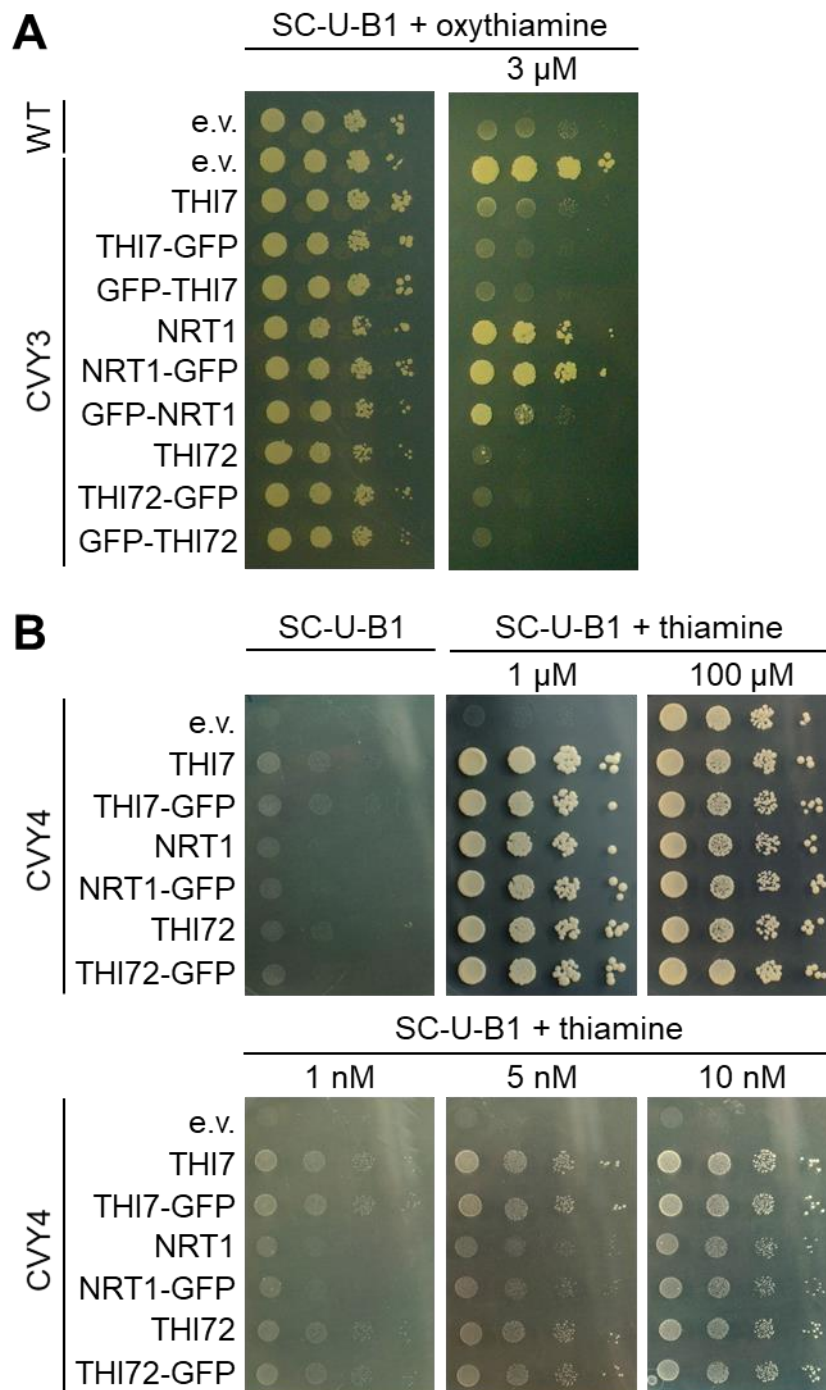


Figure 2 – N- and C-terminal GFP fusions of *THI7*, *NRT1* and *THI72* are active. (A) Phenotypic growth test of CVY3 (*thi7Δnrt1Δthi72Δ*) strains expressing native, N- and C-terminal GFP-fused version of *THI7*, *NRT1* and *THI72* transporters on thiamine-free medium and thiamine-

free selective (SC-U-B1) medium supplemented with oxythiamine at a final concentration of 0.6 and 3 μ M (B) Phenotypic growth test of CVY4 (*thi7 Δ nrt1 Δ thi72 Δ thi4 Δ*) strains expressing native and C-terminal GFP-fused version of *THI7*, *NRT1* and *THI72* transporters on thiamine-free selective (SC-U-B1) medium or supplemented with 1 nM, 5 nM, 10 nM, 1 μ M and 100 μ M thiamine. (e.v.: empty vector)

2.4 Endocytosis of Thi7-GFP is triggered by cycloheximide and is impaired in *art2 Δ*

We sought to confirm that the decrease in abundance of Thi7 observed upon CHX treatment by immunoblotting (Figure 1) was the results of Thi7 degradation rather than an effect on gene expression. The twelve *art Δ* strains, a WT and the *rsp5* strain were transformed with a plasmid expressing THI7-GFP under the control of its own promoter. The *rsp5* strain is characterized by an impaired expression (approximately 10 times lower compared to the WT level) of the ubiquitin ligase Rsp5 due to the insertion of the KanMX (geneticin resistance) cassette in the promoter of *RSP5* (Hein et al., 1995; Springael and André, 1998). Strains were grown in a thiamine-free medium in order to maximize *THI7-GFP* expression and to stabilize it at the PM before mock- or CHX-treatment.

To compare our methodology with the previous experiment, we monitored the degradation of Thi7-GFP upon CHX addition in all *art Δ* strains by immunoblotting (Figure 3A). Thi7-GFP, whose theoretical size is 94 kDa, is composed of the two previously-observed bands and a third upper but less intense one. The observed size is approximatively the same as in Figure 1, the middle band being slightly lower. We did not determine the nature of the different bands. Nevertheless, the third upper band is barely visible in several strains (*art1/2/5/9 Δ* and *bul2 Δ*) and absent in the *rsp5* strain, suggesting a ubiquitylated form of Thi7-GFP. In some strains, a fourth weaker band is detected at even higher molecular weight (115 kDa). Moreover, a single band is present at approximatively 27 kDa and corresponds to free GFP, known to be resistant in some extent to vacuolar proteases, unlike the EYFP version we used in Figure 1.

We observed a decrease in Thi7-GFP abundance in a WT, but its extent was lower than the one observed for Thi7-EYFP. We detected free GFP, marker of vacuolar degradation of Thi7-GFP. We monitored the basal amount of free GFP and its accumulation during the incubation with CHX in the different strains. We observed that free GFP basal level was very low in *art2Δ* compared to a WT and absent in the *rsp5* strains. This result suggests that the CHX-induced downregulation of Thi7-GFP is Rsp5 and Art2-dependent. At a lower extent, free GFP level in *art8Δ* and *art9Δ* was also lower than in a WT, but no effect was seen by fluorescence microscopy.

We followed the localization of Thi7-GFP after addition of cycloheximide by fluorescence microscopy. We confirmed that Thi7-GFP is targeted to the vacuole after addition of CHX to a WT strain, confirming that Thi7-GFP is endocytosed. Indeed, a strong fluorescent signal is localized in the lumen of the vacuole after two hours. However, we observed that some fluorescent signal was still localized at PM even after three hours of incubation. The same profile was observed in all *artΔ* strains (data not shown), including *art9Δ*, but not in *art2Δ* (Figure 3B). In absence of *Art2*, Thi7-GFP is exclusively localized at PM like in mock-treated strains and the *rsp5* strain.

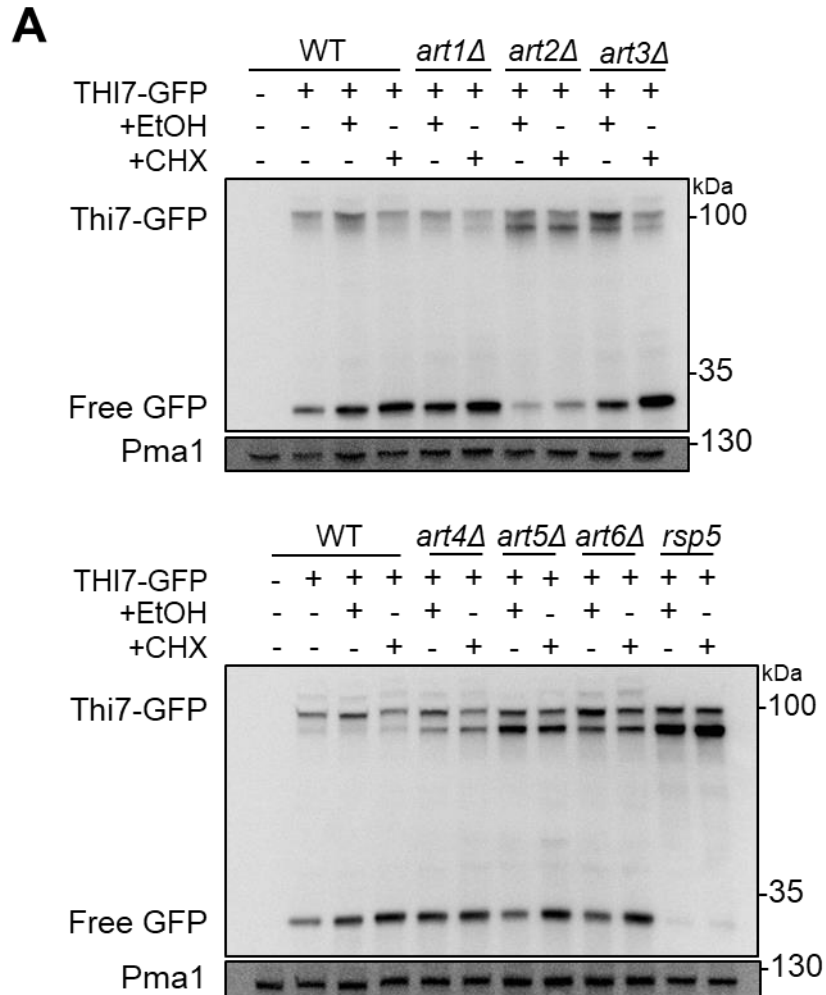


Figure 3 – Endocytosis of Thi7-GFP is triggered by cycloheximide in a WT strain and all *artΔ* except for *art2Δ*. (A) WT and *artΔ* strains expressing THI7-GFP under its endogenous promoter were grown in thiamine-free selective medium up to early-log phase and harvested after 120 min incubation with cycloheximide at a final concentration of 50 μ g/ml or mock-treated with ethanol for total protein extractions. Extracts were immunoblotted with anti-GFP and anti-Pma1 as a loading control. Free GFP is the results of the degradation of Thi7-GFP in the vacuole.

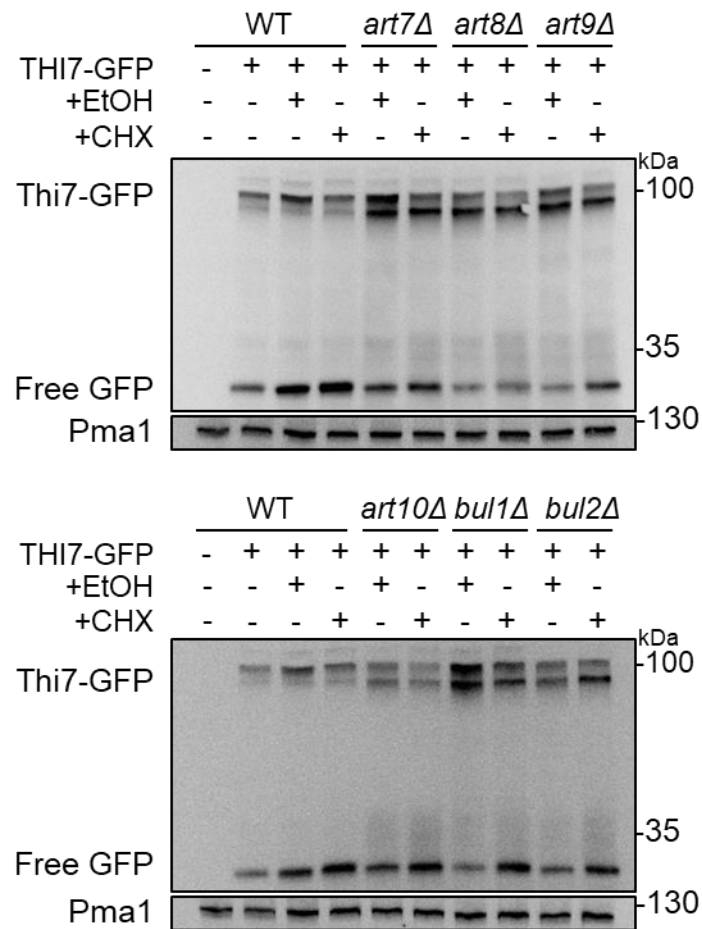


Figure 3 (continued) – Endocytosis of Thi7-GFP is triggered by cycloheximide in a WT strain and all *artΔ* except for *art2Δ*. (A) WT and *artΔ* strains expressing THI7-GFP under its endogenous promoter were grown in thiamine-free selective medium up to early-log phase and harvested after 120 min incubation with cycloheximide at a final concentration of 50 μ g/ml or mock-treated with ethanol for total protein extractions. Extracts were immunoblotted with anti-GFP and anti-Pma1 as a loading control. Free GFP is the results of the degradation of Thi7-GFP in the vacuole.

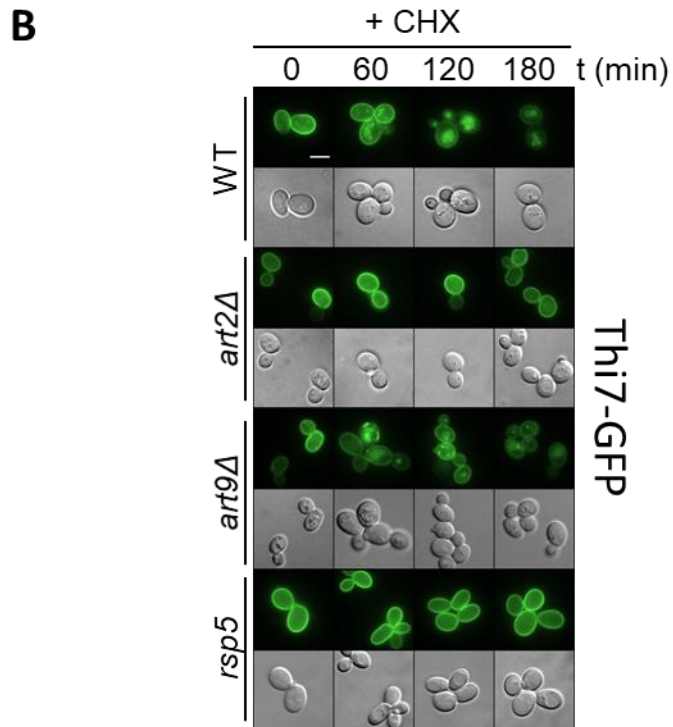


Figure 3 (continued) – Endocytosis of Thi7-GFP is triggered by cycloheximide in a WT strain and all *artΔ* except for *art2Δ*. (B) Localization of Thi7-GFP in WT, *art2Δ*, *art9Δ* and *rsp5* strains after mock- and CHX-treatment. Shown cells are the most representative of the population (Scale bar represents 5 μ m)

2.5 Addition of thiamine triggers the Rsp5-dependent endocytosis of Thi7

Cells were initially grown in a thiamine-free medium in order to stimulate *THI7-GFP* expression. Thiamine at a final concentration of 100 μ M was added to the cell culture and cells were either harvested after 120 min for total protein extraction or observed after 90 and 180 min. The addition of an excess of thiamine is known to quickly inhibit the expression of *THI7* (Mojzita and Hohmann, 2006).

By immunoblotting, we can still observe the third upper band in the WT and *art9 Δ* , but not in *rsp5* or *art2 Δ* , in which only the two bands observed in Figures 1 and 3A are visible. The incubation with thiamine triggers a 76% decrease of the abundance of Thi7-GFP after two hours (Figure 4A, first panel). The decrease of abundance is not only due to the dilution of the Thi7-GFP pool during cell division, following the inhibition of Thi7-GFP expression by thiamine, since the decrease is strongly reduced in the *rsp5* mutant strain (Figure 4A, second panel). Indeed, Thi7-GFP abundance is only 29% reduced in CHX-treated condition when expression of *RSP5* is low. It suggests that thiamine-induced decrease of abundance observed in a WT relies on the ubiquitin ligase Rsp5.

Looking at microscopy results, before addition of thiamine, Thi7-GFP is stabilized at the PM due to the absence of thiamine in the medium. After 90 min incubation with 100 μ M thiamine, a strong fluorescence signal is localized in the vacuolar lumen, supporting that an excess of substrate triggers endocytosis of the transporter. Eventually, Thi7-GFP is almost totally targeted to the vacuole after three hours of incubation with thiamine (Figure 4B).

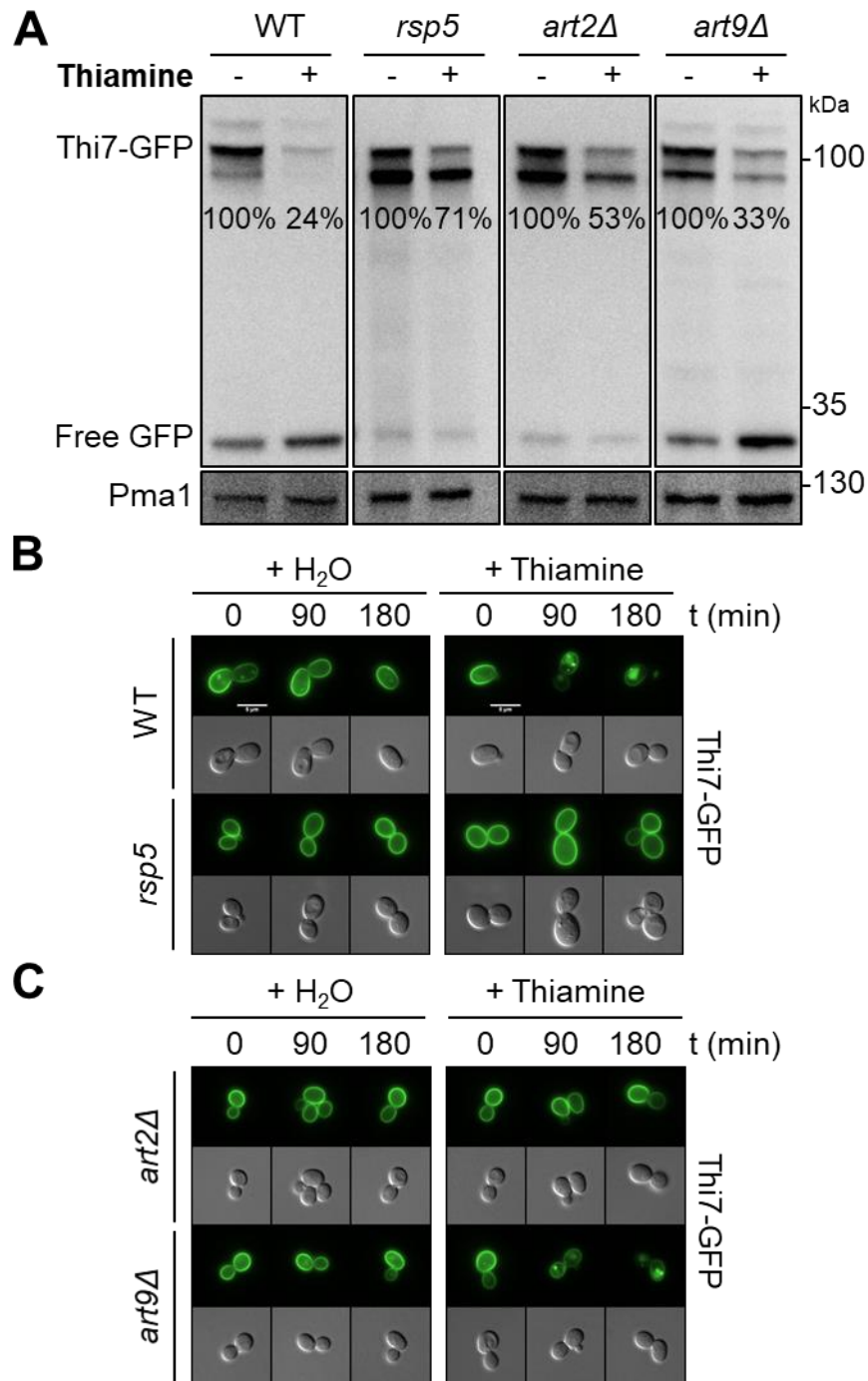


Figure 4 -The degradation of Thi7-GFP observed in a WT after addition of an excess of thiamine is impaired in *art2Δ* strain. (A) Strains expressing Thi7-GFP under its endogenous promoter were grown in thiamine-free medium up to early-log phase and

harvested after 120 min incubation with thiamine at a final concentration of 100 μ M for total protein extraction. Extracts were immunoblotted with anti-GFP and anti-Pma1 as a loading control. Free GFP is the result of the degradation of Thi7-GFP in the vacuole. Values below Thi7-GFP bands are quantification of the relative band intensity between mock-treated and thiamine-treated condition, normalized by the intensity of Pma1-corresponding band. These immunoblot crops were taken from Figure 5 (B) Localization of Thi7-GFP in a WT and *rsp5* strain after addition of an excess of thiamine into culture grown in thiamine-free medium. (C) Localization of Thi7-GFP in *art2 Δ* and *art9 Δ* strains after addition of 100 μ M thiamine into culture grown in thiamine-free medium. Shown cells are the most representative of the population. (Scale bar represents 5 μ m)

2.6 Art2 is essential for the substrate-induced endocytosis of Thi7

The degradation of Thi7-GFP in all *art Δ* strain except for *art2 Δ* behaved like in a WT (Figure 5). Notably, the intensity of Thi7-GFP bands before addition of thiamine is lower in the WT than in *art Δ* strains. After 120 min incubation with thiamine, a decrease of about 50% of Thi7-GFP total amount is observed in an *art2 Δ* strain (vs 75% in a WT). The impairment is not as strong as in the *rsp5* strain, in which downregulation is almost abolished (Figure 4A). The degradation of Thi7-GFP in *art9 Δ* is similar to the one in a WT strain. The level of free GFP is also a way to determine the basal level of Thi7-GFP degradation. The free GFP amount is about 40% lower in an *art2 Δ* strain than in a WT strain, while Thi7-GFP total amount is the same in both strains. The accumulation of free GFP after addition of thiamine in *art2 Δ* is also lower than the one in a WT. The free GFP level in *art9 Δ* is not reduced compared to a WT neither with or without thiamine induction of endocytosis.

