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Vestiges of Ent3p/Ent5p function in the giardial Epsin homolog

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Abstract

An accurate way to characterize the functional potential of a protein is to analyze recognized protein domains encoded by the genes in a given group. The epsin N-terminal homology (ENTH) domain is an evolutionarily conserved protein module found primarily in proteins that participate in clathrin-mediated trafficking. In this work, we investigate the function of the single ENTH-containing protein from the protist *G. lamblia* by testing its function in *S. cerevisiae*. This protein, named GlENTHp (for *G. lamblia* ENTH protein), is involved in *Giardia* in endocytosis and in protein trafficking from the ER to the vacuoles, fulfilling the function of the ENTH proteins epsin and epsinR, respectively. There are two orthologs of epsin, Ent1p and Ent2p, and two orthologs of epsinR, Ent3p and Ent5p in *S. cerevisiae*. Although the expression of GlENTHp neither complemented growth in the *ent1 ent2* mutant nor restored the GFP-Cps1 vacuolar trafficking defect in *ent3 ent5*, it interfered with the normal function of Ent3/5 in the *wild-type* strain. The phenotype observed is linked to a defect in Cps1 localization and α-factor mating pheromone maturation. The finding that GlENTHp acts as dominant negative epsinR in yeast cells reinforces the phylogenetic data showing that GlENTHp belongs to the epsinR subfamily present in eukaryotes prior to their evolution into different taxa.

Keywords

ENTH motif; vacuole; endocytosis; *Giardia lamblia*; yeast; vesicle transport

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1.0 Introduction

In eukaryotic cells, the most extensively studied and best characterized type of vesicular transport is clathrin-mediated trafficking. In this process, clathrin-coated vesicles (CCVs) are also covered by scaffolding proteins, and mediate cargo delivery to endosomal/lysosomal compartments. Clathrin alone has no affinity for biological membranes and needs the action of clathrin-associated adaptor proteins (APs) to be recruited to membranes and to capture transmembrane cargo. There are two classes of endocytic adaptors that participate in the formation of the CCVs: multimeric adaptor proteins and monomeric adaptor proteins [1–4]. Specific monomeric adaptor regulators of the endosomal/lysosomal system are the ENTH/ANTH/VHS superfamily protein containing an ENTH (Epsin N-terminal homology), an ANTH (AP180 N-terminal homology) or a VHS (Vps27, Hrs and STAM) domain at their N-terminus [5]. Among the ENTH family, the epsin subfamily possesses an ENTH module that binds PIP_2 and participates in clathrin-mediated endocytosis [6]. In mammalian cells, epsin 1 and epsin 2 are ubiquitously expressed, while epsin 3 is specifically expressed in keratinocytes induced by type I collagen [7]. While the ENTH domain is well defined, the C-terminal of epsin proteins does not contain any secondary structure [8] but has multiple short peptide motifs that mediate interactions with endocytic proteins such as clathrin, alpha appendage domain of the adaptor protein complex 2 (αAP-2) and Eps15-homology domains [9]. Epsin proteins also contain ubiquitin-interaction motifs (UIMs) that interact with polyubiquitins and may capture ubiquitinated cargo receptors at the plasma membrane for internalization [10–12]. The association of epsin proteins with clathrin drive the formation of CCVs at the cell surface, while another member of this family, epsinR (epsin Related), was found enriched in CCVs associated with Golgi-to-endosome trafficking [13]. The Golgi/endosome EpsinR contains an ENTH domain that binds to PI4P and a C-terminal sequence that interacts with clathrin, the gamma appendage domain of the adaptor protein complex 1 (γAP-1), and the monomeric adaptors GGAs.

The epsin proteins have crucial physiological roles. Whereas in metazoans epsin proteins are required for proper embryo development [14], in yeast they are essential for cell viability [15]. The yeast *S. cerevisiae* contains two orthologs of epsin, Ent1p and Ent2p [16], and two orthologs of EpsinR proteins, Ent3p and Ent5p [15]. Ent1p and Ent2p sequences have the globular N-terminus ENTH domains, through which bind $PI4,5P_2$ at the plasma membrane, and contain clathrin-binding motifs (CBM), two UIM, two tripeptide NPF sequences, and other regions with unknown functions within the C-terminus. It was demonstrated that these two proteins are involved in clathrin-mediated endocytosis [8]. *ENT1* and *ENT2* are two redundant genes but it has been shown that the ENTH domain itself is both necessary and sufficient for the viability of strains lacking *ENT1* and *ENT2* genes *(ent1 ent2* cells) [16]. On the other hand, Ent3p and Ent5p are involved in protein trafficking between the *trans*-Golgi Network (TGN) and endosomes [15]. Like epsinR, the N-terminal region of Ent3p contains the ENTH module with a different lipid binding pocket, suggesting that epsinR and Ent3p could bind different phosphoinositides (PIs), facilitating membrane curvature at the TGN. In contrast, Ent5p possesses an ANTH domain that mediates binding to PI. In the C-terminal region, Ent3p and Ent5p contain short peptide motifs and domains that mediate interactions with clathrin, AP-1, and GGAp [17]. Ent3p as Ent5p are required for the

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recruitment of clathrin *in vivo*, confirming the overlapping functions of the Ent3 and Ent5 proteins in the assembly of clathrin coated pits and in the CCVs-mediated transport between the TGN and the endosomes in yeast. In the double mutant *ent3 ent5* , the ENT3 and ENT5 genes have been deleted, resulting in non-lethal cells with defects in intracellular clathrin-mediated trafficking [17–20].

The yeast *S. cerevisiae* and most vertebrates (including primates, rodents, and zebrafish) contain at least two epsin paralogs. In contrast, only one epsin gene is present in the unicellular parasites *Toxoplasma gondii, Plasmodium falciparum*, and *Giardia lamblia* [21]. *G. lamblia* belongs to the *Excavata* group, which is among the earliest branches in the phylogeny, distantly related to the well-studied model organisms of animals and fungi, and is a tractable microorganism from which to compare function across deep evolutionary time. In *Giardia*, we have identified the protein GlENTHp (for *Giardia lamblia* ENTH protein), which contains an ENTH domain and is able to bind clathrin, ubiquitin, αAP-2 and γAP-1, holding an *epsin-epsinR* role in this parasite [22]. These findings are consistent with the evidence that *Giardia* possesses many proteins performing multiple tasks and that simplified trafficking machinery is utilized by this parasite, allowing lipids and proteins to reach their intended organelles.

Because many components of the endocytic machinery are structurally and functionally conserved between eukaryotes, we used the yeast system here to explore the similarities as well as the particularities of GlENTHp as a member of the epsin family. We showed that GlENTHp was not able to accomplish the role of Ent1/2p in endocytosis or of Ent3/5p in the vacuolar protein trafficking in *S. cerevisiae*. Interestingly, we found that GlENTHp interfered with the function of Ent3/5p but not of Ent1/2p when it was overexpressed in *wild-type* cells, reflecting that a higher conservation of ENTH-protein sequences across divergent species is a requirement for proper protein function and that subtle and complex regulatory interactions with other species-specific components of the clathrin-coated machinery are also necessary.

2.0 Materials and methods

2.1 Strains and plasmids

The strains and plasmids used in this study are listed in Tables 1 and 2, respectively. As previously described for Ent1/2p and Ent3/5p [23, 24], GlENTHp, giardial epsin ENTH domain (gENTH) and the mutant GlENTH$_{K75A}$p were tagged with mCherry at their Cterminus. Yeast strains were grown in standard yeast extract–peptone–dextrose (YPD) or synthetic medium [yeast nitrogen base (YNB) supplemented with amino acids required for plasmid maintenance and 2% dextrose] at 30°C for 3–4 days. For liquid culture assays, 10^5 cells were inoculated in 10 ml of selective media, incubated at 30°C for 48 h, and the OD at 600 nm was measured.

2.2 GlENTHp expression in yeast cells

The plasmids used in this study were constructed by amplifying the *glenth* coding sequence from giardial genomic DNA and cloned in multicopy *URA3* or *HIS3* vectors. Expression

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was driven by the *MET25* promoter. DNA manipulations were performed using standard techniques, employing T4 DNA polymerase-mediated ligations in *Escherichia coli*. Amino acid substitutions were made using the QuikChange XL site-directed mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions. All restriction enzymes were purchased from New England Biolabs (Ipswich, MA). Yeast was transformed by the Li-Acetate method following the Clontech yeast handbook.

2.3 Preparation of cell extracts

Each cell strain was grown ON at 30°C and diluted to a density of 0.3–0.4 OD/mL before incubation at 30°C until a density of 0.7–0.8 OD/mL. Cell were then collected and washed in phosphate-buffered saline, prior to suspension in Laemmli's protein sample buffer containing 6 M urea. Cells were lysed by grinding with 0.5 mm glass beads, and proteins were then resolved by SDS–PAGE and detected by immunoblotting using rabbit antimCherry antibody. Briefly, protein samples were separated on 12% polyacrylamide mini gels at 20 mA constant current in SDS/PAGE running buffer (3 mM SDS/25 mM Tris/192 mM glycine) and transferred onto nitrocellulose membrane in transfer buffer (1 mM SDS/48 mM Tris/400 mM glycine/10% methanol) at 80 V for 90 min. Blots were blocked overnight in Blotto-Tween (PBS/0.2% Tween-20/5% nonfat dried milk) and incubated with the appropriate primary antibody and dilution for 1 h at room temperature and with a secondary antibody conjugated with horseradish peroxidase for 1 h at room temperature. After washing, specific bands were detected by chemiluminescence using Supersignal West Pico (Pierce) as a substrate and visualized using an Alpha-Innotech imaging system (San Leandro, CA).

