Identification of Suppressor of Clathrin Deficiency-1 (SCD1) and Its Connection to Clathrin-Mediated Endocytosis in Saccharomyces cerevisiae

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ABSTRACT Clathrin is a major coat protein involved in vesicle formation during endocytosis and transport in the endosomal/trans Golgi system. Clathrin is required for normal growth of yeast (Saccharomyces cerevisiae) and in some genetic backgrounds deletion of the clathrin heavy chain gene (CHC1) is lethal. Our lab defined a locus referred to as “suppressor of clathrin deficiency” (SCD1). In the presence of the scd1-v allele (“v” – viable), yeast cells lacking clathrin heavy chain survive but grow slowly, are morphologically abnormal and have many membrane trafficking defects. In the presence of scd1-i (“i” - inviable), chc1Δ causes lethality. As a strategy to identify SCD1, we used pooled linkage analysis and whole genome sequencing. Here, we report that PAL2 (YHR097C) is the SCD1 locus. pal2Δ is synthetic lethal with chc1Δ; whereas a deletion of its paralog, PAL1, is not synthetic lethal with clathrin deficiency. Like Pal1, Pal2 has two NPF motifs that are potential binding sites for EH domain proteins such as the early endocytic factor Ede1, and Pal2 associates with Ede1. Also, GFP-tagged Pal2p localizes to cortical patches containing other immobile phase endocytic coat factors. Overall, our data show that PAL2 is the SCD1 locus and the Pal2 protein has characteristics of an early factor involved in clathrin-mediated endocytosis.

Movement of proteins within the secretory and endocytic pathways is initiated by the binding of coat proteins to the cytosolic surface of the membrane followed by capture of cargo molecules and vesicular budding (McMahon and Mills 2004, Gomez-Navarro and Miller 2016). Clathrin and its associated proteins form a major class of vesicular transport coats (Kirchhausen et al. 2014, Robinson 2015). Clathrin-coated vesicles (CCVs) are involved in receptor mediated endocytosis, recycling of membranes, transcellular transport and transport between the TGN and endosomes (Kirchhausen et al. 1989, Pearse and Robinson 1990, Bonifacino and Glick 2004, Boettner et al. 2011, Kirchhausen et al. 2014, Robinson 2015, Elkin et al. 2016, Lu et al. 2016). Clathrin, which forms the striking polygonal surface lattice on CCVs, is a trimeric molecule, or triskelion, containing three heavy chains (HC) of ~180 kD that radiate from a vertex and three light chains (LC) of 30 - 40 kD, which bind noncovalently near the vertex of the triskelion, one per heavy chain arm (Kirchhausen et al. 1989, Edeling et al. 2006).

CCVs have been found in virtually every eukaryotic organism that has been examined, including the yeast Saccharomyces cerevisiae (Mueller and Branton 1984, Payne and Schekman 1985, Lemmon and Jones 1987, Silveira et al. 1990). Previous studies in yeast found that deletion of the clathrin HC gene, CHC1, is lethal in some genetic backgrounds, but not in others (Payne and Schekman 1985, Lemmon and Jones 1987, Payne et al. 1987, Schekman and Payne 1988, Munn et al. 1991). The viable clathrin HC deficient cells exhibit a number of phenotypes, including slow growth, abnormal morphology and polyplody, and defects in mating, sporulation, endocytosis and sorting in the endosomal/Trans Golgi Network (TGN) system (Payne and Schekman 1985, Lemmon and Jones 1987, Payne et al. 1987, Payne et al. 1988, Payne and Schekman 1989).
localizes to cortical patches, similar to Pal1-GFP and other endocytic knockouts of its paralog, has been used in yeast to identify different methods to identify the gene were complicated by the fact that traditional complementation and other chromosomal mapping (Kovar 1998). For the triple tagged Pal1-3xGFP, the strains were streaked from a single colony onto 1% yeast extract + 2% peptone (YEP) containing 2% galactose (YEP-Gal) and YEP + 2% Dextrose (YEPD) for the triple tagged Pal1-3xGFP, (pFA6a 3xGFP-KanMX6 was used as a template (Kovar et al. 2005). For growth tests shown in Figure 2 and Figure S2 strains were streaked from a single colony onto 1% yeast extract + 2% peptone (YPE) containing 2% galactose (YPE-Gal) and YEP + 2% Dextrose (YPED) plates and incubated at 30° (Figure 2), 34° and 37° (Figure S2) for 4 days. For serial dilution spotting in Figure 2B, the strains were grown overnight in YEP liquid medium containing 2% galactose (YEP-Gal) and YEPD. Cells were veriﬁed by restriction digestion and sequencing. Plasmids were generated by PCR ampliﬁcation of PAL2 (YHR097C) with ~500 base pairs (bp) upstream and ~300 bp downstream of the open reading frame (ORF) using clone no. 671 (plate no. 7; F12 well) from the multicopy Tiling library collection (gift of G. Prelich (Jones et al. 2008)) as a template. The PCR product was sequenced to conﬁrm it encoded the wild type Pal2 protein (scd1-v background). The ampliﬁed PCR fragment was digested with Kpn1 (‘S’) and BamH1 (‘J’), which had been encoded in the primers, and cloned into the pRS426 vector cut with Kpn1 and BamH1. Plasmid pRS316-PAL2 was generated in the similar way as pRS426-PAL2 except that both the vector and PCR fragment were digested with Xho1 (‘S’) and Kpn1 (‘J’). Plasmid clones were veriﬁed by restriction digestion and sequencing.

