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The arrestin-like protein ArtA is essential for ubiquitination and endocytosis of the UapA transporter in response to both broad-range and specific signals

Mayia Karachaliou,† Sotiris Amillis,† Minoas Evangelinos, Alexandros C. Kokotos, Vassilis Yalelis and George Diallinas*
Faculty of Biology, University of Athens, Panepistimiopolis 15784, Athens, Greece.

Summary

We investigated the role of all arrestin-like proteins of *Aspergillus nidulans* in respect to growth, morphology, sensitivity to drugs and specifically for the endocytosis and turnover of the uric acid-xanthine transporter UapA. A single arrestin-like protein, ArtA, is essential for HulA^{Rsp5}-dependent ubiquitination and endocytosis of UapA in response to ammonium or substrates. Mutational analysis showed that residues 545–563 of the UapA C-terminal region are required for efficient UapA endocytosis, whereas the N-terminal region (residues 2–123) and both PPxY motives are essential for ArtA function. We further show that ArtA undergoes HulA-dependent ubiquitination at residue Lys-343 and that this modification is critical for UapA ubiquitination and endocytosis. Lastly, we show that ArtA is essential for vacuolar turnover of transporters specific for purines (AzgA) or L-proline (PrnB), but not for an aspartate/glutamate transporter (AgtA). Our results are discussed within the frame of recently proposed mechanisms on how arrestin-like proteins are activated and recruited for ubiquitination of transporters in response to broad range signals, but also put the basis for understanding how arrestin-like proteins, such as ArtA, regulate the turnover of a specific transporter in the presence of its substrates.

Introduction

Plasma membrane transporters constitute primary targets of cellular regulatory circuits controlling cell communication and signalling (Dupré *et al.*, 2004; Sorkin and von Zastrow, 2009). Most transporters traffic to the plasma membrane embedded in exocytic vesicles, but under certain physiological conditions, stress stimuli, or in response to development signals, they can be re-routed to the vacuole/lysosome for degradation, either directly or through the MVB pathway (late endosome), or recycle between the Golgi, the endosome and the plasma membrane (Dupré *et al.*, 2004; Sorkin and von Zastrow, 2009; Foley *et al.*, 2011). Signals triggering transporter endocytosis include shifts in the nitrogen or carbon source availability of the growth medium, stress or the presence of excess substrate, (Hicke and Dunn, 2003; Dupré *et al.*, 2004; Sorkin and von Zastrow, 2009). Transporter endocytosis, recycling and direct sorting into the MVB/vacuolar pathway depend on alternating cycles of different types of ubiquitination and deubiquitination, named the 'ubiquitin code' (Belgareh-Touzé *et al.*, 2008; Risinger and Kaiser, 2008; Lauwers *et al.*, 2010).

In *S. cerevisiae*, in response to various physiological signals, several plasma membrane transporters are ubiquitinated by the HECT domain E3 ligase Rsp5 and subsequently removed from the cell surface, or directly diverted from the Golgi to the endovacuolar system (Hicke and Dunn, 2003; Dupré *et al.*, 2004; André and Haguenauer-Tsapis, 2004; Risinger *et al.*, 2006; Rubio-Texeira and Kaiser, 2006; Cain and Kaiser, 2011). Recent studies have contributed in the understanding of how Rsp5 recognizes a wide variety of substrates under various physiological signals. Rsp5 contains three WW domains, which recognize PY motives with the typical sequence PPxY or LPxY. Several adaptor proteins containing such motives have been shown to facilitate the ubiquitination of particular proteins or sets of proteins (Léon and Haguenauer-Tsapis, 2009). These adaptors include the membrane proteins Bsd2 (Hettema *et al.*, 2004), Tre1/2 (Stimpson *et al.*, 2006), Ear1 and Ssh4 (Léon *et al.*, 2008) or members of a family of soluble α-arrestins or arrestin-like proteins (Lin *et al.*, 2008; Léon and Haguenauer-Tsapis, 2009; Nikko and Pelham, 2009; Nikko *et al.*, 2009; Hatakeyama *et al.*, 2010; O' Donnell *et al.*, 2010; MacGurn *et al.*, 2011; Becuwe *et al.*, 2012) and their distant homologues Bul1 and Bul2 (Helliwell *et al.*, 2001; Soetens *et al.*, 2001; Merhi and André, 2012). All yeast α-arrestins, including Bul1 and Bul2, have been studied

Accepted 12 February, 2013. *For correspondence. E-mail diallina@biol.uoa.gr; Tel. (+30) 21 0727 4649, Fax (+30) 21 0727 4702. †These authors contributed equally.

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systematically in respect to their role on the ubiquitination and endocytosis of several transporters and the general model emerging is that different arrestin-like proteins recognize different transporters, or the same transporter in response to different stimuli.

Recently, three reports (MacGurn *et al.*, 2011; Becuwe *et al.*, 2012; Merhi and André, 2012) put the basis on how arrestin-like proteins are post-translationally regulated in response to nutrient signalling. In the absence of preferred carbon or nitrogen sources, arrestin-like proteins Art4/Rod1, Bul1/2 or Art1, which control the ubiquitination and turnover of the acetate transporter Jen1p, the arginine transporter Can1p or the general amino acid permease Gap1p, respectively, are phosphorylated and remain inactive. In the case of Art4/Rod1 and Bul1/2, it was shown that under such poor nitrogen conditions the relevant arrestin-like proteins bind to 14-3-3 proteins, which inhibit their capacity to elicit Jen1p or Gap1p downregulation. Upon a shift to rich carbon or nitrogen sources, Art4/Rod1, Art1 or Bul1/2 are dephosphorylated, probably released from 14-3-3 proteins and recruited for catalysing the ubiquitination of Jen1p, Can1p or Gap1p respectively. In the case of the Art1 and Bul1/2, phosphorylation of the arrestin adaptors involves the Npr1 kinase, which is itself negatively regulated by the TOR pathway (MacGurn *et al.*, 2011), whereas in the case of Art4/Rod1, the AMPK homologue Snf1 seems to be implicated. Still, another arrestin-like protein, Aly2/Art3, which might localize in the endosomes, was recently found to be phosphorylated by Npr1 (Hatakeyama *et al.*, 2010). In the case of Bul1/2 and Art4, dephosphorylation of the arrestin-like proteins depends on the Sit4 and the PP1 phosphatases Glc7/Reg1 respectively.

Another aspect of the emerging mechanism underlying the control of transporter ubiquitination by specific arrestin-like protein adaptors is that the arrestins themselves are ubiquitinated, and this seems to be part of the mechanism regulating their action. In all cases tested (Art1, Art2, Art3/Aly2, Art4/Rod1, Art8/Rim8 and Art9), arrestin-like protein ubiquitination is Rsp5-dependent and essential for their function (Kee *et al.*, 2006; Nikko *et al.*, 2009; Hatakeyama *et al.*, 2010; Herrador *et al.*, 2010; MacGurn *et al.*, 2011; Becuwe *et al.*, 2012; Merhi and André, 2012; O'Donnell, 2012). Ubiquitination of PalF, an arrestin-like protein involved in pH sensing in the filamentous ascomycete *Aspergillus nidulans*, has been proposed to be the sole molecular trigger required for transmitting the alkaline pH signal to the downstream elements of the pathway (Hervás-Aguilar *et al.*, 2010). Studies on Art1, Art4/Rod1 and Bul1 revealed that this ubiquitination is required for proper permease downregulation and that there seems to be a cross-talk between the phosphorylation–dephosporylation status and the ubiquitination levels of arrestin-like proteins, but the mechanisms controlling the ubiquitination of Art proteins and its exact role in transporter downregulation remain poorly known.

In some cases, specific transporters appear unaffected in single arrestin-like protein mutants, possibly because of functional redundancy of the arrestins (Léon and Haguenauer-Tsapis, 2009; Nikko and Pelham, 2009). In addition, it seems that not all α-arrestins regulate endocytosis, as described in a recent study, where two arrestin-like proteins, Aly1 and Aly2, regulate intracellular trafficking of the general amino acid permease Gap1 (O' Donnell *et al.*, 2010). Notably, Aly1 and Aly2 co-purify with clathrin and clathrin-adaptor protein (AP) complexes (McMahon and Boucrot, 2011) *in vivo* and interact directly with the γ-subunit of AP-1 *in vitro*, suggesting that, like their β-arrestin relatives (Goodman *et al.*, 1996), α-arrestins promote cargo incorporation into clathrin-coated vesicles (O' Donnell *et al.*, 2010).