As we did with CHX, the localization of Thi7-GFP was monitored in all *art Δ* strain after addition of thiamine (Figure 6). Thi7-GFP endocytosis was only blocked in *art2 Δ* , all the GFP signal being localized at the PM even after 3h of thiamine treatment (Figure 4C). No significant difference between a WT and the *art9 Δ* strain was detected.

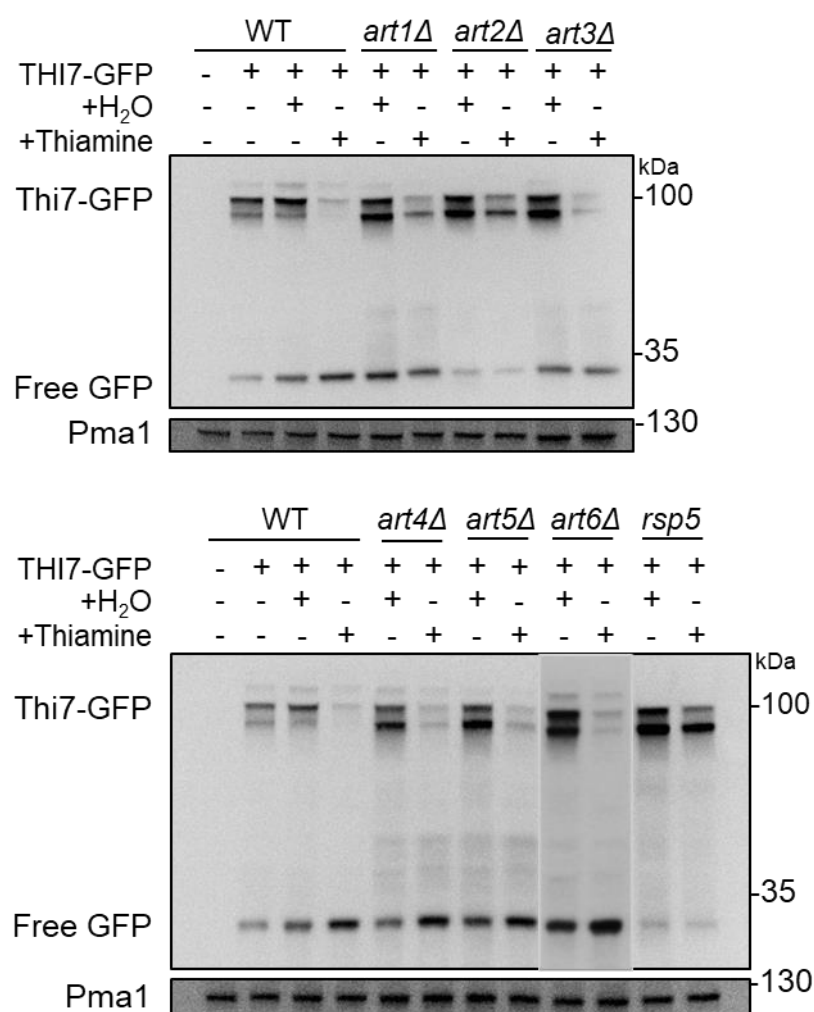


Figure 5 – Thi7-GFP degradation is triggered by thiamine in all *artΔ* except for *art2Δ*. WT and *artΔ* strains expressing THI7-GFP under its endogenous promoter were grown in thiamine-free medium up to early-log phase and harvested after 120 min incubation with thiamine at a final concentration of 100 μ M for total protein extractions. Extracts were immunoblotted with anti-GFP and anti-Pma1 as a loading control. Free GFP is the results of the degradation of Thi7-GFP in the vacuole. The wells corresponding to the mock- and CHX-treated *art6Δ* strain were inverted.

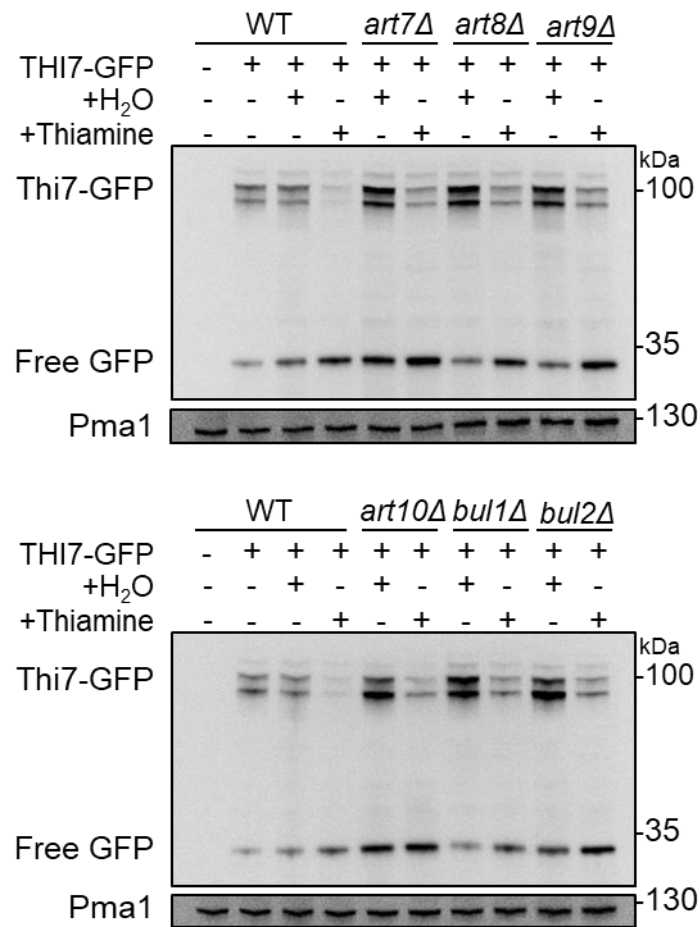


Figure 5 (continued) – Thi7-GFP degradation is triggered by thiamine in all *artΔ* except for *art2Δ*. WT and *artΔ* strains expressing THI7-GFP under its endogenous promoter were grown in thiamine-free medium up to early-log phase and harvested after 120 min incubation with thiamine at a final concentration of 100 μ M for total protein extraction. Extracts were immunoblotted with anti-GFP and anti-Pma1 as a loading control. Free GFP is the result of the degradation of Thi7-GFP in the vacuole.

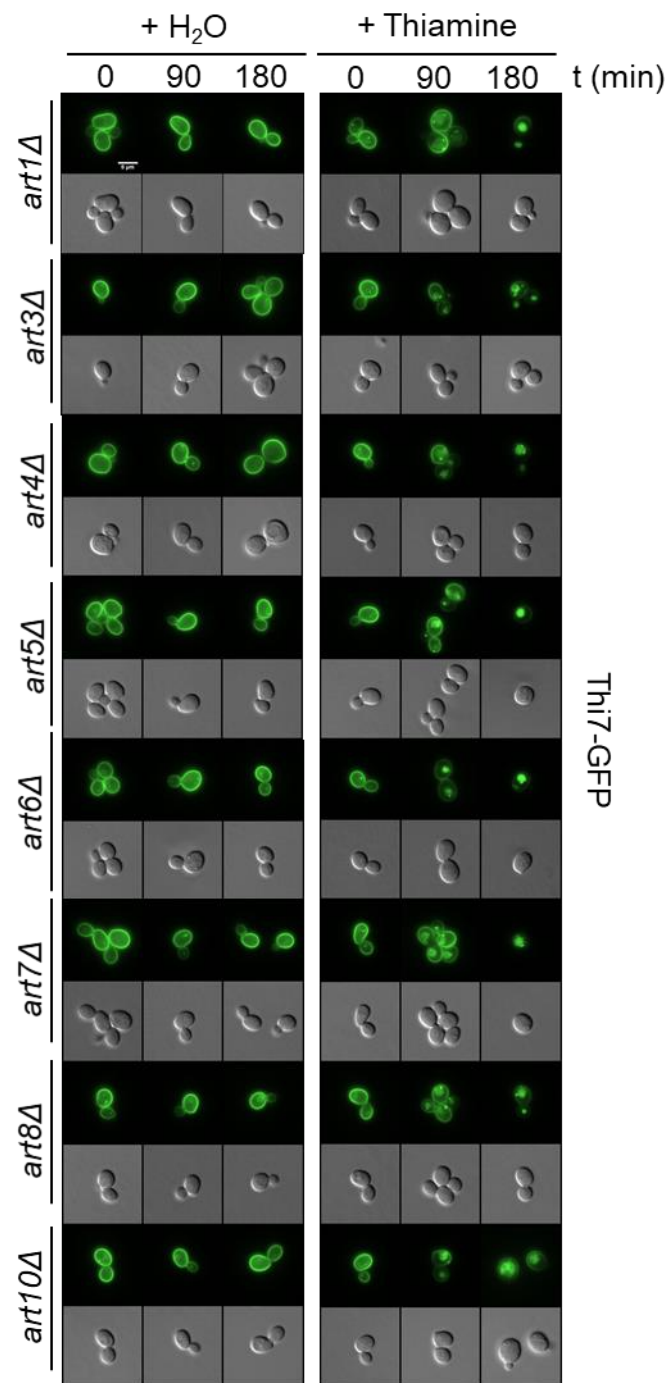


Figure 6 – Excess of thiamine in the external medium triggers the endocytosis of Thi7-GFP. (see legend on page 100)

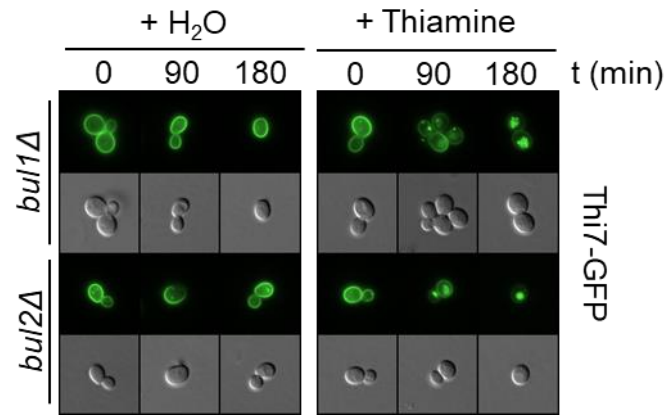


Figure 6 (continued) – Excess of thiamine in the external medium triggers the endocytosis of Thi7-GFP. WT, *artΔ* and *rsp5* strains expressing *THI7-GFP* under the control of its promoter were grown in thiamine-free medium to early exponential phase before addition of thiamine at a final concentration of 100 μ M or water. Localization of Thi7-GFP was monitored before as well as 90 and 180 min after treatment. Shown cells are the most representative of the population. (Scale bar represents 5 μ m)

The stabilisation of Thi7 at the PM even after addition of thiamine is specific to *ART2* deletion and not the result of any dysregulation linked to the deletion of the genomic locus. Indeed, expression of a tagged version of Art2 from a plasmid, 3xHA-Art2, under the control of its endogenous promoter rescued Thi7-GFP endocytosis (Figure 7). Notably, the 3xHA fused Art2p version seems to be slightly more active than its native counterpart. Our results indicate that Thi7-GFP endocytosis is faster in an *art2Δ* strain expressing 3xHA-Art2 than in a WT. Indeed, several 3xHA-Art2 - dependent fluorescent intracellular foci are already visible without addition of thiamine in that strain, while in a WT, Thi7-GFP is exclusively localized at the PM in a thiamine-free medium.

The thiamine- and CHX- induced endocytosis and degradation of Thi7-GFP are therefore both dependent on Art2.

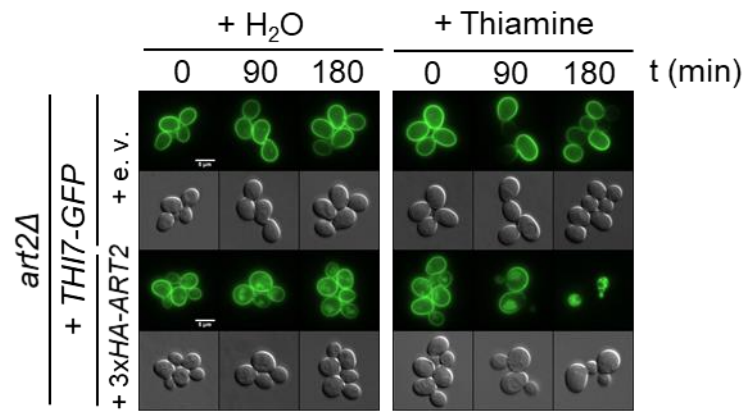


Figure 7 – Expression of 3xHA-ART2 restores the endocytosis of Thi7-GFP in the *art2Δ* background. *THI7-GFP* and either 3xHA-ART2 or its corresponding empty vector (e.v.) are expressed in the *art2Δ* strain, grown in thiamine-free selective medium. Endocytosis was triggered using thiamine at final concentration of 100 μ M. Shown cells are the most representative of the population. (Scale bar represents 5 μ m)

2.7 The deletion of *ART2* and *ART9* impairs the endocytosis of the two putative low affinity thiamine transporters, Nrt1 and Thi72

We extended our study to two homologs of Thi7, the putative low affinity PM thiamine transporters, Nrt1 and Thi72, which are highly similar to Thi7 (87% and 85%, respectively). We monitored the endocytosis of these transporters after addition of CHX and thiamine (Figure 8). The size of Nrt1-GFP and Thi72-GFP are theoretically equal to the one of Thi7-GFP, i.e. 94 kDa. However, Nrt1-GFP and Thi72-GFP bands are more diffuse than the triplet observed for Thi7-GFP. Nrt1-GFP also appears as a triplet. The two upper bands are very close to each other and slightly higher than the 100 kDa-corresponding band. The lower band is more intense than the two others and should be between 90 and 100 kDa. The profile of Thi72-GFP is more diffuse than Nrt1-GFP and more bands are visible. The highest band is slightly higher than 100 kDa, while the band just below is slightly lower. Three additional lower bands are distinguishable with the upper one being the most intense. This upper band should correspond to a molecular weight between 90 and 100 kDa, while the two other bands are lower than 90 kDa but are clearly visible in only some strains. Interestingly, the free GFP signal is globally less intense than in the other experiments.

Again, the addition of thiamine triggers the degradation of the two transporters. Thiamine approximatively triggers a 60% decrease of abundance of Nrt1-GFP and Thi72-GFP in a WT strain, while it is only reaching 20 % in the *art2Δ* strain. The deletion of *ART9* also seems to reduce the abundance of Nrt1-GFP and slightly the one of Thi72-GFP, while no effect was observed for Thi7-GFP. The abundance of Nrt1-GFP and Thi72-GFP is about 25 % and 35% lower in *art9Δ* after addition of thiamine, respectively.

We calculated the ratio of free GFP abundance on Nrt1-GFP and Thi72-GFP abundance in the different strains thanks to the intensity of the corresponding bands by immunoblotting (Figure 8). This ratio somehow reflects the proportion of transporters

targeted to the vacuole and degraded. The proportion of degraded Nrt1-GFP and Thi72-GFP is almost twice higher in a thiamine-supplemented WT culture than in the mock-treated one. On the contrary, the ratio is decreasing with thiamine addition in *art2Δ*, being twice lower. It validates the stabilization of the transporter at the PM and the slow degradation of free GFP in the vacuole added to the dilution effect. Concerning *art9Δ*, thiamine did not significantly change the proportion of Nrt1-GFP degradation (Figure 8A). Moreover, Thi72-GFP degradation is decreased by 20 % (Figure 8B).

Since an impairment of Nrt1 and Thi72 endocytosis was detected in the *art9Δ* strain, we also monitored the degradation of the two transporters in an *art2Δart9Δ* background to determine if endocytosis was totally abolished. However, the extent of the impairment was not markedly different from the one in the *art2Δ* strain (Figure 8A and 8B, two last wells).

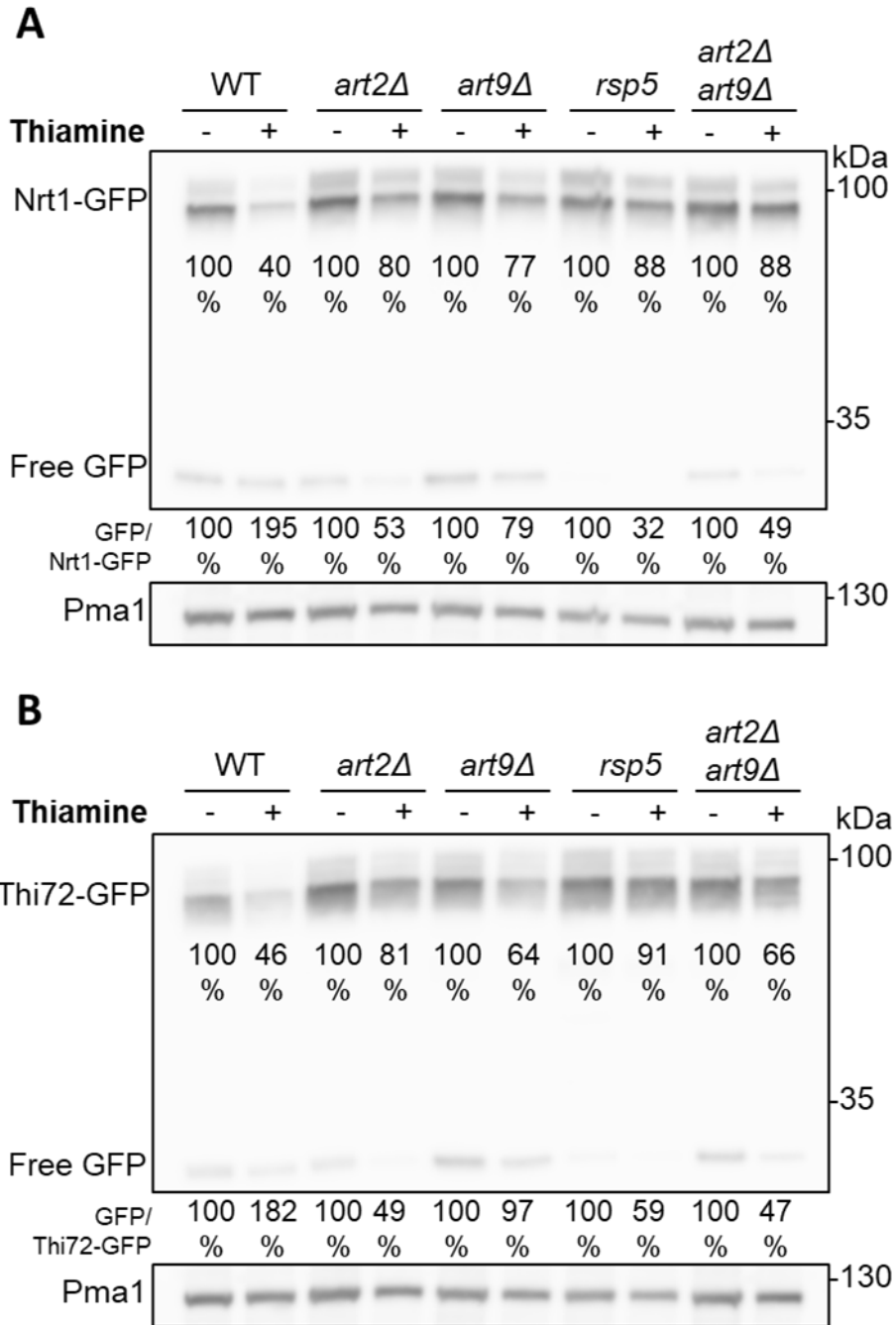
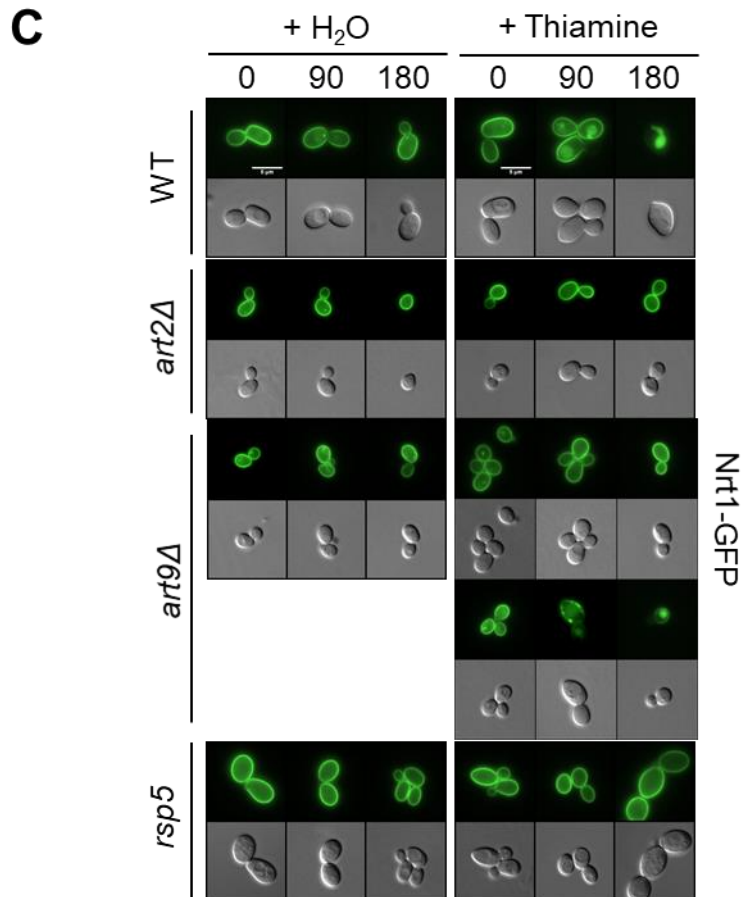