2.4 Microscopy and protein transport assays

For assessing GlENTHp localization in yeast cells, *wild-type* (WT) cells were transformed with **p**GlENTHp-mCherry(*URA3*). For colocalization assays, the strain BWY5912 (GFPCLC) was transformed with **p**GlENTHp-mCherry(*URA3*). The NDB-ceramide assay was performed by washing the cultures in PBS and incubating with 5 μM of NBD-ceramide (Molecular Probe) in PBS for 10 min at 37°C. Cells were observed in confocal microscope after the three washes with PBS. To test the localization of GFP-Cps1, e*nt3* *ent5* cells were co-transformed with a **p**GFP-Cps1(*URA*3) and an empty **p**MET25.423(*HIS3*), with **p**GlENTHp-mCherry(*HIS3*) or **p**ENT5(*LEU2*) vector. WT cells transformed with **p**GFPCps1(*URA*3) were used as a control. The same procedure was used to transform WT cells with **p**GlENTHp-mCherry(*URA3*), **p**GlENTH$_{K75A}$p-mCherry(*URA3*) or **p**gENTH-mCherry(*URA3*) and an empty **p**MET25.426 to evaluate if GlENTHp was acting like an epsinR dominant negative mutant in yeast cells. For Ste3 localization, the strain BWY2858 (Ste3-GFP) was transformed with an empty **p**MET25.426(*URA3*) or with **p**GlENTHp-mCherry(*URA*3). To analyze the actin cytoskeleton, the strain BWY2776 (Abp1-GFP) was transformed with an empty **p**MET25.426(*URA3*) or with **p**GlENTHp-mCherry(*URA3*). BWY2784 [ent1 ent2 yap1801 yap1802 , ABP1-GFP (G418) + pBW0778 ENTH1.414(*TRP*1)] was used as positive control. To test the misslocalization of Ent3p and/or Ent5p, the strain QAY480 (Ent3-GFP) or QAY188 (Ent5-GFP), respectively, was transformed with **p**GlENTHp-mCherry(*URA3*), **p**GlENTH$_{K75A}$p-mCherry(*URA3*) or

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pgENTH-mCherry(*URA3*). In all cases, the cells were maintained in synthetic medium lacking uracil (YNB-URA3), histidine (YNB-HIS3), and also tryptophan (YNB-TRP) for BWY2784, to maintain viability and grown to mid-logarithmic phase prior to analysis. For live-cell imaging, cells were grown to early log phase on rich medium plates at 30°C. Cells were placed in 2 μl of minimal media on an uncoated glass coverslip and then inverted onto a glass slide. Imaging was carried out at room temperature. Images were captured using an Axiovert 200 inverted microscope (Carl Zeiss) equipped with a Cooke Sensicam (Cooke), an X-Cite 120 PC fluorescence illumination system, a 100×, 1.4 numerical aperture (NA) Plan Apochromat oil immersion lens and SlideBook 4.2 software. All images were captured using identical exposure conditions

2.5 Plasmid Shuffle

ent1 ent2 expressing ENT1, from a plasmid containing an auxotrophic marker (*TRP1*), were used for counter-selection on 5-fluoroanthranilic acid (5-FAA) [25]. Cells co-transformed with ENT1(*URA3*) (as a positive control), with an empty **p**MET25.426 (negative control), with GlENTHp, gENTH or GlENTH$_{N107Y}$ (*URA3*) plasmids were grown on 5-FAA plates (to evict the *TRP1* plasmid) at 30°C for 3 days.

2.6 Amino acid sequence alignment

All sequences were aligned using the CLUSTAL 2.1 program [26]. The ENTH domain protein sequences from *Saccharomyces cerevisiae* (Ent1$^{1-160}$ and Ent2$^{1-160}$, protein accession numbers NP_010120 and NP_013307, respectively) and *Giardia lamblia* (GlENTHp$^{1-160}$, GenBank accession no.: GL50803_3256) were used. For Ent3p and Ent5p alignments, the protein sequence NP_012659.1 and NP_010437.3 were used, respectively. For protein structure and function prediction, the I-TASSER (Iterative Threading ASSEmbly Refinement) server was used [27].

2.7 Halo pheromone assays

Halo assays were performed essentially as described [28]. Briefly, the function of GlENTHp was evaluated from the diameter of clear halos of growth inhibition formed in confluent supersensitive α-factor cells (see Table 1). These cells were spread on a yeast extract-peptone-dextrose (YPD) plate containing. Single colonies of the test samples, were suspended in YPD broth, diluted to 0.5 OD$_{600}$/ml and spotted (5 μl) onto the plate containing the lawns of supersensitive cells. Testing cells included *ent3 ent5* strain containing an empty **p**MET25.426 (*URA3*), **p**GlENTHp-mCherry (*URA3*) (pBW2701), or **p**ENT5 (*URA3*) vector and *wild-type* strain containing an empty **p**MET25.426 (*URA3*), **p**GlENTHp-mCherry(*URA3*) (pBW2701), **p**GlENTH$_{K75A}$p-mCherry(*URA3*), or **p**gENTH-mCherry(*URA3*) vector (see Table 2). For positive and negative controls, *wild-type MAT*α or *MAT***a** were used, respectively. Plates were incubated at 30°C ON and photographed. This experiment was repeated three times with similar results.

2.8 Fluorescence resonance energy transfer (FRET) by Acceptor Photobleaching analysis

GFP-strains transformed with pGlENTHp-mCherry(*URA3*) were scraped from the 60 mm Petri dishes, resuspended in 3 μl of water and plated on concanavalin A-coated coverslips,

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which were then placed (up-side-down) on top of glass coverslips. An Olympus IX-71 inverted microscope equipped with a 100 W mercury arc lamp, a 60 × plan apochromatic objective (NA=1.4), a standard GFP–mCherry FRET filter set, and an ANDOR iXon3 camera (DU-888E-C00-#BV) was used for acceptor photo bleaching FRET imaging. GFP–mCherry fluorophores were selected because there is a large separation between donor and acceptor excitation frequencies and therefore an insignificant crosstalk. Repeated scans were applied during a 3 min period to achieve complete photobleaching of the GlENTHp-mCherry acceptor protein. FRET signal was estimated as an increase of GFP intensity, (captured using a 505 nm filter cube) after the photobleaching of mCherry [29]. FRET maps were generated and analyzed using ImageJ (http://imagej.nih.gov/ij/). CLC-GFP cells transformed with the empty **p**mCherry(*URA3*) vector were used as negative control. All images were equally processed.

3.0 Results

3.1 GlENTHp is localized in patches at the plasma membrane in *S. cerevisiae*

When the strain stably expressing the GFP-clathrin light chain (GFP-CLC) was transformed with the plasmid expressing pGlENTHp-mCherry(*URA3*), only partial colocalization of both proteins was observed close to the plasma membrane (Fig 1A, top panels). No colocalization between GlENTHp-mCherry and the TGN-marker NBD-ceramide [30] was observed when *wild-type* cells (SEY6210) were transformed with pGlENTHp-mCherry(*URA3*) and treated with NBD-ceramide (Fig 1A, bottom panels). Expression of the ENTH domain of GlENTHp fused with mCherry (gENTH), showed a cytoplasmic localization (Fig 1B).

3.2 GlENTHp is unable to rescue the *ent1 ent2* phenotype

Homologs to the yeast *ENT1/2* genes have been reported in a variety of organisms, ranging from humans to parasites [21]. To examine if GlENTHp can provide the functions of the budding yeast *ENT1/2* genes, we tried to complement a *S. cerevisiae* lethal *ent1 ent2* mutant, in which the redundant genes *ENT1* and *ENT2* had been deleted but complemented with the *wild-type* **p**ENT1(TRP1) plasmid for viability [16]. *ent1 ent2* -ENT1 cells were transformed with the pGlENTHp-mCherry(*URA3*) plasmid or the vector pgENTH-mCherry(*URA3*) (containing only the giardial ENTH domain). *ent1 ent2* cells transformed with **p**ENT1(*URA3*) or with an empty **p**MET25.426 were used as a positive and negative controls, respectively. Complementation assays showed that neither GlENTHp nor the gENTH was able to rescue the lethality when the cells evicted ENT1 on plates containing 5-FAA (Fig. 2A). The expression of GlENTHp and gENTH was tested by immunoblotting (Fig. 2B).

It was reported that the ENTH domain alone of yeast epsin is necessary and sufficient for viability of *ent1 ent2* cells [31], with the essential function requiring a patch of the conserved residues Y_{100}/T_{104} that interact with yeast homologues of RalBP1/RLIP76, a GTPase-activating protein (GAP) for Cdc42 and Rac1 [32]. The Cdc42 (a small GTPase protein that belongs to the Rho family of Ras GTPases superfamily) and Rac GTPases are key regulators of the actin cytoskeleton [33], thus suggesting that this complex links signaling, endocytosis, and actin cytoskeleton regulation. When we compared the amino

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acid sequences of *S. cerevisiae* Ent1/2p ENTH domains with the homologous domains from *G. lamblia*, we found that the conserved T (T_{111} from *G. lamblia*) but not the Y residue was present in gENTH sequence (Fig. S1). Because the ENTH1^{Y100R} and ENTH1^{T104D} domains did not complement *ent1 ent2* cells [31], we analyzed whether the inefficacy of GlENTHp and gENTH for complementing *ent1 ent2* cells relied on the absence of the Y_{100}/T_{104} patch. Thus, we produced a mutant GlENTH$_{N107Y}$p and gENTH$_{N107Y}$, in which N_{107} of the gENTH domain was changed to Y_{107}, and analyzed whether these mutants could fulfill the essential function of the ENTH domain in *ent1 ent2* cells. Our results showed that neither GlENTH$_{N107Y}$p nor gENTH$_{N107Y}$p was able to complement *ent1 ent2* cells (Fig. 2C), suggesting that a conserved Y/T patch is not sufficient for gENTH domain recognition and functionality in *S. cerevisiae*. Immunoblotting was performed to verify the expression of GlENTH$_{N107Y}$p and gENTH$_{N107Y}$ in *S. cerevisiae ent1 ent2* cells (Fig. 2D).