Our lab has used the extensively studied uric acid-xanthine transporter UapA (reviewed in Diallinas and Gournas, 2008; Gournas *et al.*, 2008; Amillis *et al.*, 2011; Kosti *et al.*, 2011) of *A. nidulans* to approach questions concerning the mechanisms underlying endocytosis, triggered by ammonium or excess substrate (Gournas *et al.*, 2010). We have shown that either ammonium or substrates elicit the ubiquitination, by the HulA^{Rsp5} E3 ligase, of a single Lys residue (Lys-572) in the C-terminal region of plasma membrane-localized UapA. Ubiquitinated UapA is internalized and is directed to the MVB/vacuolar pathway for degradation. We further showed that ammonium- and substrate-triggered UapA endocytosis recruit or activate distinct mechanisms, since the latter, unlike ammonium-elicited internalization, operates only for transport-active molecules. Using UapA mutants with modified function or altered substrate affinities and/or specificities, we showed that transport-dependent UapA endocytosis occurs through a mechanism which senses subtle conformational changes associated with the transport cycle (Gournas *et al.*, 2010). Interestingly, we have also demonstrated that in the presence of substrates, non-functional UapA versions can be endocytosed *in trans* if expressed in the simultaneous presence of active UapA versions, a result that suggests that UapA oligomerizes (Gournas *et al.*, 2010).

In this work we systematically knock-out all arrestin-like genes of *A. nidulans* and identify a single protein (ArtA) as being essential for the HulA^{Rsp5}-dependent ubiquitination and subsequent endocytosis of UapA in response to apparently different signals. We provide strong evidence that ArtA interacts with a C-terminal region of UapA and show that the ArtA N-terminal region and both PPxY motives are necessary for its function. Furthermore, we show that ArtA is itself ubiquitinated at a single Lys residue and that ArtA ubiquitination is critical for UapA endocyto-

sis. Finally, we show that ArtA is specific for the turnover of some transporters but not of others. Our results are discussed in relation to how a single arrestin-like protein, ArtA, recognizes different substrates in response to broad-range or/and specific signals.

Results

Identification and in silico *analysis of genes encoding arrestin-like proteins in* A. nidulans

We investigated whether arrestin-like proteins have an analogous role in *A. nidulans* and if so, which proteins are specific for selected transporters and under which conditions. In this direction, we wanted to identify the arrestin-like protein(s) responsible for UapA endocytosis in response to different signals. A BlastP analysis showed that *A. nidulans* has 10 genes coding for putative arrestin-like proteins, most of which contain PY elements (see Fig. 1B). Three of them, *palF*, *creD* and *apyA*, have been previously described. PalF (gene: ANID_01844.1) is a positive-acting arrestin-like protein which, together with the seven-transmembrane receptor PalH, acts as a key molecular sensor that mediates activation of an intracellular signalling cascade by alkaline ambient pH in *A. nidulans* and other ascomycete fungi (Herranz *et al.*, 2005; Hervás-Aguilar *et al.*, 2010). PalF ubiquitination suffices to trigger alkaline pH signalling to downstream elements of the pathway (Hervás-Aguilar *et al.*, 2010). The *creD* gene (ANID_04170.1) has been genetically defined by a mutation (*creD34*) that suppresses the phenotypic effects of mutations in *creC* and *creB*, two genes encoding a de-ubiquitinating enzyme and a WD40-motif-containing protein, respectively, which form a complex essential for carbon catabolite regulation (Boase and Kelly, 2004). Finally, the *apyA* gene (ANID_03265.1) has been recognized as an arrestin-like protein through BlastP analysis, but its physiological role has not been studied. CreD and ApyA have been shown by a bacterial two-hybrid system to interact with the HulA ubiquitin ligase (Boase and Kelly, 2004). Seven more arrestin-like genes were identified herein and named *artA* (ANID_00056.1), *artB* (ANID_01089.1), *artC* (ANID_01743.1), *artD* (ANID_09105.1), *artE* (ANID_02447.1), *artF* (ANID_03302.1), *artG* (ANID_05453.1). The genomes of other Aspergilli have 7–12 arrestin-like proteins (http://www.broadinstitute.org/annotation/genome/aspergillus_group/).

We compared the *A. nidulans* arrestin-like proteins with the arrestin-like proteins of *S. cerevisiae* and among themselves (Tables S2 and S3). ArtA is significantly more similar to the Art1p/Ldb19p/Cvs7p (21.4% identity) than to any other arrestin-like protein of *S. cerevisiae*. Art1p is an arrestin-like protein that is necessary for the endocytosis of several nitrogen-containing compounds, such as amino acids and uracil (Lin *et al.*, 2008; Léon and Haguenauer-Tsapis, 2009; Nikko *et al.*, 2009; Nikko and Pelham, 2009; MacGurn *et al.*, 2011). CreD is mostly similar to Art4p/Rod1p and Art7p/Rog3p (24.1–26.7% identity), the former being involved in the endocytosis of the glucose transporter Htx6p and of the lactate permease Jen1 (Nikko and Pelham, 2009; Becuwe *et al.*, 2012). The remaining Art proteins of *A. nidulans* share less clear-cut similarities with the *S. cerevisiae* arrestin-like proteins (identities up to 19.1%). *A. nidulans* arrestin-like proteins share low similarity among themselves (< 18.9%), with a single exception being CreD and ApyA (26.3% identity). This contrasts the case in *S. cerevisiae*, where six out of the ten arrestin-like proteins can be classified in pairs (Art2-Art8, Art3-Art6, Art4-Art7), an indication of redundancy due to relatively recent duplication events. Thus, *A. nidulans* might prove to employ arrestin-like proteins in processes not present in yeasts.

Construction and phenotypic analysis of null mutants of genes encoding arrestin-like proteins

Using a standard gene knock-out procedure (see *Experimental procedures*), we constructed knock-out alleles of nine genes encoding arrestin-like proteins (*artA, artB, artC, artD, artE, artF, artG, apyA, creD*). The knock-out mutant of the tenth arrestin-like protein, *palF*Δ, was a gift from Prof. H. Arst. All knock-out null mutants were viable and could thus be tested directly for their morphology and rate of growth in different temperatures (25°C and 37°C), pH values, nitrogen or carbon sources and toxic analogues of purines, pyrimidines and amino acids. Highlights of this analysis are shown in Fig. 1A. Increased sensitivity towards toxic compounds has been used to identify arrestin-like genes in *S. cerevisiae* (Lin *et al.*, 2008; Nikko *et al.*, 2009). Among the ten arrestin-like protein knock-outs, *artE*Δ showed an inability to produce coloured asexual conidiospores decorating the surface of the colony. Several of the knock-out mutants showed different growth rates on various nitrogen or carbon sources and especially in respect to resistance or sensitivity to the toxic analogues tested.

In regard to UapA, which is the primary subject of this work, *artA*Δ showed increased sensitivity to allopurinol, a well established substrate of this transporter (Diallinas and Scazzocchio, 1989). *artA*Δ also showed increased sensitivity to 8-azaguanine, a substrate of the AzgA purine transporter (Cecchetto *et al.*, 2004). As will be shown below, ArtA is indeed responsible for the endocytic turnover of both UapA and AzgA, in full accordance with the increased sensitivity observed for the *artA*Δ mutant to allopurinol and 8-azaguanine (see Fig. 1A).

