Regulation of clathrin coat assembly by Eps15 homology domain–mediated interactions during endocytosis

Ryohei Suzuki*, Junko Y. Toshima*, and Jiro Toshima*<sup>a</sup>,<sup>b</sup>,<sup>*</sup>

*Department of Biological Science and Technology, Tokyo University of Science, 2641 Yamazaki, Noda, Chiba 278-8510, Japan; <sup>b</sup>Research Center for RNA Science, Research Organization for Information Science and Technology, Tokyo University of Science, 2641 Yamazaki, Noda, Chiba 278-8510, Japan

ABSTRACT Clathrin-mediated endocytosis involves a coordinated series of molecular events regulated by interactions among a variety of proteins and lipids through specific domains. One such domain is the Eps15 homology (EH) domain, a highly conserved protein–protein interaction domain present in a number of proteins distributed from yeast to mammals. Several lines of evidence suggest that the yeast EH domain–containing proteins Pan1p, End3p, and Ede1p play important roles during endocytosis. Although genetic and cell-biological studies of these proteins suggested a role for the EH domains in clathrin-mediated endocytosis, it was unclear how they regulate clathrin coat assembly. To explore the role of the EH domain in yeast endocytosis, we mutated those of Pan1p, End3p, or Ede1p, respectively, and examined the effects of single, double, or triple mutation on clathrin coat assembly. We found that mutations of the EH domain caused a defect of cargo internalization and a delay of clathrin coat assembly but had no effect on assembly of the actin patch. We also demonstrated functional redundancy among the EH domains of Pan1p, End3p, and Ede1p for endocytosis. Of interest, the dynamics of several endocytic proteins were differentially affected by various EH domain mutations, suggesting functional diversity of each EH domain.

INTRODUCTION

Clathrin-mediated endocytosis is a coordinated series of molecular events, including cargo loading, formation and invagination of coated pits, and vesicle formation (Geli and Riezman, 1998; Engvist-Goldstein and Drubin, 2003; Sorkin, 2004; Toret and Drubin, 2006). Recent live-cell imaging studies revealed the detailed timing of protein recruitment to sites of clathrin-mediated endocytosis in budding yeast and mammalian cells (Merrifield et al., 2002; Kaksonen et al., 2003, 2005; Ehrlich et al., 2004; Newpher et al., 2005). In yeast, Syp1p, a SGI1-α–related protein, and Ede1p, a Eps15-related protein, are the earliest proteins known to arrive at sites of endocytosis (Kaksonen et al., 2005; Toshima et al., 2006; Newpher et al., 2005). Ent1/2p and Yap1801/2p, which are related to the mammalian clathrin adaptors epsin and AP180/CALM, respectively, have both clathrin- and phosphatidylinositol 4,5-bisphosphate–binding domains and may function to recruit clathrin to sites of endocytosis (Wendland and Emr, 1998; Wendland et al., 1999; Newpher et al., 2005). One to two minutes after Syp1p and Ede1p arrive at such sites, the late coat module, including the Pan1p complex, appears and couples coat formation to actin polymerization in cooperation with Las17p, a yeast WASP, and Myo5p (Kaksonen et al., 2003; Sun et al., 2006). These coordinated endocytic events are regulated by interactions among a variety of proteins and lipids through specific domains (Engvist-Goldstein and Drubin, 2003). One such domain is the Eps15 homology domain (EH domain), which is an evolutionarily conserved protein–protein interaction domain present in a number of proteins distributed from yeast to mammals (Di Fiore et al., 1997;
The EH domain was originally identified as a domain comprising repeats of ~100 amino acids in the N-terminal region of Eps15, which is a binding partner of ε-adaplin, a component of the AP-2 complex and part of the clathrin-coated pit (Benmerah et al., 1995, 1996; van Delft et al., 1997). Several experiments, including phage-display screens and screening of a human fibroblast expression library, demonstrated that the EH domain binds to peptides containing the Asn-Pro-Phc (NPF) sequence (Salcini et al., 1997; Paoluzi et al., 1998). Structural analyses demonstrated that NPF residues are almost completely embedded in a conserved hydrophobic pocket within the EH domain, allowing close contact between the asparagine residue of the tripeptide and a highly conserved tryptophan residue in the EH domain (de Beer et al., 1998, 2000). Mutation of this conserved tryptophan residue dramatically impairs binding of the EH domains to NPF motifs, and the mechanism of EH domain/NPF motif interaction is likely conserved among most EH domains (Salcini et al., 1997; de Beer et al., 1998; Grant and Caplan, 2008).

In yeast, three EH domain–containing proteins (EHDPs)—Pan1p, End3p, and Ede1p—have been reported to play important roles during endocytosis. Pan1p is another yeast Eps15-related protein. It contains two EH domains and forms a stable complex with End3p, which also has two EH domains (Raths et al., 1993; Benedetti et al., 1994; Tang et al., 1997; Wendland and Emr, 1998; Toshima et al., 2007). Previous studies showed that Pan1p and Ede1p bind to NPF motifs of the clathrin adaptors Yap1801/2p and/or Ent1/2p and that the dynamics of Pan1p and Ede1p depends on these interactions (Wendland and Emr, 1998; Howard et al., 2002; Aguilar et al., 2003; Maldonado-Baez et al., 2008). These observations suggest that the EH domain/NPF interaction between Eps15-related proteins and clathrin adaptors is conserved from yeast to mammals. Although genetic and cell-biological studies of EHDPs suggest a role for the EH domain in clathrin-mediated endocytosis, functional differences among the EH domains of Pan1p, End3p, and Ede1p and how these EHDPs regulate clathrin coat assembly are not fully understood.

In the present study we find that the EH domains of Pan1p, End3p, and Ede1p function redundantly for efficient assembly of the clathrin coat. By live-cell imaging of cortical patch dynamics, we demonstrate that the patch lifetimes of Pan1p, End3p, and Ede1p are increased when their EH domains are mutated. We also demonstrate that mutations of the EH domains cause a defect of cargo internalization and delay assembly of the clathrin coat without affecting assembly of the actin patch. Furthermore, the EH domains of Pan1p, End3p, and Ede1p are required for proper localization of the endocytic coat proteins Slp1p and Yap1801p, suggesting that the EH domains target these proteins to endocytic sites in order to facilitate clathrin coat assembly.