Figure 8 - The endocytosis of the putative low affinity thiamine transporters, Nrt1 and Thi72-GFP, is also mediated by Art2. Strains expressing (A) Nrt1-GFP and (B) Thi72-GFP under the *THI7* endogenous promoter were grown in thiamine-free medium up to early-log phase and harvested after 120 min incubation with thiamine at a final concentration of 100 μ M for

total protein extraction. Extracts were immunoblotted with anti-GFP and anti-Pma1 as a loading control. Values correspond to the quantification of the average band intensity from two independent experiments, which are normalized by the corresponding Pma1 band intensity.



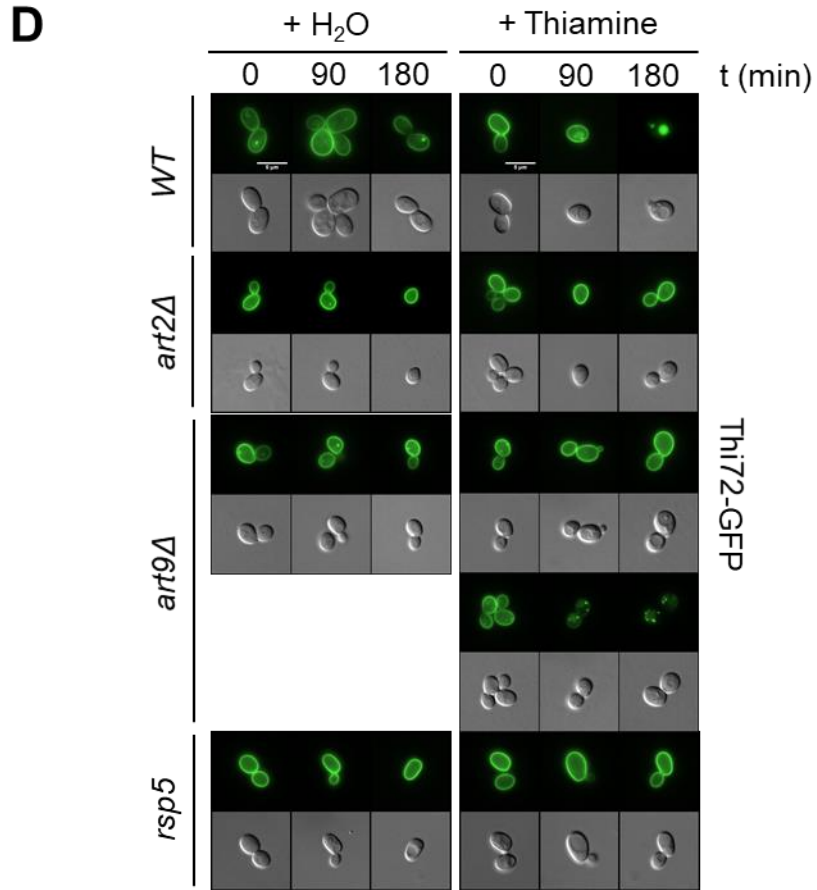


Figure 8 (continued) - The endocytosis of the putative low affinity thiamine transporters Nrt1 and Thi72-GFP is also mediated by Art2. Localization of (C) Nrt1-GFP or (D) Thi72-GFP in a WT, *art2Δ*, *art9Δ* and *rsp5* strain after addition of an excess of thiamine into culture grown in thiamine-free medium. Shown cells are the most representative of the population. (Scale bar represents 5 μ m)

Nrt1-GFP and Thi72-GFP are also localized at the PM in absence of thiamine in the medium. Addition of an excess of thiamine triggers the targeting of both transporters to the vacuole in a WT strain. The deletion of *ART2* strongly reduced the extent of Nrt1 and Thi72 degradation by stabilizing the transporters at the PM even 180 min after induction of endocytosis (Figure 8C and 8D). We found that the deletion of *ART9* somehow affects the endocytosis of Nrt1 and Thi72, unlike Thi7. In *art9Δ*, Thi71-GFP and Thi72-GFP are endocytosed and targeted to the vacuole in about half of the cells, while a strong signal is mainly localized at the PM in others, for still an unknown reason. On the contrary, Nrt1-GFP and Thi72-GFP were exclusively localized at the PM in *art2Δart9Δ* after addition of thiamine (data not shown), like in *art2Δ*. Nrt1-GFP and Thi72-GFP localization after addition of thiamine was also monitored in all the other *artΔ* strains in which endocytosis behaves like in a WT strain (Figure 9 and 10).

The conclusion for the CHX-induced endocytosis of Nrt1-GFP and Thi72-GFP are approximatively the same. The endocytosis is impaired by the deletion of *ART2* and, in a lesser extent, *ART9*. Nevertheless, Thi72-GFP seems to be less affected by CHX than thiamine (Figure 11).

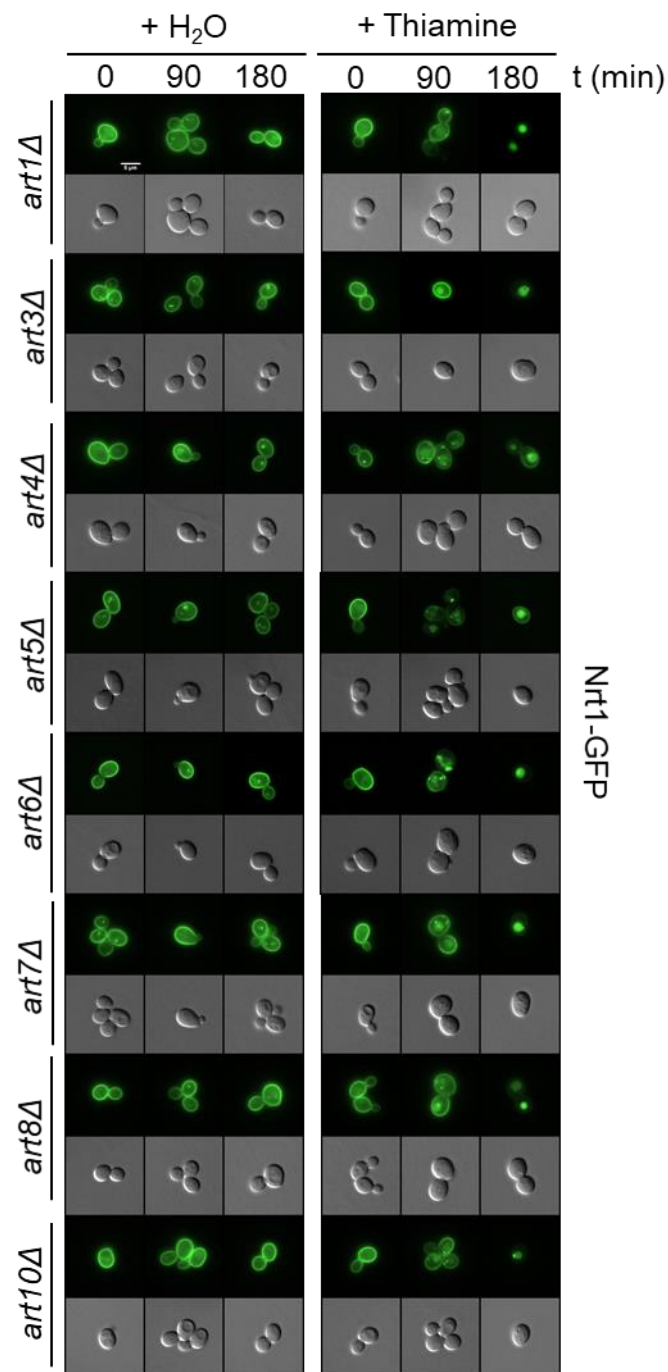


Figure 9 – Endocytosis of Nrt1-GFP is triggered by thiamine in all *artΔ* except for *art2Δ* and *art9Δ*. (see legend p. 109)

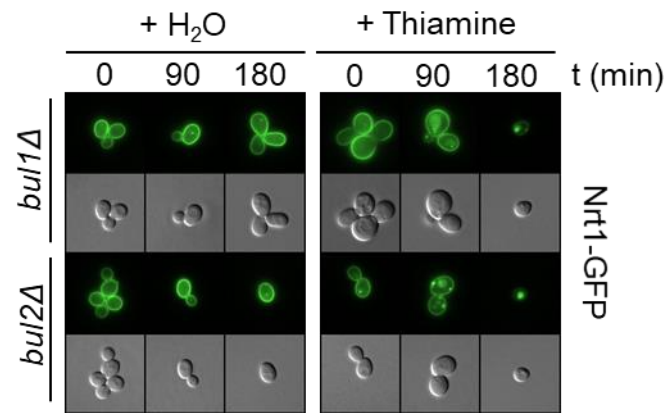


Figure 9 (continued) – Endocytosis of Nrt1-GFP is triggered by thiamine in all *artΔ* except for *art2Δ* and *art9Δ*. WT, *artΔ* and *rsp5* strains expressing *NRT1-GFP* under the control of the *THI7* promoter were grown in a thiamine-free medium up to early exponential phase before addition of thiamine at a final concentration of 100 μ M or water. Localization of Nrt1-GFP was monitored before as well as 90 and 180 min after treatment. Shown cells are the most representative of the population. (Scale bar represents 5 μ m)

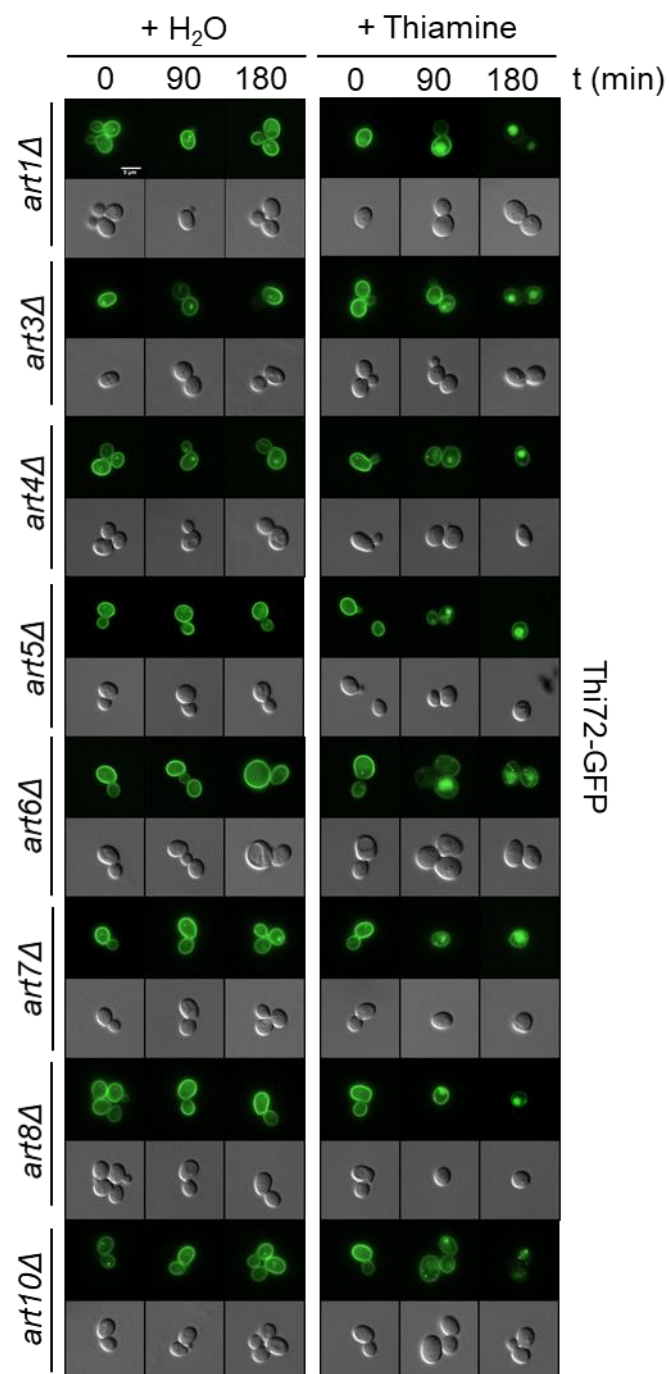


Figure 10 – Endocytosis of Thi72-GFP is triggered by thiamine in all *artΔ* except for *art2Δ* and *art9Δ*. (see legend p. 111)

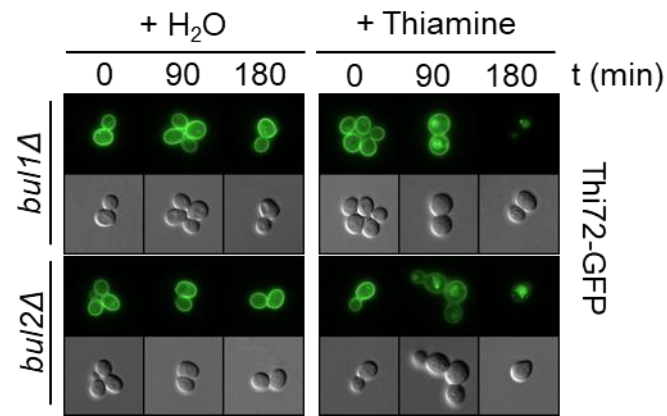


Figure 10 (continued) – Endocytosis of Thi72-GFP is triggered by thiamine in all *artΔ* except for *art2Δ* and *art9Δ*. WT, *artΔ* and *rsp5* strains expressing *THI72-GFP* under the control of the *THI7* promoter were grown in a thiamine-free medium up to early exponential phase before addition of thiamine at a final concentration of 100 μ M or water. Localization of Thi72-GFP was monitored before as well as 90 and 180 min after treatment. Shown cells are the most representative of the population. (Scale bar represents 5 μ M)

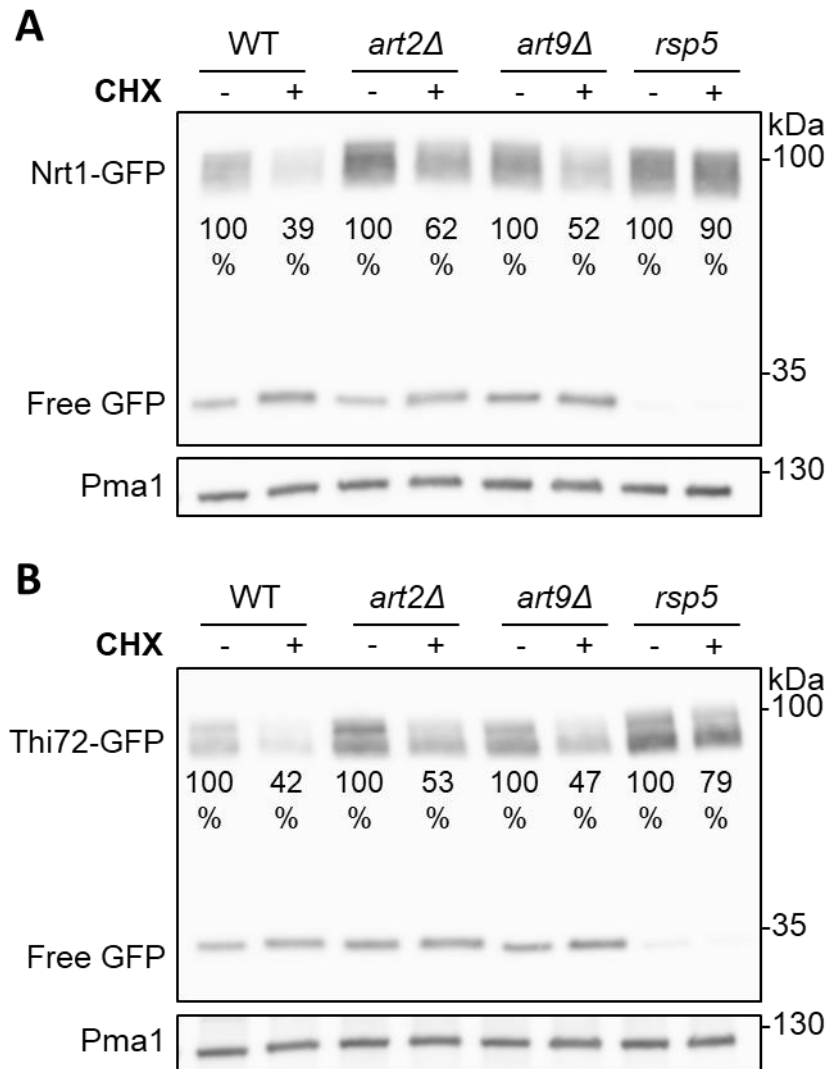


Figure 11 – The CHX-induced endocytosis of Nrt1-GFP and Thi72-GFP is slightly impaired in *art2Δ* and *art9Δ*. Strains expressing (A) *NRT1-GFP* or (B) *THI72-GFP* under the *THI7* endogenous promoter were grown in a thiamine-free medium up to early-log phase and harvested after 180 min incubation with CHX at a final concentration of 50 μ g/ml for total protein extraction. Extracts were immunoblotted with anti-GFP and anti-Pma1 as a loading control. Free GFP is the results of the degradation of Thi7-GFP in the vacuole. Values correspond to the quantification of band intensity, from which background was subtracted, normalized by the corresponding Pma1 band intensity. The experiment was performed once.

2.8 Do the three thiamine transporters physically interact with Art2?

We probed the interaction between the three C-terminal GFP fused thiamine transporters and HA-fused Art2 (3xHA-Art2) or Art9-HA. After immunoprecipitation of Thi7-GFP, Nrt1-GFP or Thi72-GFP using the GFP-trap technology (Chromotek), we showed that Art2 was co-purified without using cross-linking agents (Figure 12). Unfortunately, a repetition of this immunoprecipitation very recently pointed out that a significant amount of 3xHA-Art2 was also eluted in absence of thiamine transporters (data not shown), while it was not the case in the first experiment. Therefore, without reproducing it again, we cannot conclude to an interaction between Art2 and Thi7, Nrt1 or Thi72.

