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# Yeast epsins contain an essential N-terminal ENTH domain, bind clathrin and are required for endocytosis

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**The mammalian protein epsin is required for endocytosis. In this study, we have characterized two homologous yeast proteins, Ent1p and Ent2p, which are similar to mammalian epsin. An essential function for the highly conserved N-terminal epsin N-terminal homology (ENTH) domain was revealed using deletions and randomly generated temperature-sensitive *ent1* alleles. Changes in conserved ENTH domain residues in *ent1$^{ts}$* cells revealed defects in endocytosis and actin cytoskeleton structure. The Ent1 protein was localized to peripheral and internal punctate structures, and biochemical fractionation studies found the protein associated with a large, Triton X-100-insoluble pellet. Finally, an Ent1p clathrin-binding domain was mapped to the final eight amino acids (RGYTLIDL*) in the Ent1 protein sequence. Based on these and other data, we propose that the yeast epsin-like proteins are essential components of an endocytic complex that may act at multiple stages in the endocytic pathway.**

*Keywords*: actin/clathrin/endocytosis/ENTH domain/epsin

## Introduction

Endocytosis is an essential process in eukaryotic cells, in which extracellular fluids and portions of the plasma membrane are internalized. It is through endocytosis that cells take up nutrients, down-regulate the signaling activities of receptor/ligand complexes and allow viruses to gain entry. Of the multiple endocytic pathways that have been described, the most well characterized is the clathrin-dependent receptor-mediated endocytic pathway (reviewed in Schmid, 1997). In this pathway, the cytosolic tails of receptor proteins are recognized by an adaptor complex, which in turn recruits clathrin. The adaptor complex promotes the polymerization of clathrin triskelions into baskets, providing a mechanical deformation of the plasma membrane that results in the formation of a clathrin-coated pit. Following the activity of several proteins, most notably the GTPase dynamin, the clathrin-coated pit undergoes a fission event culminating in the release of a clathrin-coated vesicle into the cytosol. There are a number of homologous proteins required for endocytosis in yeast and mammalian cells, including clathrin (Tan *et al*., 1993) and accessory factors (Geli and Riezman, 1998; Wendland *et al*., 1998).

Despite years of study by many laboratories, many questions persist regarding the precise functions of proteins that mediate the complex process of clathrin-dependent endocytosis. Furthermore, additional factors that are required for this and other endocytic pathways remain to be identified. We have taken the approach of using the budding yeast *Saccharomyces cerevisiae* as a model for endocytosis for use in genetic screens to identify novel endocytic factors. In a screen for yeast mutants defective in the uptake of the fluorescent lipophilic dye FM4-64, we found that the Pan1 protein is required for endocytosis in yeast (Wendland *et al*., 1996). Interestingly, the structure of the Pan1 protein is remarkably similar to that of the mammalian endocytosis protein Eps15 (Wong *et al*., 1995; Wendland *et al*., 1996; Carbone *et al*., 1997; Benmerah *et al*., 1998). Both proteins contain multiple protein–protein interaction modules including two or three N-terminal Eps15 homology (EH) domains, a central region rich in heptad repeats and proline-rich C-termini.

A number of proteins associated with the endocytic pathway have been found to contain EH domains, thus spurring interest in determining the function of this conserved protein module and the identity of interacting proteins. Several recent studies of EH domains have determined the NMR structure of the EH domain (de Beer *et al*., 1998) and demonstrated that the ligand is the tripeptide asparagine–proline–phenylalanine (NPF) (Salcini *et al*., 1997). In addition, two-hybrid and other protein–protein interaction screens with the EH domains of both Eps15 and Pan1p have revealed a number of cellular ligands that contain the NPF motif (Haffner *et al*., 1997; Salcini *et al*., 1997; McPherson *et al*., 1998; Wendland and Emr, 1998). In this way, De Camilli and colleagues found a novel protein called epsin that contains three NPF motifs that bind to the EH domains of Eps15 (Chen *et al*., 1998). In addition, they found that, like Eps15, epsin is also required for endocytosis in mammalian cells.

In this study, we describe the characterization of yeast homologs of epsin, which we identified based on two-hybrid screen interactions with the EH domains of Pan1p (Wendland and Emr, 1998), and show that they are required for endocytosis in yeast. The primary region of similarity lies in the N-terminal domain, which recently was termed the epsin N-terminal homology (ENTH) domain (Kay *et al*., 1999). Using deletion mutants and temperature-sensitive alleles, we show that the ENTH domain is essential for normal endocytic function and actin cytoskeleton structure. We also demonstrate that the extreme C-terminus of Ent1p contains a clathrin-binding domain.

## Results

In a previously published two-hybrid study to identify factors that interact with the EH domains of Pan1p, we

identified yAP180A and yAP180B, yeast homologs of the mammalian AP180 clathrin assembly polypeptide. In addition, we found a weakly positive clone corresponding to open reading frame (ORF) YDL161w (Wendland and Emr, 1998). Another yeast gene, YLR206w, predicts a protein that is 66% similar to YDL161w. The proteins predicted by these genes were of interest to us for several reasons: (i) they contained two copies of the tripeptide motif NPF that is a ligand for EH domains (Salcini *et al*., 1997); (ii) they shared similar C-terminal sequences with the yAP180A and yAP180B proteins; and (iii) there was a low but significant degree of similarity with the N-terminal sequences of the yAP180 proteins. This N-terminal sequence homology is also found in two additional yeast ORFs, as well as in the mammalian protein epsin. Epsin, which participates in the endocytosis process, was identified due to its interaction with Eps15 (Chen *et al*., 1998), a protein similar to Pan1p. The conserved N-terminal domain that is shared among these proteins and others has been described recently and is termed the ENTH domain (Kay *et al.*, 1999). An alignment of the ENTH domains from the four ENTH domain-containing yeast proteins, epsin and yAP180B is shown in Figure 1, and five absolutely conserved residues are indicated with asterisks. Thus, in spite of the weak interaction between the Pan1p EH domain and YDL161w, the presence of two NPF motifs as well as the shared homology with epsin prompted us to study YDL161w and YLR206w. These genes have been renamed *ENT1* and *ENT2*, respectively, since they contain the ENTH domain, and the additional yeast ORFs, YJR125c and YLL038c, that also contain this domain have been renamed *ENT3* and *ENT4*, respectively.

### The ENTH domains of Ent1p and Ent2p are essential

To begin investigating the function(s) of Ent1p and Ent2p, we next performed single-step gene replacement to delete the *ENT1* and *ENT2* genes. Cells lacking either gene alone were viable and able to grow at both low and high temperatures. Proper function of the secretory and vacuolar protein sorting pathways was also confirmed (data not shown). Interestingly, however, cells in which both *ENT1* and *ENT2* are deleted were inviable, suggesting that these genes encode proteins with redundant functions. Viable double deletion cells can be recovered only if a plasmid bearing a functional version of either *ENT1* or *ENT2* is also present. This allowed us to use a standard plasmid shuffle technique (see Materials and methods) to ask which domains of the Ent proteins are necessary for viability. For these experiments, plasmids encoding internal deletions of Ent1p or Ent2p were constructed in which regions corresponding either to the ENTH domains or to more C-terminal segments were deleted. The ability of these plasmids to maintain viability of the double deletion strain was tested. Deletion of either protein's C-terminal segment sustained viability, whereas deletion of the ENTH domain resulted in cell death (Figure 2). These data indicate that the ENTH domain performs an essential function and that at least one ENTH domain from either Ent1p or Ent2p is necessary for cell viability and that the ENTH domains from Ent3p and Ent4p cannot act as substitutes.