**RESULTS**

**Mutations in the EH domains of Pan1p, End3p, and Ede1p increase their lifetime at cortical patches**

Several lines of evidence indicate that the yeast EHDPs Pan1p, End3p, and Ede1p play important roles during endocytosis (Miliaras and Wendland, 2004). The EH domain is frequently present as tandem repeats within a single protein (Santolini et al., 1999; Grant and Caplan, 2008), and Pan1p, End3p, and Ede1p contain two or three EH domains, respectively, in their N-terminal regions (Figure 1A). Previous studies showed that mutation of a highly conserved tryptophan residue in the EH domain impairs its ability to bind to the NPF motif (Salcini et al., 1997; de Beer et al., 2000). To explore in detail the role of the EH domain in yeast endocytosis, we mutated all of the conserved tryptophan residues in the EH domains of Pan1p, End3p, or Ede1p to alanine and integrated them into their endogenous loci (termed pan1EH, end3EH, and ede1EH, respectively). Although pan1EH and ede1EH cells were viable at both 37 and 39°C, end3EH cells were temperature sensitive for growth at 39°C (Figure 1B). Because the second EH domain of End3p has only limited similarity and it is unclear whether it really functions as an EH domain (Santolini et al., 1999), we created additional mutants that were mutated in only the first or second EH domain of End3p. We found that both of the mutants were temperature sensitive for growth at 39°C (Supplemental Figure S1A), suggesting that the second EH domain might be important for the function of End3p. To examine the effects of EH domain mutation on EHDP dynamics, we tagged Pan1p, End3p, and Ede1p with green fluorescent protein (GFP) and examined the resulting effects. The GFP-tagged EH domain mutants were expressed at similar levels to the wild-type proteins (Supplemental Figure S1B). All of the GFP-tagged mutants were localized to cortical patches (Supplemental Figure S1, C–E), suggesting that the EH domain/NPF motif interactions are unnecessary for primary targeting of the EHDPs to cortical patches. Consistent with previous reports, Pan1-GFP and End3-GFP patches formed at the cell cortex with lifetimes of 26.5 ± 5.5 and 28.6 ± 4.5 s, respectively, culminating in inward movement (Figure 1C; Kaksenson et al., 2005). Pan1EH-GFP and End3EH-GFP patches formed and disappeared with the typical inward movement, but their lifetimes were increased to 34.6 ± 10.5 and 42.4 ± 7.8 s, respectively (Figure 1C). Tracking of individual patches showed that the distance and timing of the inward movement of Pan1EH-GFP or End3EH-GFP patches was almost the same as that of the wild-type proteins but that the time required to reach the maximum fluorescence intensity was increased (Figure 1D and Supplemental Figure S2), suggesting that mutation in EH domains might cause a delay in accumulation of EHDPs to endocytic sites. Ede1p is an early-arriving endocytic protein that appears at the cell cortex before Sla1p and has a lifetime ranging widely from ~30 to 150 s (Figure 1E; Toshima et al., 2006; Stimpson et al., 2009). Similar to Pan1p and End3p patches, Ede1EH-GFP patches had a significantly increased lifetime, and ~34% of them remained stationary at the plasma membrane for >240 s (Figure 1E). The EH domain therefore seems to be important for efficient recruitment of EHDPs to sites of endocytosis.

**Dynamics of the Sla1p patch and actin patch in EH domain mutants**

We next examined whether the dynamics of the late coat module and actin patch were affected in the EH domain mutants. We used Sla1-GFP and Abp1-mRFP to analyze the dynamics of the late coat module and actin patch, respectively (Kaksenson et al., 2003). First, we measured the mean lifetimes of both Sla1-GFP and Abp1-mRFP in each mutant. The GFP-tagged EH domain mutants showed significantly increased lifetime, and ~34% of them remained stationary at the plasma membrane for >240 s (Figure 1E).
major EH domain functions in Pan1p and Ede1p. In contrast, in comparison to the end3$^{\Delta EH}$ mutant, the end3$^{\Delta EH}$ mutant exhibited much more severe phenotypic features, which resembled those of the end3$^{-}$null mutant shown previously (Supplemental Figure S3, D–F; Kaksonen et al., 2005). Although Pan1p and Ede1p are relatively large proteins composed of 1480 and 1381 amino acids, respectively, and contain several domain structures, End3p is a small protein (349 amino acids) in which more than half of the structure is occupied by the EH domains. Thus the function of End3p besides the EH domain might be disrupted in the end3$^{\Delta EH}$ mutant because of structural instability. In addition to the end3$^{\Delta EH}$ mutant, we also examined the phenotype of another end3$^{-}$ mutant, end3 K47E/172E, in which two lysine residues that are potential sites for lipid binding (Naslavsky et al., 2007) are mutated. The end3 K47E/172E mutant exhibited almost the same phenotype as wild-type cells (Supplemental Figure S3, B and E–G), indicating that these sites are not critical for the function of End3.

Functional redundancy of EH domains for endocytic patch formation

To investigate functional redundancy among EHDPs, we analyzed the effect of double or triple mutants on the dynamics of Sla1p or Abp1p. Although pan1$^{\Delta EH}$ end3$^{\Delta EH}$ cells were temperature sensitive for growth at 37°C, the growth of pan1$^{\Delta EH}$ ede1$^{\Delta EH}$ cells was almost

were observed, except that the time required to reach the maximum fluorescence intensity was increased in end3$^{3EH}$ mutants or decreased in ede1$^{3EH}$ mutants (Figure 2B). Taken together, these data indicate that the EH domain is required for regulation of Sla1p coat assembly but not for actin-driven membrane invagination and vesicle internalization.

To clarify whether the NPF-binding pocket is responsible for most of the functions of the EH domain or whether the EH domain has other functionally important interactions, such as lipid binding (Naslavsky et al., 2007), outside the NPF-binding pocket, we deleted the entire EH domains of Pan1p, End3p, and Ede1p (termed pan1$^{\Delta EH}$, end3$^{\Delta EH}$, and ede1$^{\Delta EH}$, respectively) and tested the phenotypic consequences in these mutants. All of the EH deletion mutants were localized to cortical patches, although parts of End3$^{\Delta EH}$-GFP formed a large aggregate in the cytosol, confirming that the EH domains are unnecessary for primary targeting of the EHDPs to cortical patches (Supplemental Figure S3A). The ede1$^{\Delta EH}$ mutants exhibited almost the same phenotype as the ede1$^{3EH}$ mutant in terms of growth, localization, and Sla1p and Abp1p dynamics (Supplemental Figure S3, B, D–F). Similar to the pan1$^{\Delta EH}$ mutant, the pan1$^{\Delta EH}$ mutant also showed no marked phenotype, except that lifetimes of Pan1p and Sla1p were slightly decreased compared with wild-type cell (Supplemental Figure S3, B, D–F). The NPF-binding pocket therefore seems to be responsible for the major EH domain functions in Pan1p and Ede1p. In contrast, in comparison to the end3$^{3EH}$ mutant, the end3$^{3EH}$ mutant exhibited much more severe phenotypic features, which resembled those of the end3-null mutant shown previously (Supplemental Figure S3, D–F; Kaksonen et al., 2005). Although Pan1p and Ede1p are relatively large proteins composed of 1480 and 1381 amino acids, respectively, and contain several domain structures, End3p is a small protein (349 amino acids) in which more than half of the structure is occupied by the EH domains. Thus the function of End3p besides the EH domain might be disrupted in the end3$^{3EH}$ mutant because of structural instability. In addition to the end3$^{\Delta EH}$ mutant, we also examined the phenotype of another end3$^{-}$ mutant, end3 K47E/172E, in which two lysine residues that are potential sites for lipid binding (Naslavsky et al., 2007) are mutated. The end3 K47E/172E mutant exhibited almost the same phenotype as wild-type cells (Supplemental Figure S3, B and E–G), indicating that these sites are not critical for the function of End3.