3.3 GlENTHp does not interfere with the normal function of Ent1/2p

The results obtained so far indicated that GlENTHp was unable to replace the function of Ent1/2p in *S. cerevisiae*. When the GlENTHp structure was analyzed, no defined structure was found for the protein except for the alpha helices of its ENTH domain (http://zhanglab.ccmb.med.umich.edu/I-TASSER/output/S253398/fufv5n/). Although, we were unable to detect canonical protein-binding motifs at the C-terminus of GlENTHp (Fig. S2), the presence of a conserved ENTH domain prompted us to evaluate whether GlENTHp might act as a dominant non-functional counterpart of Ent1/2p. Thus, to test the possibility that the addition of GlENTHp to *wild-type* cells would inhibit the normal function of Ent1/2p, we studied the localization of the Ste3 receptor or Abp1 (Actin binding protein 1) fused with GFP in *wild-type* cells overexpressing GlENTHp-mCherry. The transmembrane receptor Ste3 is expressed in α mating type cells and, in the absence of ligand, it is constitutively internalized to the vacuole and degraded [34, 35]. By using live-cell confocal microscopy, we analyzed the trafficking of Ste3-GFP, since changes in Ste3-GFP localization will indicate a defect in endocytosis. Our results showed that GlENTHp expression did not interfere with Ste3 transport when the strain Ste3-GFP over-expressed GlENTHp-mCherry (Fig. 3A). Transformation of the Ste3-GFP strain with the empty pMET25.426 vector was performed as a control (Fig. 3A). As expected, in *ent1 ent2 yap1801 yap1802* cells (also lacking Yap1801 and Yap1802, homologues of the mammalian endocytic clathrin assembly proteins CALM/AP180), Ste3-GFP accumulated mostly at the plasma membrane (Fig. 3A) [35]. GlENTHp-mCherry expression in *wild-type* cells expressing Ste3-GFP was verified by immunoblotting (Fig. 3B).

A common phenotype observed among endocytosis mutants is a defect in the structure or polarity of the actin cytoskeleton [36]. Filamentous actin in yeast is present in both actin cables and cortical patches. The patches are located at the plasma membrane and are transient structures, with the localization of these patches varying as cells proceed through the cell cycle [37]. The fluorescent protein Abp1p-GFP has been used as a marker for actin patch dynamics [38–40] since Abp1p is a branched F-actin-binding protein and is found exclusively in actin patches associated with sites of endocytosis [41, 42]. Because a defect occurs in the structure and localization of the cortical actin patches in the absence of normal Ent1/2p function, either in *ent1 ent2* [16] or in the mutant e*nt1 ent2 yap1801 yap1802*

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cells [43], we analyzed the actin cytoskeleton by live-cell confocal microscopy in *wild-type* cells expressing Abp1-GFP when GlENTHp is over-expressed. Thus, changes in Abp1-GFP localization would indicate a defect in actin patch dynamics. When the strain expressing Abp1-GFP was transformed with **p**GlENTHp-mCherry(*URA3*), we found normal punctate cortical actin patches with no defect in Abp1-GFP localization (Fig. 3C). Co-expression of GlENTHp-mCherry and Abp1-GFP showed only limited colocalization at the sites of endocytosis (Fig. 3C). Transformation of the Abp1-GFP strain with the empty pMET25.426 vector was performed as control (Fig. 3B). As expected, *ent1 ent2 yap1801 yap1802* cells showed comet tails of Abp1-GFP associated with the cell cortex (Fig. 3C)[43]. Immunoblotting showed GlENTHp-mCherry expression in *wild-type* cells expressing Abp1-GFP (Fig. 3D). These results indicated that GlENTHp was not acting as a dominant negative Ent1/2p protein.

3.4 GlENTHp does not complement the function of Ent3/5p

To assess whether GlENTHp might play an Ent3/5p role in *S. cerevisiae*, we evaluated clathrin-mediated TGN/endosome trafficking in cells lacking Ent3/5p (*ent3 ent5*). *ent3 ent5* cells are viable but the intracellular trafficking of carboxypeptidase S (Cps1), which is transported as a precursor from the TGN to the vacuole and processed to a smaller active form, is affected in these cells [20]. In *wild-type S. cerevisiae* cells, GFP-Cps1 is localized to the vacuolar lumen while in the *ent3 ent5* cells, GFP-Cps1 localized outside the vacuole [20]. To evaluate if GlENTHp can rescue the Cps1 transport defect in *ent3 ent5* cells, these cells were co-transformed with the following plasmid combinations: *i*) **p**GFP-Cps1 and an empty **p**MET25.423, *ii*) **p**GFP-Cps1 and **p**GlENTHp-mCherry, or *iii*) **p**GFP-Cps1 and **p**ENT5 (positive control). The results showed that the expression of GlENTHp did not re-establish the localization of GFP-Cps1 to the vacuolar lumen in *ent3 ent5* mutant cells, as was observed for the positive control cells expressing *ENT5* (Fig. 4A). We also assayed the maturation and activity of α-factor mating pheromone, which is initiated by the TGN protease Kex2p. The localization of Kex2p depends on clathrin cycling Kex2p between the TGN and endosomes, and defects in Kex2p localization reduce the efficiency of α-factor maturation [44]. Thus, the halo bioassay for α-factor activity provides an indirect measure of whether α-factor maturation has been completed, since this small peptide pheromone is secreted and can arrest the cell cycle and cell growth of *MAT**a*** yeast cells in preparation for mating [45]. It has been reported that pheromone maturation was considerably reduced in *ent3 ent5* cells, compared with single-mutant or *wild-type* cells [17]. To test if the expression of GlENTHp in *ent3 ent5* cells can restore α-factor maturation, *wild-type* (*MATα*) and *ent3 ent5* cells, containing pGlENTHp-mCherry, the empty **p**MET25.426, or **p**ENT5, were spotted on a lawn of supersensitive *MAT**a*** cells. The results showed a defect of mature α-factor secretion from the *ent3 ent5* mutant expressing GlENTHp-mCherry or mutant cells containing the empty vector, as measured by the smaller zone of inhibition of the tester strain growth, compared to what was observed for the *ent3 ent5* mutant containing ENT5 and for *wild-type MATα* cells (Fig. 4B). These experiments provided evidence that GlENTHp cannot restore the maturation of the α-factor and it is thus unable to perform the function of Ent3/5p. GlENTHp-mCherry expression in *ent3 ent5* cells was verified by immunoblotting (Fig. 4C).

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3.5 GlENTHp interferes with the function of Ent3/5p

Sequence protein analysis shown only a putative DLL clathrin-binding motif at the C-terminus of GlENTHp but no other conserved protein-binding motifs observed for Ent3/5p (Fig. S2, C–D). To test whether GlENTHp could act as a protein whose function interferes with Ent3/5p, we reevaluated Cps1 localization and α-factor mating pheromone maturation in *wild-type* cells expressing GFP-Cps1 and overexpressing GlENTHp-mCherry, gENTH-mCherry or the mutant GlENTH$_{K75A}$p-mCherry [22]. We previously showed that the mutant GlENTH$_{K75A}$p is unable to bind PIs and thus useful to test the importance of this binding in the analysis of the function of GlENTHp in *S. cerevisiae*. By using live-cell confocal microscopy, we observed that GFP-Cps1 was excluded from the vacuole lumen in *wild-type* cells co-transformed with a **p**GFP-Cps1(*URA*3) and **p**GlENTHp-mCherry(*HIS3)* (Fig. 5A). Similar to the transformation of *wild-type* cells with pGFP-Cps1(*URA*3) and the empty pMET25.423(*HIS3*) vector as control, the overexpression of gENTH-mCherry or the mutant GlENTH$_{K75A}$p-mCherry showed the correct Cps1 localization inside the vacuole (Fig. 5A). To confirm the interference of GlENTHp in Ent3/5p function, the maturation and secretion of the α-factor mating pheromone was assayed as described above, except that GlENTHp-mCherry was overexpressed in *wild-type* (*MATα*) cells in this assay. The halo bioassay for α-factor activity revealed that the expression of GlENTHp in *MATα* cells considerably diminished the level of mature α-factor secretion, as measured by the smaller zone of inhibition of the tester strain relative to that seen around *MATα* cells with an empty pMET25.426 vector (Fig. 5B). Conversely, gENTH-mCherry and the mutant GlENTHK75Ap-mCherry showed a halo development comparable to that seen around *MAT*α cells with an empty pMET25.426 vector (Fig. 5C). These observations suggest that the whole protein GlENTHp is required to impair the correct function of Ent3/5p and to act as a non-functional epsinR dominant-negative protein. GlENTHp-mCherry, gENTH-mCherry and GlENTH$_{K75A}$p-mCherry expression in cells expressing GFP-Cps1 was verified by immunoblotting (Fig. 5D).

3.6 GlENTHp overexpression cause Ent3/5p misslocalization

It was reported that two sequential waves of clathrin-coat assembly that originate at the TGN are distinguished by adaptor type. Gga2p, Ent3p and a minor population of Ent5p assemble in the first wave. AP-1 and most Ent5p are recruited in the second wave with the progression between coat types being controlled by PI4P [24]. Changes in PI4P levels alter adaptor progression resulting in synthetic growth and partial α-factor maturation defects [46]. Moreover, when PI4P levels at the Golgi were lowered, a significant fraction of Ent5p was redistributed to the cytoplasm and an increase of PI4P synthesis at the TGN led to a significantly higher Ent3/5p colocalization at the first wave [46], suggesting that the access of Ent3/5p to PI4P is critical for their localization and function. These results prompted us to test whether the dominant-negative effect of GlENTHp in *S. cerevisiae* might be related to a misslocalization of Ent3p and/or Ent5p. For this, we used *wild-type* cells expressing Ent3p-GFP or Ent5p-GFP, and mCherry-tagged GlENTHp, gENTH or GlENTH$_{K75A}$p. GFP-tagged Ent3p localized as puncta at internal sites in *wild-type* cells (Fig. 6A) but changed its localization to the cytoplasm when GlENTHp was overexpressed (Fig. 6A). By comparison, Ent3p maintained punctate localization when gENTH or GlENTH$_{K75A}$p were overexpressed

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(Fig. 6A). Similar results were obtained in Ent5p-GFP expressing cells with a significant fraction of Ent5p redistributed to the cytoplasm only when GlENTHp was overexpressed (Fig. 6B). Together our findings support an interfering role for GlENTHp in Ent3/5p recruitment to the TGN, which could only be accomplished if the whole protein is overexpressed.