Additional evidence for the importance of the ENTH domain in the function of Ent1p was obtained by generating temperature-sensitive (ts) alleles of *ENT1*. Error-prone PCR mutagenesis was used to introduce nucleotide substitutions randomly throughout the coding segment of *ENT1*, and then products of the PCR were incorporated into yeast vectors by gapped plasmid repair. Using the plasmid shuffle technique as before, the plasmids bearing *ent1* temperature-sensitive alleles were identified in colonies that could grow at 26°C but not at 37°C (Figure 3). Nine *ent1$^{ts}$* alleles were identified, and the plasmids bearing these alleles isolated. To test whether the ENTH domain was the region containing the mutation of importance, the mutations were mapped by replacing the C-terminal half of the mutated gene with wild-type sequences. These mapping data revealed that of nine original *ts* alleles that were selected for analysis, eight were still sensitive to high temperature, indicating that the relevant mutation within the gene resided in the 5′ end of the gene corresponding to the ENTH domain. These plasmids were then sequenced on both strands to determine the specific nucleotide substitutions that had occurred. Three alleles that each contained mutations resulting in the exchange of single amino acids (Figures 1 and 3) were subjected to further analysis. Interestingly, one of these alleles, *ent1-9*, encodes a single amino acid substitution in one of the absolutely conserved residues within ENTH domains (G87S). The temperature sensitivity of each of the *ts* alleles was suppressed by osmotic support on plates containing 1 M sorbitol. In contrast, the double deletion cells were still inviable (data not shown). Since each of the mutant Ent1 proteins were stably expressed at elevated temperatures (data not shown), the 1 M sorbitol may rescue the *ts* phenotype at non-permissive temperature by stabilizing the folding of the mutant proteins, or by supporting cell wall defects.

### Endocytic defects in ent1$^{ts}$ cells

The endocytic function of double deletion cells bearing *ent1$^{ts}$* alleles was assessed by examining the internalization of the fluorescent lipophilic dye FM4-64. This dye inserts into the plasma membrane of cells and is internalized in a time-, temperature- and energy-dependent process. Using fluorescence microscopy, FM4-64-labeled membranes can be followed as they transit from the plasma membrane to punctate cytosolic intermediate compartments, and ultimately are delivered to the lysosome-like vacuole of yeast (Vida and Emr, 1995). At the permissive temperature of 26°C, double deletion cells with an *ent1$^{ts}$* plasmid were able to internalize FM4-64 similarly to double deletion cells containing a plasmid bearing the wild-type *ENT1* gene (Figure 4). In contrast, *ent1$^{ts}$* cells that were pre-shifted to the non-permissive temperature of 37°C for 15 min prior to FM4-64 labeling exhibited a marked defect in endocytosis (Figure 4). In this case, the vacuolar membranes were less intensely labeled and instead the label appeared to accumulate in punctate structures within the cytoplasm and at the cell periphery. Essentially identical staining patterns were observed with each of the *ent1$^{ts}$* alleles tested (*ent1-1*, *ent1-2*, *ent1-5*, *ent1-6*, *ent1-8* and *ent1-9*). Cells in which the *ent1$^{ts}$* allele was complemented with an additional plasmid encoding wild-type *ENT1* were able to internalize FM4-64 normally. The reduced vacuolar

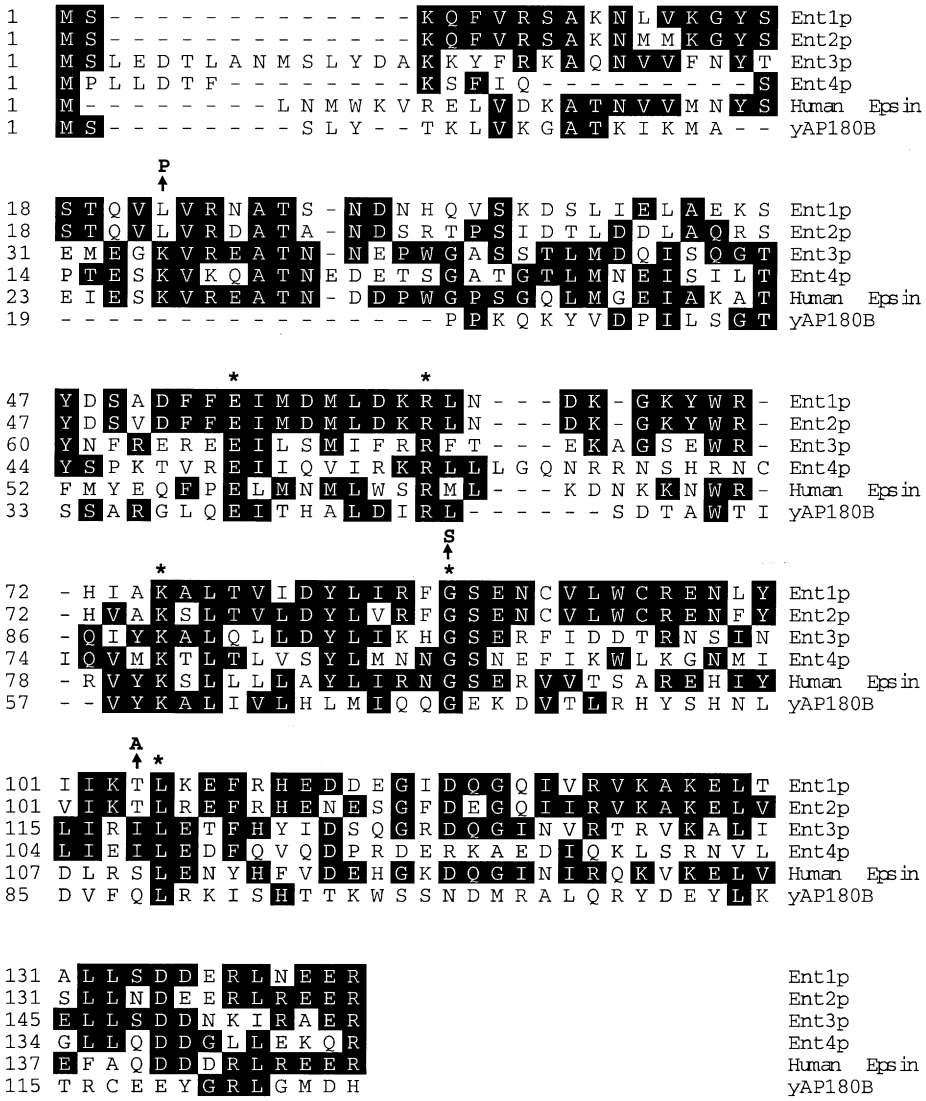


**Fig. 1.** Sequence alignment of the ENTH domains in yeast proteins. The N-terminal region of six proteins (Ent1p, YDL161w; Ent2p, YLR206w; Ent3p, YJR125c; Ent4p, YLL038c; human epsin, DDBJ/EMBL/GenBank accession No. D79993; and yAP180B, YGR241c) was aligned using MegAlign. Residues that are identical in at least two proteins (other than when two are identical only in Ent1p and Ent2p) are indicated by black boxes. Residues that are identical in all six proteins are designated by an asterisk (*). Amino acid substitutions in *ent1*$^{ts}$ alleles are shown with arrows at the substituted position.

membrane labeling and the accumulation of labeled structures within the *ent1*$^{ts}$ cells at the non-permissive temperature support a role for the Ent proteins in an internalization step, and/or a post-internalization step prior to vacuolar delivery.

As an additional measure of endocytic function in *ent1*$^{ts}$ cells, the stabilization of Ste6p was examined. Ste6p is the **a**-factor transporter, which is a member of the ABC transporter family that is localized at the plasma membrane of cells (reviewed in Michaelis, 1993). The half-life of Ste6p is ~15 min at 37°C, and its turnover occurs by endocytosis and delivery to the vacuole where it is degraded (Kolling and Hollenberg, 1994; Berkower *et al*., 1996). Ste6p degradation is slowed or blocked in endocytosis mutants due to defects in internalization from the plasma membrane or in delivery from an endosome to the vacuole. Ste6p turnover was examined by shifting the cells to 37°C for 10 min, then adding cycloheximide and collecting cells at various chase times, followed by immunoblotting. We found normal turnover in double deletion cells expressing wild-type *ENT1*, but even at prolonged chase times the Ste6p was stabilized in the *ent1-1* and *ent1-9* alleles (Figure 5). Higher molecular mass protein is also evident in the samples from the *ent1*$^{ts}$ cells; the precise nature of this material presently is unknown and could represent aggregates or protein that has been modified by phosphorylation, ubiquitination or some other post-translational modification. These data are consistent with an endocytic block at either the internalization step or the endosome-to-vacuole delivery step of endocytosis.