**FIGURE 1:** Generation and characterization of EH mutants in Pan1p, Ede1p, and End3p. (A) Sequence alignments of the EH domains in Pan1p, End3p, Ede1p, and mouse Eps15. Residues chosen for mutagenesis are highlighted in red. Residues conserved highly and moderately throughout the family are indicated in black and gray, respectively. (B) The end3$^{3EH}$ mutant was temperature sensitive for growth at 39°C. A dilution series of cells was plated on YPD medium and incubated at 25, 37, or 39°C. (C) Average lifetimes of Pan1-GFP and End3-GFP ± SD in wild-type and mutant cells. Data were taken from 2-min movies with a 1-s frame interval. n = 50 patches for each strain. **p < 0.001. (D) Quantification of fluorescence intensity (red) and distance from site of patch formation (blue) as a function of time for patches of indicated GFP-tagged proteins. Each curve represents data from one patch. Behavior of three independent patches was plotted for each strain. (E) Distribution of Ede1-GFP and Ede1$^{3EH}$-GFP patch lifetimes. Movies were taken with a 1-s frame interval. n = 50 patches for each strain.
that the lifetimes of Sla1p patches were decreased in double mutants, including the \textit{ede1}^{EH} mutation, even in \textit{end3}^{EH} cells, which showed a markedly increased Sla1p lifetime (Figures 2A and 3B), indicating that the EH domains of Ede1p have a dominant function in determining the lifetime of Sla1p patches. As shown in kymographs and particle-tracking analysis, inward movement of Sla1p appeared to be normal, but the times required to reach the maximum fluorescence intensity were changed in these double or triple mutants, similar to single mutants (Figure 3C).

We next examined the dynamics of non-mutated Pan1p, End3p, and Ede1p in cells expressing mutant forms of the other EH-DPs. Of interest, the dynamics of Pan1p and End3p were quite similar to those of Sla1p: the Pan1p and End3p patch lifetimes were increased in the \textit{end3}^{EH} or \textit{pan1}^{EH} mutants and decreased in the \textit{ede1}^{EH} mutant (Figure 4, A–D). This suggests that Pan1p and End3p are able to form a complex with Sla1p, components of the late endocytic coat module, and act together as reported previously (Tang et al., 2000), even though the functions of the EH domains are disrupted. Ede1p patch lifetimes were increased in all of the \textit{pan1}^{EH}, \textit{end3}^{EH}, and \textit{pan1}^{EH} \textit{end3}^{EH} mutants (Figure 4, E and F).

We further examined the effect on endocytic internalization by assessing the internalization of \textit{35}S-labeled \(\alpha\)-factor. \textit{end3}^{EH} cells exhibited a modest defect of \(\alpha\)-factor internalization, whereas \textit{pan1}^{EH} or \textit{ede1}^{EH} cells had only a slight defect (Figure 5A), consistent with the finding that \textit{end3}^{EH} cells had a more severe phenotype in terms of growth and Sla1p-GFP dynamics (Figures 1B and 2A). The relative \(\alpha\)-factor internalization rates of the mutants are compared in the histogram shown in Figure 5B. Unexpectedly, combination of the \textit{end3}^{EH} and \textit{pan1}^{EH} mutations had no additive effect, although they exhibited a synthetic growth defect (Figures 3A and 5B). The \textit{end3}^{EH} mutant also had little additive effect on \(\alpha\)-factor internalization when combined with \textit{ede1}^{EH} cells but a remarkable effect when combined with \textit{end3}^{EH} cells (triple mutant; Figure 5B). This result suggested that \textit{pan1}^{EH} and \textit{ede1}^{EH} cells exhibited a redundant function only when combined with \textit{end3}^{EH} cells. These results clearly demonstrate the presence of functional redundancy among these three EHDPs.

**Dynamics of the early endocytic component in EH domain mutants**

Similar to mammalian cells, clathrin in yeast is localized to cortical patches (Merrifield et al., 2002; Kaksonen et al., 2003, 2005;
Figure 3: Redundant function of EH mutants in endocytic patch formation. (A) The pan\(^{EH}\) end3\(^{EH}\) and triple mutants were temperature sensitive for growth at 37°C. A dilution series of cells was plated on YPD medium and incubated at the indicated temperature. (B) Patch lifetimes of Sla1-GFP (top) and Abp1-mRFP (bottom) ± SD in the indicated strains. Data were obtained from 2-min movies taken with a 1-s frame interval. \(n = 50\) patches for each strain. **p < 0.001. (C) Left, localization of Sla1-GFP and Abp1-mRFP from the strains indicated. Middle, kymograph representations of Sla1-GFP and Abp1-mRFP from the boxed area for the indicated times. Right, quantification of fluorescence intensity (blue) and distance from site of patch formation (red) as a function of time for patches of Sla1-GFP. Each curve represents data from one patch. Behavior of three independent patches was plotted for each strain. All movies were taken with a 1-s frame interval for both Sla1-GFP and Abp1-mRFP. Scale bars, 2 μm.