Up to now, no interaction was found between thiamine transporters and Art9-HA or Art9-FLAG

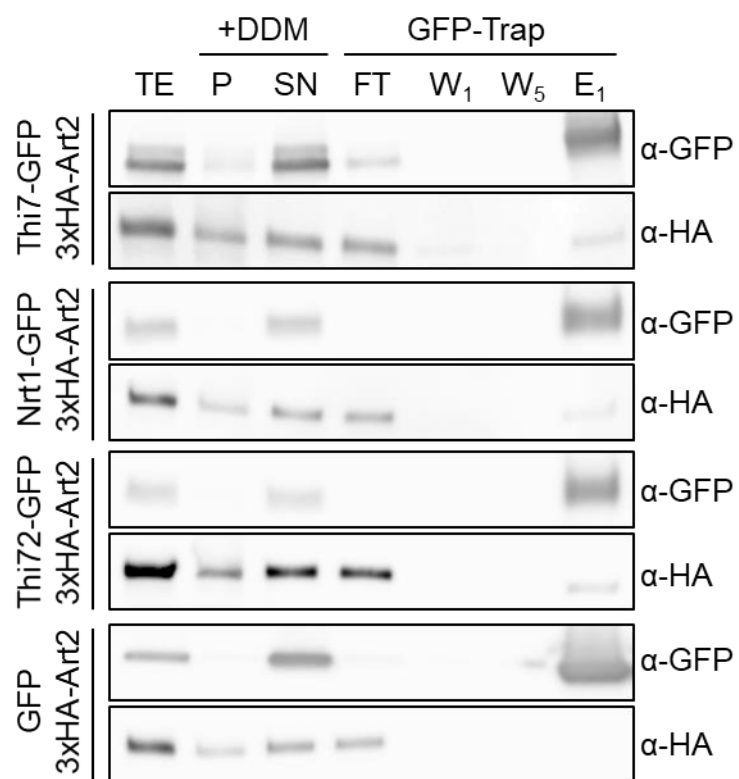


Figure 12 – Is Art2 co-immunoprecipitated with Thi7-GFP, Nrt1-GFP and Thi72-GFP? GFP-trap-based immunoprecipitation assay on total protein extracts from the *art2Δ* strain transformed with two plasmids, one expressing 3xHA-ART2 and the second expressing either (A) Thi7-GFP, (B) Nrt1-GFP, (C) Thi72-GFP or (D) GFP as a control. Cells were grown in a thiamine-free medium before addition of thiamine at a final concentration of 100 μM for 30 min. Up to now, this experiment has not been confirmed yet. (DDM: Dodecyl Maltoside; TE: Total Extract, P: Pellet, SN: Supernatant, FT:Flow Through, W: Washing, E: Elution)

2.9 The ubiquitylation of Thi7 takes place at its cytosolic carboxy-terminal part

We showed that the downregulation of the three thiamine transporters was dependent on the ubiquitin ligase Rsp5. Therefore, we designed various lysine-to-arginine (KR) mutants of Thi7-GFP to determine which lysine residues were essential for its ubiquitylation and endocytosis (Figure 13 A). A phenotypic growth test on oxythiamine-containing medium of a CVY3 strain expressing these KR mutants was performed to confirm that the mutations were not affecting transport activity (Figure 13 B).

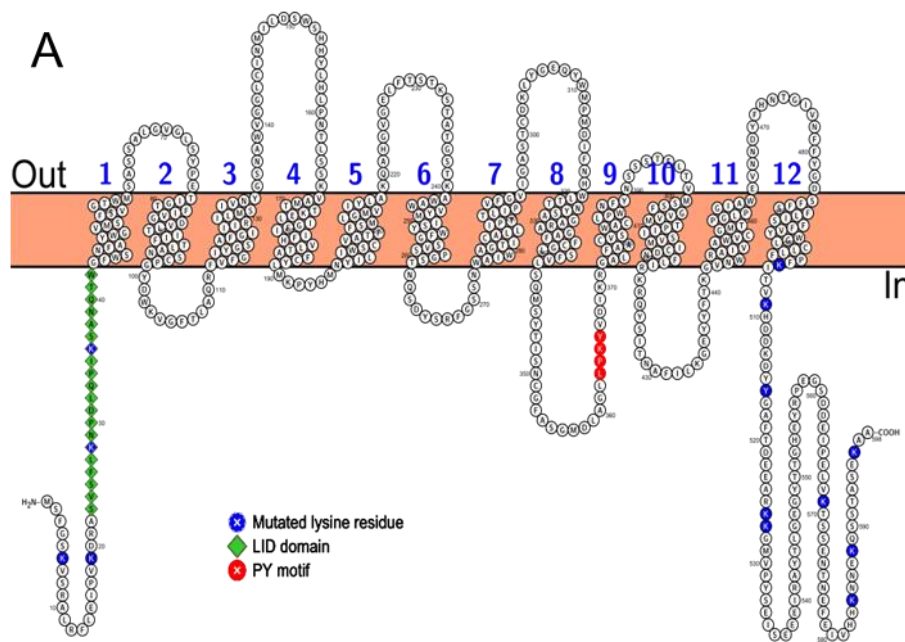


Figure 13 – All the KR mutant versions of Thi7-GFP transports oxythiamine. (A) Scheme of (A) Thi7 topology, done with the Protter software (ETH Zurich, Switzerland), and of (B) the different engineered KR mutants of Thi7. (C) The GFP-tagged KR mutants' versions were expressed in a *thi7Δnrt1Δthi72Δ* strain and grown in a thiamine free medium. Serial 10-fold dilutions of yeast cells in early log phase were spotted on thiamine-free oxythiamine-containing medium. The expression of pRS416 (Empty vector) and the non-tagged version were used as controls.

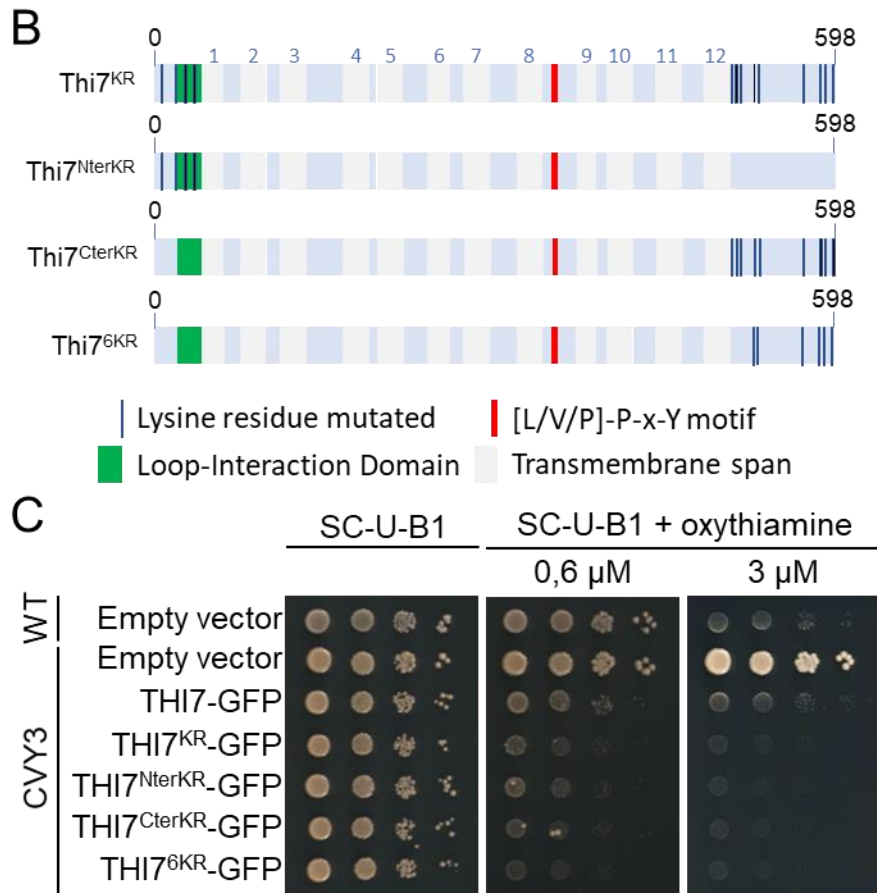


Figure 13 (continued) – All the KR mutant versions of Thi7-GFP transports oxythiamine. (A) Scheme of (A) Thi7 topology, done with the Protter software (ETH Zurich, Switzerland), and of (B) the different engineered KR mutants of Thi7. (C) The GFP-tagged KR mutants' versions were expressed in a *thi7Δnrt1Δthi72Δ* strain and grown in a thiamine free medium. Serial 10-fold dilutions of yeast cells in early log phase were spotted on thiamine-free oxythiamine-containing medium. The expression of pRS416 (Empty vector) and the non-tagged version were used as controls.

We first engineered a Thi7-GFP version in which all the lysine (K) residues of the cytosolic N-terminal (i.e. 4 K residues) and the cytosolic C-terminal parts (i.e. 9 K residues) are mutated in arginine residues, termed Thi7^{KR}-GFP. This mutant version of Thi7-GFP is stabilized at the PM even after addition of thiamine (Figure 14A), supporting that ubiquitylation is mainly taking place either at N- or at C-terminus. Moreover, the decrease in

abundance of Thi7^{KR}-GFP in the *art2Δ* strain after thiamine addition approximatively equals the decrease in a WT strain, i.e. 50 % decrease after 180 min (Figure 14B). As a control, we also checked that the KR mutations did not affect Thi7 interaction with Art2. Using GFP-Trap, we showed that 3xHA-ART2 was co-purified with Thi7-GFP (Figure 14C). Unfortunately, as previously stated, this immunoprecipitation will need to be repeated to check whether the interaction is specific or not.

On the contrary, when all the lysine residues of the cytosolic N-terminal part are mutated into arginine (Thi7^{NterKR}-GFP), endocytosis still occurs. The mutation of lysine residues of the C-terminal part (Thi7^{CterKR}-GFP) abrogated the endocytosis of Thi7-GFP, as the Thi7^{KR}-GFP version did, suggesting that ubiquitylation is occurring at the C-terminus. Based on the prediction of UbPred (Radivojac et al., 2010), we also engineered a version mutated for the six C-terminal lysine residues identified as the most likely to be ubiquitylated in Thi7. This Thi7^{6KR}-GFP (K526; K527R; K569R; K584R; K588R; K596R) version is also poorly endocytosed after addition of an excess of thiamine in the medium (Figure 14D). This suggests that among these lysine residues, some are essential for Thi7 ubiquitylation. Since all these lysine residues but one are conserved in Nrt1 and Thi72 (K596 is absent in Nrt1), we suspect that these lysine residues are also involved in their ubiquitylation.

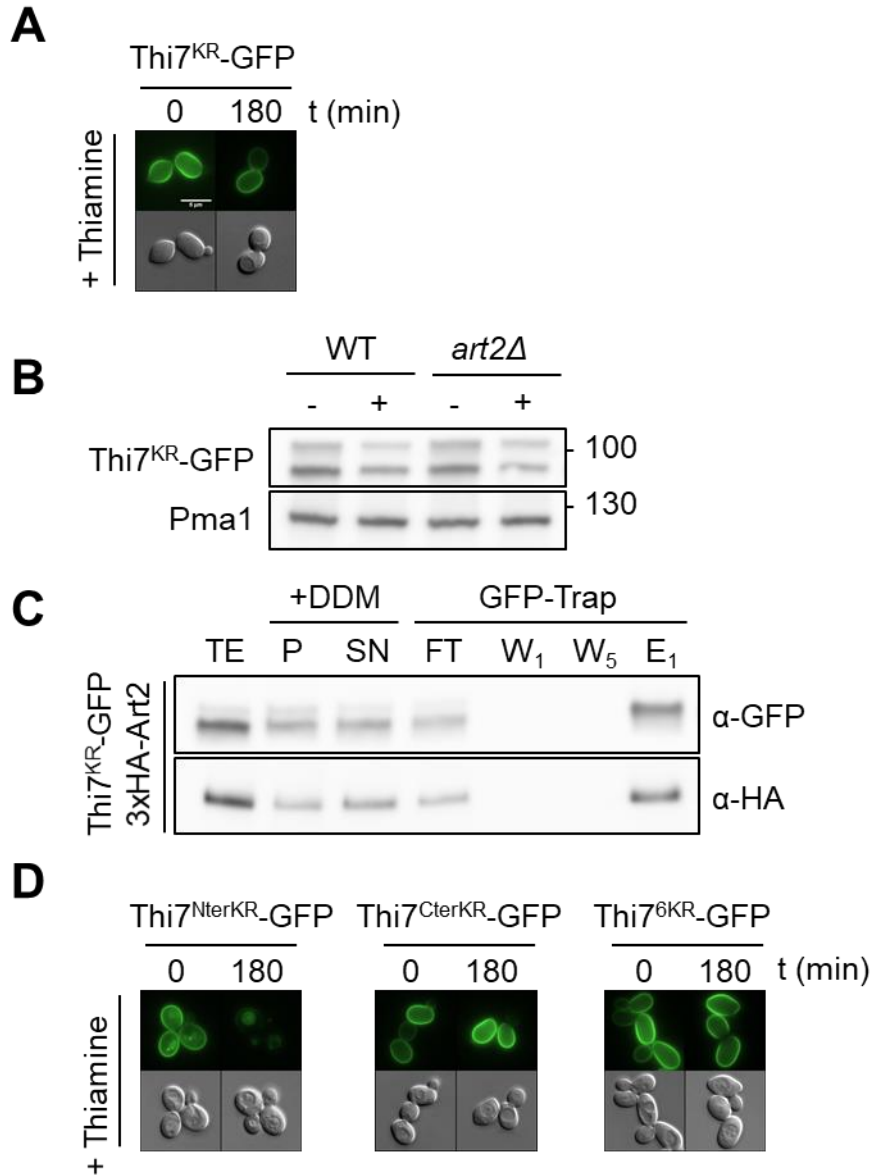


Figure 14 – Thi7-GFP ubiquitylation takes place at the C-terminus. (A) Localization of Thi7^{KR}-GFP after incubation with thiamine at a final concentration of 100 μ M. (B) Monitoring of Thi7^{KR}-GFP endocytosis in WT and *art2Δ* (C) Immunoprecipitation assay of Thi7^{KR}-GFP in 3xHA-Art2-expressing *art2Δ*. (D) Monitoring the endocytosis of different KR mutant version of Thi7-GFP after incubation with thiamine at a final concentration of 100 μ M: Thi7-NterKR-GFP, Thi7-CterKR-GFP and Thi7-Cter6KR-GFP. Shown cells are the most representative of the population. (DDM: Dodecyl-maltoside; TE: Total Extract, P: Pellet, SN: Supernatant, FT:Flow Through, W: Washing, E: Elution)

2.10 Art2 is required for the ubiquitylation of Thi7

ART adaptors are thought to be adaptors of the ubiquitin ligase Rsp5. We showed that the low abundance of Rsp5 in the *rsp5* strain led to the stabilization of transporters at the PM, as did the deletion of *ART2* (Figure 4C). In an *end3Δ* strain deficient for endocytic vesicle formation, the ubiquitylated plasma membrane proteins are not internalized anymore, thus stabilizing their ubiquitylated forms. In *end3Δ* background, a single strong band of ubiquitin is detected at higher molecular mass than Thi7-GFP (Figure 15, first lane). The intensity of the band is 68 % lower in the *end3Δart2Δ* background, confirming that Art2 is involved in the ubiquitylation of Thi7 (Figure 15, second lane). However, the slight remaining band suggests that some Art2-independent ubiquitylation is still possible. When Thi7^{KR}-GFP is expressed in the *end3Δ* background, the strong ubiquitylated band is absent, ensuring that the ubiquitylated band we observed is Thi7-specific (Figure 15, last two lanes).

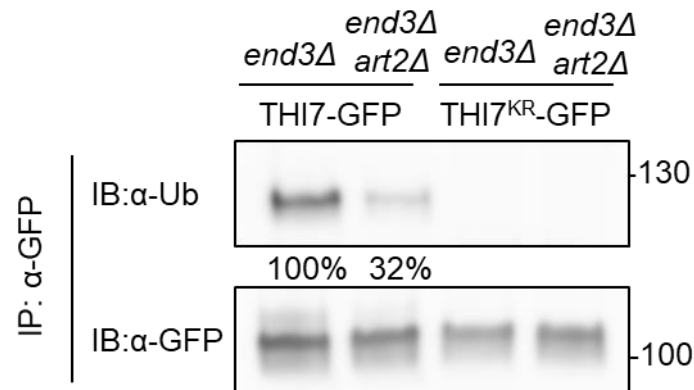


Figure 15 – Art2 is required for Thi7-GFP ubiquitylation. Thi7-GFP or Thi7^{KR}-GFP were expressed in *end3Δ* and *end3Δart2Δ* strains and grown in a thiamine-free medium to early exponential phase before a 30-min incubation with thiamine (at a final concentration of 100 μM). After solubilization, immunoprecipitation of transporters was performed using the GFP-Trap kit. Samples were immunoblotted with anti-GFP and anti-Ub. The ubiquitin and GFP immunodetection were performed on the same blot with the same extracts but on different wells. Quantification is based on the mean grey value of the ubiquitin band, from which the background is subtracted, and normalized to the intensity of the corresponding GFP signal. This experiment was performed twice. (IP: Immunoprecipitation; IB: immunoblotting)

These results indicate that Thi7-GFP would mainly undergo an Art2-mediated monoubiquitylation, since a single intense ubiquitin band seems to be affected by the absence of Art2.

Using total protein extracts that were immunoblotted with anti-GFP, we also observed that the upper band of Nrt1-GFP and Thi72-GFP was less intense in *end3Δart2Δ* than in *end3Δ*. Unfortunately, the intensity of Nrt1- and Thi72-GFP was too low after the 180-min incubation with 100 μM thiamine to observe that upper band. Although the immunoprecipitation of these two transporters followed by ubiquitin immunodetection would be required to conclude, that preliminary experiment suggest that Nrt1-GFP and Thi72-GFP could be also monoubiquitylated by Art2 (Figure 16).

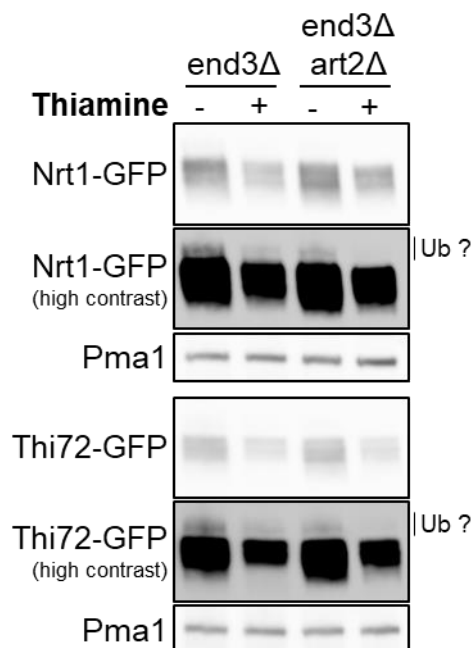


Figure 16 – Nrt1-GFP and Thi72-GFP could also be ubiquitylated by Art2. *NRT1-GFP* and *THI72-GFP* are expressed in an *end3Δ* and *end3Δart2Δ* strain, which were grown in thiamine-free medium to early exponential phase. Total protein extraction was performed after a 180-min incubation with thiamine. Samples were immunoblotted with anti-GFP, and anti-Pma1, as a loading control. (Ub: ubiquitylated band)

DISCUSSION

1 Proteomic screening

We developed an innovative approach to explore the high complexity of the alpha-arrestin adaptors – PM proteins system using mass spectrometry-based quantitative proteomics. The screening only covers a small part of all the PM-localized proteins. Indeed, all the proteins are not expressed at the same time and triggering the global endocytosis of many proteins is challenging due to the specificity of the ubiquitylation process. Yet, we found 28 new potential targets of the α -arrestin adaptor family using this approach.

This screening is supporting the view that several adaptors can be involved in the endocytosis of a given PM protein (e.g. Fks1 or Hxt1 in Table 1). This phenomenon is particularly intriguing and cannot be seen as full redundancy. The fact that several α -arrestin gene deletion independently affects their abundance suggests that the endocytosis defect triggered by the absence of one adaptor is not or not completely rescued by the remaining adaptors. One explanation is that α -arrestin adaptors are involved at different steps of cargo sorting and targeting to the vacuole. For example, some α -arrestins could be required at the PM, mediating ubiquitylation to trigger protein endocytosis, while other α -arrestins would act at the endosome or the GA level to promote the incorporation of their target in intraluminal vesicles of MVBs. Supporting this idea, it was shown that α -arrestin were required at the GA, consolidating the fate of proteins by ubiquitylating them and leading to their targeting to the vacuole (Becuwe and Léon, 2014). Alternatively, such profile of ratios could illustrate that several α -arrestins form complexes in order to mediate ubiquitylation of some PM proteins. Therefore, we suspect an additive and complementary effect of several α -arrestin adaptors, ensuring a completely functional endocytic process.