3.7 GlENTHp interacts with AP-1 and GGAs

No conserved γ-ear/GAE-domain but a putative clathrin-binding motif was detected by sequence analysis of GlENTHp (Fig. S2). However, we were able to experimentally prove that it interacts with the giardial AP-1 and clathrin [22]. To test if it might also be the case in *S. cerevisiae*, strains containing CLC, the γAP-1 adaptor subunit (APL4) or the GGAs (GGA1 and GGA2), tagged with GFP at their C-terminus, were transformed with the pGlENTHp-mCherry(*URA3*) vector and the interaction between each GFP(donor)/ mCherry(acceptor) pair was determined by FRET in live budding yeast. Cells expressing GFP-CLC and the empty vector pMET25.426::-mCherry(*URA3*) was used as negative control resulting in a FRET value of 0.05 (n=60). Values below to this negative baseline was observed for GFP-CLC and GlENTHp-mCherry (FRET value of 0.03, n=50) (Fig. 7). Conversely, FRET was observed after GlENTHp-mCherry photobleaching, as increased emission in the GFP channel for APL4, GGA1 and GGA2 with a FRET value of 0.15 (n=48), 0.18 (n=60) and 0.26 (n=60), respectively, suggesting that a direct interaction between GlENTHp and the adaptor proteins occurred (Fig. 7).

4.0 Discussion

Previously, we showed that GlENTHp contains an ENTH domain and has a fundamental role in lipoprotein-receptor mediated endocytosis and in hydrolase trafficking to giardial lysosomal vacuoles, performing the functions of both epsin and epsinR, respectively [22]. In *Giardia*, GlENTHp binds to clathrin, ubiquitin, γAP-1 and αAP-2 adaptor subunits and the phosphoinositides $PI3,4,5P_3$ and PI4P, with the characteristics of both epsin and epsinR all-in-one [22]. The ENTH family has been found in almost all eukaryotes, with Ent1/2p and Ent3/5p proteins functioning as epsin or epsinR adaptors, respectively, in yeast cells [17, 47]. A recent report supported the hypothesis that the ENTH domain of epsinR is the foundation of the ENTH/ANTH/VHS superfamily, since it is present in 80 out of the 84 studied eukaryotic organisms, positioning GlENTHp at the beginning of the family [21]. This finding encouraged us to analyze whether GlENTHp might be a key factor in the evolution of the ENTH family by testing its function in the yeast *S. cerevisiae*. We found that GlENTHp was unable to replace the function of either Ent1/2p or Ent3/5p in *S. cerevisiae*, most probably because of the restricted degree of homology between the protein sequences. These results underscored that neither a similar protein-folding domain nor the presence of structural amino acids are sufficient to perform a similar protein function. It is possible that the lipid binding preference of GlENTHp for $PI3,4,5P_3$ but not for $PI4,5P_2$ impaired its interaction with the appropriate target at the yeast plasma membrane, resulting in the failure to rescue endocytosis in *ent1 ent2* cells. Because the ENTH domain of Ent1/2p was able to replace the function of the whole protein in *ent1 ent2* cells, we did not analyze the possibility that interaction with clathrin partners was critical for the epsin-like

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function. However, another possibility might be the inability of GlENTHp C-terminus to bind yeast clathrin or poly-ubiquitinated proteins since no canonical binding domains are present in GlENTHp. Also, it was clear that GlENTHp (or the gENTH domain) cannot perform the function of native Ent3/5p in *S. cerevisiae* cells. However, keeping with the *in vivo* function of this protein in *G. lamblia* and the phylogenetic analysis of GlENTHp, we decided to analyze if this protein might act as a non-functional version of Ent1/2p or Ent3/5p. Our results showed that this was the case only for Ent3/5p in *S. cerevisiae* cells, suggesting that the vestiges of the epsinR function observed in *G. lamblia* were sufficient to interfere with the wild Ent3/5p function but insufficient to perform the epsinR role in the yeast cells. How is GlENTHp impairing the function of Ent3/5p? Because we previously showed that GlENTHp is able to bind PI4P, it is then possible that this protein, when overexpressed in *wild-type* cells, competes for the PI4P concentrated at the membrane of the Golgi apparatus, inhibiting the binding of Ent3/5p and restricting their function. However, neither the gENTH domain alone nor the $GlENTH_{K75A}p$ (mutant unable of binding phosphoinositides) impaired Ent3/5p function or localization, suggesting that PI4P binding is necessary but not sufficient to restrict the Ent3/5p functions. These findings reinforce the view that the A/ENTH domain and C-terminus of the protein contribute to epsin targeting and function [48–50]. The simplest interpretation of these data is that GlENTHp transiently bind to PI4P at the TGN-membrane, inhibiting the binding of GGAs to the membrane and avoiding the binding of Gga2p/Ent3p. GlENTHp might also directly compete with Ent3/5p by binding to AP-1 and the GGAs but not clathrin, altering the packaging of these proteins into the CCVs (Fig. 8). These events might probably explain the clear misslocalization of Ent3/5p in cells overexpressing GlENTHp and Ent3/5p malfunction. Considering that *G. lamblia* possesses AP-1 adaptor protein but lacks GGAs adaptors, it was interesting to find that GlENTHp recognize the GGAs yeast proteins. This evidence that the ancestor of *Giardia* probably possessed GGAs proteins in agreement with the new view in which the trafficking machinery in this parasite seems to be a consequence of a secondary reduction process (reviewed by [51]). Altogether, our findings reinforces the notion that although a conserved sorting mechanism exists between *G. lamblia* and *S. cerevisiae*, a greater degree of protein sequence similarity is required to replicate the function of proteins from different organisms.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

GlENTHp	*Giardia lamblia* ENTH protein
CCVs	clathrin-coated vesicles
ENTH	Epsin N-terminal homology domain
ANTH	AP180 N-terminal homology domain
VHS	Vps27, Hrs and STAM domain
UIMs	ubiquitin-interaction motifs
epsinR	epsin Related
AP-1	adaptor protein complex
CBM	clathrin-binding motifs
TGN	*trans*-Golgi Network
5-FAA	5-Fluoroanthranilic Acid
Abp1	Actin binding protein 1
Cps1	carboxypeptidase S.

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Highlights

- The protein GlENTHp (for *Giardia lamblia* ENTH protein) acts an Ent3/5p dominantnegative protein in *Saccharomyces cerevisiae*.
- GlENTHp impairs the correct clathrin-mediated trafficking between the TGN and the vacuole.
- GlENTHp might belong to the epsinR subfamily.
- A higher conservation of the protein sequence is required for proper function of the clathrin-coated machinery in yeast cells.

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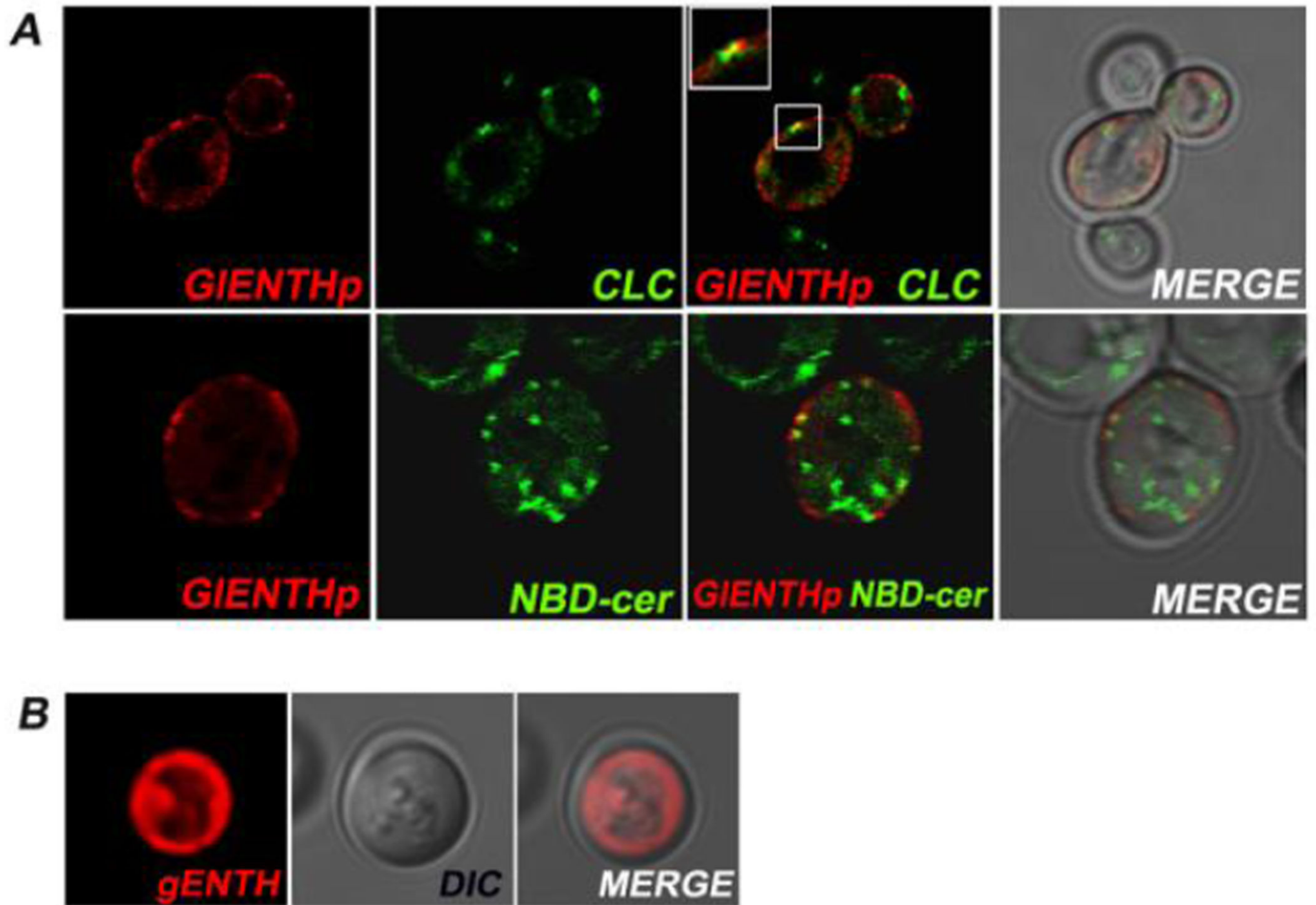

Figure 1. GlENTHp and gENTH expression in *S. cerevisiae* cells

(A) GlENTHp-mCherry is observed in patches at the plasma membrane in cells expressing the GFP-tagged CLC (upper panels) and *wild-type* cells (lower panels) incubated with NBD-ceramide. Limited colocalization is observed only between GlENTHp-mCherry and GFP-CLC (inset). Panels on the right show fluorescence and DIC merge. **(B)** The giardial ENTH domain alone (gENTH) shows cytoplasmic localization. DIC: differential interference contrast. Cells were visualized by confocal microscopy.