Newpher et al., 2005). Clathrin appears early at all sites of endocytosis, preceding Sla1p, and has a mean lifetime of ~73.8 s (Kaksonen et al., 2005; Newpher et al., 2005). Therefore we next examined whether the overall process of clathrin coat assembly was affected by EH domain mutations. Because a large proportion of clathrin localizes to intracellular compartments, such as the trans-Golgi network, in addition to cortical patches and it is difficult to analyze the cortical localization precisely (Kaksonen et al., 2005; Newpher and Lemmon, 2006), we used another early-arriving endocytic protein, Syp1p, which has a long and variable lifetime similar to that of clathrin, as a marker of clathrin-coat assembly (Stimpson et al., 2009). To analyze extremely long lifetimes of Syp1p in the EH mutants (Figure 6), we tagged Syp1p with three tandem tagged copies of GFP (3GFP). The 3GFP-tagged Syp1p showed similar localization to Syp1-GFP at the cortical patches in addition to the bud neck, as reported previously (Supplemental Figure S4, A and B; Stimpson et al., 2009). The lifetime of Syp1p patches labeled by Syp1-3GFP in wild-type cells ranged from 50 to 350 s, with an average of 118.1 ± 51.0 s (Figure 6, A and C). The lifetime of Syp1-3GFP patches was a little longer compared with that of single GFP-tagged Syp1p (Stimpson et al., 2009) because of the enhanced fluorescence (Supplemental Figure S4C). All single mutants showed increased Syp1p patch lifetimes relative to wild-type cells, even in ede1\(^{EH}\) cells (Figure 6, A and C, and Supplemental Figure S4D). These effects were enhanced modestly in the pan1\(^{EH}\) end3\(^{EH}\) and clearly in the triple mutant (Figure 6, B and C). For other double mutations, such as pan1\(^{EH}\) ede1\(^{EH}\) or end3\(^{EH}\) ede1\(^{EH}\), no additive effect was observed (Figure 6C). It was noteworthy that these results were well consistent with those of α-factor internalization, shown in Figure 5B. Kymographs for these mutants showed that majority of Syp1p patches had extended lifetimes but regularly disappeared from the cell cortex in all mutants (Figure 6D).

To determine the precise timing of Syp1p and Sla1p recruitment to the cortical patches, we imaged the cells expressing Syp1-3GFP and Sla1-mCherry. In wild-type cells, Syp1p appeared ~61.4 s prior to Sla1p and stayed with Sla1p for ~28.9 s at the cortical patch (Figure 7, A and B). Mutations in the EH domains of Pan1p caused the lifetime of Syp1p to increase but had almost no effect on the lifetimes of Sla1p and Abp1p (Figure 2A and 6B). In agreement with these results, in the pan1\(^{EH}\) mutant, only the timing of Sla1p recruitment...
Syp1-3GFP–labeled endocytic sites in the triple mutants are capable of being internalized into the cytosol. Having previously reported that ede1Δ mutants form fewer Sla1p-labeled endocytic sites than wild-type cells (Stimpson et al., 2009), we assessed the number of endocytic sites in the triple mutants. Maximum-intensity Z projections of live cells were analyzed to determine the average number of patches per surface area. Patch densities were quantified only in the mother cells, in which individual patches were distinguishable. As shown in Figure 8C, in the triple mutant, there was no decrease in the number of Syp1p patches per surface area relative to wild-type cells. We further examined whether EH domain mutations could affect the frequency of endocytotic internalization by comparing the number of Abp1-mRFP patches—a marker of actin-driven membrane invagination and endocytotic vesicle internalization. Maximum-intensity projections of Z stacks revealed that the number of endocytotic internalizations labeled with Abp1-mRFP decreased by ∼41% in the triple mutants (Figure 8D). This observation suggests that the frequency of endocytotic internalization is decreased in the triple mutants and that this might cause a reduction of α-factor internalization.

Effects of EH domain mutants on proteins containing the NPF motif

Having established that EH domains interact with NPF motifs (Salcini et al., 1997; de Beer et al., 1998; Paoluzi et al., 1998), we next

FIGURE 4: Localization of EH domain proteins in EH domain mutants. (A, C, E) Localization of Pan1-GFP (A), End3-GFP (C), and Ede1-GFP (E) in cells expressing mutant forms of other EH domain proteins. Right, kymographs from the same movies. (B, D) Average lifetimes of Pan1-GFP and End3-GFP ± SD in mutant cells. Data were taken from 2-min movies with a 1-s frame interval. n = 50 patches for each strain. *p < 0.01, **p < 0.001. Scale bars, 2 μm. (F) Distribution of Ede1-GFP patch lifetimes in wild-type and indicated mutant cells. n = 50 patches for each strain. Movies were taken with a 1-s frame interval.
examine whether EH domain mutations could affect the localization or dynamics of NPF motif-containing proteins. Ent1p and Ent2p, an essential pair of genes belonging to the epsin family (Chen et al., 1998; Wendland et al., 1999; Aguilar et al., 2003), contain two NPF motifs that interact with the EH domains of Pan1p and Ede1p (Aguilar et al., 2003). To evaluate precisely differences in the localization of NPF-containing proteins, we compared the triple mutants expressing Ent1-GFP or Ent2-GFP directly alongside wild-type cells (Figure 9A). To distinguish the wild-type and mutant cells, we treated the former with the vacuolar dye 3-triethylammoniumpropyl-4-p-diethylaminophenylhexatrienyl pyridinium dibromide (FM4-64). As shown in Figure 9, A and B, the fluorescence intensity of individual Ent1p or Ent2p patches was little affected, indicating that recruitment of these proteins was not dependent on the EH domains. In contrast, the lifetimes of Ent1p patches and Ent2p patches were affected differently by the EH domain mutants: Ent2p patches had a lifetime ∼2.5-fold longer in the triple mutants (64.0 ± 19.0 s) than in the wild-type cells (25.4 ± 4.4 s), whereas the Ent1p patch lifetime was extended only modestly (Figure 9C). We also examined the corresponding effect on two other NPF motif-containing proteins, Yap1801p and Yap1802p, which are homologues of the mammalian clathrin adaptor proteins CALM/AP180 (Wendland and Emr, 1998). Yap1802-GFP patches in the triple mutant also showed no marked change in fluorescence intensity but had a slightly extended lifetime relative to those in wild-type cells (Figure 9, A–C). In contrast to these proteins, however, the fluorescence intensity and lifetime of Yap1801-GFP were markedly decreased in the triple mutants (Figure 9, A–C). These results prompted us to examine other endocytic coat proteins, such as Sla1p and Sla2p. We found that Sla2-GFP patches exhibited a phenotype similar to that of Ent2-GFP patches, whereas the fluorescence intensities of Sla1-GFP patches were decreased in the triple-mutant cells relative to the wild-type cells (Figure 9, A–C). These results suggest that EHDPs seem important for the proper accumulation and dynamics of NPF-containing proteins at an endocytic patch. Because Sla1p contains a single NPF sequence at the C terminus (amino acids 2040–2042), we examined whether this NPF sequence is responsible for Sla1p localization. The GFP-tagged Sla1pNPF-AAA mutant in which the NPF sequence was converted to three alanine residues showed normal localization and lifetimes in wild-type cells (Supplemental Figure S5A). We also found that Sla1pNPF-AAA-GFP exhibited similar dynamics to wild-type Sla1p in the end3EH and ede1EH mutants (Supplemental Figure S5B), indicating that the NPF sequence is not required for Sla1p localization.