The screening also pointed out that some PM proteins, whose abundance was lower in a WT strain treated with CHX, could be even less abundant in the absence of a specific α -arrestin. The hexose transporters Hxt4/7, two uncharacterized proteins (Rsn1, Ina1) and the GPI-anchored 1,3- β -glucanosyl-transferases Gas1/3 are in that category. The α -arrestin-dependent decrease could be the result of a decrease in the expression of genes following the stabilization of another protein at the PM. The α -arrestin adaptor could also be involved in the recycling of the endocytosed proteins from internal compartments to the PM. The lack of such adaptor would increase the proportion of the protein targeted for vacuolar degradation instead of being recycled back to the PM. Furthermore, Art3/6 were shown to promote the retrograde transport of the general amino acids permease Gap1 from endosomes to GA (O'Donnell et al., 2010), which could lead to Gap1 recycling to the PM. Considering Gas1 and Gas3, the involvement of α -arrestin was unexpected since they do not harbor any cytosolic part. Moreover, they only display blue ratios unlike another identified protein involved in cell wall synthesis, Fks1. In yeast, the mechanism underlying the endocytosis of GPI-anchored proteins (GPI-APs) is poorly characterized. However, their targeting to the vacuole is known to require proper Rsp5 activity (MacDonald et al., 2015). Moreover, GPI-APs requires membrane adaptors to traffic from the ER to the GA and ultimately to the PM (reviewed in Muniz and Zurzolo, 2014). Moreover, a recent study also reveals that they cluster with ubiquitylated Cos membrane adaptor proteins at endosomal compartment, thereby being incorporated in ILVs of MVBs (MacDonald et al., 2015). This is likely that membrane adaptors are also involved at the PM, mediating the endocytosis of GPI-APs when ubiquitylated. Alternatively, GPI-APs could be clustered in protein or PM lipid domains and endocytosed with ubiquitylated PM proteins found in these domains. Concerning our results, Gas1/3 could also interact with such integral membrane adaptors for their sorting in recycling vesicles. The proteomic screening would be the first clue that the trafficking of GPI-anchored proteins could also rely on the α -arrestin system. However, no increase of abundance has been observed for the

strains we tested (*art7Δ* to *bul2Δ*). This suggests these α -arrestins are not required and/or that they act in a fully redundant way in endocytosis of Gas1/3.

Our screening also constitutes the first link between the abundance of the GTP-binding protein Ras2, members of the eisosomes (Nce102, Pil1, Lsp1), the ER-PM tethering system (Ist2, Tcb1 and Tcb3) as well as the cell integrity pathway (Rho1, Zeo1) with CHX so far. All these proteins were more abundant in response to CHX, but their abundance is decreased in the absence of α -arrestins. Therefore, the proteomic is also the first example of an α -arrestin-dependent effect on the abundance of non-integral PM proteins. The increased abundance caused by CHX is likely to be the result of the unspecific endocytosis of many transporters at the PM, which probably affects PM protein and lipid composition. This rapid change in the PM composition could be the cause of such increase of proteins involved in PM integrity. For example, the proteomic screening reveals that the abundance of two identified proteins involved in cell wall synthesis and maintenance (Fks1, Gas1/3) is highly reduced after addition of CHX. A drastic decrease of their abundance could result in a defect of cell wall maintenance. Such a defect could trigger the upregulation of proteins of the cell integrity pathway like Rho1 and Zeo1. At the same time, the CHX-induced stimulation of endocytosis could result in a dysregulation of phosphoinositides content at the PM. Phosphoinositides are essential for organelle identity, endocytic vesicle formation and trafficking along the secretory pathway (D'Hondt et al., 2000). Indeed, this hypothesis is supported by the CHX-induced increase of Ist2, Tcb1 and Tcb3 abundance, which could result in an increase of ER-PM contact sites. At these contact sites, the phosphatase Sac1 is known to dephosphorylate PI4P in PI. PI4P is the precursor of PI(4,5)P₂, recognized by the endocytic machinery, which is dephosphorylated in PI4P during vesicle scission. Therefore, an increase activity of Sac1 at the ER-PM contact sites could also alter PM integrity. However, an increased number of contact sites between the ER and the PM does not necessarily imply an increase of Sac1 activity. Sac1 activity is

linked to the pool of PI4P at the PM, high concentration of PI4P stimulating the activity of Sac1 (Manford et al., 2012). The link between CHX or a high number of endocytic events and accumulation of PI4P has not been investigated. We could imagine that a high number of endocytic event triggers an increase of the PI4P content since PI(4,5)P₂ phosphatase (Inp51 and Inp52) activity is required at endocytic sites for vesicle scission (Conibear, 2010). Moreover, the phosphoinositide metabolism at the PM is linked to the sphingolipid metabolism and more directly to eisosomes via Pil1 (Brice et al., 2009; Kabeche et al., 2014). Moreover, proteins involved in the phosphoinositide metabolism are known to be required for the Rho1-dependent cell integrity pathway (Audhya and Emr, 2002). Therefore, an effect on phosphoinositide content could involve ER-PM membrane contact sites, MCC domains, the abundance of components of the eisosomes as well as proteins of the cell integrity pathway.

Since CHX is a potent stimulator of the TORC1 kinase complex, we could hypothesize that the expression or stability of these proteins is TORC1-dependent. Moreover, a phosphoproteomic study reveals that the phosphorylation status of Rho1 and Lsp1 is affected by the deletion of *NPR1*, a TORC1 effector (MacGurn et al., 2011). The deletion of some α -arrestins results in a lower abundance of this set of protein compared to a WT. In some extent, the deletion of α -arrestins inhibits the CHX-induced increase of abundance of these proteins. That result could suggest that the CHX-induced increase is α -arrestin-dependent. We cannot rule out that the absence of a specific α -arrestin is stabilizing some integral PM proteins, thereby preventing changes in PM organization and in the abundance of these peripheral proteins. Nevertheless, Rho1 and Ras2 were shown to physically interact with α -arrestins, suggesting a more direct role of the latter in their abundance.

Through these peripheral PM proteins, the proteomic screening brings insights into the complexity of the PM adaptation in response to environmental cues. They support a holistic regulation at many levels, suggesting that specific changes of PM

protein composition could have more global effect on protein or lipid abundance at the PM. The screening also suggests that α -arrestin are involved in these interconnections.

Although the proteomic approach applied to PM-enriched fractions revealed to be a suitable tool to study the α -arrestin network, our methodology implies some limitations. The use of CHX as an endocytosis-triggering signal allowed us to trigger the unspecific endocytosis of many transporters, but it probably restrained our screening to protein endocytosed in response to this drug. On almost 110 routinely identified proteins, only 28 were significantly affected in three biological replicates. Since CHX stimulates TORC1, this is likely that we enriched our results in proteins whose abundance is dependent on TORC1. Interestingly, the results emphasize that the abundance and putatively the endocytosis of hexose transporters would be regulated by TORC1, which has been shown to mainly affect the abundance of nitrogen-source transporters up to now. The most prominent problem of the use of CHX is that the global effect on cell physiology is not clearly defined. Indeed, although the inhibition of protein translation mimics a cytosolic accumulation of amino acids in the short-term, the consequences must be detrimental on longer term. An alternative way to trigger endocytosis of many transporters would be to apply a heat-shock to promote a global misfolding of PM proteins, accelerating their turnover rate. Obviously, all the proteins do not behave the same way upon heat-shock, some PM proteins being highly stable, which already constitutes a limitation of this approach. Although, the experiment would focus on the involvement of α -arrestins in PM quality control, the initiation of endocytosis would be stimulated by a physiological response.

2 Thiamine transporter endocytosis

The proteomic experiment led us to the study of a new family of targets of the alpha-arrestin adaptors, thiamine transporters. Our results support a common regulation of the endocytosis of the three plasma membrane thiamine transporters. The α -arrestin Art2 is the main mediator of their CHX and thiamine-induced Rsp5-dependent ubiquitylation and endocytosis. Although we were not able to explain the effect of the deletion of *ART9* on Thi7 abundance in the screening, we show that the endocytosis of Nrt1 and Thi72 was somehow affected by the absence of *ART9*.

2.1 Art2 is involved in both cycloheximide and thiamine-induced endocytosis

When comparing CHX- and thiamine-stimulated endocytosis of Thi7-GFP, we could think that CHX is less potent to trigger the downregulation of Thi7-GFP. However, the lower decrease after CHX addition we observed when working with Thi7-GFP in a thiamine-free medium is probably the consequence of the antagonistic effects of the absence of thiamine stabilizing the protein at PM on one hand, and CHX triggering its endocytosis on the other hand. The cells are growing in a thiamine-free medium, which is characterized by low growth and a poor nutritional status of the cell, while CHX mimics a high cytosolic amino acid concentration. This is confirmed by the remaining fluorescent signal observed at PM, while free GFP is accumulating inside the vacuole. The remaining signal cannot be due to newly synthesized Thi7-GFP reaching the PM, since CHX is an inhibitor of protein synthesis. The immunoblotting experiments also suggest a minor role for Art8 and Art9 in such conditions. The vacuolar accumulation of free GFP is slightly impaired in *art8 Δ* and remains unchanged in *art9 Δ* compared to a WT. However, no impairment of endocytosis was detected in *art8 Δ* and *art9 Δ* strains by microscopy. We were neither able to show the same effect using thiamine.

Except for these discrepancies, one observation of our work is that the same α -arrestin is involved after induction of endocytosis by CHX and thiamine. That result is reminiscent of the uracil permease Fur4, whose substrate and CHX-induced endocytosis was also shown to be mediated by the same α -arrestins (Nikko and Pelham, 2009).

2.2 Art2 activation following the endocytosis triggering stimulus

Although our results strongly support that Art2 is the key regulator of thiamine transporter abundance in response to thiamine and CHX addition, the underlying mechanism of Art2 activation is still under investigation. Concerning substrate-induced endocytosis, the targeting of Thi7-GFP to the vacuole is slow (i.e. 180 min), compared to the kinetics of thiamine uptake by the cell (Praekelt et al., 1994), suggesting that the Art2-mediated ubiquitylation pathway must be somehow stimulated after the entry of thiamine. Gournas and co-workers recently showed that the arginine-induced ubiquitylation of the arginine permease Can1 requires the metabolism of arginine by the cell, leading to TORC1 and subsequently Art1 activation, and the concomitant structural rearrangement of Can1 upon binding of arginine. This conformational change triggers the disruption of the interaction between Can1 N-terminal tail and cytosolic loops, leading to the exposure of a N-terminal tail-located acidic patch. This acidic-patch was shown to be an Art1-binding site (Gournas et al., 2017) (see Introduction, Figure 6).

Unlike arginine, thiamine is not metabolized but phosphorylated in thiamine pyrophosphate (TPP), which is the coenzyme of several enzymes involved in carbohydrate and amino acid metabolism (see Introduction, section 4.1). On one hand, Art2 activation could be the consequence of enzymes involved in thiamine homeostasis that could be sensitive to the cytosolic thiamine content. On the other hand, the impact of thiamine uptake, cytosolic thiamine content and TPP-dependent enzyme

activity on the carbohydrate or amino acid metabolism could stimulate the Snf1-PP1 or the TORC1-Npr1-Sit4 pathway, respectively. The Snf1 kinase is known to inhibit the activity of Art4 cells grown in non-fermentable carbon sources (like lactate) containing medium, inhibiting lactate transporter Jen1 endocytosis. On the contrary, Snf1 is inhibited upon glucose addition, while the active type 1 protein phosphatase (PP1) dephosphorylates and stimulates the activity of Art4, thereby leading to Jen1 endocytosis (Becuwe et al., 2012b). Snf1 is stimulated when the AMP or ADP on ATP ratio is high (Mayer et al., 2011; O'Donnell et al., 2015). ATP production is impaired when thiamine is scarce since the TPP content of yeast cells grown in thiamine free medium is low, potentially leading to Snf1 stimulation and subsequent inhibition of Art2. Upon thiamine addition, metabolism is stimulated, situation that could lead to an increase amount of ATP and Snf1 inhibition. In that condition, we could imagine that Art2 undergoes a PP1-dependent dephosphorylation. In the same way, the impact of thiamine depletion could affect the TORC1 kinase complex. Indeed, cell growth is strongly reduced in thiamine-free medium, condition suggesting a poorly active TORC1. Thiamine was shown to be required for branched chain amino acid synthesis via the TPP-dependent activity of acetolactate synthase Ilv2 (Falco et al., 1985). TORC1 activity could be reduced in thiamine depleted conditions since TORC1 was shown to be sensitive to the cytosolic amino acid content. Moreover, a recent study from the team of Professor B. André (ULB, Belgium) supported the idea that TORC1 would be activated by the co-transported proton in a process dependent on the H⁺-ATPase Pma1, leading to symporter endocytosis (Saliba et al., 2018). Therefore, Art2-dependent ubiquitylation of thiamine transporters could also be the result of TORC1 activation following their proton transport.

Thi7, Nrt1 and Thi72 belong to the Nucleobase-Cation Symporter family as does Fur4. Like Fur4, the three thiamine transporters have a Loop-Interaction Domain (LID) in their N-terminal tail. Uracil binding to Fur4 triggers a conformational change from an outward faced to an inward faced conformation, which disrupts

the interaction between the LID and cytosolic loops. Therefore, uracil transport leads to the exposure of a degron, composed of the PEST-like sequence that will be phosphorylated, initiating the Rsp5-dependent ubiquitylation of two nearby lysine residues (Keener and Babst, 2013). Phosphorylation of the PEST-like sequence is reminiscent of the acidic patch found on the amino acid transporters Can1 (Gournas et al., 2017) and Mup1 (Guiney et al., 2016). It is likely that thiamine transporters undergo the same structural rearrangement upon thiamine transport, also leading to the exposure of a degron. Further experiments will be required to confirm the phosphorylation of the N-terminal tail. Nevertheless, we showed in the present study that ubiquitylation of Thi7 does probably not occur at N-terminal lysine residues but rather on the C-terminal tail, which differs from the Fur4 model. Moreover, the C-terminal tail is rich in acidic amino acids (especially glutamic acid, E) as well as serine and threonine residues, suggesting a potential PEST-like sequence. However, it does not rule out the possibility that Art2 interacts with N-terminal tail, while mediating the ubiquitylation of C-terminal lysine residue. The ubiquitylation of Gap1 in response to rapamycin takes place at the N-terminal tail, while Art3/6 and Bul1/2 interacts with the C-terminal tail (Crapeau et al., 2014). Therefore, our hypothesis is that Thi7 degron is localized on the cytosolic C-terminal tail and is composed of a PEST-like sequence, including putative phosphorylated residues, and lysine residues targeted by Rsp5.

Interestingly, Art2 also mediates the CHX-induced endocytosis of thiamine transporters in the absence of thiamine in the medium (although in a lesser extent for Thi72). CHX is known to stimulate the activity of TORC1. We could imagine that upon CHX addition, the stimulated TORC1 complex stimulates the activity of Art2 by inhibiting the activity of Npr1. Nevertheless, thiamine transporters should not undergo any structural rearrangement in the absence of thiamine, hindering the availability of a potential degron. On one hand, the CHX-induced posttranslational status of Art2 could be different from the thiamine-induced one, thereby involving another constitutively available Art2-binding site on

thiamine transporters. Many phosphorylation sites were identified on Art2, but only a few are TORC1 pathway-dependent (Iesmantavicius et al., 2014). We could therefore imagine that the differences between CHX- and thiamine-induced activation of Art2 are due to the dephosphorylation of TORC1-independent phosphorylation sites. On the other hand, CHX-induced activation of TORC1 could affect the availability of the Art2-binding site by modifying directly thiamine transporters. Besides, a phosphoproteomic study reveals that some phosphorylation sites are down-regulated upon addition of rapamycin, a potent inhibitor of the TORC1 complex. However, these phosphorylation sites are localized at the C-terminal tail (Huber et al., 2009). A serine and a threonine residue of the C-terminal tail are also predicted to be localized inside a casein kinase 1 complex (Yck1/2) recognition motif, which is reminiscent of Yck1/2-dependent phosphorylation required for Fur4 endocytosis (Marchal et al., 1998, 2000). These phosphorylation sites could also be part of the regulation of thiamine transporter intrinsic activity, as it was shown for the ammonium permease Mep2 (Boeckstaens et al., 2014). Notably, the absence of thiamine-dependent conformational change in the CHX-induced endocytosis could explain the remaining PM-localized Thi7-GFP, discussed in section 2.1.

Whatever the mechanism controlling its phosphorylation, phosphorylated Art2 probably interacts with 14-3-3 proteins. Art2 was shown to interact with one of the two isoforms of 14-3-3 proteins, Bmh2, in a yeast two-hybrid screening (Llopis-Torregrosa et al., 2016). Moreover, Art2 is likely to be ubiquitylated following its dephosphorylation. Indeed, the interaction between Art2 and Rsp5 and the Rsp5-dependent ubiquitylation of Art2 were already shown in previous studies (Peng et al., 2003; Kee et al., 2005; Nikko et al., 2008).

In our immunoblotting experiments, the basal level of free GFP is used as an indicator of the non-induced degradation of Thi7-GFP, while the extent of the accumulation is more an indicator of induced endocytosis. The presence of free GFP in thiamine depleted conditions would illustrate the natural turnover of Thi7-

GFP at the PM. Deletion of *ART2* has also a strong effect on the non-induced degradation of Thi7, suggesting that Art2 is at the crossroad of many signals, including thiamine, CHX and natural misfolding of Thi7.

2.3 Ubiquitylation of the cytosolic C-terminal tail of Thi7

We showed by microscopy that all the versions of Thi7 in which the cytosolic C-terminal lysine residues were mutated in arginine were not targeted to the vacuole upon addition of 100 μ M thiamine. These results combined to the fact that the absence of Art2 strongly reduces Thi7 ubiquitylation and endocytosis support that Art2-mediated ubiquitylation of Thi7 takes place at C-terminal lysine residues and induces its endocytosis. However, our results suggest that some ubiquitylation is still taking place in the absence of Art2. Indeed, we observed that ubiquitylation of Thi7-GFP is not totally abrogated in *art2 Δ* , while the ubiquitylation is absent in the Thi7^{KR}-GFP, in which only the N- and C-terminal lysine residues are mutated. Therefore, the remaining ubiquitylation does not take place at cytosolic loops-localized lysine residues since these residues are not mutated in Thi7^{KR}-GFP. Ubiquitylation would take place at the N-terminal or C-terminal part of the protein. The mediator as well as the consequence of such ubiquitylation remains a mystery since endocytosis is strongly impaired in the *art2 Δ* strain. As previously stated, α -arrestin adaptors have been shown to act at the GA (Becuwe and Léon, 2014). In the same way, many membrane adaptors (e.g. Tre1/2 and Bsd2) of Rsp5 are known to act at endosomes (Becuwe et al., 2012b). Therefore, we could hypothesize that the remaining Art2-independent ubiquitylation of Thi7 is the consequence of these adaptors. Indeed, Thi7-GFP sorting to the vacuole in the *end3 Δ art2 Δ* strain is not completely abolished since few free GFP signal is still detected. The divalent ion transporter Smf1, which was also shown to be endocytosed in an Art2-dependent way, does not require Art2 but Tre1/2 and Bsd2 for its ubiquitylation-dependent sorting in intraluminal vesicles of the multivesicular bodies and its vacuolar degradation (Stimpson et al., 2006; Sullivan et al., 2007). Moreover, Thi7 was shown to interact with Cos membrane adaptors (Cos3/4/6) that were shown to cluster PM proteins (including Mup1 and Ste2) at the early endosomes, leading to their incorporation in the ILVs of MVBs (Miller et al., 2005; MacDonald et al., 2015). Besides,

Rsp5 and its adaptors, Tre1/2 and Bsd2, are required for Cos protein function (MacDonald et al., 2015).

Moreover, we cannot rule out the fact that Thi7-GFP could be internalized in an unspecific way in the absence of Art2 by interacting with other PM proteins and be ubiquitylated in an Art2-independent way during this process. However, in addition to Nrt1, Thi72 and the Cos proteins, the only other PM protein shown to physically interact with Thi7 is the PM sterol transporter AUS1 of the ABC transporter family (Snider et al., 2013).

Altogether, our results and data from similar proteins allowed us to draw two putative models of the Art2-mediated endocytosis of the thiamine transporter Thi7 as well as of the two putative low-affinity transporters, Nrt1 and Thi72, in response to thiamine or CHX (see Discussion, section 2.7, Figure 1 and 2).