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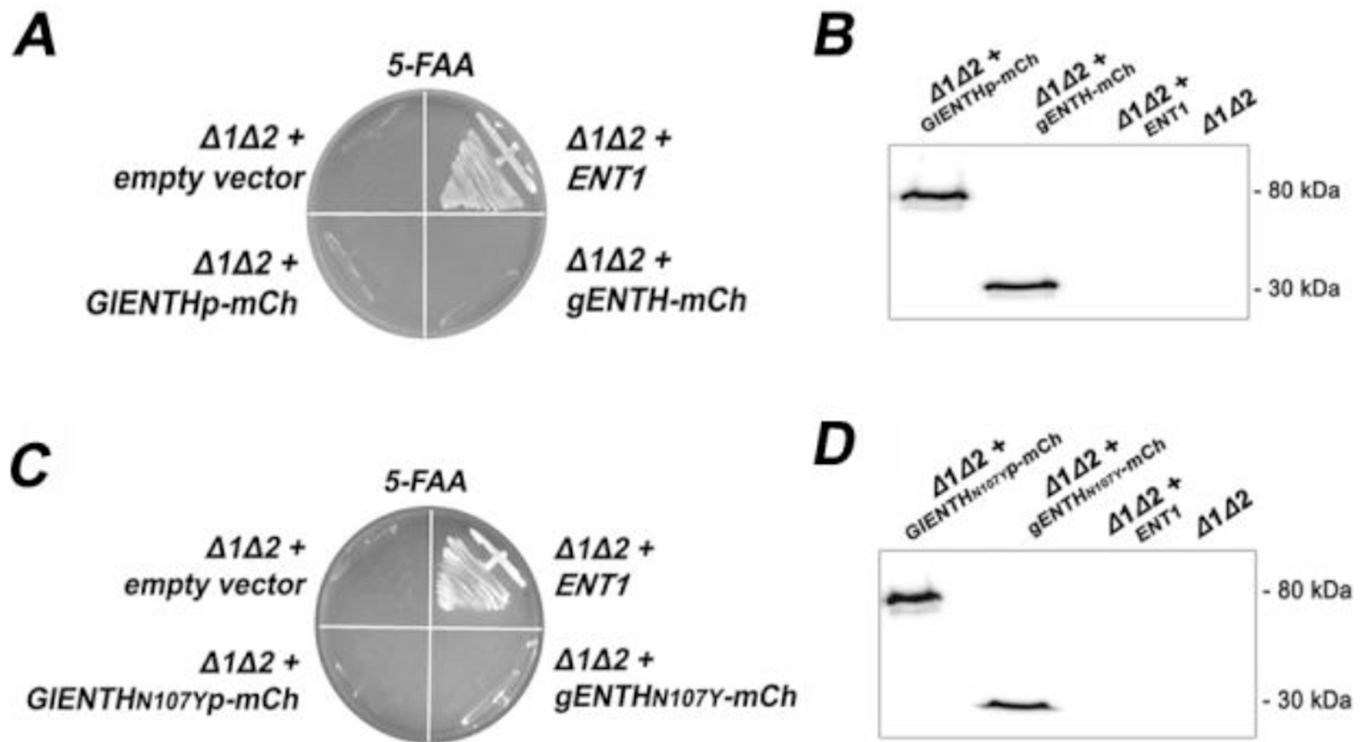

Figure 2. GlENTHp and the mutant GlENTH$_{N107Y}$p are unable to complement *ent1 ent2* cells

(A) GlENTHp or the gENTH cannot fulfill the essential function of the *wild-type* yeast epsin. *ent1 ent2* cells (***1 2***) with an ENT1(*TRP1*) plasmid and a second *URA3* empty plasmid, or encoding the full length GlENTHp, gENTH or ENT1, were grown at 30°C for 3 days on plates containing 5-FAA to evict the ENT1(*TRP1*) plasmid. **(B)** Immunoblotting shows GlENTHp-mCherry and gENTH-mCherry, expressed as a ~80 kDa or ~30 kDa band, respectively, in *ent1 ent2* cells (***1 2***) by using the anti-mCherry Ab. **(C)** GlENTH$_{N107Y}$p or its gENTH$_{N107Y}$ mutated domain cannot fulfill the essential function of the *wild-type* Ent1p. *ent1 ent2* cells (***1 2***) with an ENT1(*TRP1*) plasmid and a second *URA3* empty plasmid, or encoding the full length GlENTH$_{N107Y}$p, gENTH$_{N107Y}$ or ENT1p, were grown at 30°C for 3 days on plates containing 5-FAA to evict the ENT1 *TRP1* plasmid. **(D)** Immunoblotting shows GlENTH$_{N107Y}$p-mCherry and gENTH$_{N107Y}$-mCherry expressed as a ~80 kDa or ~30 kDa band, respectively, in *ent1 ent2* cells (***1 2***) by using the anti-Cherry Ab.

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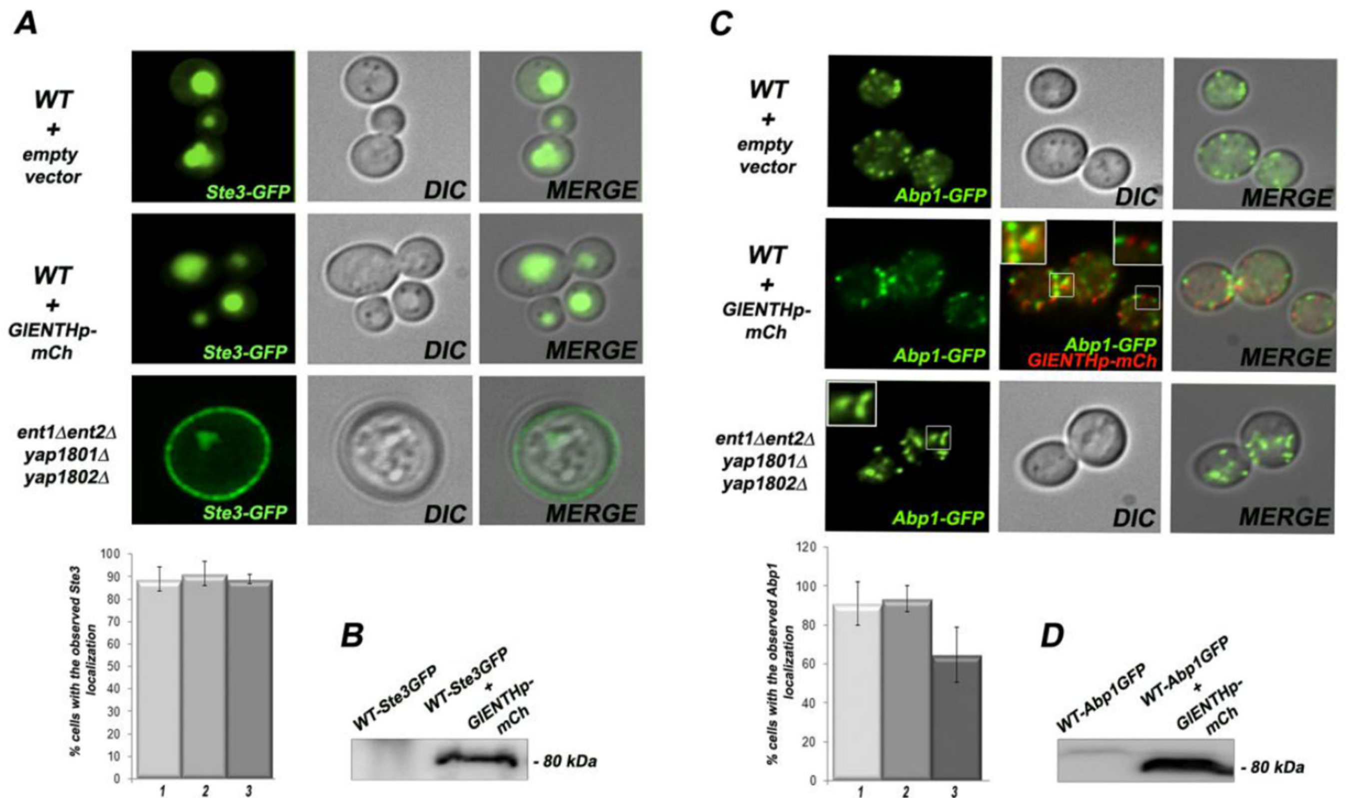

Figure 3. GlENTHp cannot act as a dominant negative mutant of epsin in *S. cerevisiae* cells

(A) Cells expressing GlENTHp show no defect on endocytosis of Ste3-GFP. The *wild-type* cells expressing Ste3-GFP was transformed with an empty pMET25.426 or with **p**GlENTHp-mCherry, grown to early log phase on rich medium plates at 30°C and visualized by confocal microscopy. *ent1 ent2 yap1801 yap1802* +Ste3-GFP +ENTH1.414] cells were used as control. The graphic shows the percentage of cells with the observed localization of Ste3. 1: WT+empty vector, 2: WT+GlENTHp-mCh, 3: *ent1 ent2 yap1801 yap1802* **(B)** Immunoblotting shows GlENTHp-mCherry, expressed as a ~80 kDa band, in Ste3-GFP cells by using the anti-Cherry Ab. **(C)** Cells expressing GlENTHp show no defect on the actin cytoskeleton. *Wild-type* cells expressing Abp1-GFP were transformed with an empty **p**MET25.426 or with **p**GlENTHp-mCherry(*URA3*). Abp1-GFP and GlENTHp-mCherry only partially colocalize (insets). [*ent1 ent2 yap1801 yap1802* +ABP1-GFP+ENTH1.414] cells were used as control. Cells were grown to early log phase on rich medium plates at 30°C and visualized by confocal microscopy. DIC: differential interference contrast. Panels on the right show fluorescence and DIC merge. Graphic showing the percentage of cells with the observed Abp1 localization. 1: WT+empty vector, 2: WT+GlENTHp-mCh, 3: *ent1 ent2 yap1801 yap1802* . **(D)** Immunoblotting shows GlENTHp-mCherry, expressed as a ~80 kDa band, in Abp-GFP cells by using the anti-Cherry Ab.