Based on the results shown in Fig. 1A, we also predicted possible relationships between arrestin-like

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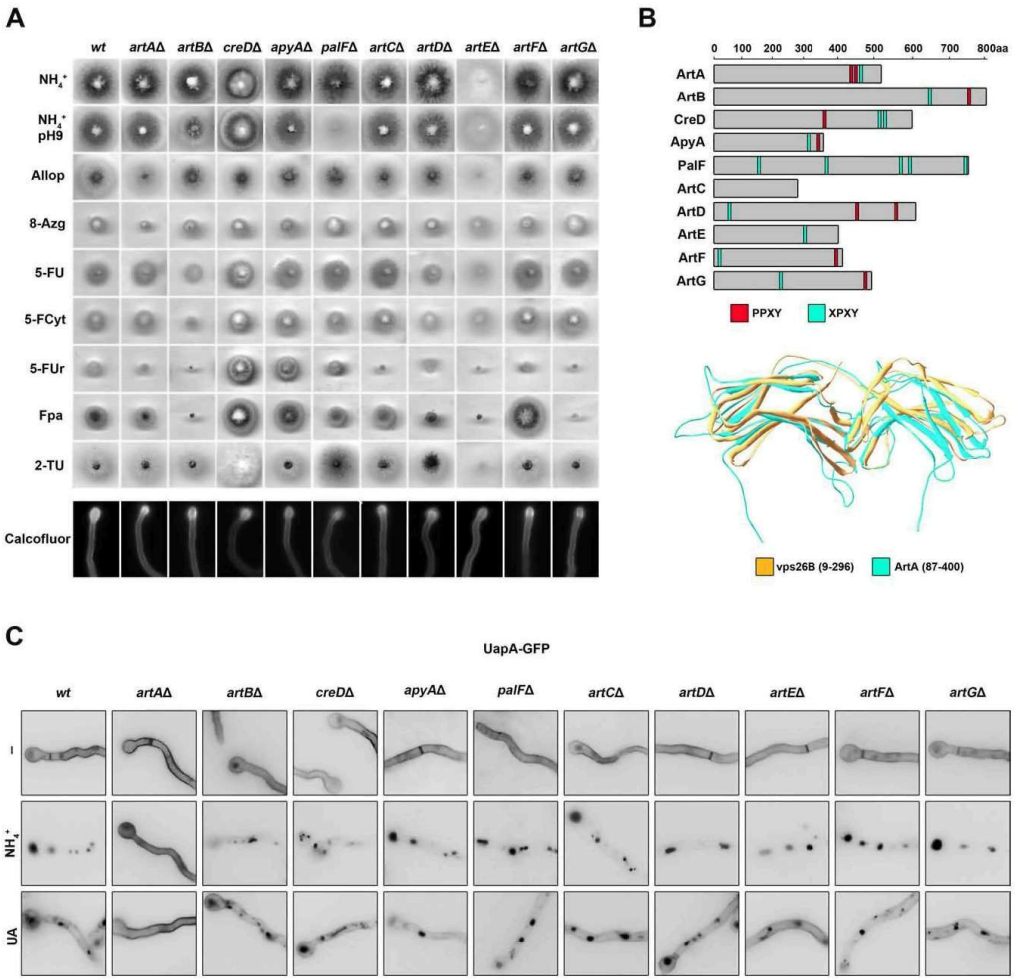

Fig. 1. Arrestin-like proteins in *A. nidulans*.

A. Growth phenotypes of arrestin null mutants. Complete genotypes are shown in Table S1. Supplemented minimal medium (MM) with 1% glucose as carbon source and 10 mM ammonium tartrate (NH_4^+) as nitrogen source was used as a growth rate control. Supplemented glucose MM with 10 mM sodium nitrate as nitrogen source was used with each of the following toxic analogues: allopurinol (Allop), 8-azaguanine (8-Azg), 5-fluorouracil (5-FU), 5-fluorocytosine (5-FCyt), 5-fluorouridine (5-FUr), p-fluorophenylalanine (FPA), 2-thiourea (2-TU). Growth tests were at 37°C and pH 6.8. In the lowest panel, vegetative microscopic samples of hyphal cells growing on MM with glucose as carbon source and ammonium tartrate as nitrogen source (16 h at 25°C) are shown after staining with Calcofluor white.

B. Upper panel: schematical representation of the actual positions of putative PY elements in the *A. nidulans* arrestin-like protein sequences. Noticeably, ArtC has no canonical PY elements. Lower panel: superimposition of the ArtA predicted structure, modelled on the crystal structure of the mouse vacuolar protein sorting-associated protein Vps26B (2r51_A), obtained from the RCSB PDB Protein Data Bank (http://www.pdb.org/pdb/home/home.do) and plotted with the SwissPdbViewer 4.0.1 software.

C. UapA subcellular localization in arrestin-like protein null mutants. Epifluorescence microscopy of UapA-GFP subcellular localization under non-endocytic (–) or endocytic conditions [NH_4^+ or uric acid (UA)] in isogenic arrestin-like protein null mutants. Growth conditions are described in *Experimental procedures*.

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proteins and different transporters, summarized in Table S4. For example, the FurD (Amillis *et al.*, 2007) and FcyB (Krypotou *et al.*, 2012) nucleobase transporters, which belong to the NCS1 family (Pantazopoulou and Diallinas, 2007), might interact with arrestin-like proteins ArtB and ArtD, a conclusion based on the increased sensitivity of *artB*Δ and *artD*Δ mutants to 5-fluorouracil (5-FU) or 5-fluorocytosine (5-FC), respectively. The resistance/sensitivity phenotypes on p-fluorophenylalanine (FPA) and 5-fluoruridine (5-FUd) or 2-thiourea (2-TU), which very probably reflect the apparent transport activities of a putative general amino acid permease, the unique *A. nidulans* nucleoside transporter CntA (Hamari *et al.*, 2009) and the major urea transporter UreA (Abreu *et al.*, 2010), respectively, seem to be affected by several arrestin-like proteins. It is also noticeable that *creD*Δ, *palF*Δ and mostly the *artB*Δ mutants show pleiotropic phenotypes. Furthermore, in some cases, some transporters seem to be affected negatively or positively by different arrestin knock-out mutations, such as the nucleoside transporter CntA. A similar situation has been observed before in *S. cerevisiae* and might be explained by the hypothesis that increased accumulation of some transporters might indirectly lead to reduced translocation of other transporters to the plasma membrane (Lin *et al.*, 2008). Finally, none of the arrestin-like protein knock-outs showed altered polar growth or hyphal morphology (Fig. 1A lowest panel).

A single arrestin-like protein, ArtA, is necessary for UapA endocytosis and vacuolar turnover in response to ammonium or excess substrate

In order to investigate the role of all arrestin-like proteins in the endocytosis and/or MVB sorting of UapA, we crossed all relevant null mutants with a strain expressing a fully functional UapA-GFP version from its endogenous promoter (Gournas *et al.*, 2010). The strain expressing UapA-GFP was deleted for the homologous *uapC* gene, encoding a secondary uric acid/xanthine transporter (Diallinas *et al.*, 1995), so that uric acid or xanthine uptake was solely mediated by UapA. Isogenic progeny was selected and analysed for UapA-GFP subcellular localization and endocytosis by epifluorescence microscopy. Results are summarized in Fig. 1C. None of the arrestin-like protein knock-out deletions had any effect on the expression or localization of UapA-GFP in the plasma membrane, visible in the hyphal periphery and in the septa, under control conditions. Under endocytic conditions, imposed by the presence of ammonium or excess substrate (uric acid), where UapA-GFP is normally internalized and sorted in MVBs/vacuoles (see wild-type control in Figs 1C and 2A), a single arrestin-like protein null mutant, *artA*Δ, showed no UapA-GFP vacuolar turnover. In all other arrestin-like protein knock-out deletion mutants UapA-GFP was turned-over similarly to the wild-type control in the presence of ammonium or excess substrate. Given that we have previously concluded that UapA-GFP vacuolar turnover occurs exclusively via endocytosis and not through direct delivery to the vacuole from the Golgi (Gournas *et al.*, 2010), our results strongly suggest that lack of a functional ArtA blocks UapA internalization from the plasma membrane.