2.4 Art9 does not affect the endocytosis of Thi7

The proteomic screening pointed out a higher abundance of Thi7 in the *art9Δ* strain than in a WT strain, while no impairment of Thi7 endocytosis was detected by immunoblotting and fluorescence microscopy. The difference obtained in the strain expressing the genomically-tagged Thi7-EYFP compared to the results obtained by mass spectrometry could be explained by the fact that these two types of analyses are not based on the same extracts. Indeed, the screening has been performed on plasma membrane-enriched fractions, while the immunoblotting was done on total protein extracts. However, a simple analysis of plasma membrane enriched fractions from *THI7-EYFP* – expressing strains by immunoblotting is unlikely to explain that difference.

We also suspected that the effect of the deletion of *ART9* was the result of the identification algorithm of the mass spectrometer software. To be identified by mass spectrometry, proteins are cleaved by trypsin and tryptic peptides are then sequenced. Identified peptides are allocated to a corresponding protein. When various proteins share common peptides, these peptides are allocated to the protein having the highest number of specific peptides. In the case of very similar proteins, it is likely that one protein is gathering peptides from other proteins. Since the quantification of identified proteins is based on the relative abundance of its constitutive peptides, these shared peptides are considered in the computation of the ratio. Nrt1 and Thi72 are sharing 84% and 87% of sequence identity with Thi7, respectively. Moreover, we showed that deletion of *ART9* somehow affects their endocytosis. Therefore, we checked whether specific peptides from these two transporters were used for quantification of Thi7. The quantification was not done using specific peptides from Nrt1 and Thi72, but common peptides from the three transporters were used for Thi7-GFP abundance quantification. We cannot rule out the possibility that the effect of the deletion of *ART9* on Thi7-GFP abundance in the screening is affected by its effect on Nrt1 and Thi72. However, when looking

at the quantification of Thi7 specific peptides, the increased abundance in *art9Δ* following the CHX-treatment is also observed.

2.5 Involvement of Art9 in the endocytosis of Nrt1 and Thi72

The deletion of *ART9* seems to affect the thiamine-induced endocytosis of Nrt1 and Thi72 although we are still looking for the underlying mechanism. Indeed, we showed that half of the cells displayed a strong signal at PM and the other half in the vacuolar lumen. The absence of physical interaction between Art9 and Nrt1-GFP or Thi72-GFP, while their endocytosis is affected in the *art9Δ*, could be a clue suggesting that the effect of *ART9* deletion is more indirect and could affect the extent of Art2 activation. Art9 is known to be involved in the sensing of external pH during its targeting to late endosomes/multivesicular bodies and interaction with Rim21 sensor (Herrador et al., 2010, 2015; Gomez-Raja and Davis, 2012). In addition to that, Art9 was shown to be involved in the downregulation of the proton pump Pma1 (Smardon and Kane, 2014; Herrador et al., 2010). The extent of Art2 activation would be dependent on Art9 through its pH regulating activity. Unfortunately, it does not explain why all cells do not show an impaired endocytosis rather than different effects according to cells. To explain this phenotype, we could think that Art2 activity is linked to the cell cycle when *ART9* is missing. *ART9* genetically interacts with genes involved in cell-cycle control. In our conditions, the cells are not synchronized, that could explain that the vacuolar targeting of Thi7-GFP is different between cells. Indeed, Art2 was proposed as a substrate of the cyclin-dependent kinase Cdc28 (also known as Cdk1) in a large scale proteomic experiment (Holt et al., 2009). Unfortunately, it does not explain why the Art2-mediated endocytosis of Thi7 is not affected in the same conditions.

Another hypothesis is that the deletion of *ART9* is also indirectly affecting genome organization at its locus, impacting the

expression of close genes, indirectly affecting the cell-cycle. However, the partitioning of Nrt1-GFP and Thi72-GFP localization between cells is only seen when thiamine is added and not in thiamine-free medium. Even if it turns out to be an indirect effect, it is somehow linked to endocytosis or thiamine homeostasis.

2.6 Use of the *THI7* promoter for expressing thiamine transporter-GFP fusions

We decided to use the *THI7* promoter in order to express THI71-GFP and NRT1-GFP. However, it is likely that these three transporters are not expressed to the same extent in the same conditions. Thi7 is the main transporter of thiamine transporter in yeast synthetic medium (Mojzita and Hohmann, 2006). Moreover, all the endocytosis experiments were performed in a background that does not carry any deletion of thiamine transporter-coding genes. Therefore, when looking at the endocytosis of Nrt1-GFP and Thi72-GFP, endogenous *THI7* is also expressed. Its transport activity could affect the endocytosis of the two homologs. Therefore, the Thi7-dependent additional transport of thiamine in strains expressing *NRT1-GFP* or *THI72-GFP* could explain the higher rate of thiamine-induced endocytosis of these two transporters. Besides, the endocytosis of Nrt1-GFP and Thi72-GFP raises the question of why these low affinity thiamine transporters are endocytosed after addition of an excess of thiamine, since they are likely to be expressed in such conditions. Indeed, low affinity transporters tend to be more stable than their high-affinity counterparts and less likely to be endocytosed in response to an excess of their substrate, as it has been shown for the low affinity tryptophan permease Tat1 compared to the high-affinity one Tat2 (Suzuki et al., 2013). We cannot rule out that Nrt1-GFP, Thi72-GFP are interacting with the endogenous Thi7 pool at the PM, and Thi7 endocytosis following thiamine addition would drag the two low-affinity transporters in the endocytic process. Nevertheless, the addition of such a saturating excess of thiamine is likely to be sufficient to trigger

the endocytosis of low-affinity transporters. Therefore, to confirm our results, monitoring the endocytosis of Nrt1-GFP and Thi72-GFP in a *thi7Δnrt1Δthi72Δ* background would be interesting.

These hypotheses rely on the fact that Nrt1 and Thi72 are considered as low-affinity thiamine transporters. However, thiamine is required at so low amount that we can wonder why the cell needs a low-affinity transporter in addition to a high affinity one. Nrt1 has already been shown to transport nicotinamide riboside (Belenky et al., 2008). Therefore, thiamine transport by Nrt1 could be considered as unspecific. The specific substrate of Thi72 could not be thiamine too. However, *NRT1* and *THI72* genetically interact with the TPP sensor Thi3 (Huang et al., 2012), and their expression was shown to be dependent on the transcription factor Thi2 (Reimand et al., 2010; Nosaka et al., 2005), suggesting a link with thiamine metabolism. Besides, the expression of *THI72* is higher when the cytosolic thiamine content is low (Nosaka et al., 2005).

2.7 Model of Art2-mediated ubiquitylation of Thi7, Nrt1 and Thi72

Figure 1 illustrates a putative model for the substrate-induced thiamine transporter endocytosis based on the discussion of sections 2.1.-2.-3. (see pp. 127-134). TORC1 was chosen as intermediate of Art2 regulation in this model since a yeast two-hybrid experiment was not able to show any interaction between Art2 and Snf1 (Llopis-Torregrosa et al., 2016). On the contrary, the phosphorylation status of Art2 turns out to be modified in a TORC1-dependent way in a phosphoproteomic study (Iesmantavicius et al., 2014).

In the absence of thiamine (Figure 1 A), the low activity of TORC1 promotes the phosphorylation of Art2 by the Npr1 kinase. Phosphorylated Art2 interacts with 14-3-3 proteins, stabilizing thiamine transporters at the PM. Upon thiamine binding, the structural rearrangement of LID-loop interaction leads to the

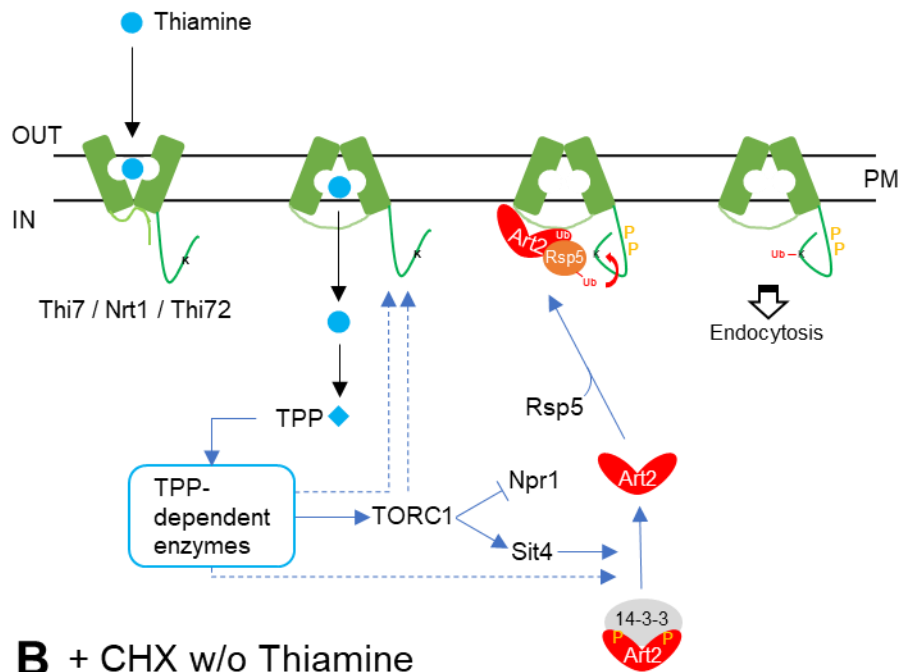
exposure of an *ART2* interaction domain. Concomitantly, thiamine uptake ensures an optimal activity of thiamine pyrophosphate (TPP) enzymes, sustaining an active metabolism and a good nutritional status. These growth conditions promote TORC1 activation, leading to Npr1 inhibition. Npr1 being inactive, Art2 is dephosphorylated by a TORC1-dependent phosphatase (e.g. Sit4), which stabilizes Art2 in a non- or poorly phosphorylated state. Art2 is therefore ubiquitylated by Rsp5. In addition to that, TORC1 promotes in a still unknown way the phosphorylation of some serine and threonine residues (of the putative PEST-like sequence) of the C-terminal tail required for the accessibility of the Rsp5-targeted lysine residue. An alternative to this model could be that TPP-sensitive proteins ensuring thiamine homeostasis would promote thiamine transporter modification and/or Art2 dephosphorylation.

In the absence of thiamine but after addition of CHX (Figure 1 B), CHX diffuses across the PM, inhibits protein translation and indirectly stimulates TORC1. Concomitantly, the absence of thiamine results in a low amount of TPP in the cytosol, impairing the activity of TPP-dependent enzymes from the carbohydrate and amino acid metabolism, leading to an inhibition of TORC1. The two antagonistic effects give rise to a pool of active ubiquitylated Art2, and another pool of still phosphorylated and inactive Art2 interacting with 14-3-3 proteins. Even in the absence of thiamine, a small pool of transporters at the PM could still perform a structural rearrangement upon the transport of other substrates than thiamine, for example. Moreover, the unspecific CHX-dependent activation of TORC1 also promotes the phosphorylation of the PEST-like sequence of the C-terminal tail of the transporters. These transporters are therefore in the right conformation to interact with the ubiquitylated Art2-Rsp5 complex, while Rsp5-targeted lysine residues are accessible. This pool of transporters is therefore endocytosed, but a proportion is recycled back at the PM due to the lack of active Art2 to further ubiquitylate them at the endosomal or GA level.

Ultimately, although we lack information to support this view, we designed an alternative model of Thi7/Nrt1/Thi72 endocytosis,

emphasizing the role of the carbohydrate metabolism, the kinase Snf1 and the type 1 phosphatase, PP-1 (Glc7/Reg1) (Figure 2). In the absence of extracellular thiamine, the only source of cytosolic thiamine is the thiamine biosynthetic pathway. In that condition, the carbohydrate metabolism is highly impaired due to the low activity of TPP-dependent enzymes. Indeed, the alcoholic fermentation pathway, the Krebs cycle as well as the pentose phosphate pathway depend on TPP-dependent enzymes. The low activity of these pathways leads to an accumulation of AMP and/or ADP, and a low ATP production, thereby leading to Snf1 activation (Mayer et al., 2011). Snf1 phosphorylates Art2, promoting its interaction with 14-3-3 proteins and preventing Art2-Rsp5 recruitment at the PM. Thi7 is therefore stabilized at the PM. Upon addition of thiamine, the carbohydrate metabolism is stimulated, decreasing the AMP:ATP and ADP:ATP concentration ratio. The reduced concentration of AMP/ADP and the increased production of ATP result in the inhibition of Snf1, while the phosphatase PP-1 is active. PP-1 promotes the dephosphorylation of Art2 and its release from the complex with 14-3-3 proteins. Art2 is then ubiquitinated in a Rsp5-dependent way and is ready to promote the ubiquitylation of the thiamine transporter at the PM. Obviously, the previously described structural rearrangement of the thiamine transporter and the phosphorylation of its C-terminal PEST-like sequence are still required for Art2 – Rsp5 binding.

A + Thiamine w/o CHX



B + CHX w/o Thiamine

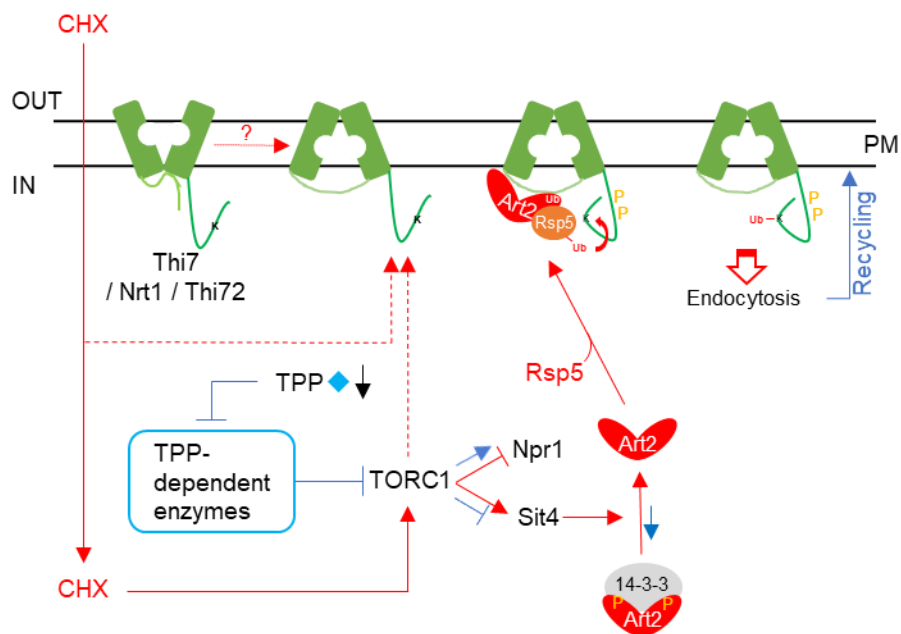


Figure 1 – Model of the (A) thiamine and (B) CHX-induced Art2-mediated ubiquitylation of thiamine transporters. Blue and red lines illustrate the thiamine

pyrophosphate (TPP)—dependent and CHX-dependent pathways, respectively. Dashed lines correspond to multiple step processes. (PM: plasma membrane; TPP: thiamine pyrophosphate; Ub: ubiquitin moiety; P: phosphate group, K: Rsp5-targeted lysine residue(s)).

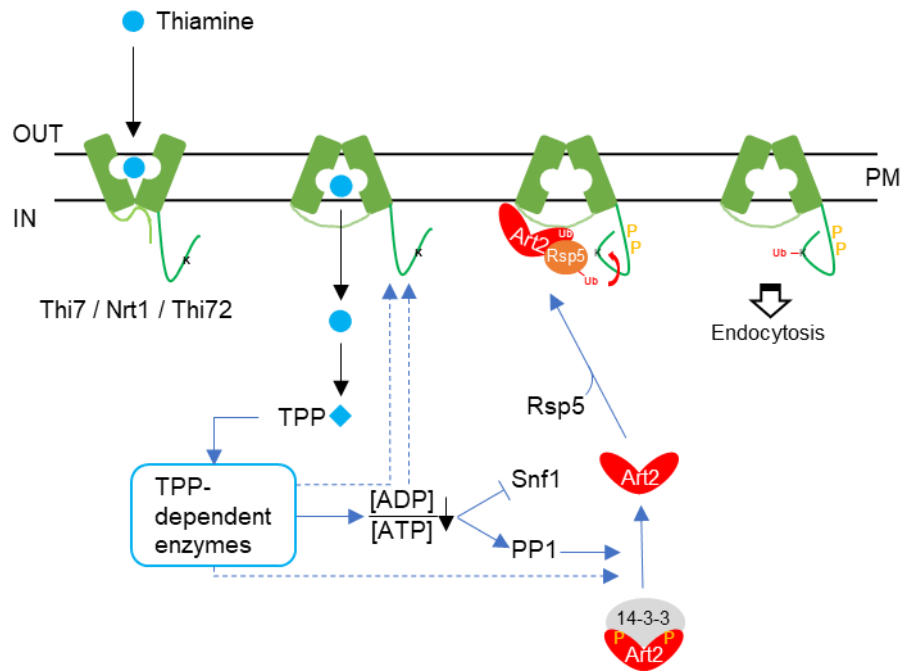


Figure 2 – Alternative model of the thiamine-induced Art2-mediated ubiquitylation of thiamine transporters. Dashed lines correspond to multiple step processes. (PM: plasma membrane; TPP: thiamine pyrophosphate; Ub: ubiquitin moiety; P: phosphate group, K: Rsp5-targeted lysine residue(s), AMP: Adenosine monophosphate; ATP: Adenosine triphosphate; PP-1: Type 1 Protein Phosphatase).

CONCLUSION

We applied a plasma membrane (PM) quantitative screening to the study of the α -arrestin-mediated ubiquitylation of PM proteins. The proteomic approach led us to the discovery of 28 potential targets of α -arrestins. Although a few of the identified targets have been already investigated, most of the effects we observed were new. Therefore, the proteomic approach turned out to be an appropriate tool to study the involvement of α -arrestin in the degradation of PM proteins.

The biggest interest of our study was to emphasize new interactions in an unbiased way. Most of the identified proteins are not model proteins. Therefore, this work is likely to open the path to new regulation mechanisms that may be independent on the current model based on either carbohydrate or amino acid metabolism. In the same way, these new targets could bridge the two current models. With that in mind, we decided to further characterize a hit from our screening, the thiamine transporter Thi7. Thiamine (vitamin B1) is a coenzyme of enzymes involved in carbohydrate and amino acid metabolism. Moreover, as a coenzyme, thiamine is not metabolized by the cell, unlike the main endocytosis-triggering stimuli studied so far (non- and fermentable carbon sources, ammonium or amino acids).

Therefore, our work raised the question on how the vitamin content is integrated by the cell and activates the ubiquitylation machinery. We showed that a single α -arrestin, Art2, is required for the thiamine and cycloheximide-induced endocytosis of the three plasma membrane thiamine transporters, Thi7, Nrt1 and Thi72. Moreover, we pointed out that Art2 mediates the ubiquitylation of Thi7 and that lysine residues of the cytosolic C-terminal tail are required for Thi7 endocytosis in response to an excess of thiamine. Therefore, Art2 turns out to be a key player in the integration of thiamine content in the cell by regulating the abundance of thiamine transporters at the PM.

Our results also suggest a role for Art9 in the regulation of thiamine transporters, especially Nrt1 and Thi72, even though we did not decipher the underlying mechanism.

This work is a first contribution to the development of three new model proteins for the study of the regulation and interaction of α -arrestin adaptors. The three highly similar transporters prove to be interesting tools to understand how the α -arrestins manage to mediate the specific endocytosis of very similar proteins. Besides, this study is going to bring new insights about vitamin homeostasis in *S. cerevisiae*. Although the baker's yeast has been a valuable tool for the study of thiamine homeostasis so far, the post-translational regulation of thiamine transporters remains a mystery in *S. cerevisiae* as well as in many other eukaryotic models. Nevertheless, our study pointed out Art2 as a new regulator of thiamine uptake in *S. cerevisiae*.

PROSPECTS

This study is only the beginning of an exciting new area about the involvement of α -arrestin in thiamine homeostasis. Therefore, much work must still be done.

Some experiments are still needed to strengthen the conclusion we drew in the current study. We should investigate whether the PY motif of Art2 is required for the endocytosis of Thi7, bringing a stronger clue that Art2 bridges Thi7 to Rsp5. In the same way, investigating the post-translational modification status of Art2 before and after addition of thiamine would help to understand the activation mechanism of Art2. A lysine residue (K670) conserved among seven α -arrestins, including Art2, was recently identified as essential for Rsp5-dependent ubiquitylation of Art2 paralog, Art8 (Hovsepian et al., 2017). Lysine-to-arginine mutation of K670 or engineering of a non-ubiquitylable version of Art2 would answer the question of whether Art2 ubiquitylation is needed for thiamine transporter ubiquitylation and endocytosis. Phosphorylation of Art2 would be also a point to consider. A recent proteomic screening has already proposed some TORC1-dependent phosphorylation sites in Art2 (Iesmantavicius et al., 2014). Concerning Art9, its role remains unclear. Transformation of the *ART9* deletion strains (*art9 Δ*) with a plasmid expressing *ART9* would clarify whether the double phenotype observed for Nrt1 and Thi72 is due to the absence of Art9 or an unspecific effect of the deletion of its genomic locus. However, the “double phenotype” observed in the *ART9* deletion strain is anyway thiamine-dependent. Synchronization of cells could be also a way to challenge the hypothesis of cell-cycle control of α -arrestins. Finally, endocytosis monitoring in a strain lacking all transporters could be a way to confirm that the Art2-dependent endocytosis of Nrt1 and Thi72 is not only due to their interaction with Thi7.