DISCUSSION

EH domains of End3p, rather than those of Pan1p, play a critical role in endocytosis

In this study we mutated all of the EH domains of yeast endocytic EHDPs—Pan1p, End3p, or Ede1p—and evaluated their effects on several endocytosis-related processes, including recruitment of endocytic
its EH domain was mutated (Maldonado-Baez et al., 2008). Maldonado-Baez et al. (2008) showed that ~60% of Pan1<sup>EH</sup>-GFP patches exhibited greatly extended lifetimes ranging from 75 to >120 s, whereas the remaining ~40% had slightly extended lifetimes (45 ± 5 s). The different lifetimes of Pan1<sup>EH</sup>-GFP patches might be caused by differences in their manner of expression; in their experiment, Pan1<sup>EH</sup>-GFP was expressed via its endogenous promoter using a single-copy plasmid in pan1Δ cells, whereas we expressed Pan1<sup>EH</sup>-GFP from an endogenous locus. Any defects in the pan1<sup>EH</sup> mutant are probably masked by End3p because they work together as a complex and have redundant functions (Tang et al., 1997; Toshima et al., 2007).

**Role of the EH domains of Ede1p in endocytosis**

As opposed to the pan1<sup>EH</sup> or end3<sup>EH</sup> mutant, we found a decrease of ~20.5% in the lifetime of the Sla1-GFP patch in the ede1<sup>EH</sup> mutant relative to wild-type cells. This effect is in agreement with earlier observations of Sla1-GFP in ede1Δ cells (Kaksonen et al., 2005; Stimpson et al., 2009). Of interest, the ede1<sup>EH</sup> mutant also decreased the lifetime of the Sla1-GFP patch even when combined with the pan1<sup>EH</sup> or end3<sup>EH</sup> mutant. Moreover, prolonged Sla1-GFP patch lifetime was observed in pan1<sup>EH</sup> end3<sup>EH</sup> double mutant (Figure 3, B and C) was reduced when the double mutant was combined with the ede1<sup>EH</sup> mutant. These results suggest that the EH domains of Ede1p have a dominant role in determining the lifetime of the Sla1p patch. Although it has not been clarified how Ede1p regulates Sla1p localization, physical interaction might be important, as the third SH3 domain of Sla1p interacts with Ede1p (Tonikian et al., 2009). In contrast to the Sla1p patch, the lifetimes of the Ede1p and Syp1p patches were increased in ede1<sup>EH</sup> cells, suggesting that the total time required for completion of clathrin coat assembly is increased in the ede1<sup>EH</sup> mutant.

The presence of several phenotypes that are distinct between ede1<sup>EH</sup> and ede1Δ cells suggests a specific function of the Ede1p EH domain. For instance, in ede1Δ cells, Syp1p was strongly concentrated at the necks of buds, and cortical Syp1p was diffusely concentrated at the necks of buds, and cortical Syp1p was diffusely localized with polarization toward the daughter cell (Stimpson et al., 2009), whereas ede1<sup>EH</sup> mutants, as was the case in wild-type cells, exhibited cortical localization of Syp1p but had a lifetime that was increased approximately twofold. Another distinct phenotype was that deletion of the EDE1 gene reduced the number of endocytic sites (Stimpson et al., 2009), whereas the ede1<sup>EH</sup> mutant had a normal number of Syp1p patches, similar to the triple mutant (data not shown). Thus these observations suggest that the EH domains of Ede1p have a role in efficient assembly of the clathrin-coated pit but not in the regulation of Syp1p localization or endocytic site formation.

**EH domains are required for early coat assembly**

Although mutation of EH domains caused defects in coat proteins dynamics, no apparent effect was observed in Abp1p, a marker of actin-driven membrane invagination. This result is of interest because mutation of endocytic proteins can often affect the entire process of endocytosis. For instance, deletion of CHC—the clathrin heavy chain—reduces the patch lifetimes of several coat proteins and, in contrast, extends the patch lifetime of Abp1p (Kaksonen et al., 2005; Newpher and Lemmon, 2006). end3Δ cells exhibited prolonged patch lifetimes for both coat proteins and Abp1p (Kaksonen et al., 2005). The pan1-15TA mutant, in which major potential Prk1p phosphorylation sites in Pan1p are mutated to alanine, exhibited a large abnormal actin structure, including several endocytic proteins (Toshima et al., 2005). Therefore our proteins to cortical patches, cargo internalization, and clathrin coat assembly. Previous studies revealed that native Pan1p and End3p form a stable complex with 1:1 stoichiometry (Tang et al., 1997; Toshima et al., 2007) and that these proteins may function as a complex containing four EH domains. Among these EH domains, those of End3p seem to be relatively important since the end3<sup>Δ</sup> mutant has more severely defective growth and endocytosis than the pan1<sup>Δ</sup> mutant. Unexpectedly, the pan1<sup>Δ</sup> mutant exhibited only minor endocytic defects, even though Pan1 is an essential gene and a variety of pan1 mutants have been reported to exhibit severe defects of the actin cytoskeleton and/or endocytosis (Tang and Cai, 1996; Tang et al., 2000; Wendland et al., 1996; Toshima et al., 2005). This result differs from a previous observation that the lifetime of Pan1p was significantly extended when...
observation suggests a specific role of EH domain–mediated interactions in the process of early clathrin coat assembly.

How does mutation of EHDPs affect early coat assembly? Because Ede1 was previously characterized as a binding partner of Syp1p (Reider et al., 2009; Stimpson et al., 2009), it seems reasonable to suppose that Syp1p lifetime is increased in ede1EH cells because Ede1EH patch lifetime is increased. However, it is still unclear why the lengthened Syp1p lifetime is also observed in the pan1EH and end3EH mutants. By tracking individual Syp1p patches in the pan1EH and end3EH mutants, we found that the time required to reach the maximum fluorescence intensity was not increased in these mutants (Supplemental Figure S4D), although Syp1p patch intensities were quite irregular, as reported previously (Stimpson et al., 2009). This observation suggests that early coat assembly might be suspended until recruitment of some additional factors might cooperatively cause defects. It was reported that some EH domains recognize other peptide motifs, including Phe–Trp (FW), Trp–Trp (WW), and Ser–Trp–Gly (SWG) (Paoluzi et al., 1998). The EH domain of End3p was also shown to interact with peptides containing the His–(Thr/Ser)–Phe (HTF/HSF) motif (Paoluzi et al., 1998). Using the PatMatch (Yeast Genome Pattern Matching) program, we found that the FW sequence(s) exists in Chc1p, yeast clathrin heavy chain, and Ap1p, a large subunit of the clathrin-associated protein complex (AP-2). We also found that two WW sequences are present in Sla1p. These motifs would be attractive targets for further investigation of how EHDPs regulate clathrin-coated pit assembly.