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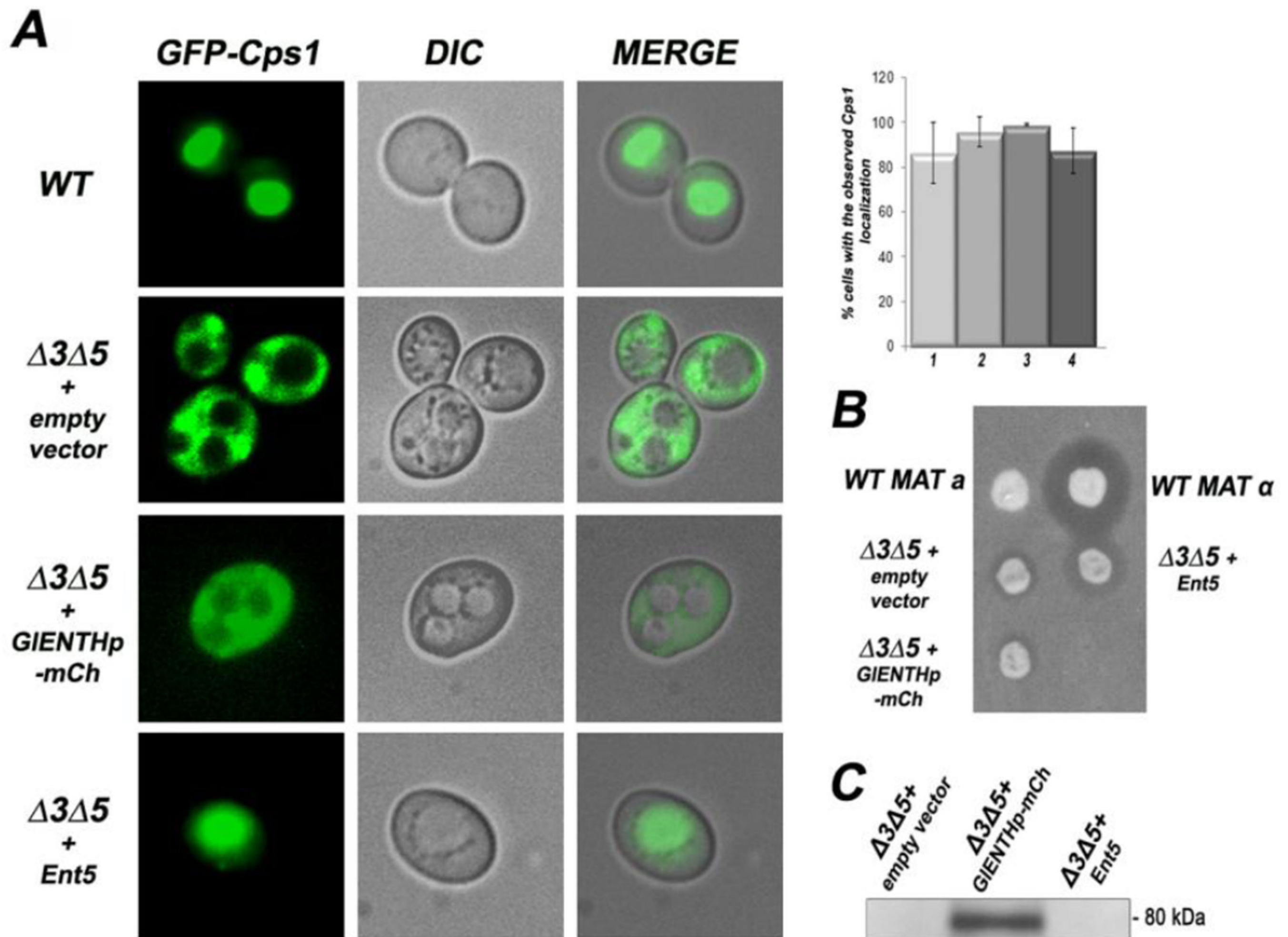

Figure 4. GlENTHp expression does not restore Golgi-to-vacuole protein trafficking in *ent3 ent5* cells

(A) GlENTHp cannot compensate the defect in Cps1 intracellular trafficking in *ent3 ent5* cells. *ent3 ent5* mutant cells (3 5) were co-transformed with a **p**GFP-Cps1 and the empty vector **p**MET25.426, **p**GlENTHp-mCherry or **p**ENT5. *ent3 ent5* live cells were observed by confocal microscopy. *Wild-type* cells transformed with **p**GFP-Cps1 were used as a control (WT). DIC: differential interference contrast. Panels on the right show fluorescence and DIC merge. Graphic showing the percentage of cells with the observed localization of Cps1. 1: WT, 2: 3 5+empty vector, 3: 3 5+GlENTHp-mCh, 4: 3 5+Ent5. **(B)** GlENTHp cannot restore α-factor maturation. Equivalent OD_{600} units of *wild-type* (*MAT*α), *wild-type* (*MAT***a**) (negative control), and *ent3 ent5* strain (**3 5**) with the empty vector **p**MET25.426, **p**GlENTHp-mCherry or **p**ENT5, were spotted on a lawn of supersensitive *MAT***a** cells. Halo assay determination of α-factor stimulated signaling and cell cycle arrest activity shows decreased growth inhibition of the underlying lawn for *ent3 ent5* +GlENTHp-mCherry. **(C)** Immunoblotting shows GlENTHp-mCherry, expressed as a ~80 kDa band, in *ent3 ent5* cells by using the anti-Cherry Ab.

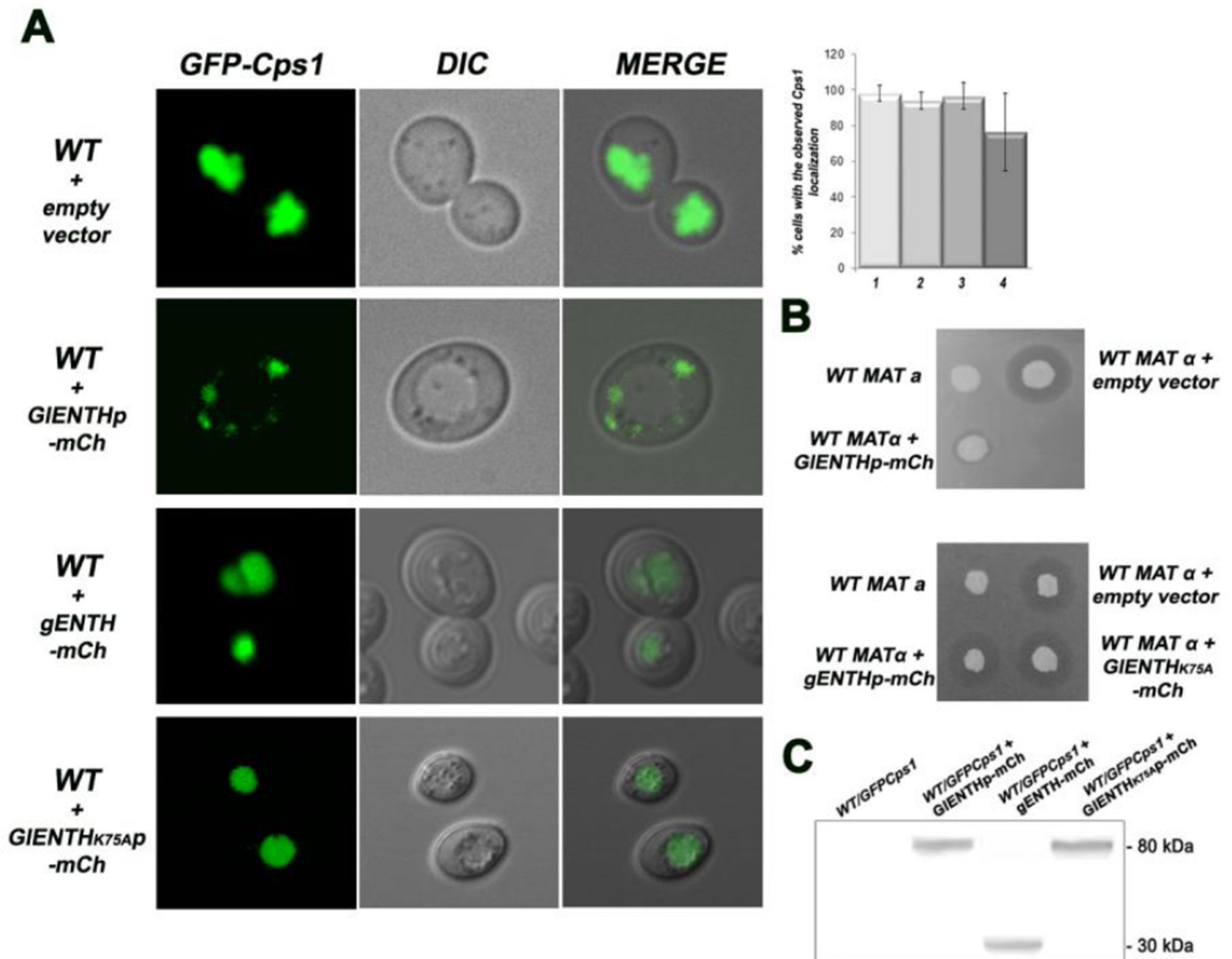

Figure 5. GlENTHp functions as an epsinR dominant negative mutant in *wild-type S. cerevisiae* cells