### *Actin cytoskeleton morphology in ent1*$^{ts}$ *cells*

A common phenotype observed among endocytosis mutants is a defect in the structure or polarity of the actin cytoskeleton (Riezman *et al*., 1996). Filamentous actin in yeast is present in both actin cables and cortical patches. The patches are located at the plasma membrane and are

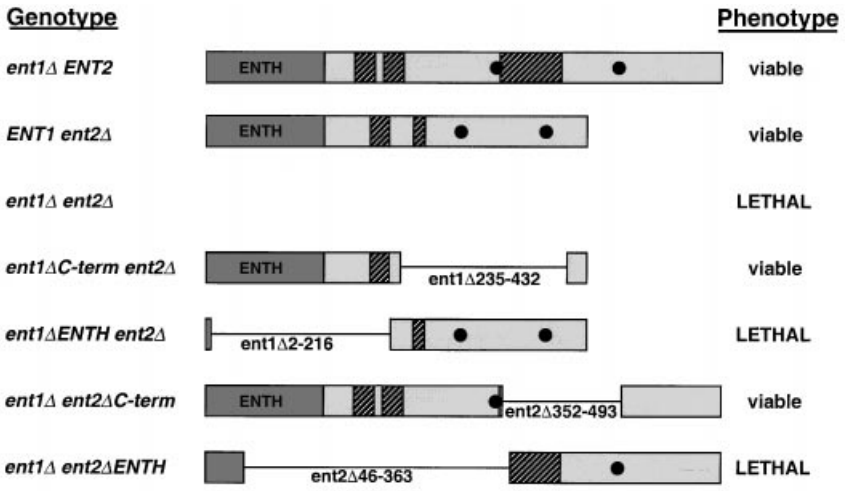


**Fig. 2.** The ENTH domain is the essential part of Ent1p and Ent2p. The genotypes of various strains are indicated on the left, and the corresponding phenotype on the right. Each of these strains have both *ENT* genes deleted and contain plasmids encoding either wild-type or internally deleted versions of *ENT1* or *ENT2*, as illustrated in the schematics. ENTH, ENTH domain; hatched boxes, heptad repeats; solid circles, NPF motifs.

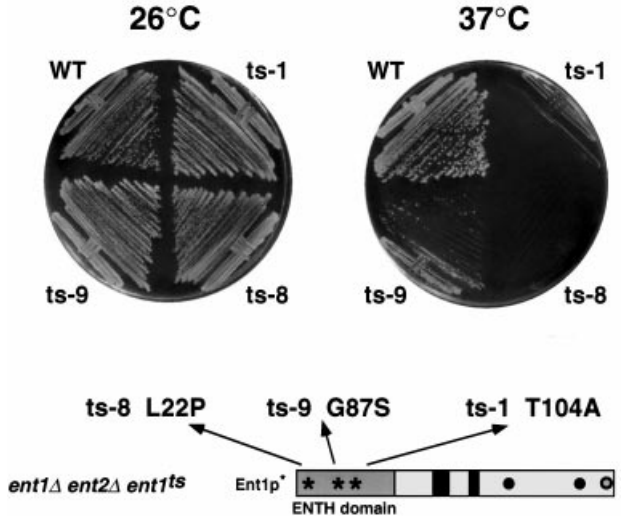


**Fig. 3.** Temperature-sensitive alleles in *ent1* are unable to grow at 37°C. Double deletion cells containing a plasmid encoding either wild-type or $ent1^{ts}$ alleles were streaked onto rich medium and grown at the permissive temperature of 26°C or at the non-permissive temperature of 37°C. The amino acid substitutions in the $ent1^{ts}$ alleles are indicated on the schematic below (wild-type residue, position number, *ts* allele residue.

mobile structures; furthermore, the localization of these patches varies as cells proceed through the cell cycle (Kilmartin and Adams, 1984). To examine the structure of the actin cytoskeleton in *ent1* mutant cells, double deletion cells containing plasmids bearing either wild-type *ENT1* or $ent1^{ts}$ alleles were incubated at 37°C for 1.5 h, followed by fixation and visualization of the filamentous actin using rhodamine-conjugated phalloidin. The nuclei were visualized using 4′,6-diamidino-2-phenylindole (DAPI). As can be seen in Figure 6, the cells with the wild-type *ENT1* displayed normal actin polarization, including the accumulation of patches within small buds and alignment of the patches between cells just prior to cytokinesis. In contrast, $ent1^{ts}$ cells exhibited actin patches that appeared to be enlarged and disorganized. Perhaps the most interesting observation is that while there is no clear cell cycle arrest phenotype, cells attempting to undergo cytokinesis do not redistribute actin patches to the mother cell for alignment at the plane between the mother and daughter cells (arrows in Figure 6). Interestingly, *pan1* mutants also exhibit cytokinesis defects (Tang and Cai, 1996) and aberrant actin structures at the necks of cells undergoing cytokinesis (Wendland *et al.*, 1996). These data indicate that in the absence of normal *ENT1/2* function, a defect in the structure and relocalization of the cortical actin patches occurs.

### *Localization of Ent1p*

The primary sequence of Ent1p predicts a cytosolic protein with no extended hydrophobic stretches. In order to determine the subcellular localization of Ent1p, a green fluorescent protein (GFP) fusion to the full-length Ent1 protein was generated. In cells expressing GFP–Ent1p, a punctate pattern of fluorescence was observed, with bright spots appearing both near the periphery of the cells and internally (Figure 7). This localization pattern is suggestive of Ent1p association with plasma membrane-associated patches and/or early endosomes. We needed to overexpress the GFP–Ent1p in order to observe its localization, and it is possible that some of these GFP spots could correspond to aggregates of protein. This is unlikely, however, since immunoelectron microscopy of Ent1p-HA expressed from a single copy vector revealed a similar localization to peripheral plasma membrane patches and to intracellular membranes (unpublished data). In addition, preliminary immunoelectron microscopy studies reveal a partial co-localization of Ent1p-HA with actin at the plasma membrane (unpublished data). This is consistent with the localization of other proteins implicated in endocytosis, including Pan1p (Tang and Cai, 1996).

Additional data to explore the localization of Ent1p were obtained through differential centrifugation fractionation studies using cells expressing an HA epitope-tagged Ent1p from a single copy vector. In these experiments, the majority of the Ent1 protein was found in the very low-speed P3 pellet (300 *g*), and a smaller fraction localized to the P13 pellet (13 000 *g*) (Figure 8) and is nearly identical to what is observed for Pan1p (unpublished data). When the fractionation was performed in the presence of

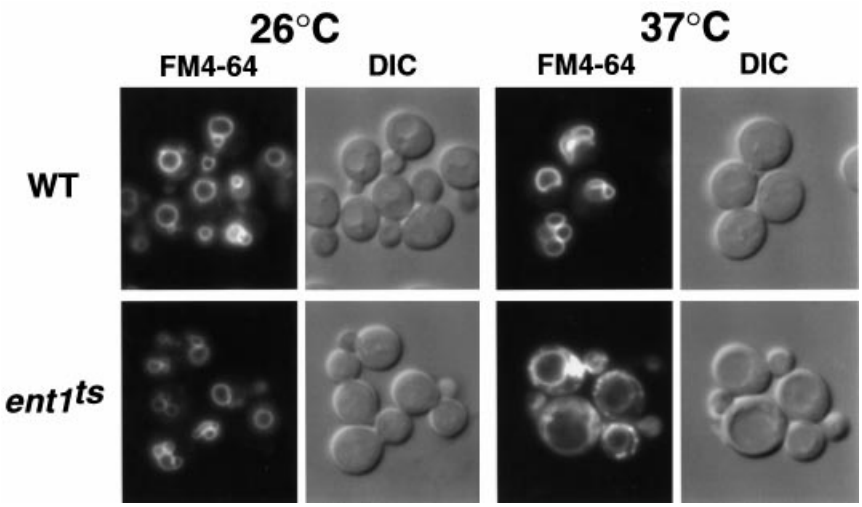


**Fig. 4.** FM4-64 internalization is defective in *ent1*$^{ts}$ cells. Double deletion cells containing a plasmid encoding either wild-type or *ent1*$^{ts}$ alleles were grown at 26°C or shifted to 37°C for 15 min. The cells were labeled with FM4-64 for 15 min and chased for 1 h at the appropriate temperature.