Function of EH domains in endocytosis is conserved in both yeast and mammals.

There are several lines of evidence to suggest that mammalian Eps15p has a similar function to yeast EHDPs. First, it was shown that clathrin-coated pit assembly is perturbed by overexpression of the Eps15 mutant lacking the second and third EH domains (Benmerah et al., 1999). A previous study demonstrated that EH domains are required but are not sufficient for targeting Eps15 to clathrin-coated pits (Benmerah et al., 2000). Knockdown of Eps15 increases the lifetime of clathrin, indicating that Eps15 is critical for efficient assembly of the clathrin coat (Mettlen et al., 2009). Thus the role of the EH domain in the endocytic pathway seems to be conserved between yeast and animal cells.

MATERIALS AND METHODS

Yeast strains, growth conditions, and plasmids

The yeast strains used in this study are listed in Table 1. All strains were grown in standard rich medium (yeast extract/peptone/dextrose [YPD]) or synthetic medium supplemented with 2% glucose and appropriate amino acids. pan1EH, end3EH, or ede1EH mutant was integrated as follows: To create a pan1 integration plasmid, the Xmrn–Dral fragment of the PAN1 gene was cloned into pBluescript II SK (pBS), and the Salt fragment of the LEU2 gene was inserted into the Salt site 154 base pairs upstream of the PAN1 open reading frame (ORF). The mutated Mscl–Nhel pan1 fragments were used to replace the PANT gene in the integration plasmid. To integrate
pan1EH mutant at the endogenous locus, the integration plasmids were digested with Sac and XbaI and transformed into pan1Δ::HIS3/PAN1 diploid strains. Integrated pan1 mutants were selected on synthetic complete plates lacking leucine and sporulated to obtain pan1 mutants. To create end3 integration plasmids, the XbaI-ClaI fragment of the END3 gene was cloned into pBS, and the Smal fragment of the URA3 gene was inserted into the EcoO109I site 150 base pairs upstream of the END3 ORF. The mutated XbaI–Xhol end3 fragments were used to replace the END3 gene in the integration plasmid. To integrate end3DH mutants at the endogenous locus, the integration plasmids were digested with XbaI and ClaI and transformed into end3Δ strains. To create ede1 integration plasmid, the SacI–Xhol fragment of the EDE1 gene was cloned into pBS, and the Smal fragment of the LEU2 gene was inserted into the EcoRV site, which was made by amino acid substitutions, at 139 base pairs downstream of the EDE1 ORF. The mutated SacI–Ndel ede1 fragment was used to replace the EDE1 gene in the integration plasmid. To integrate ede1TH mutant at the endogenous locus, the integration plasmids were digested with SacI and Xholl and transformed into ede1Δ strains. Amino acid substitutions were made using QuikChange XL Site-Directed Mutagenesis Kit (Stratagene, Santa Clara, CA). pan1ΔEH mutant was constructed by inserting the PCR-amplified fragment (nucleotides [nt] 1074–1784) into the PAN1 fragment deleting EH domains and the region between the EH domains (nt 787–2063). Similarly, end3AEH, or ede1AEH mutant was constructed by inserting the PCR-amplified fragments (END3, nt 289–366; EDE1, nt 298–381 and 664–807) into the END3 or EDE1 fragment deleting EH domains and the region between the EH domains (END3, nt 16–663; EDE1, nt 19–1107). GFP and mRFP tags were integrated at the C-terminus of each gene (Longtine et al., 1998). The triple-GFP tag was integrated at the C-terminus of the SYP1 gene as follows: The 3GFP fragment was subcloned into BamHI- and NotI-digested pBS (pBS-3GFP), and the NotI–SacI fragment, which contains the Saccharomyces cerevisiae ADH1 terminator and the HIS3MX6 module, was amplified by PCR using pFA6a-GFP (S65T)-HIS3MX6 (Longtine et al., 1998) as a template and inserted into NotI- and SacI-digested pBS-3GFP (pBS-3GFP-HIS3). To create an integration plasmid, a fragment of the SYP1 ORF (nt 2077–2610) was generated by PCR and cloned into the BamHI site of pBS-3GFP-HIS3. To integrate 3GFP at the C-terminus of the SYP1 gene, the integration plasmid was linearized by ClaI and transformed into yeast.

**Fluorescence microscopy**

Fluorescence microscopy was performed using an Olympus IX81 microscope equipped with a 100×/numerical aperture 1.40 (Olympus, Center Valley, PA) objective and an Orca-AG cooled charge-coupled device camera (Hamamatsu, Hamamatsu, Japan), using MetaMorph software (Universal Imaging, West Chester, PA). Simultaneous imaging of red and green fluorescence was performed using an Olympus IX81 microscope, as described, and an image splitter (Dual-View; Optical Insights, Santa Fe, NM) that divided the red and green components of the images with a 565-nm dichroic mirror and passed the red component through a 630/50-nm filter and the green component through a 530/30-nm filter. Rhodamine–phalloidin staining of filamentous actin and FM4-64 staining were performed as described previously (Toshima et al., 2005).