(A) GlENTHp expression shows defect in Cps1 intracellular trafficking in *wild-type* cells. *Wild-type MAT*α (WT) cells were co-transformed with a **pGFP**-Cps1 and with an empty **p**MET25.423, **p**GlENTHp-mCherry, **p**gENTH-mCherry, or **p**GlENTH$_{K75A}$p-mCherry vector. Live cells were observed by confocal microscopy. DIC: differential interference contrast. Panels on the right show fluorescence and DIC merge. Graphic showing the percentage of cells with the observed localization of Cps1. 1: WT+empty vector, 2: WT +GlENTHp-mCh, 3: WT+gENTH-mCh, 4: WT+GlENTH$_{K75A}$p-mCh. **(B)** Only GlENTHp expression reduces α-factor maturation. Equivalent OD$_{600}$ units of *wild-type MAT***a** (WT *MAT***a**) cells (negative control) cells with an empty **p**MET25.426 or **p**GlENTHp-mCherry and *wild-type MAT*α (WT *MAT*α) (positive control), were spotted on a lawn of supersensitive *MAT***a** cells. Halo assay determination shows decreased growth inhibition of the underlying lawn for WT *MAT*α cells expressing GlENTHp-mCherry (top panel) but not when gENTH-mCherry or GlENTH$_{K75A}$p-mCherry were expressed (bottom panel). **(C)** Immunoblotting shows GlENTHp-mCherry (~80 kDa), gENTH-mCherry (~30 kDa) and

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GlENTH$_{K75A}$p-mCherry (~80 kDa) expressed in *wild-type* (WT/GFP-Cps1) cells by using the anti-Cherry Ab.

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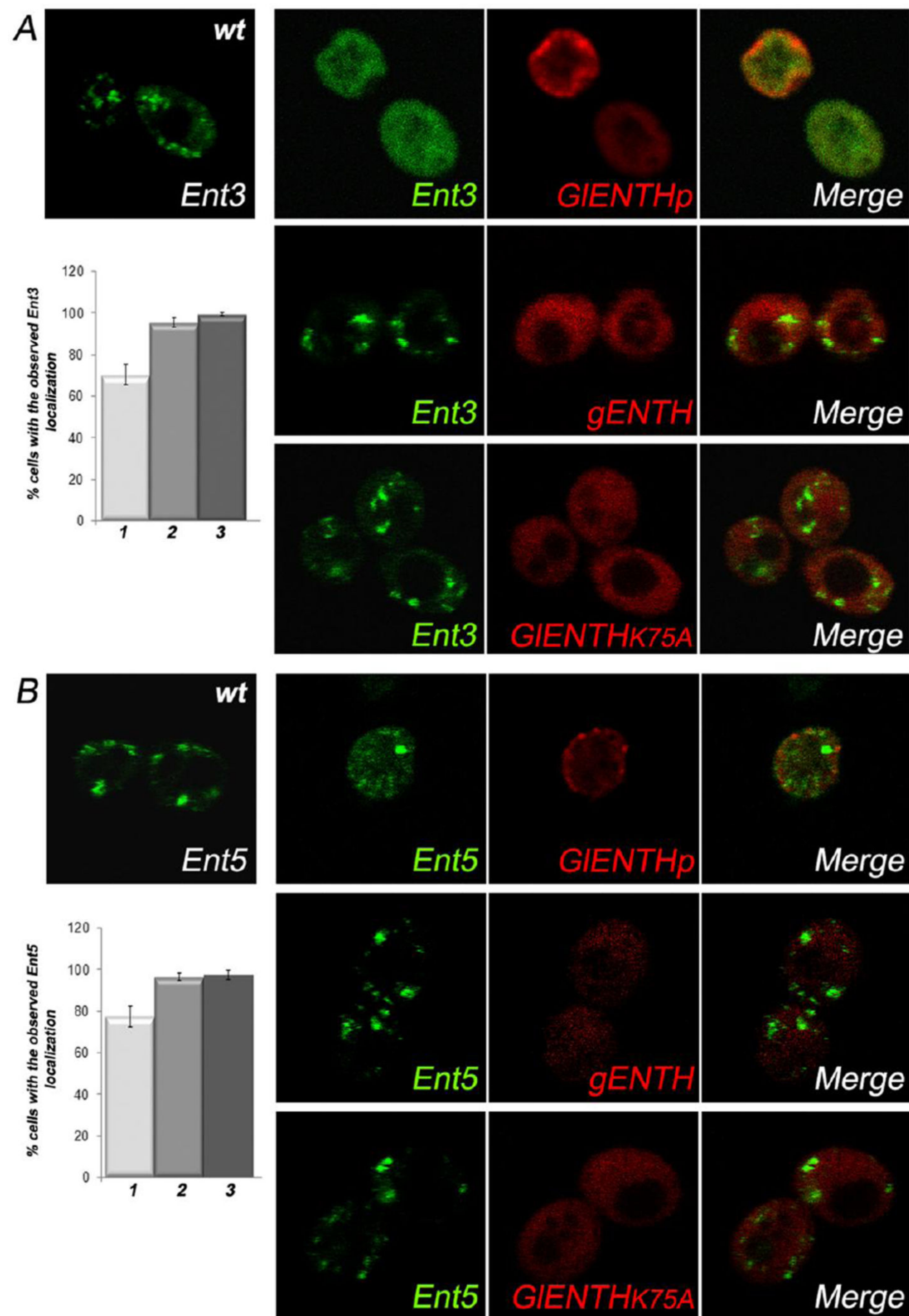

Figure 6. The whole GlENTHp produces Ent3/5p misslocalization

(A) *Wild-type* cells expressing Ent3p-GFP were transformed with pGlENTHp-mCherry, gENTH-mCherry, or pGlENTH$_{K75A}$p-mCherry vector. The expression of GlENTHp but not of gENTH or GlENTH$_{K75A}$p inhibits the correct localization of Ent3p. Graphic shows the percentage of cells with the observed localization of Ent3p. wt: *wild-type* cell expressing Ent3p. 1: GlENTHp-mCh, 3: gENTH-mCh, 4: GlENTH$_{K75A}$p-mCh. Error bars, s.e.m.; P < 0:005. **(B)** *Wild-type* cells expressing Ent5p-GFP were transformed with pGlENTHp-mCherry, gENTH-mCherry, or pGlENTH$_{K75A}$p-mCherry vector. Only the expression of

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GlENTHp causes a localization defect of Ent4p. Live cells were observed by confocal microscopy. Graphic showing the percentage of cells with the observed localization of Ent5p wt: *wild-type* cell expressing Ent3p or Ent5p. 1: GlENTHp-mCh, 3: gENTH-mCh, 4: $GlENTH_{K75A}p$-mCh. Error bars, s.e.m.; $P < 0:005$.

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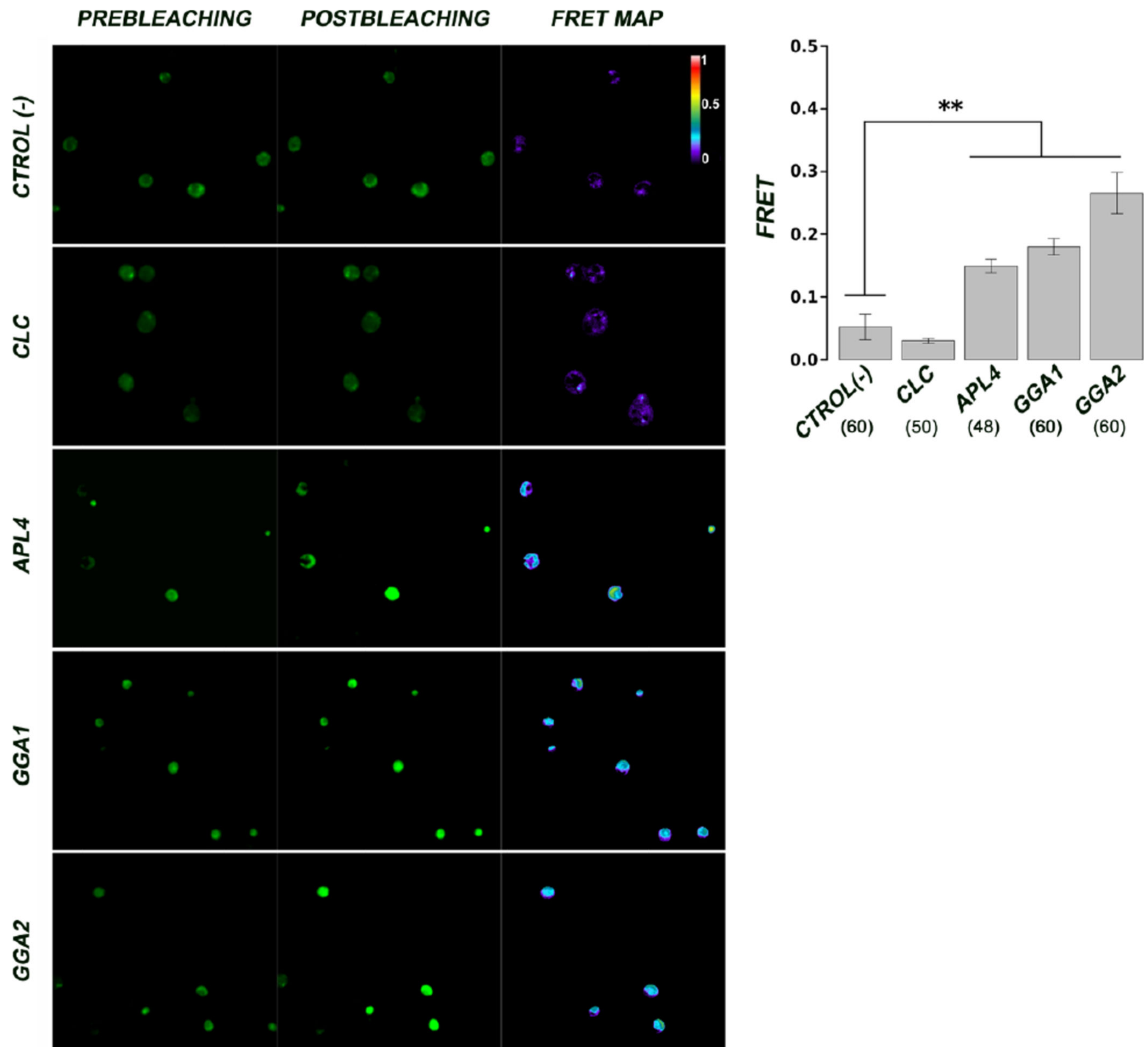