A negative point of our study is that we still cannot conclude about the interaction of Art2 with thiamine transporters. In one

experiment out of two, we observed that a significant amount of 3xHA-Art2 was binding the GFP-trap column in a Thi7-independent way. Therefore, blocking the column with bovine serum albumin could be a solution to decrease Art2 non-specific binding. If GFP-trap is eventually not appropriate for our study, we could apply other protein-protein interaction-probing techniques, such as bimolecular fluorescence complementation (BiFC) assay. Unfortunately, a big disadvantage of BiFC is that the relatively high-affinity interaction between the two parts of the fluorescent protein tends to force the interaction between the two proteins of interest. On the contrary, BiFC would allow to localize the interaction, which may also take place at other compartments than PM. Experiments based on the Förster resonance energy transfer (FRET) would therefore be more suitable but would be limited by the transient interaction of α -arrestins with their target. The development of photo-crosslinking amino acids, like para-benzoyl-phenylalanine (BPA), could help to tackle the problem of the transient interaction in minimalizing false positive interactions.

As a long-term prospect, the determination of the effective signal triggering the endocytosis should be performed.

A first hypothesis may be that Art2 could be regulated by specific proteins involved in thiamine homeostasis. Therefore, this raises the question as to whether Art2 activation is dependent on thiamine pyrophosphate (TPP). Indeed, our model proposes that once transported, thiamine is phosphorylated in TPP, which in turn mediates the activity of enzymes involved in carbohydrate and amino acid metabolism. To test this hypothesis, we could monitor thiamine-induced Thi7-GFP endocytosis in a strain defective for the thiamine pyrophosphatase Thi80, namely *thi80-1* (Nishimura et al., 1991), leading to a low TPP content in the cell, while thiamine transport is not impaired.

An alternative proposition would be that thiamine uptake somehow affects the carbohydrate and amino acid metabolism, thereby activating either the PP-1 - Snf1 or the TORC1 - Npr1 - Sit4 pathways, respectively. The PP-1 – Snf1 pathway can be

investigated by probing the phosphorylation status of Snf1, known to regulate its activity, before and after addition of thiamine. Concerning TORC1, we should test its activity in response to thiamine uptake. TORC1 activity can be followed by monitoring the post translational modification of several of its effectors, such as Sch9 or Npr1. Working in a *tco89Δ* strain or using rapamycin in addition of thiamine may bring some insights about TORC1 involvement in Thi7-endocytosis. Finding the kinases and phosphatases regulating Art2 will obviously be the key to decipher the endocytosis-stimulating signal of thiamine transporters. The involvement of Npr1/Sit4 or Snf1/PP1 (Glc7/Reg1) in Thi7 endocytosis could be easily checked using deletion strain. Nevertheless, performing a kinetic proteomic and phosphoproteomic analysis of cells grown in a thiamine-free medium upon addition of thiamine would bring many insights about thiamine homeostasis, putatively leading to the identification of other kinases and phosphatases.

Likewise, the post-translational modifications of three thiamine transporters upon thiamine addition is worth studying. A phosphoproteomic screening showed that two phosphorylation sites of Thi7 are downregulated by the addition of rapamycin (Huber et al., 2009). Although, we were able to observe Art2-dependent ubiquitylation of the three thiamine transporters, the multiple bands that we observed in both N- and C-terminally fused versions of thiamine transporters are intriguing. Some of them are still visible in a Rsp5-defective strain. Moreover, Thi7, Nrt1 and Thi72 are probably phosphorylated prior to be ubiquitylated. Therefore, thiamine-dependent phosphorylated residues and the putative PEST-like sequence could be identified by phosphorylation-focused mass spectrometry analysis of Thi7. That analysis combined with a mutagenesis approach generating phosphorylation-dead and -mimetic mutants of Thi7 could bring some clues about the binding of Art2 to thiamine transporters in response to thiamine. By the way, the role of the cytosolic loop-localized PY motif of the three transporters should be investigated although nothing has shown so far that it was needed for thiamine-induced endocytosis.

A crucial point of our model is the thiamine transport-induced conformational change of thiamine transporters and the LID-dependent exposure of an Art2-binding domain. Mutations of the LID sequence (e.g. N29A or P34A) or cytosolic loops that artificially disrupt their interaction may trigger the constitutive endocytosis of thiamine transporters even in the absence of thiamine. The challenge will be to ensure the activation of Art2 in that condition. Moreover, although the three transporters have an LID sequence, the structural rearrangement model during thiamine transport (proposed for Fur4 and Can1) cannot explain their endocytosis upon addition of cycloheximide in the absence of thiamine in the medium. It is likely that these regulation mechanisms are not mutually exclusive, and several actors are probably adding up. Currently, an oxythiamine-based mutagenesis experiment is carried out to isolate thiamine transport-defective Thi7 mutants. This approach will bring many data about the requirement of thiamine binding and transport for Art2-mediated endocytosis of Thi7. If thiamine binding to Thi7 is enough to trigger its endocytosis via a conformational change-induced exposure of a specific degron, a strong characterization of the mutant conformation would be required to draw relevant conclusions. More generally, resistance to oxythiamine and other toxic thiamine analogs could be a very interesting read-out in order to select mutant strains in which Thi7 endocytosis is constitutively triggered. Although many mutations will be found in thiamine transporter genes, some of them could target Art2-inhibiting actors.

Our results suggest that Art2 fine-tunes the abundance of thiamine transporters by ubiquitylating them at the PM. It would be very interesting to know whether Thi7 also undergoes ubiquitylation at the GA, as it was shown for Gap1 and Jen1 (De Craene et al., 2001; Soetens et al., 2001; Becuwe and Léon, 2014). The monitoring of Thi7 ubiquitylation in strains defective for GA to endosomes or endosomes to vacuole transport lacking or not *ART2* is a way to answer that question. Moreover, the opposite experiment can be performed in order to determine whether Art2 could re-direct Thi7 to the vacuole from the GA

when cytosolic thiamine concentration is high enough. More specifically, *thi7Δnrt1Δthi72Δ* (CVY3) (Vogl et al., 2008) and CVY3 *art2Δ* strains, both transformed with a plasmid expressing *THI7* under the control of a conditional promoter could be grown in 100 μM thiamine. We showed that such an excess of thiamine promotes its transport across the membrane in the absence of the three transporters. Therefore, the cytosolic concentration of thiamine should be high enough to stimulate Art2. When inducing the expression of Thi7-GFP, newly synthesized Thi7-GFP follows the secretory pathway and is likely to be directly routed to the vacuole, except in the absence of Art2, where it will reach the PM and be stabilized.

Ultimately, investigating the involvement of Art2 in Thi7, Nrt1 and Thi72 endocytosis upon addition of other endocytosis-triggering signals, like different carbon or nitrogen sources, could confirm that thiamine is at crossroads of both regulation pathways. Moreover, Art2-mediated endocytosis can be extended to the endocytosis of other vitamin transporters, whose expression is linked to thiamine content, and proteins highly similar to Thi7 like the allantoin permease Dal4 or the uridine permease Fui1.

EXPERIMENTAL PROCEDURES

1 Strains and plasmids

All the bacterial transformations were performed using *Escherichia coli* TOP10 strain (Invitrogen). The first proteomic screening was based on *Saccharomyces cerevisiae* auxotrophic BY4742 strains from the EUROSCARF consortium. Each strain was deleted for a given *ART* gene, which was replaced with a geneticin (G418) resistance cassette (kanMX4). Multiple deletant strains and genomic insertions were engineered either using PCR-based deletion cassettes or yeast crossing (and subsequent tetrad dissection). We received from Professor Pelham (MRC, UK) a strain lacking eleven α -arrestin adaptors (EN67), expressing only *ART9*, and plasmids coding for each adaptor which were used for a second screening. In order to study the thiamin transporter Thi7, Professor Stolz (from TUM, Germany) kindly provided us with two strains deleted for the three thiamin transporters (CVY3) and an essential thiamine biosynthetic gene *THI4* (CVY4) as well as a high-copy-number plasmid expressing a *THI7-GFP* fusion expressed under the control of the *THI7* natural promoter. From that plasmid, we transferred the cassette into a centromeric plasmid (pRS316). Using the Gibson assembly technology (Gibson et al., 2009), we also engineered plasmids coding for non- and (N- and C-terminally) GFP-fused versions of all thiamine transporters (Thi7, Nrt1 and Thi72). The lysine-to-arginine mutant versions of *THI7* were engineered using synthetic oligonucleotides (Integrated DNA technologies, Leuven) that were assembled using Gibson assembly. The involvement of the ubiquitin ligase Rsp5 in transporters degradation was checked using the *npi1-1* (named *rsp5* in this thesis) strain, engineered by the team of Professor André (ULB, Belgium). We also started the study of the yeast α -arrestin Art9 using two plasmids coding for *ART9* tagged versions, that we received from Pr. Vincent (IIB, Spain). Table 1

and 2 list the strains and plasmids used in this study, respectively.

Table 1 - Strains used in this study.

Strain (alias)	Genotype	Source
Wild-Type (WT)	BY4742; Mat α ; <i>his3Δ1</i> ; <i>leu2Δ0</i> ; <i>lys2Δ0</i> ; <i>ura3Δ0</i>	EUROSCARF
<i>art1Δ</i>	BY4742; Mat α ; <i>his3Δ1</i> ; <i>leu2Δ0</i> ; <i>lys2Δ0</i> ; <i>ura3Δ0</i> ; <i>YOR322c::kanMX4</i>	EUROSCARF
<i>art2Δ</i>	BY4742; Mat α ; <i>his3Δ1</i> ; <i>leu2Δ0</i> ; <i>lys2Δ0</i> ; <i>ura3Δ0</i> ; <i>YBL101c::kanMX4</i>	EUROSCARF
<i>art2Δ</i>	BY4741; Mat α ; <i>his3Δ1</i> ; <i>leu2Δ0</i> ; <i>met15Δ0</i> ; <i>ura3Δ0</i> ; <i>YBL101c::kanMX4</i>	EUROSCARF
<i>art3Δ</i>	BY4742; Mat α ; <i>his3Δ1</i> ; <i>leu2Δ0</i> ; <i>lys2Δ0</i> ; <i>ura3Δ0</i> ; <i>YJL084c::kanMX4</i>	EUROSCARF
<i>art4Δ</i>	BY4742; Mat α ; <i>his3Δ1</i> ; <i>leu2Δ0</i> ; <i>lys2Δ0</i> ; <i>ura3Δ0</i> ; <i>YOR018w::kanMX4</i>	EUROSCARF
<i>art5Δ</i>	BY4742; Mat α ; <i>his3Δ1</i> ; <i>leu2Δ0</i> ; <i>lys2Δ0</i> ; <i>ura3Δ0</i> ; <i>YGR068c::kanMX4</i>	EUROSCARF
<i>art6Δ</i>	BY4742; Mat α ; <i>his3Δ1</i> ; <i>leu2Δ0</i> ; <i>lys2Δ0</i> ; <i>ura3Δ0</i> ; <i>YKR021w::kanMX4</i>	EUROSCARF
<i>art7Δ</i>	BY4742; Mat α ; <i>his3Δ1</i> ; <i>leu2Δ0</i> ; <i>lys2Δ0</i> ; <i>ura3Δ0</i> ; <i>YFR022w::kanMX4</i>	EUROSCARF
<i>art8Δ</i>	BY4742; Mat α ; <i>his3Δ1</i> ; <i>leu2Δ0</i> ; <i>lys2Δ0</i> ; <i>ura3Δ0</i> ; <i>YPR030w::kanMX4</i>	EUROSCARF
<i>art9Δ</i>	BY4742; Mat α ; <i>his3Δ1</i> ; <i>leu2Δ0</i> ; <i>met15Δ0</i> ; <i>ura3Δ0</i> ; <i>YGL045w::kanMX4</i>	EUROSCARF
<i>art9Δ</i>	BY4741; Mat α ; <i>his3Δ1</i> ; <i>leu2Δ0</i> ; <i>lys2Δ0</i> ; <i>ura3Δ0</i> ; <i>YGL045w::kanMX4</i>	EUROSCARF
<i>art10Δ</i>	BY4742; Mat α ; <i>his3Δ1</i> ; <i>leu2Δ0</i> ; <i>lys2Δ0</i> ; <i>ura3Δ0</i> ; <i>YLR392c::kanMX4</i>	EUROSCARF
<i>bul1Δ</i>	BY4742; Mat α ; <i>his3Δ1</i> ; <i>leu2Δ0</i> ; <i>lys2Δ0</i> ; <i>ura3Δ0</i> ; <i>YMR275c::kanMX4</i>	EUROSCARF
<i>bul2Δ</i>	BY4742; Mat α ; <i>his3Δ1</i> ; <i>leu2Δ0</i> ; <i>lys2Δ0</i> ; <i>ura3Δ0</i> ; <i>YML111w::kanMX4</i>	EUROSCARF
<i>end3Δ</i>	BY4741; Mat α ; <i>his3Δ1</i> ; <i>leu2Δ0</i> ; <i>met15Δ0</i> ; <i>ura3Δ0</i> ; <i>YNL084c::kanMX4</i>	EUROSCARF

Table 1 - Strains used in this study.

Strain (alias)	Genotype	Source
EN60	BY4742; Mat α ; <i>his3Δ1</i> ; <i>leu2Δ0</i> ; <i>ura3Δ0</i> ; <i>ecm21</i> ::G418; <i>csr2</i> ::G418; <i>bsd2</i> <i>rog3</i> ::natMX <i>rod1</i> <i>ygr068c</i> <i>aly2</i> <i>aly1</i> <i>ldb19</i> <i>ylr392c</i> ::SpHIS5	H. Pelham (MRC, UK)
EN63	BY4742; Mat α ; <i>his3Δ1</i> ; <i>leu2Δ0</i> ; <i>ura3Δ0</i> ; <i>ecm21</i> ::G418; <i>csr2</i> ::G418; <i>bsd2</i> <i>rog3</i> ::natMX <i>rod1</i> <i>ygr068c</i> <i>aly2</i> <i>aly1</i> <i>ldb19</i> <i>ylr392c</i> <i>bul1</i> ::SpHIS5	H. Pelham (MRC, UK)
EN67	BY4742; Mat α ; <i>his3Δ1</i> ; <i>leu2Δ0</i> ; <i>ura3Δ0</i> ; <i>ecm21</i> ::G418; <i>csr2</i> ::G418; <i>bsd2</i> <i>rog3</i> ::natMX <i>rod1</i> <i>ygr068c</i> <i>aly2</i> <i>aly1</i> <i>ldb19</i> <i>ylr392c</i> <i>bul1</i> <i>bul2</i> ::SpHIS5	H. Pelham (MRC, UK)
<i>npi1-1</i> (<i>rsp5</i>)	BY4741; Mat α ; <i>his3Δ1</i> ; <i>leu2Δ0</i> ; <i>met15Δ0</i> ; <i>ura3Δ0</i> ; Promoter _{RSP5} ::kanMX	B. André (ULB, Belgium)
<i>thi7Δnrt1Δthi72Δ</i> (CVY3)	BY4742; Mat α ; <i>his3Δ1</i> ; <i>leu2Δ0</i> ; <i>lys2Δ0</i> ; <i>ura3Δ0</i> ; YLR237w::KanMX; YOR071c::LEU2; YOR192c::LYS2	J. Stolz (TUM, Germany)
<i>thi7Δnrt1Δthi72Δthi4Δ</i> (CVY4)	BY4742; Mat α ; <i>his3Δ1</i> ; <i>leu2Δ0</i> ; <i>lys2Δ0</i> ; <i>ura3Δ0</i> ; YGR144w::SpHis5; YLR237w::KanMX; YOR071c::LEU2; YOR192c::LYS2	J. Stolz (TUM, Germany)
<i>art2Δart9Δ</i>	BY4742; Mat α ; <i>his3Δ1</i> ; <i>leu2Δ0</i> ; <i>lys2Δ0</i> ; <i>ura3Δ0</i> ; YBL101c::kanMX4; YGL045w::kanMX4	This study
<i>end3Δart2Δ</i>	BY4741; Mat α ; <i>his3Δ1</i> ; <i>leu2Δ0</i> ; <i>met15Δ0</i> ; <i>ura3Δ0</i> ; YNL084c::kanMX4; YBL101c::natNT2	This study
WT <i>THI7-EYFP</i>	BY4742; Mat α ; <i>his3Δ1</i> ; <i>leu2Δ0</i> ; <i>lys2Δ0</i> ; <i>ura3Δ0</i> ; YLR237w- <i>EYFP</i> ::natMX6	This study
<i>art2Δ THI7-EYFP</i>	BY4742; Mat α ; <i>his3Δ1</i> ; <i>leu2Δ0</i> ; <i>lys2Δ0</i> ; <i>ura3Δ0</i> ; YBL101c::kanMX4; YLR237w- <i>EYFP</i> ::natMX6	This study
<i>art9Δ THI7-EYFP</i>	BY4742; Mat α ; <i>his3Δ1</i> ; <i>leu2Δ0</i> ; <i>met15Δ0</i> ; <i>ura3Δ0</i> ; YGL045w::kanMX4; YLR237w- <i>EYFP</i> ::natMX6	This study

Table 2 - Plasmids used in this study

Name	Description	Source
pRS316	pRS316 CEN <i>URA3</i>	(Hieter & Sikorski, 1989)
pRS416	pRS416 CEN <i>URA3</i>	(Hieter & Sikorski, 1989)
pRS315	pRS315 CEN <i>LEU2</i>	(Hieter & Sikorski, 1989)
pRS313	pRS313 CEN <i>HIS3</i>	(Hieter & Sikorski, 1989)
ART1-HA	YCplac111 (CEN <i>LEU2</i>); P _{ART1} - <i>ART1-HA</i> -T _{POL4}	H. Pelham (MRC, UK)
3xHA-ART2	YCplac111; P _{ART2} -3xHA- <i>ART2</i> -T _{POL4}	H. Pelham (MRC, UK)
ART3-HA	YCplac111; P _{ART3} - <i>ART3-HA</i> -T _{POL4}	H. Pelham (MRC, UK)
ART4-HA	YCplac111; P _{ART4} - <i>ART4-HA</i> -T _{POL4}	H. Pelham (MRC, UK)
ART5-HA	YCplac111; P _{ART5} - <i>ART5-HA</i> -T _{POL4}	H. Pelham (MRC, UK)
ART6-HA	YCplac111; P _{ART6} - <i>ART6-HA</i> -T _{POL4}	H. Pelham (MRC, UK)
ART7-HA	YCplac111; P _{ART7} - <i>ART7-HA</i> -T _{POL4}	H. Pelham (MRC, UK)

Table 2 - Plasmids used in this study

Name	Description	Source
ART8-HA	YCplac111; P _{ART8} -ART8-HA-T _{POL4}	H. Pelham (MRC, UK)
ART10-HA	YCplac111; P _{ART10} -ART10-HA-T _{POL4}	H. Pelham (MRC, UK)
THI7-GFP	YEplac195 (2μ URA3); P _{THI7} -THI7-yEGFP-T _{ADH1}	J. Stolz (TUM, Germany)
3xFLAG-ART9	pRS313; P _{ART9} -3xFLAG-ART9-T _{ART9}	O. Vincent (IIB, Spain)
ART9-3HA	pRS313; P _{ART9} -ART9-3HA-T _{ART9}	O. Vincent (IIB, Spain)
THI7	pRS416; P _{THI7} -THI7-T _{ADH1}	This study
THI7-GFP	pRS316; P _{THI7} -THI7-yEGFP-T _{ADH1}	This study
GFP-THI7	pRS316; P _{THI7} -yEGFP-THI7-T _{ADH1}	This study
NRT1	pRS416; P _{THI7} -NRT1-T _{ADH1}	This study
NRT1-GFP	pRS416; P _{THI7} -NRT1-yEGFP-T _{ADH1}	This study
GFP-NRT1	pRS416; P _{THI7} -yEGFP-NRT1-T _{ADH1}	This study
THI72	pRS416; P _{THI7} -THI72-T _{ADH1}	This study
THI72-GFP	pRS416; P _{THI7} -THI72-yEGFP-T _{ADH1}	This study
GFP-THI72	pRS416; P _{THI7} -yEGFP-THI72-T _{ADH1}	This study

Table 2 - Plasmids used in this study

Name	Description	Source
THI7 ^{fullKR} -GFP	pRS416; P _{THI7} -THI7 ^{KR} -yEGFP-T _{ADH1}	This study
THI7 ^{NterKR} -GFP	pRS416; P _{THI7} -THI7 ^{K6;19;28;36R} -yEGFP-T _{ADH1}	This study
THI7 ^{CterKR} -GFP	pRS416;P _{THI7} -THI7 ^{K505;509;513;526;527;569;584;588;596R} -yEGFP-T _{ADH1}	This study
THI7 ^{6KR} -GFP	pRS416; P _{THI7} -THI7 ^{K526;527;569;584;588;596R} -yEGFP-T _{ADH1}	This study

2 Media

Yeast strains used for the first proteomic screening and its validation by immunoblotting were grown in Yeast Dextrose (YD) rich medium, composed of 2% glucose, 2% KAT yeast extract (Ohly) (final pH at 5.2). Transformed strains were grown in the corresponding selective thiamine-free synthetic complete (SC) medium. The latter medium is made up of 2% glucose, 0.67% Yeast Nitrogen Base (YNB) without amino acids and without thiamine (ForMedium) and 1.12 g/l of Drop-Out powder lacking the amino acid(s) required for selection (pH adjusted at 5.2 with NaOH). The composition of the Drop-Out powder is defined in Table 3.