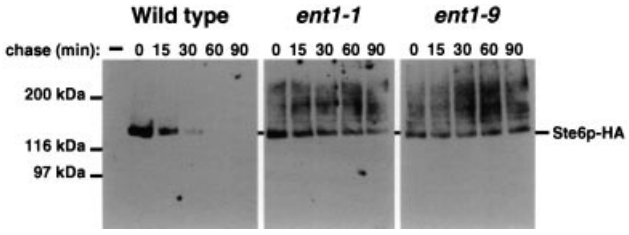


**Fig. 5.** The plasma membrane protein Ste6p is stabilized in *ent1*$^{ts}$ cells. Wild-type cells and double deletion cells containing a plasmid encoding either wild-type or *ent1*$^{ts}$ alleles and a single copy plasmid encoding Ste6p-HA were grown at 26°C, then shifted to 37°C for 10 min, cycloheximide added and cells collected at chase periods of 0, 15, 30, 60 and 90 min. Each sample was converted to a cell lysate and the Ste6p-HA visualized by immunoblotting. The negative control lane (–) corresponds to wild-type cells with an untagged Ste6p plasmid at 0 min chase.

the detergent Triton X-100, the Ent1 protein is still found exclusively in the P3 and P13 fractions, suggestive of a tight association with a large protein complex or subcellular structure such as the cytoskeleton. Similar Triton X-100 low-speed pellet fractionation has been observed for other endocytosis proteins including End3p (Benedetti *et al*., 1994) and Pan1p (Tang and Cai, 1996).

### *Ent1p binds clathrin*

We previously found a clathrin-binding domain in the yAP180A protein contained within the final 50 amino acids of the protein (Wendland and Emr, 1998). The C-terminal half of both yAP180A and yAP180B bind clathrin, and the primary homology within these regions is restricted to the final five amino acids. This suggested that these five amino acids (NLIDØ*) might correspond to the clathrin-binding domain in these proteins. Since the C-termini of the Ent proteins are also homologous to the yAP180 proteins, we asked if the C-terminus of Ent1p also contains a clathrin-binding activity. To determine if Ent1p is also a clathrin-binding protein, the C-terminal half of Ent1p was fused to GST, and the resulting fusion protein was bound to glutathione–agarose beads. Yeast extracts containing clathrin were then incubated with the beads, washed and the bound proteins separated by SDS–PAGE and analyzed by Western blotting (SKL1 monoclonal; Lemmon *et al*., 1988). As shown in Figure 9A, no clathrin binding was observed with a fusion protein corresponding to the non-clathrin-binding N-terminal half of yAP180A (neg. cont.), whereas clathrin bound the C-terminal half of Ent1p (Ent1p amino acids 323–454).

The sequence of the putative clathrin-binding domain in the Ent and yAP180 proteins is remarkably similar to the clathrin-binding motif defined in amphiphysin II (LLDLDFDP) and to a slightly shorter motif in the βadaptin3A protein (LLDLD) and the non-visual arrestin3 (LIEFE) (Krupnick *et al*., 1997; Dell'Angelica *et al*., 1998; Ramjaun and McPherson, 1998). Amphiphysin is a nerve terminal enriched protein that interacts with dynamin and synaptojanin; this protein complex has been implicated in synaptic vesicle endocytosis (David *et al*., 1996; Shupliakov *et al*., 1997). Arrestins are implicated in the down-regulation of signaling through G-protein-coupled receptors (Kuhn *et al*., 1984). βadaptin3A is a large subunit of the AP-3 adaptor-like complex (Dell'Angelica *et al*., 1997) that plays a role in membrane traffic from the Golgi to lysosomes/vacuoles (Cowles *et al*., 1997; Dell'Angelica *et al*., 1999). A requirement for clathrin in the AP-3-mediated pathway is controversial; however, it is clear from *in vitro* studies that the βadaptin3A subunit appendage region binds the N-terminal domain of clathrin (Dell'Angelica *et al*., 1998). The βadaptin3A motif ends with an acidic aspartate residue (...LLDLD...), whereas the motif in the four yeast proteins ends with the acidic C-terminus of the polypeptide chain (...LIDØ*) (Figure 9B). In order to demonstrate that the extreme C-terminal amino acids were sufficient for clathrin binding, and that the acidic C-terminus could substitute for the requirement for the aspartate residue in the previously defined clathrin-binding motif, two additional GST fusion proteins were generated. The first protein corresponded to the final eight amino acids of Ent1p fused to GST, and Figure 9A shows that these eight amino acids were sufficient for clathrin binding (Ent1p, RGYTLIDL*). In addition, the final eight amino acids of yAP180A were also fused to GST, and this fusion protein also bound clathrin (data not shown). As a control, another GST fusion was produced corresponding to the last eight amino

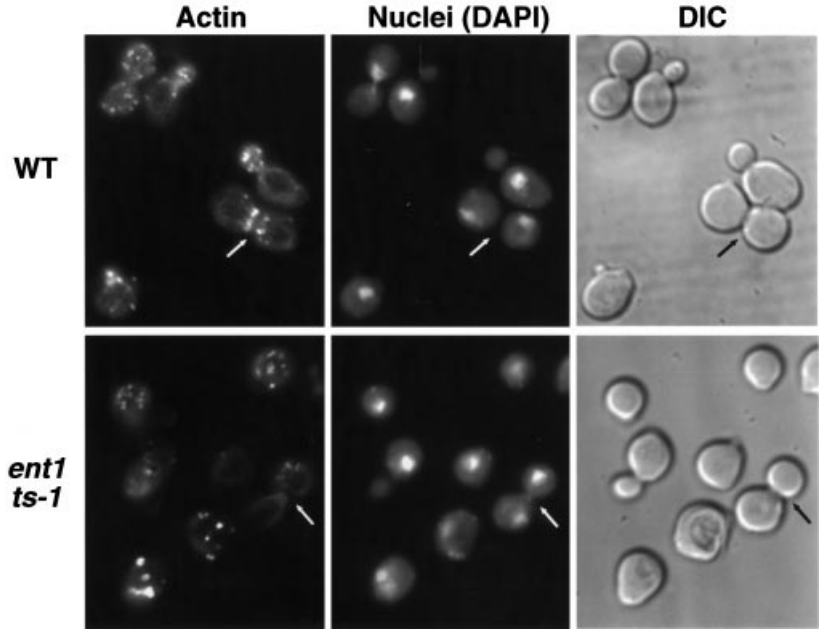


**Fig. 6.** The actin cytoskeleton is abnormal in *ent1*$^{ts}$ cells. Double deletion cells containing a plasmid encoding either wild-type or *ent1*$^{ts}$ alleles were grown at 26°C, then shifted to 37°C for 90 min followed by fixation with 4% formaldehyde. Filamentous actin was visualized using rhodamine–phalloidin and nuclei were detected with DAPI. Arrows indicate the neck between cells about to undergo cytokinesis.