Figure 7. GlENTHp interacts with adaptor proteins

Strains expressing CLC, APL4, GGA1 and GGA2 transformed with GlENTHp-mCherry were subject to FRET analysis. Representative fluorescence of GFP pre- and postphotobleaching for each strain is showed. Pseudocolor images illustrating FRET efficiency is shown in the right panels. Pseudocolor scale bar is displayed. Bar graphs show the mean FRET efficiency for each pair. Data are presented as mean ± SEM. The number of cells for each group is indicated in parentheses. The pairs GlENTHp/APL4, GlENTHp/GGA1 and GlENTHp/GGA2 showed significant positive FRET when comparing with the negative control of CLC/empty mCherry vector.

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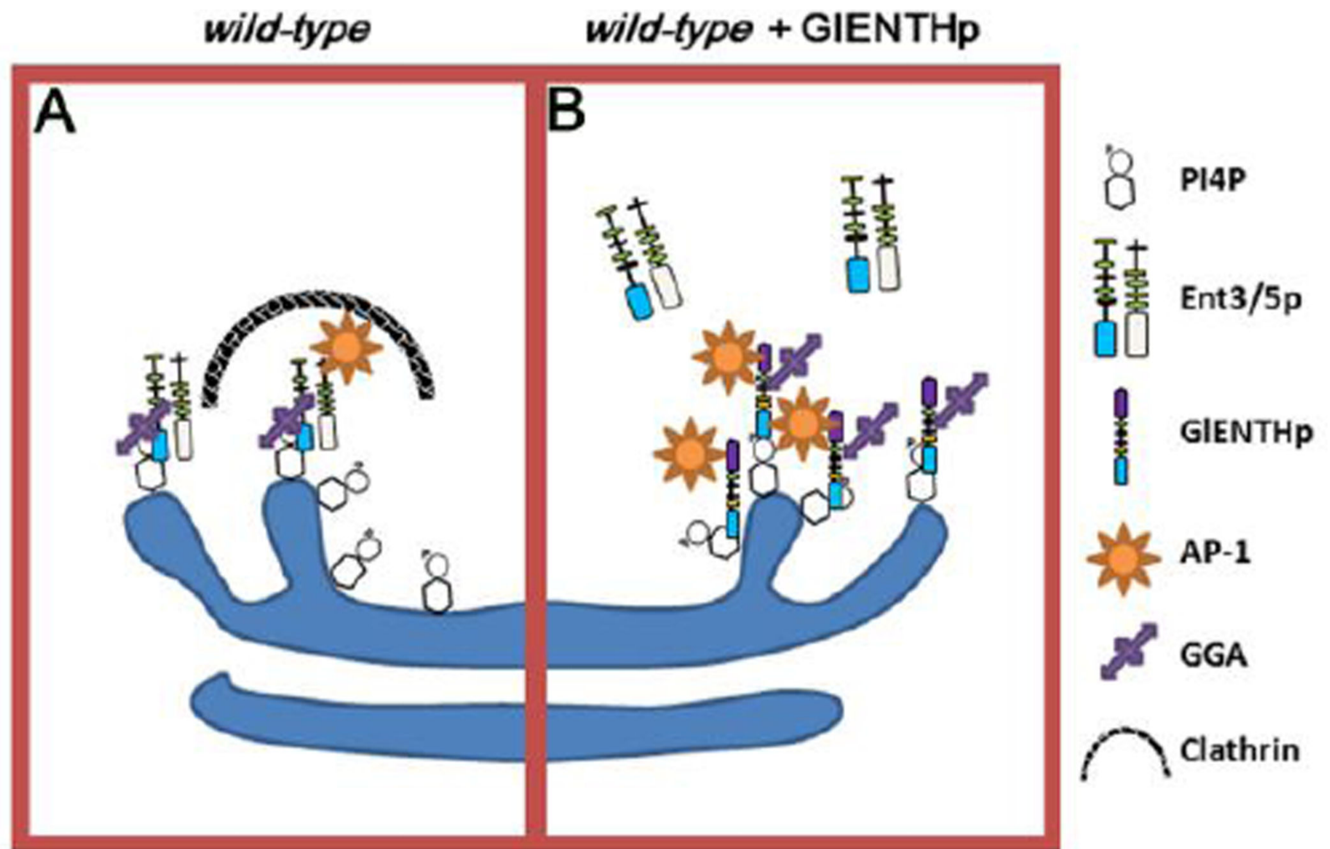

Figure 8. Hypothetical model showing how GlENTHp might interfere with the Ent3/5p function
(A) Membrane association of Ent3/5p requires binding of GGAs to PI4P localized in the trans cisternae of the Golgi apparatus (in blue) in yeast and the interaction with AP-1 and clathrin to participate in vesicular Golgi-to-vacuole trafficking (*wild-type* cells). (B) The whole GlENTH protein competes for PI4P, AP-1 and GGAs binding producing misslocalization of Ent3/5p. However, because GlENTHp do not bind to clathrin, impairs CCV formation and thus unable to complement the Ent3/5p function (*wild-type* cells overexpressing GlENTHp).

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Table 1

Strains used in this study

STRAIN	GENOTYPE	SOURCE
SEY6210	MAT α his3- 200 trp1- 901 leu2-3 ura3-52 lys2-801 suc2- 9	Wendland Laboratory's strain
SEY6210**a**	MAT**a** his3- 200 trp1- 901 leu2-3 ura3-52 lys2-801 suc2- 9	Wendland Laboratory's strain
BWY1170	SEY6210;ent1::HIS3ent2::HIS3 + pBW0768	Wendland Laboratory's strain
BWY2858	SEY6210; Ste3-GFP::KAN	Wendland Laboratory's strain
BWY5908 (MD256)	SEY6210; ent3::TRP1ent5::TRP1	Duncan Laboratory's strain
BWY2776	SEY6210; ABP1-GFP::G418	Wendland Laboratory's strain
BWY2784	SEY6210; ent1 ::LEU2 ent2 ::HIS3 yap1801 ::HIS3 yap1802 ::LEU2, ABP1-GFP(G418)+pBW0778 ENTH1.414 (TRP)	Wendland Laboratory's strain
BWY3033	SEY6210; ent1::LEU2 ent2::HIS3 yap1801::HIS3 yap1802::LEU2 bni1::NATMX4 STE3-GFP::KANMX6 + pBW0778 (pENTH1 CEN, TRP1)	Wendland Laboratory's strain
BWY5912	SEY6210 alpha, GFP-CLC::URA3	Wendland Laboratory's strain
αTESTER	GPY-1796 MAT**a** sst1 ::LYS2 ura3-52, leu2-3, 112 his3-D200 trp1-D901 lys2-801 suc2-D9 GAL-MEL	Wendland Laboratory's strain
QAY480	Mat a his3 1 leu2 0 ura3 0 lys2 0 ENT3-GFP(S65T)::KanMX6	Duncan Laboratory's strain
QAY188	Mat a his3D1 leu2D0 ura3D0 lys2D0 ENT5 - GFP::Kan	Duncan Laboratory's strain
APL4-GFP	BY4741 MATa his3 1 leu2 0 met15 0 ura3 0 APL4-GFP::HIS3	Invitrogen
GGA1-GFP	BY4741 MATa his3 1 leu2 0 met15 0 ura3 0 GGA1-GFP::HIS3	Invitrogen
GGA2-GFP	BY4741 MATa his3 1 leu2 0 met15 0 ura3 0 GGA2-GFP::HIS3	Invitrogen

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Table 2

Plasmids used in this study

PLASMID	DESCRIPTION	DETAILS	SOURCE
pBW0279	p ENT1 (*URA3*)	pMET25.426::ENT1(URA)	Wendland's lab
pBW0768	p ENT1 (*TRP1*)	pRS.414::ENT1(TRP)	Wendland's lab
pBW0778	p ENTH1 (*TRP1*)	pRS.414::ENTH1(TRP)	Wendland's lab
pBW2715	pGlENTHp-mCherry (*HIS3*)	pMET25.423:: GlENTHp- mCherry (HIS)	This study
pBW2701	pGlENTHp-mCherry (*URA3*)	pMET25.426::GlENTHp-mCherry (URA)	This study
pBW2702	pgENTH-mCherry (*URA3*)	pMET25.426::gENTH-mCherry (URA)	This study
pBW2720	pGlENTHN107Y-mCherry (*URA3*)	pMET25.426::GlENTHN107Yp-mCherry (URA)	This study
pBW2721	pgENTHN107Y-mCherry (*URA3*)	pMET25.426::gENTHN107Y-mCherry (URA)	This study
pBW2703	pGlENTHK75Y-mCherry (*URA3*)	pMET25.426::GlENTHK75Yp-mCherry (URA)	This study
pBW0405	pGFP-CPS1 (*URA3*)	pRS416::GFP-CPS1(URA)	Wendland's lab
pBW2713(MD151)	pENT5 (*URA3*)	pRS316::ENT5(URA)	Duncan's lab
pBW2712(MD150)	pENT5 (*LEU2*)	pRS315::ENT5(LEU)	Duncan's lab