**35S-labeled α-factor internalization assay**

Preparation and internalization of 35S-labeled α-factor was performed as described previously (Toshima et al., 2005). Briefly, cells were grown to an OD600 of 0.3 in 50 ml of YPD, briefly centrifuged, and resuspended in 4 ml of YPD containing 1% (wt/vol) bovine serum albumen, 50 mM KH2PO4, pH 6.0, and 20 μg/ml uracil, adenine, and histidine. After addition of 35S-labeled α-factor, cell aliquots
| Strain          | Genotype                                                                 | Source            |
|-----------------|---------------------------------------------------------------------------|-------------------|
| JTY0011         | Mata his3-Δ200 leu2-3, 112 ura3-52 lys2-801 pan1Δ::PAN1::LEU2              | Toshima lab       |
| JTY0059         | Mata his3-Δ200 leu2-3, 112 ura3-52 bar1Δ::LEU2                            | Toshima lab       |
| JTY0147         | Mata his3-Δ200 leu2-3, 112 ura3-52 lys2-801 pan1Δ::PAN1::LEU2 PAN1-GFP::HIS3 | Toshima lab       |
| JTY0318         | Mata his3-Δ200 leu2-3, 112 ura3-52 lys2-801 END3-GFP::HIS3                | Toshima lab       |
| JTY0369         | Mata his3-Δ200 leu2-3, 112 ura3-52 lys2-801                               | Toshima lab       |
| JTY0393         | Mata his3-Δ200 leu2-3, 112 ura3-52 SLA1-GFP::KanMX6 ABP1-RFP::HIS3       | Toshima lab       |
| JTY0943         | Mata his3-Δ200 leu2-3, 112 ura3-52 pan1Δ::pan1FH::LEU2                   | This study        |
| JTY0950         | Mata his3-Δ200 leu2-3, 112 ura3-52 pan1Δ::pan1FH::LEU2 PAN1-GFP::HIS3    | This study        |
| JTY0954         | Mata his3-Δ200 leu2-3, 112 ura3-52 lys2-801 pan1Δ::pan1FH::LEU2 SLA1-GFP::KanMX6 ABP1-mRFP::HIS3 | This study        |
| JTY0958         | Mata his3-Δ200 leu2-3, 112 ura3-52 lys2-801 pan1Δ::pan1FH::LEU2 bar1Δ::HIS3 | This study        |
| JTY0960         | Mata his3-Δ200 leu2-3, 112 ura3-52 lys2-801 end3Δ::END3::URA3            | This study        |
| JTY0962         | Mata his3-Δ200 leu2-3, 112 ura3-52 lys2-801 end3Δ::end3FH::URA3          | This study        |
| JTY0972         | Mata his3-Δ200 leu2-3, 112 ura3-52 lys2-801 end3Δ::end3FH::URA3 SLA1-GFP::KanMX6 ABP1-mRFP::HIS3 | This study        |
| JTY0976         | Mata his3-Δ200 leu2-3, 112 ura3-52 lys2-801 end3Δ::end3FH::URA3 bar1Δ::LEU2 | This study        |
| JTY0979         | Mata his3-Δ200 leu2-3, 112 ura3-52 pan1Δ::pan1FH::LEU2 end3Δ::end3FH::URA3 | This study        |
| JTY0982         | Mata his3-Δ200 leu2-3, 112 ura3-52 pan1Δ::pan1FH::LEU2 end3Δ::end3FH::URA3 SLA1-GFP::KanMX6 ABP1-mRFP::HIS3 | This study        |
| JTY0983         | Mata his3-Δ200 leu2-3, 112 ura3-52 pan1Δ::pan1FH::LEU2 end3Δ::end3FH::URA3 bar1Δ::LEU2 | This study        |
| JTY1074         | Mata his3-Δ200 leu2-3, 112 ura3-52 lys2-801 ede1Δ::EDE1::LEU2             | This study        |
| JTY1075         | Mata his3-Δ200 leu2-3, 112 ura3-52 lys2-801 ede1Δ::ede1FH::LEU2           | This study        |
| JTY1077         | Mata his3-Δ200 leu2-3, 112 ura3-52 ede1Δ::ede1FH::LEU2 SLA1-GFP::KanMX6 ABP1-mRFP::HIS3 | This study        |
| JTY1079         | Mata his3-Δ200 leu2-3, 112 ura3-52 lys2-801 ede1Δ::ede1FH::LEU2 bar1Δ::HIS3 | This study        |
| JTY1080         | Mata his3-Δ200 leu2-3, 112 ura3-52 pan1Δ::pan1FH::LEU2 ede1Δ::ede1FH::LEU2 SLA1-GFP::KanMX6 ABP1-mRFP::HIS3 | This study        |
| JTY1081         | Mata his3-Δ200 leu2-3, 112 ura3-52 lys2-801 end3Δ::end3FH::URA3 ede1Δ::ede1FH::LEU2 SLA1-GFP::KanMX6 ABP1-mRFP::HIS3 | This study        |
| JTY1083         | Mata his3-Δ200 leu2-3, 112 ura3-52 end3Δ::end3FH::URA3 ede1Δ::ede1FH::LEU2 | This study        |
| JTY1085         | Mata his3-Δ200 leu2-3, 112 ura3-52 lys2-801 pan1Δ::pan1FH::LEU2 end3Δ::end3FH::URA3 ede1Δ::ede1FH::LEU2 | This study        |
| JTY1087         | Mata his3-Δ200 leu2-3, 112 ura3-52 pan1Δ::pan1FH::LEU2 ede1Δ::ede1FH::LEU2 | This study        |
| JTY1088         | Mata his3-Δ200 leu2-3, 112 ura3-52 pan1Δ::pan1FH::LEU2 ede1Δ::ede1FH::LEU2 bar1Δ::LEU2 | This study        |
| JTY1089         | Mata his3-Δ200 leu2-3, 112 ura3-52 end3Δ::end3FH::URA3 ede1Δ::ede1FH::LEU2 bar1Δ::HIS3 | This study        |
| JTY1090         | Mata his3-Δ200 leu2-3, 112 ura3-52 lys2-801 pan1Δ::pan1FH::LEU2 end3Δ::end3FH::URA3 ede1Δ::ede1FH::LEU2 SLA1-GFP::KanMX6 ABP1-mRFP::HIS3 | This study        |
| JTY1091         | Mata his3-Δ200 leu2-3, 112 ura3-52 lys2-801 pan1Δ::pan1FH::LEU2 end3Δ::end3FH::URA3 ede1Δ::ede1FH::LEU2 bar1Δ::HIS3 | This study        |
| JTY1227         | Mata his3Δ1 leu2A0 ura3Δ0 lys2A0 YAP1802-GFP::KanMX6                       | This study        |
| JTY1255         | Mata his3-Δ200 leu2-3, 112 ura3-52 lys2-801 end3Δ::end3FH::URA3 END3-GFP::URA3 | This study        |
| JTY1256         | Mata his3-Δ200 leu2-3, 112 ura3-52 lys2-801 EDE1-GFP::HIS3                | This study        |
| JTY1257         | Mata his3-Δ200 leu2-3, 112 ura3-52 lys2-801 ede1Δ::ede1FH::LEU2 EDE1-GFP::HIS3 | This study        |
| JTY1258         | Mata his3-Δ200 leu2-3, 112 ura3-52 lys2-801 ENT1-GFP::HIS3                | This study        |
| JTY1259         | Mata his3-Δ200 leu2-3, 112 ura3-52 lys2-801 pan1Δ::pan1FH::LEU2 end3Δ::end3FH::URA3 ede1Δ::ede1FH::LEU2 ENT1-GFP::HIS3 | This study        |

TABLE 1: Yeast strains.