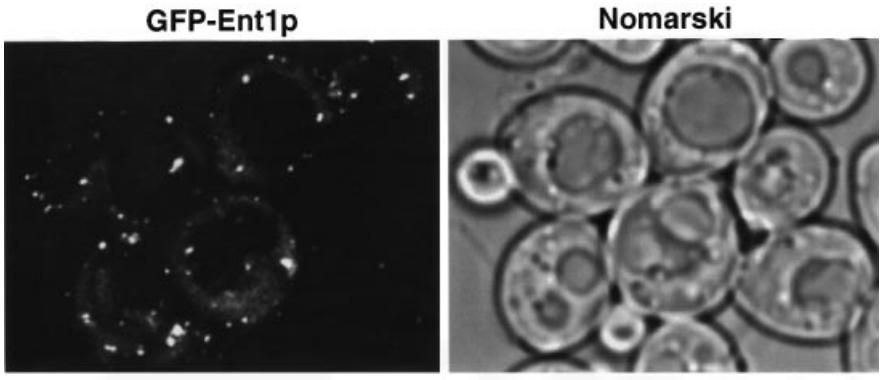


**Fig. 7.** GFP–Ent1p is localized to peripheral and internal punctate structures. Double deletion cells containing a plasmid encoding GFP–Ent1p were grown at 26°C and observed using a DeltaVision deconvolving microscope. A series of z-sections were collected and then collapsed into a single projection.

acids of Ent1p, but the acidic C-terminus was displaced from the LIDL sequence with five intervening alanine residues. In this case, clathrin binding was no longer observed (Ent1p, RGYTLIDLAAAAA*), supporting the requirement for an acidic moiety immediately following the LIDL sequence for clathrin binding. The ability of Ent1p to bind clathrin is additional evidence in support of an endocytic role for Ent1p.

## Discussion

In this study, we have characterized Ent1p and Ent2p, yeast homologs of the mammalian endocytosis protein epsin. The primary region of protein similarity lies at the N-termini, within the ENTH domains. Internal deletions and specific mutations within the ENTH domains revealed an as yet unknown, but essential function of this highly conserved motif. In addition, the yeast Ent proteins and rat epsin also share in common the presence of multiple NPF tripeptide motif ligands for EH domains, as well as direct or indirect binding to clathrin. Biochemical experiments suggested an association of Ent1p with a large protein complex. As with other endocytosis mutants, cells expressing a mutant allele of Ent1p also exhibit defects in the structure of the actin cytoskeleton. Together, these data suggest that Ent1p participates in endocytosis and actin regulation, presumably through interactions in a complex with other proteins.

### *The ENTH domain is required for the essential functions of Ent1p/Ent2p*

The ENTH domain is highly conserved from yeast to humans. The precise role of this protein domain currently is unknown; it may be a protein–protein interaction module, a protein–lipid interaction module, or perform an unrecognized enzymatic activity. ENTH domains are ~140 amino acids in length and generally appear at the N-terminus of a variety of proteins implicated in the regulation of endocytosis or cytoskeletal machinery (Kay *et al*., 1999). Sixteen very highly conserved amino acids are

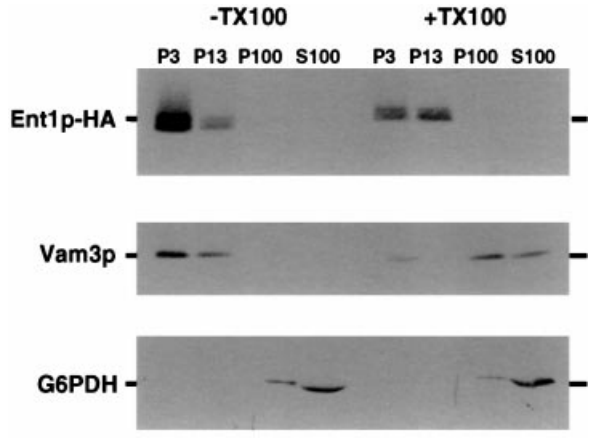


**Fig. 8.** The Ent1 protein associates with a large pelletable fraction that is Triton X-100 insoluble. Double deletion cells containing a plasmid encoding an epitope-tagged Ent1p were spheroplasted, lysed in buffer with or without 1% Triton X-100 and then subjected to sequential differential centrifugation. The vacuolar membrane protein Vam3p and the cytosolic protein glucose-6-phosphate dehydrogenase were used as markers. P3, 300 *g* pellet; P13, 13 000 *g* pellet; P100, 100 000 *g* pellet; S100, 100 000 *g* supernatant.

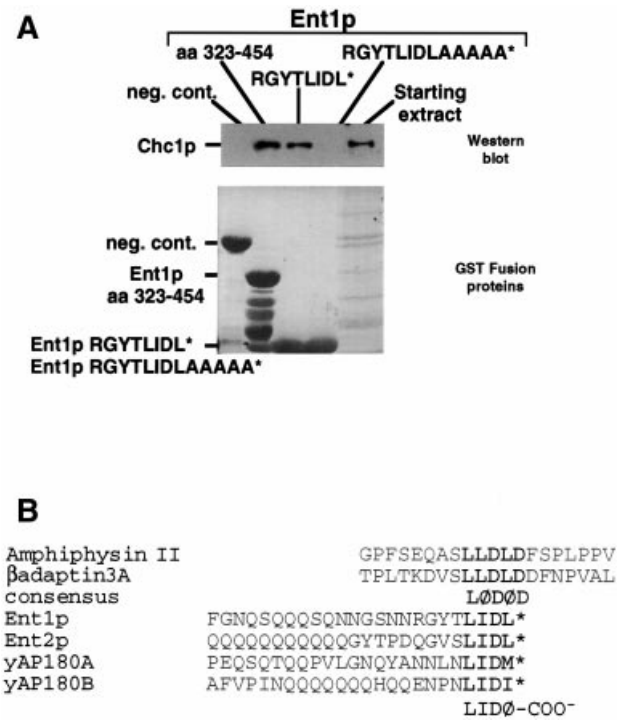


**Fig. 9.** The C-terminus of Ent1p binds clathrin. (**A**) GST fusion proteins were immobilized to glutathione–agarose beads which were incubated with yeast extracts, washed, and the bound proteins separated by SDS–PAGE. The gel was transferred to nitrocellulose and stained with amido black to reveal total protein (lower gel) or developed for an immunoblot with anti-clathrin heavy chain antibodies (upper gel). One-tenth of the starting extract was loaded for comparison. (**B**) The clathrin-binding motif of amphiphysin II and βadaptin3A is aligned to similar sequences at the C-termini of the yeast proteins Ent1p, Ent2p, yAP180A and yAP180B.

found distributed across the length of ENTH domains with a consensus sequence N-$x_{11–13}$-V-$x_2$-A-T-$x_{34–36}$-R-$x_{7–8}$-W-R-$x_3$-K-$x_{11}$-G-x-E-$x_{15}$-L-$x_{11–12}$-D-x-G-$x_3$-R-$x_{11}$-D-$x_7$-R. Cells in which the gene encoding either Ent1p or Ent2p has been deleted exhibit no obvious phenotypes or growth defects; however, cells in which both genes have been entirely deleted are inviable. This suggests that these proteins are capable of mediating a redundant function that is essential for cellular viability.

Two lines of evidence support the idea that the ENTH domain is the 'essential' part of the Ent proteins. First, double deletion cells in which a single, partially deleted version of either Ent1p or Ent2p is expressed reveal a requirement for at least one intact ENTH domain, whereas the C-terminal regions appear to be dispensable for viability. Secondly, in experiments in which the entire *ENT1* gene was subjected to error-prone PCR mutagenesis followed by selection for temperature-sensitive alleles, eight of the nine alleles isolated were found to have the relevant mutation within the ENTH domain. It is important to note that two other yeast genes, *ENT3* and *ENT4*, are predicted to encode ENTH domain-containing proteins. If these genes are expressed, their corresponding proteins are apparently functionally non-redundant with Ent1p or Ent2p. This is perhaps not surprising, since the ENTH domains of Ent1p and Ent2p are 76.9% identical, while their identities with the ENTH domains of Ent3p and Ent4p range from 24 to 34.4%. It will be interesting to study further the *ENT3* and *ENT4* genes to obtain additional information about the cellular role(s) of ENTH domains. Possible ENTH domain-like functions may also be performed by the N-terminal domains of the yAP180 proteins and, by extension, by their mammalian homologs AP180 and CALM (Zhou *et al*., 1992; Morris *et al*., 1993; Dreyling *et al*., 1996). These various ENTH domains may differ in their sites of function or interacting partners. To help elucidate the functions of ENTH domains, it also may be useful to identify dominant alleles for further study in yeast and mammalian cells.