Table 3- Composition of a complete Drop-Out powder.

Constituents	Amount (mg) for 1 L of final medium
Adenine sulfate (hemisulfate salt)	20
Uracil	20
L-tryptophane	20
L-histidine (HCl)	20
L-arginine (HCl)	20
L-methionine	20
L-tyrosine	30
L-leucine	100
L-isoleucine	30
L-lysine (HCl)	30
L-phenylalanine	50
L-glutamic acid	100
L-aspartic acid	100
L-valine	150
L-threonine	200
L-serine	400

3 Yeast transformation

Yeast cells were grown at 28°C to early log phase (OD = 1.15) in 10 ml of YD medium. Cells were harvested by centrifugation at 2,500 *g* for 5 min in microcentrifuge and re-suspended in 1 ml of Li-TE (100 mM lithium acetate, 10 mM Tris-HCl, 0.1 M Na₂EDTA, pH 7.5). Cells were once again harvested by centrifugation at 2,500 *g* for 5 min and resuspended in 100 µl Li-TE. Then, 5 µl of each plasmid, 5 µl of single-stranded herring sperm competitor DNA (at a final concentration of 10 mg/ml) and 100 µl of 70% polyethylene glycol (PEG) were added to cells. Each sample was mixed by five short bursts on a vortex mixer at full speed and then incubated for 30 min at 30°C by mixing occasionally. Twenty microliters of dimethyl sulfoxide (DMSO) were added to each sample before heat shock. Cells were incubated at 42°C for 15 min and then left at room temperature for 30 min. Cells were harvested by centrifugation at 6,000 *g* for 30 s and re-suspended in 200 µl of water. Finally, 100 µl of each sample were plated on 2% agar-containing selective medium and incubated at 28°C.

4 Proteomic screening

4.1 Experimental design

For the first screening, WT and twelve knock-out strains (*artΔ*) were grown at 28°C to an optical density (OD_{600nm}) of 7 (3x10⁷ cells/ml) in 2 L of YD. Cultures of the twelve *artΔ* strains were incubated with cycloheximide (solubilized in 100% ethanol) at a final concentration of 50 µg/ml for 90 min. Cycloheximide is a protein translation inhibitor, known to trigger the endocytosis of many yeast transporters. As a control, an additional WT strain was mock-treated with the same volume of 100% ethanol. The final concentration of ethanol in the medium was 0.5%. Concerning the second screening, EN67 strains transformed with each α-arrestin coding plasmids as well as EN60 and EN63

(for the involvement of BUL adaptors) were used. These strains were not treated with cycloheximide since the expression of a single α -arrestin in the EN67 background was enough to observe strong differences in their corresponding proteome.

Cells were then harvested, homogenized and the plasma membrane (PM) enrichment performed. PM enriched fractions were then analyzed by mass spectrometry. In the first screening, the PM proteome of the mock-treated WT strain was compared with the one of the cycloheximide (CHX)-treated one to select PM proteins whose abundance is affected (decreased or increased) by the addition of cycloheximide. Then, the abundance of these proteins was compared between the cycloheximide-treated *artΔ* strains and WT to determine if the deletion of a given α -arrestin gene would disrupt the effect of CHX.

4.2 Plasma membrane enrichment

The PM enrichment protocol was previously developed in our laboratory (Goffeau and Dufour, 1988). Cells were harvested by centrifugation at 5,000 *g* for 5 min (JLA.9100, Beckman), washed with 0.5 l of cold distilled water, and re-suspended in 15 ml of cold homogenization buffer per 10 g of cells. The homogenization buffer (adjusted at pH 7.5 with HCl) is composed of 250 mM sorbitol, 50 mM imidazole, 1 mM MgCl₂, and supplemented with 1 mM phenylmethylsulfonyl fluoride (PMSF) and 0.1% of protease inhibitor cocktail from Roche (containing 2 μ g/ml of each of the following inhibitor: leupeptin, aprotinin, antipain, pepstatin, and chymostatin). The suspension was poured into a 75 ml homogenizer glass flask. Glass beads were added at a ratio of 15 g per 10 g of cells. The suspension was homogenized in a Braun MSK homogenizer (Helsungen, Germany) for 3 min with cooling each 30 s. Cell debris and unbroken cells were eliminated by two 5 min centrifugations at 1,000 *g* and a 5 min centrifugation at 4,000 *g* (JA-30.50Ti, Beckman). The supernatant was then centrifuged for 40 min at 27,000 *g* (JA-30.50Ti, Beckman) to pellet microsomal fractions, which were re-suspended in 0.5 ml of suspension buffer per g of cells. The suspension buffer (adjusted at pH 7.5 with HCl) is composed of

10 mM imidazole, 2 mM MgCl₂, and supplemented with 1 mM PMSF and 0.1% of the protease inhibitor cocktail (Roche). The microsomal fraction-containing suspension was homogenized and brought to pH 4.8 with 1 M acetic acid. Precipitated internal membranes were removed by centrifugation at 8,000 *g* for 2 min (JA-30.50Ti, Beckman). The supernatant was rapidly brought back to pH 7.5 with 1 M NaOH and centrifuged for 30 min at 180,000 *g* (70Ti, Optima-Beckman). The pellet was re-suspended in 50 mM triethyl ammonium bicarbonate buffer (TEAB; pH 8.5) and stored at -80°C until use. Plasma membranes were then stripped of peripheral proteins by mixing samples with stripping buffer (0.1 M sodium carbonate, 50 mM TEAB, adjusted at pH 11.5 with NaOH), followed by an incubation of 30 min on ice. Samples were then centrifuged at 150,000 *g* for 30 min (TLA55, Optima-Beckman) to pellet the stripped plasma membranes, which were then re-suspended in 50 mM TEAB buffer.

Protein concentration was determined using the bicinchoninic acid (Sigma) protein assay. Finally, PM-enriched fractions (5 µg of proteins) were mixed with an equal volume of sample buffer (100 mM Tris HCl, pH 6.8, 4 mM EDTA, 4% SDS, 20% glycerol, 0.002% bromophenol blue) containing 1% dithiothreitol (DTT) and incubated at room temperature for 10 min. Proteins were separated by SDS-PAGE to check whether degradation occurred during the process.

4.3 iTRAQ labelling

Stripped PM fractions (equivalent of 20 µg of protein) were solubilized in 50 mM TEAB, 0.1 % Rapigest (Waters) (final pH should be 8) by 5 min sonication in a bath sonicator (Bioruptor, Diagenode). Proteins were reduced by 60 min incubation at 60°C with 25 mM tris(2-carboxyethyl) phosphine (TCEP), then alkylated with 250 mM methyl-methanethiosulfonate (MMTS) for 10 min at room temperature in the dark. Reduced and alkylated proteins were digested overnight (15 h) at 37°C using sequencing grade modified trypsin (Promega) at a

protease/protein ratio of 1/20. The Rapigest was lysed by incubating the protein sample in 0.5% trifluoroacetic acid (TFA) for 60 min at 37°C. After centrifugation of the sample at 180,000 *g* (TLA55, Optima-Beckman) for 45 min at 4°C, the supernatant was recovered and centrifuged again for 20 min at 180,000 *g* (TLA55, Optima-Beckman). The peptide-containing supernatant was vacuum dried (Speedvac SC 200, Savant) and iTRAQ labelling performed according to the manufacturer's protocol (Applied Biosystems). Peptides were labelled with iTRAQ reactants kit during 1 h.

4.4 Mass spectrometry analysis

Labeled peptides were resuspended in 78 µl of a 2% (v/v) acetonitrile (ACN) and 0.1% (v/v) formic acid solution in order to obtain a final concentration of 2 µg/µl. Peptide separation was performed on an ultra-high-performance liquid chromatography (UHPLC) (Eksigent 2D nano-ultra, Sciex). Two micrograms of labeled peptides were diluted in 5 µl of a 2% ACN - 0.5% formic acid solution and injected on a C18 pre-column (Acclaim PepMap 100 C18, 2 cm, 100 µm, Thermo Scientific) with the aim of desalting the sample. Eluted peptides were immediately subjected to reverse phase (RP) chromatography on a C18 analytical column (Acclaim PepMap 100 C18, 75 µm id x 25 cm, Thermo Scientific) for 150 min at a flow rate of 300 nl/min using a gradient of ACN as illustrated in Figure 1. The gradient was performed by mixing a solution A (0.1% formic acid in ULC/MS water) and a solution B (0.1% formic acid in 100% ACN). The iTRAQ-labeled peptides were analyzed by an electrospray ionization (ESI) - TripleTOF 5600+ System (AB SCIEX) in the laboratory of Professor R. Wattiez (University of Mons, Belgium).

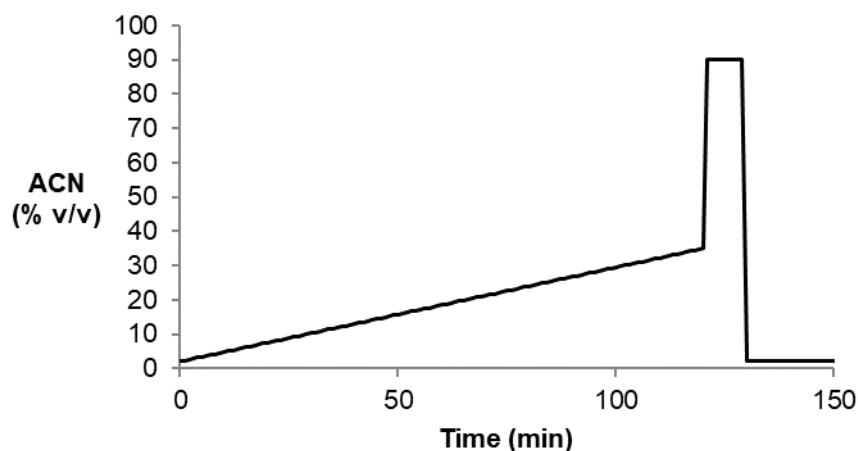


Figure 1 – Gradient of acetonitrile (ACN) performed for the elution of iTRAQ-labeled peptides in the reverse phase analytical column preceding the analysis on ESI-TripleTOF mass spectrometer.

4.5 MS data analysis

The analysis of MS data was performed using the proprietary ABSciex software, ProteinPilot 5. The MS/MS data were analyzed thanks to the UniProtKB database (database retrieved in 2007) using the “thorough ID” search option and the *S. cerevisiae* taxonomy. The “iTRAQ 8plex peptide labelled” sample type and “a biological modification ID focus” were selected in the analysis method. Trypsin was selected as the digestion enzyme, with cysteine alkylation by MMTS as a modification. Data were normalized by ProteinPilot algorithm. All reported data were based on 99% confidence for protein identification as determined by ProteinPilot (ProtScore ≥ 2). Protein identification confidence was expressed as the “Unused ProtScore”, which takes into account peptides from spectra that have not already been used to identify higher scoring proteins. Proteins were further annotated based on the yeast genome database (SGD; <http://www.yeastgenome.org>), UniProtKB (<http://www.uniprot.org>) and Organelle database (<http://labs.mcdb.lsa.umich.edu/organelledb/>).

The iTRAQ labelling method allows the relative quantification of the amount of a specific PM protein in various samples (up to eight at the same time). These differences are expressed by ratios. Ratios give the fold change of abundance of a given protein in a sample compared to another one. The fold change of a protein is calculated based on the relative amount of its constitutive peptides in the two considered samples. This information is obtained thanks to the intensity of peaks corresponding to iTRAQ reporter parts in the MS/MS spectra. The corresponding ProtScores, i.e. the quality of identification, of these peptides are considered for the protein quantification. Indeed, a peptide identified with more confidence contributes more to the protein quantification than a peptide with a low score. Each ratio is associated with a p-value, ensuring that it is significantly different from one or not. A statistical hypothesis test is carried out by the quantification software, in which the null hypothesis is that the ratio is equal to one. The p-value gives the probability that the test concludes that the null hypothesis is false although it is true, i.e. the chance that the test makes an error. The p-value should be compared to a probability threshold that represents the accepted maximal probability of error. In our study, the chosen probability threshold is 5%. Therefore, if the p-value of a ratio is lower than 0.05, the software concludes that the ratio differs significantly from one with at least 95% confidence. Final ratios for each identified protein were calculated using the geometrical mean of ratios obtained in three independent biological repetitions. Only proteins that have been identified twice on the three experiments were conserved in the result.

5 Total protein extraction

Yeast cells were grown at 28°C to an OD_{600nm} of 0.5 (about 2 x 10⁶ cells/ml) in 5 to 50 ml of their corresponding medium. Endocytosis was triggered by addition of either cycloheximide (at a final concentration of 50 µg/ml) or thiamine (at a final concentration of 100 µM) to the culture. After 2 h or 3 h

incubation, cells were harvested by centrifugation at 3,000 *g* for 5 min at 4°C, washed with 1 ml cold distilled water and resuspended in 200 µl homogenization buffer (250 mM sorbitol, 50 mM imidazole, 0.2 g/l MgCl₂.6H₂O, adjusted at pH 7.5 with HCl, supplemented with 1 mM PMSF, 0.1% (w/v) cocktail of protease inhibitor). In the ubiquitylation study of thiamine transporters, a deubiquitinase inhibitor, N-ethylmaleimide (NEM), was added to the homogenization buffer at a final concentration of 10 mM. Cells were lysed by mixing samples on a vortexer with glass beads eight times for 30 s, each time separated by a break of 30 s on ice. Cells debris were eliminated by centrifugation at 1,000 *g* for 1 min at 4°C in a microcentrifuge. A volume of 100 µl of supernatant was mixed with an equal volume of Laemmli buffer (100 mM Tris-HCl, pH 6.8, 4 mM EDTA, 4%(w/v) SDS, 20% (w/v) glycerol, 0.002% (w/v) bromophenol blue, 1% (w/v) DTT) for immunoblotting. The remaining volume of supernatant was used to determine the protein concentration. Samples were stored at -20°C.

For bigger culture volumes (500 mL to 2 L), total protein extraction was performed according to the first step of the PM enrichment protocol.

6 Immunoblotting

Twenty µg of protein from each sample were mixed with an equal volume of Laemmli buffer (with 1% DTT). Then, proteins were separated by SDS-PAGE and transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore) using a semi-dry transfer system (Bio-Rad) in 50 mM Tris, 40 mM glycine, 0.0375% SDS, and 20% methanol (pH 9.2 with NaOH). In some experiments, proteins were separated using SDS-PAGE mini-PROTEAN precast gels (Bio-Rad) and transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore) using the Trans-Blot Turbo Transfer System (Bio-Rad). The blot was saturated overnight at 4°C using 3% low fat dried milk in Tris-buffered saline (TBS; 50 mM Tris, 150 mM NaCl, adjusted at pH 7.6 with HCl)

supplemented with 0.5 % Tween 20 (TBS-T). The saturated PVDF membrane was incubated with antibodies (Table 3) at room temperature for a given time according to the targeted protein. Primary antibodies were diluted in incubation buffer (0.5% low fat dried milk-containing TBS-T). Membranes were then washed with TBS-T and incubated for 60 min with the corresponding horseradish peroxidase-coupled secondary antibody 10,000 times diluted in the same incubation buffer. Membranes were then washed and incubated with the chemiluminescent substrate (Roche). Chemiluminescence was captured using either KODAK 4000R Image Station or Amersham Imager 600 (GE Healthcare). Signals were quantified using the ImageJ package, Fiji (Schindelin et al., 2012).

Table 3 – List of the antibodies used in this study.

Antibody	Dilution	Incubation time	Origin	Source
α -Pma1	1:4,000	60 min	rabbit	Our laboratory
α -GFP	1:4,000	120 min	mouse	Roche
α -HA	1:1,000	120 min	rat	Roche
α -Ubiquitin	1:1,1000	120 min	mouse	Thermo Fisher Scientific
α -FLAG	1:1,1000	120 min	mouse	Sigma-Aldrich

7 Phenotypic growth tests

7.1 Phenotypic growth tests on thiamine-containing medium

Transformed CVY4 strains were grown overnight in SC-U liquid medium. The cultures were then diluted to reach an OD_{600nm} of 0.7 after two doubling times. After two doubling times, cells were harvested by centrifugation and resuspended with SC-U-B1 liquid medium to exactly adjust the OD_{600nm} at 0.7. Then, 10-fold serial dilutions were spotted onto SC-U-B1 solid medium containing thiamine at different concentrations.

7.2 Phenotypic growth tests on oxythiamine-containing medium

Transformed CVY3 strains were grown overnight in SC-U-B1 liquid medium. The cultures were then diluted to reach an OD_{600nm} of 0.7 after two doubling times. After two doubling times, cultures were diluted with SC-U-B1 to adjust the OD_{600nm} at 0.7. Then, four 10-fold serial dilutions were spotted onto SC-U-B1 solid medium containing oxythiamine (Sigma-Aldrich, Merck) at different concentrations.

8 Fluorescence microscopy

Yeast strains were grown at 28°C to an OD_{600nm} of 0.5 (2×10^6 cells/ml) in the corresponding medium. For each strain, cultures were made in duplicate. Endocytosis was triggered in one culture using thiamine (at a final concentration of 100 µM) or CHX (50 µg/ml). The other replicate was mock-treated with either water or 100% ethanol. After 90 and 180 minutes, 5 µl of culture were dropped on a microscope slide covered with agarose 1.5% (w/v). Microscopy was performed using the Leica DMR microscope equipped with a 100X oil-immersion objective (PLAN APO) and an Orca-ER digital camera (Hamamatsu). Cells were visualized at room temperature, using a GFP filter (excitation wavelength: 450-490 nm; emission wavelength: 500-550 nm). For each sample, a corresponding Nomarski (Differential Interference Contrast) picture was also taken. Images were processed with HOKAWO! Imaging pf07 2.1 software.

9 Immunoprecipitation

Yeast cells were grown until reaching an OD_{600nm} of approximately 1 (4×10^6 cells/ml) in 500 ml to 1 l of the corresponding medium. Total protein extraction was performed as described in Section 5 but using a Triton X-100 containing

breaking buffer (1% Triton X-100, 50 mM Tris, 150 mM NaCl, adjusted at pH 7.4 with HCl), supplemented with 1 mM PMSF and 0.1% of the protease inhibitor cocktail. Protein concentration of the extracts was quantified by the bicinchoninic acid assay. Total protein extracts were incubated with n-Dodecyl β -D-maltoside (DDM) at a detergent:protein ratio of 2:1 (w/w) for 3 h at 4°C in order to solubilize thiamine transporters. After centrifugation, the pellet was kept to test whether transporters were well solubilized. The supernatant was recovered, and immunoprecipitation was then carried out according to GFP-trap manufacturer's protocol (Chromotek). Equilibration and washing buffers have the same composition as the breaking buffer and were also supplemented with 1 mM PMSF, 0.1% of the protease inhibitor cocktail and DDM. DDM was added at the same concentration as for solubilization step in the equilibration buffer and 10 times diluted in the washing buffer. A volume of 25 μ l of GFP-Trap beads were mixed with 500 μ l of equilibration buffer and separated from the supernatant, which was discarded. The protein lysate was added to the equilibrated beads and incubated 2 h at 4°C with agitation. Beads were harvested and the supernatant (the flow-through) kept for immunoblotting. Beads were washed five times in 500 μ l of washing buffer. Elution was carried out using a 0.1 M glycine solution at pH 2.5, supplemented with 1 mM PMSF, 0.1% of the protease inhibitor cocktail and DDM (10 times diluted compared to the solubilization step). The eluate was then quickly neutralized with 5 μ l of 1 M Tris-containing neutralization solution.

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