To show more rigorously that ArtA controls UapA endocytosis and vacuolar turnover, we constructed *artA*$^+$ and *artA*Δ isogenic strains expressing UapA-GFP from the strong controllable $alcA_p$ promoter (Gournas *et al.*, 2010; for details see *Experimental procedures*). These strains lack the genomic copies of *uapA* and *uapC* (i.e. *uapA*Δ *uapC*Δ) so that uric acid or xanthine uptake takes place through the plasmid borne $alcA_p$-UapA-GFP, expressed solely under de-repressed conditions (fructose as sole carbon source). In the presence of glucose (repressing carbon source) no UapA-GFP expression or transport activity can be detected. Using the $alcA_p$ system had two advantages. First, we could uncouple ammonium-elicited repression of *uapA* transcription from UapA endocytic turnover (Pantazopoulou *et al.*, 2007), and second, we could regulate UapA *de novo* synthesis prior or after imposing endocytic conditions (Gournas *et al.*, 2010).

We examined the effect of ammonium or excess substrate into already synthesized UapA-GFP or to *de novo* made UapA-GFP in *artA*$^+$ and *artA*Δ isogenic strains. In the first case, $alcA_p$-UapA-GFP expression was induced (4–6 h) in the presence of fructose/ethanol, then repressed by addition of glucose (1 hour), prior to ammonium or substrate addition. In the second case, ammonium or substrate was added to cultures in which $alcA_p$-UapA-GFP expression was repressed by glucose, and then (> 30 min) UapA-GFP expression was induced by shifting the cells in fructose/ethanol (4–6 h). In both conditions the result was identical, showing that lack of a functional ArtA blocked UapA-GFP sorting into early endosomes and abolished vacuolar turnover (Fig. 2A). Early endosomes marked with UapA-GFP were identified by their unique bidirectional motility observed in an inverted microscope and colocalization with FM4-64, whereas vacuoles marked with UapA-GFP were identified by FM4-64 and CMAC (not shown). A Western blot analysis confirmed that under both endocytic conditions UapA-GFP vacuolar turnover is significantly reduced in the *artA*Δ mutant (Fig. 2B).

Further evidence for the involvement of ArtA in UapA turnover was obtained by direct transport assays with radiolabelled xanthine. Figure 2C shows that, under endocytic conditions (presence of ammonium), in the wild-type control (*artA*$^+$), the apparent xanthine uptake drops to 60%, whereas in the isogenic strain lacking ArtA (*artA*Δ) xanthine uptake remains close to 100%. We also obtained inde-

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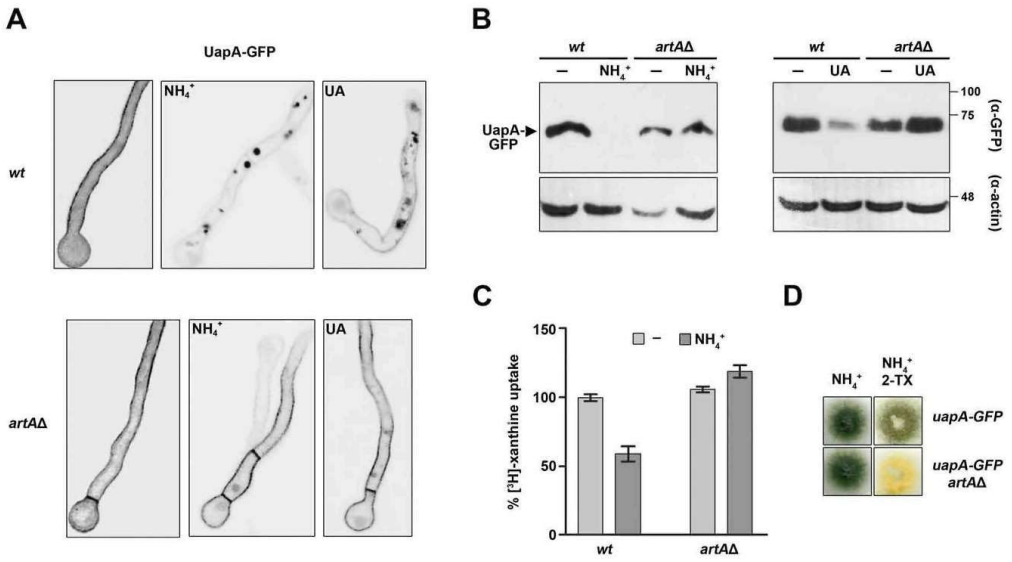

Fig. 2. ArtA is involved in UapA endocytosis and vacuolar turnover.
A. Confocal laser microscopy of UapA-GFP subcellular localization under non-endocytic (–) or endocytic conditions (NH_4^+ or UA) in isogenic *artA*$^+$ (wt) and *artA*Δ strains expressing UapA-GFP. Growth conditions are described in *Experimental procedures*.
B. Western blot analysis of total protein extracts from *artA*$^+$ (wt) and *artA*Δ strains, expressing UapA-GFP from the $alcA_p$, using anti-GFP antibody. Conditions were identical to (A).
C. Uptake rate of ^{3}H-xanthine in *artA*$^+$ (wt) and *artA*Δ strains under non-endocytic (–) or endocytic conditions (NH_4^+).
D. The 2-thioxanthine effect in the presence of NH_4^+ as nitrogen source in *artA*$^+$ (wt) and *artA*Δ strains (see text).

pendent *in vivo* evidence for an apparent increase in UapA activity in an *artA*Δ genetic background under endocytic conditions by a simple growth test using 2-thioxanthine. This xanthine analogue is taken-up by UapA and is metabolized to 2-thiouric acid, which inhibits a laccase necessary for the conversion of yellow to green pigment in conidiospores (Darlington and Scazzocchio, 1967). As a result, strains expressing UapA from its native promoter produce yellow conidiospores in media containing 2-thioxanthine and a non-repressing nitrogen source (e.g. nitrate, L-proline). In the presence of NH_4^+ however, UapA transcription is repressed and thus 2-thioxanthine is not taken up by the cells, and consequently conidiospores remain green. In media containing NH_4^+ as a nitrogen source, a strain expressing UapA from the $alcA_p$ promoter, which is not repressible by ammonium, shows a leaky phenotype (i.e. mixture of green and yellow spores), apparently due to NH_4^+-elicited UapA turnover by endocytosis. Figure 2D shows that in an *artA*Δ genetic background the effect of 2-thioxanthine is very strong (non-leaky appearance of yellow conidiospores) even in NH_4^+-containing media, strongly suggesting that lack of ArtA reduces dramatically the turnover of UapA by endocytosis.

ArtA is essential for UapA ubiquitination

We investigated whether ArtA is involved in the ubiquitination of UapA, as all evidence predicted. For that, we performed Western blot analyses under conditions inhibiting the rapid de-ubiquitination of cargoes (see *Experimental procedures*). Figure 3A shows that in the *artA*$^+$ strain the anti-GFP antibody detects a less motile form of UapA-GFP only after a relatively short shift in media containing NH_4^+ or substrate (uric acid), whereas in the isogenic *artA*Δ mutant such a form is not visible. Similar less motile UapA-GFP-specific molecules have been previously detected and shown to correspond to UapA-GFP/ubiquitin conjugates (Gournas *et al.*, 2010). To further confirm this, we purified UapA-His molecules, through Ni^{2+} affinity chromatography, expressed in isogenic strains *artA*$^+$ and *artA*Δ (see *Experimental procedures*) and the purified UapA-His fraction was immunoblotted with anti-His- and anti-ubiquitin-specific antibodies (Fig. 3B). Our results confirm that a functional ArtA is necessary for the formation of UapA-ubiquitin conjugates, similar to the need for a fully functional HulA ubiquitin ligase or the presence of Lys-572 in the tail of UapA (Gournas *et al.*, 2010).