### *Ent1p/Ent2p are required for endocytosis*

Cells expressing *ent1$^{ts}$* alleles exhibit a temperature-dependent defect in endocytosis as assayed by FM4-64 internalization and Ste6p stabilization. The block in endocytosis imposed by *ent1$^{ts}$* alleles is strikingly similar to that observed in *pan1$^{ts}$* alleles, in which the FM4-64 appears to accumulate in punctate cytosolic structures adjacent to the plasma membrane (Wendland *et al*., 1996). We do not know yet if these structures are still in continuity with the cell surface, representing a block in the earliest stages of endocytosis, or if these are internalized compartments, indicative of a post-internalization block. It is quite possible that the Ent1 protein could function at multiple points along the endocytic pathway, as has been proposed recently for Eps15 in mammalian cells (Torrisi *et al*., 1999). In fact, the localization of the GFP–Ent1p at both the cell periphery and internal compartments is consistent with this notion.

In addition to influencing the process of endocytosis, the *ent1$^{ts}$* alleles also affect the localization of the actin cytoskeleton, in particular at cytokinesis. There is a long standing correlation between endocytosis mutants and perturbations of the actin cytoskeleton, and it is interesting to note that both *ent1$^{ts}$* and *pan1$^{ts}$* alleles result in distinct defects at cytokinesis, although neither mutation causes arrest at a particular stage of the cell cycle (Tang and Cai, 1996). Each of these proteins, both in yeast and mammals, bind clathrin either directly or indirectly (van Delft *et al*., 1997; Chen *et al*., 1998). Thus, it is intriguing to recall

the cytokinesis defects observed in *Dictyostelium* clathrin mutants (Niswonger and O'Halloran, 1997), as well as yeast clathrin mutants which can become multinucleated (Lemmon and Jones, 1987). Perhaps the assembly of actin at the site of cytokinesis or some aspect of cell cycle checkpoint control during cytokinesis is particularly sensitive to alterations in certain components of the endocytic machinery. Consistent with this, it has been shown that rat epsin is phosphorylated by the cell cycle regulatory kinase Cdc2 (Chen *et al*., 1999), and the *Xenopus* homolog, MP90, was discovered originally due to its phosphorylation during mitosis (Stukenberg *et al*., 1997). We currently are investigating potential cell cycle-dependent post-translational modifications and regulation of Ent1p, as phophorylation has been shown to be important for the assembly and regulation of endocytic complexes (Slepnev *et al*., 1998). This is particularly relevant since the recently identified protein kinase Prk1p regulates Pan1p–End3p interactions through phosphorylation of Pan1p on threonine residues with a consensus site LxxQxTG (Zeng and Cai, 1999). Ent1p, Ent2p and yAP180B each contain two of these sites, and preliminary data indicate that Ent1p is phosphorylated on two threonine residues (unpublished data). We are now testing whether Ent1p is a substrate for Prk1p. In addition, our two-hybrid data indicate that there may be transient associations between Ent1p and Pan1p or other EH domain-containing proteins in yeast that are difficult to detect biochemically, given the low affinity of the NPF tripeptide–EH domain binding (de Beer *et al*., 1998; Paoluzi *et al*., 1998). This low affinity binding suggests that dynamic and regulated complexes can be formed, and this may be facilitated by the presence of multiple ligands on the binding proteins.

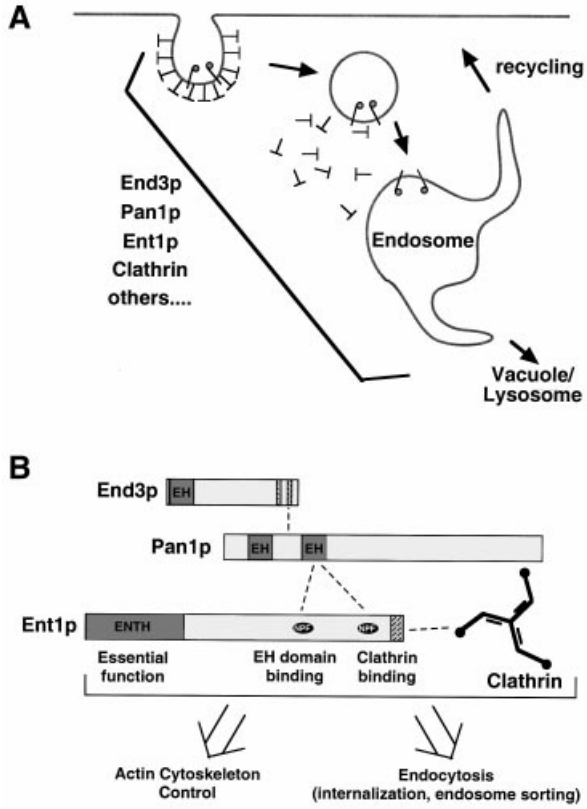


**Fig. 10.** A working model for Ent1p function. (**A**) A schematic of the endocytic process depicts the potential sites of action of Ent1p. T = clathrin triskelion. (**B**) Interactions between Pan1p, End3p and Ent1p are shown, with potential regulatory influences by Prk1p activity. Regulation of the endocytic process and the actin cytoskeleton depends upon the activity of these proteins.

### *Ent1p binds clathrin through a C-terminal motif*

A clathrin-binding motif corresponding to the amino acids LLDLD recently was defined within the protein sequences of amphiphysin II and βadaptin3A (Dell'Angelica *et al*., 1998; Ramjaun and McPherson, 1998). Interestingly, this sequence is reminiscent of the conserved sequence at the extreme C-termini of the yAP180 and Ent proteins. This was of particular interest to us since the clathrin-binding domain of yAP180A had been mapped to the final 50 amino acids (Wendland and Emr, 1998). In this study, we found that the C-terminal region of Ent1p also contained a clathrin-binding activity. The primary difference is that the sequence in the yeast proteins is seen at the extreme C-terminus and lacks the terminal aspartate residue (LIDØ*), which suggested to us that the acidic C-terminus might serve as a substitute for the final D residue in the βadaptin3A motif. This appears to be the case since GST fusion proteins encoding the final eight amino acids of either yAP180A or Ent1p could bind clathrin, but when an additional five alanines preceded the stop codon, thereby displacing the site of the acidic C-terminus, clathrin binding was no longer observed. When the entire yeast genome is searched for candidate proteins that contain this C-terminal clathrin-binding motif, only six proteins are identified: yAP180A, yAP180B, Ent1p, Ent2p, Kar5p (Beh *et al*., 1997) and the uncharacterized ORF YEL015w. It will be interesting to determine if YEL015w and Kar5p also bind clathrin, as well as the role of each of these proteins in clathrin-related and membrane trafficking functions.

The structure of the N-terminal domain of clathrin was solved recently, and it is a seven-bladed β-propeller in which the clathrin-binding domain of arrestin3 (LIEFE) binds in between two blades of the propeller (ter Haar *et al*., 1998). When this binding site in clathrin is mutated, other proteins can still bind (Goodman *et al*., 1997), suggesting that perhaps distinct binding partners are recognized at individual pairs of blade interfaces (ter Haar *et al*., 1998). Thus, it is possible that clathrin could bind simultaneously to several proteins. It is noteworthy to compare the complexes that could form between clathrin and C-terminal motif clathrin-binding partners with complexes formed between proteins containing multiple PDZ domains bound to their corresponding C-terminal motif binding partners (Songyang *et al*., 1997; Tsunoda *et al*., 1997).