(Continues)
were withdrawn at various time points and subjected to a wash in pH 1 buffer to remove surface-bound α-factor so internal α-factor could be measured or in pH 6 buffer to determine the total (internal and bound) α-factor. The amount of cell-associated radioactivity after each wash was determined by scintillation counting. Each experiment was performed at least three times.

| Strain                  | Genotype                                      | Source   |
|-------------------------|-----------------------------------------------|----------|
| JTY1262                 | Mata his3Δ20 leu2-3, 112 ura3-52 lys2-801 YAPLafer-GFP::HIS3 | This study |
| JTY1263                 | Mata his3Δ20 leu2-3, 112 ura3-52 lys2-801 pan1Δ::pan1Δ::LEU2 end3Δ::end3Δ::URA3 ede1Δ::ede1Δ::LEU2 YAP1801-GFP::HIS3 | This study |
| JTY1928                 | Mata his3Δ20 leu2-3, 112 ura3-52 lys2-801 SYP1-3GFP::HIS3 ABP1-mRFP::KanMX6 | This study |
| JTY1929                 | Mata his3Δ20 leu2-3, 112 ura3-52 pan1Δ::pan1Δ::LEU2 SYP1-3GFP::HIS3 ABP1-mRFP::KanMX6 | This study |
| JTY1930                 | Mata his3Δ20 leu2-3, 112 ura3-52 lys2-801 end3Δ::end3Δ::URA3 SYP1-3GFP::HIS3 ABP1-mRFP::KanMX6 | This study |
| JTY1931                 | Mata his3Δ20 leu2-3, 112 ura3-52 lys2-801 ede1Δ::ede1Δ::LEU2 SYP1-3GFP::HIS3 ABP1-mRFP::KanMX6 | This study |
| JTY1932                 | Mata his3Δ20 leu2-3, 112 ura3-52 pan1Δ::pan1Δ::LEU2 end3Δ::end3Δ::URA3 SYP1-3GFP::HIS3 ABP1-mRFP::KanMX6 | This study |
| JTY1933                 | Mata his3Δ20 leu2-3, 112 ura3-52 pan1Δ::pan1Δ::LEU2 ede1Δ::ede1Δ::LEU2 SYP1-3GFP::HIS3 ABP1-mRFP::KanMX6 | This study |
| JTY1934                 | Mata his3Δ20 leu2-3, 112 ura3-52 end3Δ::end3Δ::URA3 ede1Δ::ede1Δ::LEU2 SYP1-3GFP::HIS3 ABP1-mRFP::KanMX6 | This study |
| JTY1935                 | Mata his3Δ20 leu2-3, 112 ura3-52 lys2-801 pan1Δ::pan1Δ::LEU2 end3Δ::end3Δ::URA3 ede1Δ::ede1Δ::LEU2 SYP1-3GFP::HIS3 ABP1-mRFP::KanMX6 | This study |
| JTY1936                 | Mata his3Δ20 leu2-3, 112 ura3-52 lys2-801 ENT2-GFP::HIS3 | This study |
| JTY1937                 | Mata his3Δ20 leu2-3, 112 ura3-52 lys2-801 pan1Δ::pan1Δ::LEU2 end3Δ::end3Δ::URA3 ede1Δ::ede1Δ::LEU2 SYP1-3GFP::HIS3 ABP1-mRFP::KanMX6 | This study |
| JTY1938                 | Mata his3Δ20 leu2-3, 112 ura3-52 lys2-801 SLA2-GFP::HIS3 | This study |
| JTY1939                 | Mata his3Δ20 leu2-3, 112 ura3-52 lys2-801 pan1Δ::pan1Δ::LEU2 end3Δ::end3Δ::URA3 ede1Δ::ede1Δ::LEU2 SYP1-3GFP::HIS3 ABP1-mRFP::KanMX6 | This study |
| JTY1940                 | Mata his3Δ20 leu2-3, 112 ura3-52 lys2-801 pan1Δ::pan1Δ::LEU2 end3Δ::end3Δ::URA3 ede1Δ::ede1Δ::LEU2 YAP1802-GFP::HIS3 | This study |
| JTY2421                 | Mata his3Δ20 leu2-3, 112 ura3-52 lys2-801 SYP1-3GFP::HIS3 Sla1-mCherry::KanMX | This study |
| JTY2422                 | Mata his3Δ20 leu2-3, 112 ura3-52 lys2-801 pan1Δ::pan1Δ::LEU2 Syp1-3GFP::HIS3 Sla1-mCherry::KanMX | This study |
| JTY2423                 | Mata his3Δ20 leu2-3, 112 ura3-52 lys2-801 end3Δ::end3Δ::URA3 Syp1-3GFP::HIS3 Sla1-mCherry::KanMX | This study |
| JTY2424                 | Mata his3Δ20 leu2-3, 112 ura3-52 lys2-801 ede1Δ::ede1Δ::LEU2 Syp1-3GFP::HIS3 Sla1-mCherry::KanMX | This study |
| JTY2426                 | Mata his3Δ20 leu2-3, 112 ura3-52 pan1Δ::pan1Δ::LEU2 END3-GFP::HIS3 | This study |
| JTY2427                 | Mata his3Δ20 leu2-3, 112 ura3-52 pan1Δ::pan1Δ::LEU2 EDE1-GFP::HIS3 | This study |
| JTY2428                 | Mata his3Δ20 leu2-3, 112 ura3-52 lys2-801 ede1Δ::ede1Δ::LEU2 PAN1-GFP::HIS3 | This study |
| JTY2429                 | Mata his3Δ20 leu2-3, 112 ura3-52 ede1Δ::ede1Δ::LEU2 END3-GFP::HIS3 | This study |
| JTY2430                 | Mata his3Δ20 leu2-3, 112 ura3-52 lys2-801 end3Δ::end3Δ::URA3 SYP1-GFP::HIS3 | This study |
| JTY2431                 | Mata his3Δ20 leu2-3, 112 ura3-52 lys2-801 end3Δ::end3Δ::URA3 EDE1-GFP::HIS3 | This study |
| JTY2432                 | Mata his3Δ20 leu2-3, 112 ura3-52 pan1Δ::pan1Δ::LEU2 end3Δ::end3Δ::URA3 EDE1-GFP::HIS3 | This study |
| JTY2433                 | Mata his3Δ20 leu2-3, 112 ura3-52 pan1Δ::pan1Δ::LEU2 ede1Δ::ede1Δ::LEU2 END3-GFP::HIS3 | This study |
| JTY2434                 | Mata his3Δ20 leu2-3, 112 ura3-52 end3Δ::end3Δ::URA3 ede1Δ::ede1Δ::LEU2 PAN1-GFP | This study |

TABLE 1: Yeast strains. (Continued)
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