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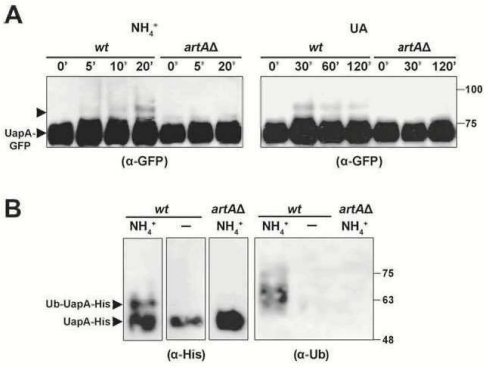

Fig. 3. ArtA is essential for UapA ubiquitination.
A. Western blot analysis of membrane-enriched protein extracts from *artA*$^+$ (wt) and *artA*Δ strains, expressing UapA-GFP from the *alcA*$_p$ using anti-GFP antibody, under conditions detecting ubiquitination of UapA (see *Experimental procedures*). Notice the ArtA-dependent appearance of less motile bands of UapA-GFP under endocytic conditions in membrane enriched fractions, which are not detected in the absence of a fully active HulA ubiquitin ligase (*hulA*Δ*C2*) or with a UapA mutant lacking Lys-572 (K572R) (Gournas *et al.*, 2010).
B. The less motile ArtA-dependent UapA-GFP signals can also be detected with anti-Ub antibody in purified UapA-His after 20 min growth in NH_4^+ (+).

The N-terminus and the PPxY motives are essential for ArtA function

We constructed, by standard oligonucleotide mutagenesis, a series of mutations to test the function of specific regions, motives or residues of ArtA. The mutations made were the following: (i) a deletion of residues 2–123 corresponding to the ArtA N-terminus, which contains several putative Ser phosphorylation sites that might have a regulatory role analogous to that found for Art1 (Lin *et al.*, 2008). (ii) Ala substitutions of the two canonical PPxY motives. (iii) Ala substitutions of two well conserved residues (Gly185 and Phe191) within the arrestin motif, which have been shown to be critical for Art1 function in *S. cerevisiae* (Lin *et al.*, 2008). ArtA mutations were inserted to the genomic *artA* locus in a strain expressing UapA-GFP, through standard reverse genetics (see *Experimental procedures*). Corresponding mutants were viable showing wild-type growth and morphology, as expected, given that the *artA*Δ mutant shows no mutant phenotype.

All mutants were analysed microscopically in respect to *alcA*$_p$-UapA-GFP endocytosis by ammonium or substrate. Figure 4A shows that Ala substitutions of Gly185 and Phe191 had no significant effect on UapA stimulus-elicited endocytosis, whereas deletion of the N-terminus or either one of the two PPxY motives (PY1 or PY2) totally blocked UapA endocytosis, similar to an *artA*Δ mutation. *In vivo* evidence supporting the functionally essential role of the two PPxY motives or the N-terminal region of ArtA was obtained using the 2-thioxanthine sensitivity test (not shown).

We obtained additional evidence that the PPxY motives are necessary and sufficient for HulA-dependent ubiquitination and subsequent turnover of UapA by constructing and analysing mutants expressing chimeric fusions of UapA with a conserved 38-amino-acid sequence of ArtA including the two PPxY motives, either in their wild-type (UapA-PY$_{wt}$) or in a mutated version (UapA-PY$_{ala}$). Figure 4B shows that a UapA-PY$_{wt}$ chimera is not functional (lack of growth on uric acid) due to constitutive targeting to the vacuole, whereas a UapA-PY$_{ala}$ chimera or UapA-PY$_{wt}$ chimera expressed in a *hulA*Δ*C2* background are functional (growth on uric acid), showing normal targeting to the plasma membrane.

The essentiality of the PPxY for ArtA-mediated UapA endocytosis was directly confirmed by Western blot analysis, which shows that, unlike the result obtained in *artA*$^+$ genetic background, UapA-GFP protein steady state levels were not reduced in the presence of either NH_4^+ or uric acid, an observation also associated with low level of UapA-GFP vacuolar turnover, similar to the level obtained under non-endocytic conditions, as judged by the low amount of free GFP detected (Fig. 4C). The requirement of the PPxY motives for HulA-dependent UapA ubiquitination was subsequently shown by an independent western analysis where no UapA-Ub conjugates could be detected in the strain expressing the *artA* allele mutated in its PPxY elements (Fig. 4D).

HulA-dependent ubiquitination of ArtA at Lys-343 is critical for ArtA function

We investigated whether ArtA itself is ubiquitinated and whether this has a role on UapA endocytosis. Figure 5A shows that anti-GFP antibody detects less motile forms of ArtA-GFP, which probably correspond to ArtA-ubiquitin conjugates. The steady state levels of ArtA-ubiquitin conjugates seemed moderately increased in response to ammonium, compared to control conditions or in response to substrates. The increase in ArtA ubiquitination levels in response to the presence of NH_4^+ for increasing periods of time was confirmed by quantitative measurements of the relative ratios of ArtA-Ub/ArtA (Fig. 5B). We subsequently showed that the less motile forms of ArtA-GFP, as expected, cross-react with anti-Ubiquitin antibody (Fig. 5C). Finally, we showed that ArtA ubiquitination requires an interaction with a fully functional HulA ligase, as judged by the non-appearance of ArtA-Ub forms in *hulA*Δ*C2* genetic background or when using an ArtA version mutated in its PPxY motives (see Fig. 5A).

Based on sequence alignments of ArtA and Art1, we predicted that Lys-343 might be the residue acting as an

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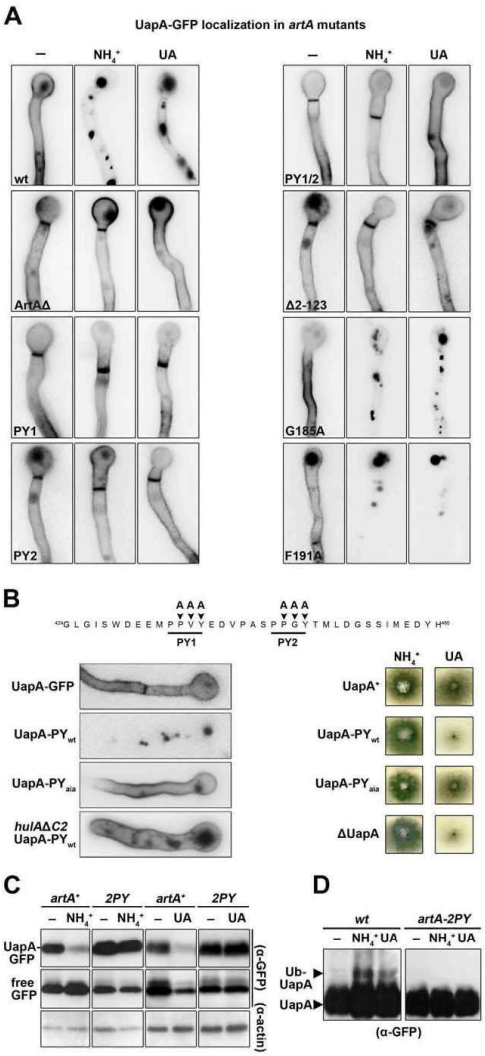

Fig. 4. The N-terminus and the PY motives are essential for ArtA function.
A. Epifluorescence microscopy of UapA-GFP subcellular localization under non-endocytic (–) or endocytic conditions (NH_4^+ or UA) in isogenic *artA* mutants expressing UapA-GFP driven under the *alcA* regulable promoter. PY1 stands for ArtA P435A/V436A/Y437A and PY2 for ArtA P445A/G446A/Y447A. Δ2–123 stands for the N-terminal truncation of amino acids 2–123. Growth conditions are described in *Experimental procedures*.
B. Epifluorescence microscopy of UapA-PY_{wt} and UapA-PY_{ala} chimeras under non-endocytic conditions in *hulA*$^+$ and in a *hulAΔC2* background, and UapA-mediated growth on UA as sole nitrogen source.
C. Western blot of total protein extracts of a wt (*artA*$^+$) and an *artA* mutant strain carrying both PY1 and PY2 substitutions (2PY), expressing UapA-GFP under non-endocytic (–) or in the presence of UA or NH_4^+ for 2 h.
D. Western blot analysis of UapA-GFP ubiquitination in membrane enriched fractions of a wt (ArtA$^+$) and an ArtA-2PY strain, grown under endocytic (30 min, NH_4^+) or control conditions.

acceptor of ubiquitination in ArtA. To test this, we constructed a strain expressing ArtA-K343-GFP. Results, also shown in Fig. 5A, confirm that Lys-343 is indeed the acceptor residue for ubiquitination. These results show that HulA-dependent ArtA ubiquitination at a single Lys residue occurs through the involvement of its PPxY elements, very probably through a direct interaction with the WW motives of HulA.