Based on these and other data, we suggest that Ent1p is a participant at multiple steps in the endocytic pathway in concert with clathrin, End3p and Pan1p (Figure 10), perhaps acting at both internalization and endosomal sorting. The precise role of this complex(es) of proteins is not yet known; however, it is likely that these protein interactions are subject to regulation that ultimately affects endocytic function. Similar hypotheses have been put forth for Eps15 and other mammalian EH domain-containing proteins including intersectin and Ese (Coda *et al*., 1998;

**Table I.** Strains used in this study

| Strain | Genotype | Source |
|---|---|---|
| SEY6210 | *MAT*α *leu2-3 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9* | Emr laboratory strain |
| SEY6210**a** | *MAT*α *leu2-3 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9* | Emr laboratory strain |
| SEY6210**a**/α | *MAT***a** *leu2-3 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9*<br>*MAT*α *leu2-3 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9* | Emr laboratory strain |
| BWY500 | *MAT***a** *leu2-3 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 ENT1*<br>*MAT*α *leu2-3 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 ent1::LEU2* | this study |
| BWY501 | *MAT***a** *leu2-3 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 ENT1 ent2::HIS3*<br>*MAT*α *leu2-3 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 ent1::LEU2 ENT2* | this study |
| BWY502 | *MAT***a** *leu2-3 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 ent1::LEU2 ent2::HIS3 pENT1.416* | this study |
| BWY503 | *MAT***a** *leu2-3 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 ent1::LEU2 ent2::HIS3 pENT2.416* | this study |
| BWY504 | *MAT*α *leu2-3 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 ent1::LEU2 ent2::HIS3 pENT1.416* | this study |
| BWY505 | *MAT*α *leu2-3 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 ent1::LEU2 ent2::HIS3 pENT2.416* | this study |
| BWY506 | *MAT***a** *leu2-3 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 ent1::LEU2 ent2::HIS3 pENT1.414* | this study |
| BWY507 | *MAT***a** *leu2-3 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 ent1::LEU2 ent2::HIS3 pENT2.414* | this study |
| BWY508 | *MAT*α *leu2-3 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 ent1::LEU2 ent2::HIS3 pENT1.414* | this study |
| BWY509 | *MAT*α *leu2-3 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 ent1::LEU2 ent2::HIS3 pENT2*.414 | this study |
| BWY510 | *MAT***a** *leu2-3 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 ent1::LEU2 ent2::HIS3 pENT1 Δ*ENTH | this study |
| BWY511 | *MAT***a** *leu2-3 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 ent1::LEU2 ent2::HIS3 pENT2 Δ*ENTH | this study |
| BWY512 | *MAT***a** *leu2-3 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 ent1::LEU2 ent2::HIS3 pENT1 Δ*C-term | this study |
| BWY513 | *MAT***a** *leu2-3 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 ent1::LEU2 ent2::HIS3 pENT2 Δ*C-term | this study |
| BWY514 | *MAT***a** *leu2-3 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 ent1::LEU2 ent2::HIS3 pent1-1* | this study |
| BWY515 | *MAT***a** *leu2-3 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 ent1::LEU2 ent2::HIS3 pent1-8* | this study |
| BWY516 | *MAT***a** *leu2-3 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 ent1::LEU2 ent2::HIS3 pent1-9* | this study |
| BWY517 | *MAT*α *leu2-3 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 ent1::LEU2 ent2::HIS3 pent1-1* | this study |
| BWY518 | *MAT*α *leu2-3 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 ent1::LEU2 ent2::HIS3 pent1-8* | this study |
| BWY519 | *MAT*α *leu2-3 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 ent1::LEU2 ent2::HIS3 pent1-9* | this study |
| BWY520 | *MAT***a** *leu2-3 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 ent1::LEU2 ent2::HIS3 pENT1-GFP.426* | this study |
| BWY521 | *MAT***a** *leu2-3 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 ent1::LEU2 ent2::HIS3 pENT1.1HA.414* | this study |
| TVY614 | *MAT*α *leu2-3 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9pep4::LEU2 prb1::HIS3 prc1::HIS3* | Emr laboratory strain |

Yamabhai *et al*., 1998; Sengar *et al*., 1999). A new family of actin-regulatory protein kinases was described recently (Cope *et al*., 1999), and one of these, Prk1p, phosphorylates Pan1p in the same region where End3p binds (Zeng and Cai, 1999). When End3p is bound, Pan1p is no longer phosphorylated; this suggests that phosphorylation of Pan1p may prevent (negatively regulate) End3p binding. Interestingly, the candidate Prk1p phosphorylation sites in Ent1p occur on either side of the second NFP tripeptide motif. It is possible that Prk1p activity could result in a competition for binding at this site of Pan1p, and may be in part responsible for the selection of Pan1p of other EH domain-binding partners.

Studies of the complex process of endocytosis in the complementary systems of yeast and mammalian cells are revealing interesting information about the required proteins and their functions, interactions and regulation. As new proteins are discovered, and as additional tools are generated to study known proteins, targets for small molecule therapeutics to control aberrant signaling in cancer cells or to block viral infection ultimately may be realized.

## Materials and methods

### *Media and materials*

Yeast strains were grown in standard yeast extract–peptone–dextrose (YPD) or synthetic medium with dextrose supplemented with the appropriate amino acids as required for plasmid maintenance. Bacterial strains were grown on standard media supplemented with 100 μg/ml ampicillin or 30 μg/ml kanamycin, as appropriate, to maintain plasmids. Materials were purchased from Fisher Scientific Co. (Fairlawn, NJ) or Sigma Chemical Co. (St Louis, MO) unless stated otherwise.

### *Plasmid and strain construction*

The strains used in these studies are listed in Table I. Standard recombinant DNA techniques were performed as previously described (Maniatas *et al*., 1982) with reagents obtained from Boehringer Mannheim Corp. (Indianapolis, IN) or New England Biolabs Inc. (Beverly, MA).

The *ENT1* and *ENT2* genes were obtained by PCR amplification of chromosomal DNA and cloning into pRS414 and pRS416 to produce pENT1.414, pENT1.416, pENT2.414 and pENT2.416. The *ent1::LEU2* deletion construct was produced by cloning the *ENT1 Pst*I–*Hin*dIII fragment in pBlueScript(KS)II–, then inserting the *LEU2* gene into the *Bgl*II–*Bst*EII sites of *ENT1*, deleting amino acids 6–431 of Ent1p. The DNA was linearized with *Sca*I and *Ssp*I and transformed into yeast for one-step gene replacement, and confirmed by PCR. To delete *ENT2*, oligos corresponding to 50 nucleotides upstream or downstream of the coding sequence flanked by 20 nucleotides of 5′ or 3′ *HIS3* sequences were used to PCR amplify the *HIS3* gene. The PCR product was then transformed into yeast for one-step gene replacement, and confirmed by PCR. The deletion strains were generated by sequential deletion of the SEY6210**a**/α diploid, followed by sporulation and tetrad dissection.