To investigate the role of ArtA ubiquitination, we constructed a strain expressing UapA-GFP in an ArtA-K343R genetic background. In this strain, mutation ArtA-K343R severely inhibited UapA endocytosis in response to both in NH_4^+ and substrates (Fig. 6A). Western blot analysis showed that ArtA-K343R is a practically loss-of function mutation in respect to UapA turnover, as intact UapA-GFP levels remain high under endocytic conditions. In addition the ratio of intact UapA-GFP/free vacuolar GFP is significantly higher to the ratio found in an *artA*$^+$ background (Fig. 6B). Notably, however, some UapA-GFP turnover was observed in the ArtA-K343R background, suggesting that ArtA ubiquitination is critical, but not absolutely essential for some UapA turnover. This observation was in agreement with a subsequent Western blot analysis showing that, although UapA ubiquitination is significantly reduced in an ArtA-K343R mutant, some minor fraction of UapA can still be ubiquitinated (Fig. 6C).

The C-tail of UapA contains a region essential for ArtA binding

Previous studies have shown that the UapA C-tail includes the single Lys residue (Lys-572) necessary for HulA-dependent ubiquitination under endocytic conditions (Gournas *et al.*, 2010). This suggested that ArtA might interact with the C-terminal region of UapA. To investigate this assumption, the C-terminal region of UapA was fused into the C-terminal region of AzgA, a purine transporter, which is fairly insensitive to NH_4^+ -triggered endocytosis (Pantazopoulou *et al.*, 2007), and the resulting chimeric molecule was used for testing whether the UapA C-terminal region confers ArtA-dependent internalization of AzgA. Results in Fig. 7A confirm that the UapA C-terminal region promotes enhanced ammonium-elicited AzgA endocytosis and that this phenomenon is dependent on a functional ArtA protein. This strongly suggested that the C-terminal region of UapA contains a domain necessary and sufficient for ArtA binding.

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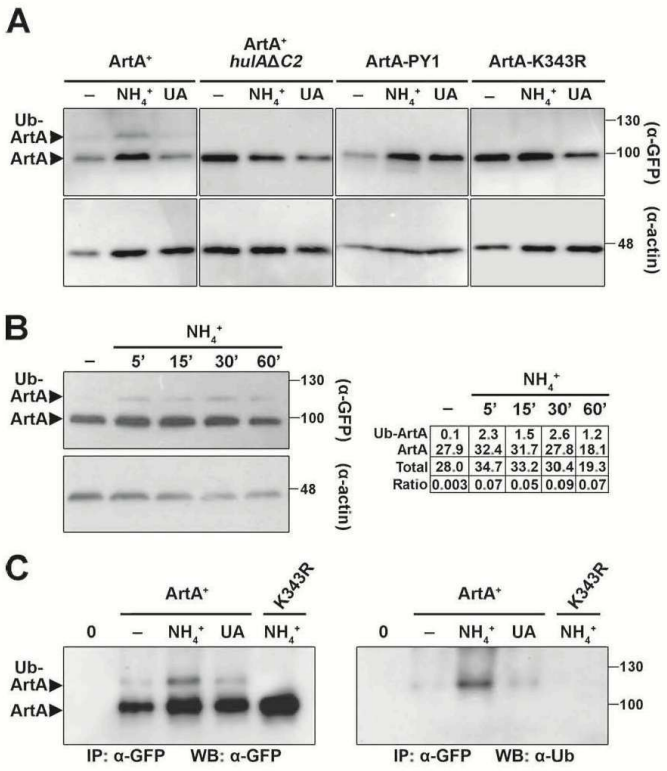

Fig. 5. HulA-dependent ubiquitination of ArtA at Lys-343 is critical for ArtA function.
A. Western blots of total protein extracts in isogenic wt, *hulAΔC2* and *artA* mutants PY1 and K343R under non-endocytic (–) or endocytic conditions (NH_4^+ or UA).
B. Time-course (left) and ImageJ semi-quantitative estimation (right) of NH_4^+-dependent increase in ArtA ubiquitination.
C. Western blots of immunoprecipitated ArtA-GFP and ArtA-K343R-GFP under denaturing conditions in the presence (60 min, NH_4^+ or substrate) or absence (–) of endocytic stimuli, probed with anti-GFP (left panel) or anti-ubiquitin (right panel) antibodies.

To further identify the region responsible for ArtA binding, we searched for UapA residues upstream from Lys-572, which might prove necessary for UapA endocytosis. For this, we constructed several Ala substitutions in the region 545–571 and two deletions corresponding to residues 564–571 and 547–571 respectively (see upper part of panel B in Fig. 7). Microscopic analysis of corresponding mutants showed that solely the longer deletion (residues 547–571) or Ala substitutions of a di-acidic motif (E^{545}-V-E^{547}) led to a severe block of ammonium- or substrate-elicited UapA endocytosis (Fig. 7B). Interestingly, di-acidic motives are known to be involved in membrane cargo trafficking and in particular in ER-exit or Golgi-to-vacuole transfer (Bonifacino and Traub, 2003; Renard *et al.*, 2010; Starr *et al.*, 2012), but are not known to interact with arrestin-like proteins or be related to ubiquitination of cargoes. In this direction, we showed that an intact E^{545}-V-E^{547} element was necessary for UapA-GFP ubiquitination, and thus might be part of a putative ArtA binding site on the C-tail of UapA (Fig. 7C). On the whole, our results showed that the region corresponding to residues 545–563 is required for UapA endocytosis, which in turn suggested that it might host the ArtA binding site.

The function of ArtA is a prerequisite for the formation of UapA-specific, SagA-dependent, pre-endocytic cortical puncta

Considering that ArtA is involved in ubiquitination of UapA and that this modification constitutes the molecular signal for UapA endocytosis, we tested whether the effect of the *artA*Δ mutation is epistatic to a mutation blocking endocytosis at a step downstream from cargo ubiquitination.

For this, we decided to knock-out SagA (ANID_01023.1), the single End3 homologue of *A. nidulans* (38% amino acid identity). In *S. cerevisiae*, End3p belongs to the family of proteins possessing an EH domain, members of which are implicated in endocytosis, vesicle transport, and signal transduction. End3p is part of the coat module protein complex along with

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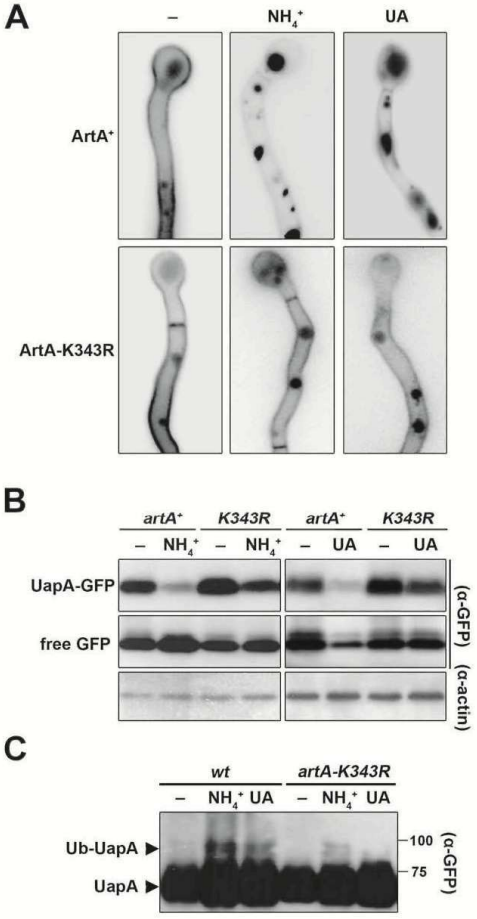

Fig. 6. Role of ArtA ubiquitination in UapA endocysis and turnover.
A. Epifluorescence microscopy of UapA-GFP subcellular localization under non-endocytic (–) or endocytic conditions (NH_4^+ or UA) in a wt or an ArtA-K343R mutant expressing UapA-GFP.
B. Western blot of total protein extracts of a wt (*artA*$^+$) and ArtA-K343R mutant strain, expressing UapA-GFP under non-endocytic (–) or in the presence of UA or NH_4^+ for 2 h.
C. Western blot analysis of UapA-GFP ubiquitination in membrane enriched fractions of a wt (ArtA$^+$) and an ArtA-K343R strain, grown under endocytic (30 min, NH_4^+) or control conditions.

Pan1p, Sla1p and Sla2p, otherwise known as the Pan1 complex, which acts downstream of cargo ubiquitination, but upstream of actin organization at endocytic sites (Tang *et al.*, 2000). Furthermore, End3p has been shown to be necessary for the internalization of all transporters tested up-to date. The *sagA* gene has been genetically identified as a gene that only affects sensitivity to DNA-damaging agents (Jones *et al.*, 1999). An apparent loss-of-function mutation in *sagA* has no detectable mutant phenotype, other than an increase in DNA alkylating agent sensitivity. A knock-out *sagA* mutant constructed for this work (see *Experimental procedures*) has a moderately delayed rate of growth, increased resistance to neomycin and enhanced frequency of bipolar emergence of germ tubes (Fig. S1A). Finally, a functional GFP-tagged SagA protein shows punctuate cortical subcellular localization (Fig. S1B), typical of other endocytic markers (Araujo-Bazán *et al.*, 2008).

We compared UapA-GFP expression in *artA*Δ, *sagA*Δ or *artA*Δ*sagA*Δ null mutants by constructing the appropriate isogenic strains (see *Experimental procedures*). Figure 8A shows that upon imposing an endocytic signal, either by NH_4^+ or substrates, there was a clear difference in the plasma membrane localization of UapA-GFP in the wild-type and in *artA*Δ, *sagA*Δ or *artA*Δ*sagA*Δ mutant backgrounds. In wild-type, as expected, UapA-GFP was internalized into mobile structures, apparently early endosomes, and sorted to the MVB/vacuole for degradation. As a consequence the amount of UapA-GFP remaining in the plasma membrane was reduced. In the *artA*Δ mutant UapA-GFP remained stable in the plasma membrane, marking the periphery of cells in a relatively homogeneous manner, similar to the picture obtained in all three strains under non-endocytic conditions. In the *sagA*Δ mutant, under endocytic conditions, UapA-GFP remained largely in or close to the plasma membrane, but in contrast to *artA*Δ, it also formed very distinctive cortical foci. Using an inverted fluorescent microscope we noticed that these puncta, which are very probably pre-endocytic membrane invaginations, are relatively static and remain attached to the plasma membrane, in mark contrast to the mobile early endosomes, seen in the wild-type strain. In the double mutant *artA*Δ *sagA*Δ, UapA-GFP remained stable in the plasma membrane, without forming cortical patches, similar to the single *artA*Δ mutant. This result strongly suggested that ArtA is implicated in UapA endocytosis at a step taking place in the plasma membrane, upstream of the action of SagA and the formation of pre-endocytic invaginations containing UapA-GFP.

To further confirm the above idea, we also tested whether blocking UapA ubiquitination by mutation K572R would have an effect on the formation of SagA-dependent, UapA-GFP-specific pre-endocytic invaginations. Figure 8B shows that blocking UapA ubiquitination also blocked the formation of pre-endocytic invaginations containing UapA-GFP in the *sagA*Δ background. Our results confirm that UapA ubiquitination takes place in the plasma membrane rather than in an early endosomal compartment, such as early endosomes.

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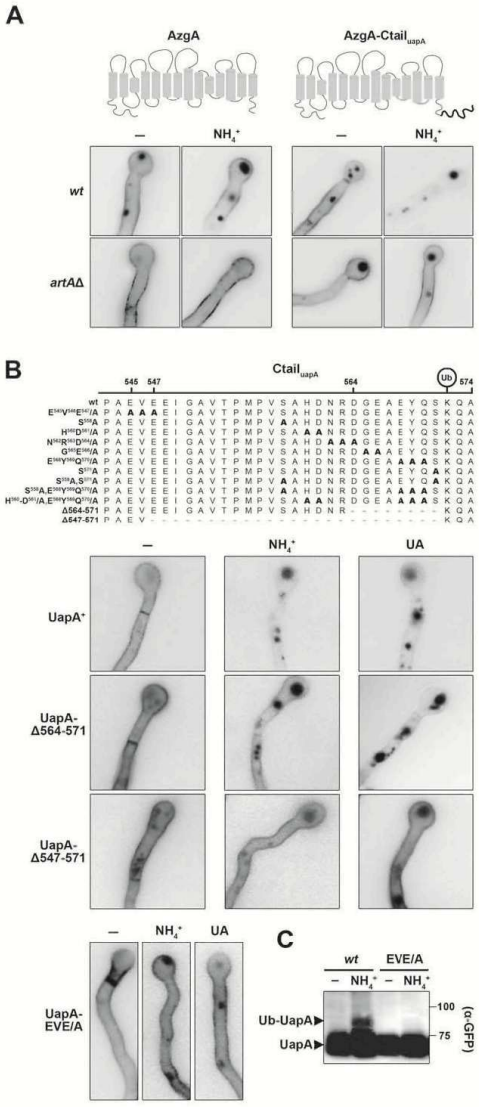

Fig. 7. The C-tail of UapA contains a region essential for ubiquitination and endocytosis.
A. ArtA-dependent, NH_4^+-elicited, endocytosis of an AzgA-GFP version including the C-terminus of UapA, as shown by epifluorescence microscopy.
B. Upper panel: schematic representation of UapA C-tail mutations analysed for UapA endocytosis. Lys-572 acting as ubiquitin acceptor is indicated. Lower panels: Epifluorescence microscopy of UapA C-terminal truncations (Δ564–571, Δ547–571) and mutation UapA-E545A/V546A/E547A (UapA-EVE/A) under non-endocytic (–) or endocytic conditions (NH_4^+ or UA).
C. Western blot analysis of UapA-GFP ubiquitination in membrane enriched fractions of a wt and a UapA-EVE/A strain, grown under endocytic (30 min, NH_4^+) or control conditions.

Specificity of ArtA in respect to transporter endocytosis

We also investigated the substrate specificity of ArtA by examining what is the effect of deleting the *artA* gene on other transporters. We constructed *artA*Δ mutants expressing GFP-tagged transporters for L-proline (PrnB), L-glutamate (AgtA) or purines (AzgA), proteins that undergo ammonium-elicited (PrnB and AgtA; Tavoularis *et al.*, 2001 and Apostolaki *et al.*, 2009) or substrate-triggered (AzgA; G. Diallinas, unpubl. obs.) endocytosis. Notably, all these transporters belong to structurally and evolutionary distinct transporter families (Diallinas, 2008). Figure 9 shows that ArtA is necessary for PrnB and AzgA endocytosis, but does not affect AgtA internalization (see also Fig. S2).

Discussion

Arrestin-like proteins have proved to be major adaptors of Rsp5/Nedd4-like ubiquitin ligases controlling the turnover of transporters through the control of the rate of ubiquitination, which is the primary molecular signal for cargo endocytosis (Lin *et al.*, 2008; Nikko *et al.*, 2009; Nikko and Pelham, 2009; Léon and Haguenauer-Tsapis, 2009; Hatakeyama *et al.*, 2010; MacGurn *et al.*, 2011; Becuwe *et al.*, 2012). Here we show that *A. nidulans* is not an exception. ArtA is involved in the endocytosis of UapA by mediating its ubiquitination via the HulA ubiquitin ligase, in response to the presence of ammonium or substrates. We further showed that a small fraction of ArtA is constitutively ubiquitinated and that ArtA ubiquitination is critical for efficient UapA ubiquitination and internalization from the plasma membrane. Furthermore, we have detected a small but repeatable increase in the fraction of ubiquitinated ArtA in response to NH_4^+, but not in response to substrates.