The internal ENTH domain *ENT1* deletion construct was generated by dropping out the 587 bp *Pfl*MI–*Bsm*I fragment, filling in with T4 DNA polymerase to correct the reading frame, and religating the vector. The internal C-terminal *ENT1* deletion construct was generated by dropping out the 591 bp *Bst*EII fragment and religating the vector. The internal ENTH domain *ENT2* deletion construct was generated by dropping out the 957 bp *Bgl*II–*Sph*I fragment, chewing back with T4 DNA polymerase to correct the reading frame, and religating the vector. The internal C-terminal *ENT2* deletion construct was generated by dropping out the 430 bp *Nco*I–*Afl*II fragment, filling in with Klenow enzyme to correct the reading frame, and religating the vector. The junctions were sequenced to confirm that the reading frame was correct. As part of this sequencing effort, we discovered some differences in our YDL161w gene as compared with the gene in the *Saccharomyces* Genome Database (http://genome-www.stanford.edu/Saccharomyces/). These differences result in several in-frame deletions and insertions spanning coding sequence nucleotides 743–798. This changes amino acids 248–266 in the database to **L**LQ**Q**QQQQQQQQ**QQ**LQQQPMY**C**, where changed amino acids are in bold and two inserted residues are underlined. These plasmids were introduced into BWY502 cells, followed by plasmid shuffle. For plasmid shuffle, cells contain two plasmids; a wild-type copy of the gene on a *URA3* plasmid and a second *TRP1* plasmid with either a wild-type or a mutant form of the gene. Cells that express the Ura3p enzymatic activity convert the compound 5-fluoro-orotic acid (5-FOA) into a toxic substance and thus are inviable on

5-FOA plates; this allows for selection of viable cells that have eliminated the *URA3* plasmid. In addition, in the *Δent1 Δent2* strain, a plasmid encoding a functional copy of either *ENT1* or *ENT2* is required for viability. If the *TRP1* plasmid does not contain a functional version of *ENT1* or *ENT2*, the cells cannot lose the *URA3* plasmid with the wild-type *ENT1* gene and thus will be inviable on 5-FOA.

The *ts ent1* alleles were produced using error-prone PCR mutagenesis with oligos corresponding to 20 bp 5′ and 3′ to the start and stop codons, respectively, and pENT1.416 as the template. Gapped pENT1.414 was prepared by cutting with *Bst*BI and *Bst*EII and co-transformed with the PCR product into BWY502, and plated onto –URA –TRP medium. The colonies were replica plated to two 5-FOA plates and incubated at 26 or 37°C. Plasmid linkage was confirmed for colonies that grew at low but not at high temperature by isolating the candidate pENT1.414-ts plasmid, retransforming into BWY502 cells and restreaking to 5-FOA plates incubated at 26 or 37°C. Mapping of the mutation to the ENTH domain was done by replacing sequences 3′ of the *Bsm*I site with the corresponding sequences from the wild-type pENT1.414.

The GFP–Ent1p fusion protein plasmid was made using PCR to convert the start codon of *ENT1* to a *Sal*I site, filling in with Klenow enzyme and ligating the fragment in-frame to the Klenow-filled *Eco*RI site of pGOGFP.426. The HA-tagged Ent1p fusion protein plasmid was generated by using PCR to convert the stop codon of *ENT1* to a *Sma*I site, and performing a triple ligation between pENT1.414 with the *Bsm*I–*Sac*II (polylinker site) fragment removed, the *Bsm*I–*Sma*I fragment from the PCR product and a *Sma*I–*Sac*II fragment from the $p(HA)_3$ vector. The GST–Ent1p C-terminal fusion protein plasmid was made by in-frame ligation of the *Spe*I–*Sac*I fragment with the *Xba*I–*Sac*I sites of pGEX-KG-KAN. The GST fusion proteins with the final eight amino acids of Ent1p were made by annealing two complementary oligos with overhanging sticky ends complementary to and in-frame with the *Eco*RI and *Hin*dIII sites of pGEX-KG-KAN, and were confirmed by sequencing.

### *FM4-64 internalization*

Cells were grown to mid-log phase in YPD at 26°C, the cultures split and incubation continued at either 26 or 37°C for 15 min. One ml of cells were pelleted at 300 *g* for 30 s and resuspended in 50 ml of pre-warmed FM4-64 dye diluted in YPD [FM4-64 stock is 1 mg/ml in dimethylsulfoxide (DMSO), diluted 1:50 for 26°C and 1:100 for 37°C labeling]. After 15 min labeling, the cells were washed and chased for 40–60 min and observed. All images were acquired at identical exposures and processed identically using Adobe Photoshop®5.0.

### *Ste6p-HA stabilization*

SEY6210**a**, BWY514 and BWY516 cells were tranformed with pSM192 and pSM786 (Berkower *et al*., 1996) and grown in selective medium to early-log phase, shifted to 37°C for 10 min, and 1 μg/ml cycloheximide added. Aliquots of 2 $OD_{600}$ cells were collected at 0, 15, 30, 60 and 90 min chase points into 10 mM $NaN_3$ and NaF on ice to stop further metabolism. Cells were converted to lysates in 0.25 M NaOH, 140 mM β-mercaptoethanol on ice for 15 min, then trichloroacetic acid (TCA) precipitated and solubilized in protein sample buffer at 37°C for 30 min. The proteins were separated by 7.5% SDS–PAGE and visualized by immunoblotting with anti-HA monoclonal antibodies and Supersignal reagents (Pierce, Rockford, IL).

### *Filamentous actin and GFP–Ent1p localization*

BWY506, BWY514, BWY515 and BWY516 were grown to mid-log phase at 26°C, shifted to 37°C for 90 min and fixed with 4% formaldehyde. The cells were washed in phosphate-buffered saline (PBS) and permeabilized with 0.02% Triton X-100 for 10 min, washed and incubated with 1.5 mM rhodamine–phalloidin (Molecular Probes, Eugene, OR) for 2 h, washed, mounted in antifade solution (DABCO plus DAPI) and observed using a DeltaVision Deconvolving microscope (Applied Precision, Seattle, WA). SEY6210 cells expressing the GFP–Ent1p fusion protein were grown to mid-log phase at 26°C and observed as above.

### *Ent1p differential centrifugation fractionation*

SEY6210 cells expressing the Ent1p-HA fusion were grown to mid-log phase, spheroplasted and homogenized with 10 strokes in a Kontes dounce homogenizer. This lysate was subjected to sequential centrifugations of 300 *g* for 5 min, 13 000 *g* for 10 min and 100 000 *g* for 1 h. The supernatant and pellet fractions were precipitated on ice with 10% TCA, washed twice with ice-cold acetone, and the protein pellets solubilized in protein sample buffer. The proteins were separated by SDS–PAGE and transferred to nitrocellulose for immunoblotting with antibodies against the HA epitope (12CA5, Boehringer Mannheim, Indianapolis, IN), Vam3p (generated in the Emr laboratory) or glucose-6-phosphate dehydrogenase (Sigma, St Louis, MO), and the immunoblot was developed with ECL reagents (Amersham, Piscataway, NJ).

### *Clathrin binding experiments*

TVY614 cells were grown in YPD to $OD_{600}$ = 2, 1000 OD harvested, and cells lysed in 2 ml of MES lysis buffer [0.1 M MES pH 5.5, 0.5 mM $MgCl_2$, 1 mM EGTA, 0.2 mM dithiothreitol (DTT), 0.1 mM AEBSF, 0.02% $NaN_3$] with 3 g of 0.4–0.6 mm glass beads in a 30 ml Corex tube by vortexing 15 times for 30 s each with 1 min on ice between vortex episodes. The supernatant was recovered by centrifugation at 3000 *g* for 10 min at 4°C, then at 100 000 *g* for 1 h at 4°C. The pellet was solubilized in MES lysis buffer plus protease inhibitors and 1% Triton X-100 for 2 h at 4°C, then the extract was spun at 21 000 *g* for 5 min, and the supernatant collected at a final protein concentration of ~15–20 mg/ml. After binding GST fusion proteins to glutathione–agarose beads, 20 ml of 50% beads were incubated with 100–300 μg of extract for 1 h at 4°C with mixing. The beads were washed three times with 1 ml of ice-cold PBS + 0.1 mM AEBSF, dried and resuspended in 40 ml of protein sample buffer. The bound proteins were separated by SDS–PAGE and analyzed by immunoblotting. The nitrocellulose filter was stained with amido black to visualize total proteins and the bound clathrin detected using an anti-clathrin heavy chain monoclonal antibody [SKL1 (Lemmon *et al*., 1988), provided by G.Payne; UCLA, Los Angeles, CA].

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