The fact that ArtA controls UapA ubiquitination and endocytosis in response to both ammonium and substrates leads to an apparent paradox. Ammonium-elicited endocytosis is a broad range physiological response concerning probably all transporters involved in the uptake of nitrogenous compounds that can be used as secondary nitrogen sources, such as purines, amino acids or nitrate (Dupré *et al.*, 2004; Pantazopoulou and Diallinas, 2007). The physiological rationale for this is that when ammonium is present in the media as a primary nitrogen source, there is no need for taking up other nitrogenous compounds through their specific transporters, which are consequently internalized and turned-over. In contrast to ammonium-elicited endocytosis, substrate-elicited endo-

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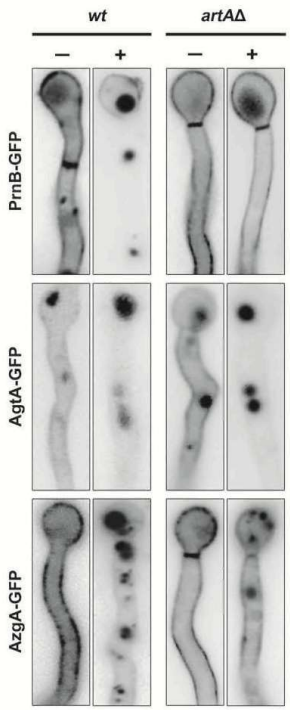

Fig. 9. Specificity of ArtA in respect to the endocytosis of different transporter cargoes. Epifluorescence microscopy of PrnB-GFP, AgtA-GFP, AzgA-GFP in *artA*Δ and *artA*$^{+}$ (wt) backgrounds under non-endocytic (–) or endocytic conditions (+). Endocytic conditions for PrnB and AgtA indicate addition of NH_4^+ and for AzgA addition substrate (Hypoxanthine) for 2 h. AgtA-GFP consistently gives a lower fluorescent signal compared to the other transporters tested. Notice that, unlike UapA-GFP or AzgA-GFP, AgtA-GFP and PrnB-GFP show a degree of constitutive turnover (appearance of GFP-labelled vacuoles) under non-endocytic conditions. For AgtA, this was recently shown to occur by direct sorting from the Golgi to the vacuole (S. Amillis, unpubl. obs.).

recruiting HulA on transporters. In the absence of a signal for activation (e.g. absence of NH_4^+), ArtA remains probably phosphorylated and little ubiquitinated, showing very low affinity for cargoes. In the presence of a substrate however, when UapA becomes active, conformational changes associated with transport catalysis might increase the affinity of the transporter for non-ubiquitinated or/and for the small fraction of constitutively ubiquitinated ArtA, and thus elicit its enhanced ubiquitination and internalization.

This scenario also predicts that activation of ArtA is related to its ability to find its cargoes and not to a catalytic activation *per se*. In line with this, Lin *et al.* (2008) showed that Rsp5-mediated ubiquitination is required for the 'correct' plasma membrane subcellular localization of Art1p in *S. cerevisiae*, while Hervás-Aguilar *et al.* (2010) showed that PalF ubiquitination is a key molecular trigger required for transmitting the alkaline pH signal from the plasma membrane to downstream elements of a pH-responding pathway in *A. nidulans*. Interestingly, overexpression of ArtA using the *alcA* promoter leads to relatively increased constitutive UapA endocytosis in the absence of ammonium or substrate (Fig. S3), an observation that is also in line with the idea that arrestin-like proteins can act on their cargoes in the absence of any physiological or stress signal.

We could not obtain any rigorous evidence of ArtA recruitment to the plasma membrane upon imposing endocytic conditions for UapA (not shown). We do not have an explanation on the reasons of the very low and diffuse fluorescent signal of ArtA-GFP expressed under native or strong promoters, but it seems that detecting the subcellular localization of arrestin-like proteins is not an easy task. To our knowledge, there is only a single case, that of Art1p in *S. cerevisiae*, where the subcellular localization of arrestin-like protein has been reported (Lin *et al.*, 2008; MacGurn *et al.*, 2011). In that case Art1p was shown to be present in cytosolic foci colocalizing with a Golgi marker under control conditions, but associate transiently with the plasma membrane under endocytic conditions. We failed to obtain similar evidence for ArtA. However, ArtA ubiquitination does not take place in a HulA allele missing the C2 domain and thus unable to be recruited to the plasma membrane (*hulA*Δ*C2*). This suggests that ArtA ubiquitination might occur in the plasma membrane.

At least two other *A. nidulans* transporters, specific for the uptake of nitrogenous compounds, PrnB (L-proline) and AzgA (purines) seem to be substrates of ArtA, either in response to ammonium (PrnB) or substrate (AzgA). The observation that overexpression of ArtA leads to reduced growth rates (not shown) suggests that several other transporters might also be substrates of this arrestin-like adaptor. Is there a recognizable common motif in these transporters that might act as a possible ArtA binding site? We showed that the ArtA putative binding site in UapA lies in its C-terminal region (residues 545–561), and interestingly, a di-acidic motif (E^{545}-V-E^{547}) in this region is essential for ArtA-dependent UapA ubiquitination and endocytosis. Similar di-acidic motives are present in the C- or N-terminal regions of other transporters under ArtA control, such as PrnB or AzgA.

Experimental procedures

Strains, classical and reverse genetics, media and growth conditions

Aspergillus nidulans strains used are listed in Table S1. Newly made null mutant strains and in locus gene tagging were constructed by transformation in an *nkuA* DNA helicase defi-

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His purification was carried out as in Lemuh *et al.* (2009) using Protino Ni-NTA Columns (Macherey-Nagel GmbH, Lab Supplies Scientific SA). For immunoprecipitation under denaturing conditions, total protein extracts were first resuspended in extraction buffer, containing 50 mM Tris-HCl, pH 7.5, 2 mM EDTA, 100 mM NaCl, 2% SDS, protease inhibitor cocktail (PIC) (Sigma-Aldrich, Life Science Chemilab SA) and 20 mM *N*-ethylmaleimide (NEM). Immunoprecipitation buffer (IP: 50 mM Tris-HCl, pH 7.5, 2 mM EDTA, 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, PIC and 20 mM NEM) was added, and lysates were incubated with 4 μg anti-GFP with gentle rotation at 4°C for 2 h, followed by addition of A-Protein Sepharose CL-4B beads (Sigma-Aldrich, Life Science Chemilab SA) and incubation with gentle rotation at 4°C for 12 h. The beads were washed twice with IP buffer, once with a buffer containing 50 mM Tris-HCl, pH 7.5, 2 mM EDTA, 250 mM NaCl, 0.5% Triton X-100, 0.05% sodium deoxycholate, PIC and 20 mM NEM, once with a buffer containing 50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 500 mM NaCl, 0.1% Triton X-100, PIC and 20 mM NEM and once with a buffer containing 50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 100 mM NaCl, and PIC and were finally boiled for 5 min at 95°C in protein sample buffer. Detection of ubiquitinated UapA-GFP was achieved in membrane-enriched protein fractions according to Galan *et al.* 1996. Protein concentrations were determined by the method of Bradford. In each case 30–50 μg protein were fractionated on 8–10% SDS-PAGE gel and electroblotted (Mini PROTEAN™ Tetra Cell, BIO-RAD) onto a PVDF membrane (Macherey-Nagel GmbH, Lab Supplies Scientific SA). Immunodetection was performed using a primary mouse anti-GFP monoclonal antibody (Roche Diagnostics), a mouse anti-actin monoclonal (C4) antibody (MP Biomedicals Europe, Lab Supplies Scientific SA), an Anti-His (PentaHis HRP Conjugate; Qiagen, SafeBlood BioAnalytica SA), an Anti-Ubiquitin (Ub-P4D1 HRP Conjugate; Santa Cruz Biotechnology, SafeBlood BioAnalytica SA) and a secondary goat anti-mouse IgG HRP-linked antibody (Cell Signaling Technology Inc., Bioline Scientific SA) and detected by the chemiluminescent method using the LumiSensor Chemiluminescent HRP Substrate kit (GenScript USA Inc, Lab Supplies Scientific SA).

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