Plasmid pET28c-EDE1 (EH1-3) [pBW1161] (gift of B. Wendland), contains 1261 bp of the coding sequence of the N-terminal region of EDE1 for EH domains 1, 2 and 3 inserted into the BamH1 (‘J’) and Xho1 (‘S’) sites of pET28c. This was used for bacterial expression of a N-terminal His6-tagged EH1-3 domain. The coding sequence of GFP was inserted between the Nde1 (‘S’) and Not1 (‘J’) sites of pET22b, to generate pET22b-GFP to express His6-GFP (gift of D. Patel & F. Zhang). Plasmids were veriﬁed by restriction digestion and sequencing.

Whole genome sequencing

The strains used in this analysis were generated from parents and spore segregants previously described in (LeNNom and Jones 1987) (see Table S1 for the list of strains used for SCD1 identiﬁcation). Parent strains BJ2700 (CHC1 leu2 scd1-i) and BJ2738 (CHC1 leu2 scd1-v) were crossed to generate the diploid BJ3068 (CHC1/CHC1 leu2/leu2 scd1-i/scd1-v). Then CHC1 was disrupted with LEU2 to generate chc1Δ LEU2/CHC1 scd1-i/scd1-v transfectants BJ3119 and BJ3120. These were subjected to tetrad analysis and wild type chc1 scd1-i or scd1-v spores were identiﬁed based on their segregation from tetrads with four viable spores or 2 viable spores, respectively. The parents of BJ3068 (BJ2700 and BJ2738) and 10 each of CHC1 scd1-i or CHC1 scd1-v spore segregants were analyzed.

Individual cultures of 10 segregants bearing the scd1-v allele or 10 bearing the scd1-i allele were grown in YEPD. Equal numbers of cells (1x10⁷) from each pool member were mixed and genomic DNA extracted using the Yeast Genomic DNA Kit (Zymo Research, Irvine, CA). Cultures of parents were treated similarly except 1x10⁶ cells were harvested for DNA isolation.

Illumina sequencing of parents and pooled strains and alignment of the resulting reads were performed as described previously (Birkeland et al. 2010, Yau et al. 2014) by the Center for Genome Technology Sequencing Core at the Hussman Institute for Human Genomics, University of Miami, Miller School of Medicine. All samples were prepped via “TruSeq DNA SamplePrep Guide 15026486 C” (Illumina) with an input of 700–800ng DNA and 12 cycles of PCR. Five hundred bp long libraries were created and the two pools and parent samples were sequenced in a single multiplexed HiSeq lane with 2x101 nt paired end reads. Read alignment to the sacCer3 (R64.1.1) (Engel et al. 2014) version of the yeast genome was performed using BWA-MEM using default parameters (Li and Durbin 2010). Because of the much higher read depth in the current study, a novel approach to data analysis used samtools (samtools mpileup -DSu -d 1000 -L 1000) followed by bcftools (bcftools view -bvcg -T pair, with a ploidy of 2) to identify variants for which there was a high likelihood that the composite genotype was different for the scd1-i and scd1-v pools. Randomly segregating alleles would each appear as heterozygous in each pool while those linked to the causative mutant allele would appear as either homozygous reference or variant in different pools. The same analysis with a ploidy of 1 compared the two starting haploid strains, where candidate alleles must again have a high likelihood of having a different
genotype. The two outputs were filtered for variants with a Phred-scaled log ratio of genotype likelihoods (CLR score) > 225 in each of the pool and haploid strain comparisons, which resulted in two variants that proved to have only a 2 bp separation on chrVIII. Read counts (forward/reverse strand, sacCer3 coordinates) for chrVIII:298485, T > C were: wild-type pool (scdl-i), 157/90 reference and 15/12 mutant; mutant pool (scdl-i), 0/0 reference and 206/113 mutant. Read counts for chrVIII:298487, A > C were: wild-type pool (scdl-i), 157/90 reference and 20/14 mutant; mutant pool (scdl-i), 0/0 reference and 237/115 mutant. The most likely explanation for the presence of rare mutant reads in the wild-type pool was imperfect scoring of contributing spore clones.

**Microscopy and image analysis**

For most experiments, cells were grown to log phase at 30°C in synthetic medium, concentrated, and immobilized on Concavalin-A coated coverslips. Coverslips were then mounted on slides, and imaged at 25x as indicated below.

Localization of Pal2-GFP or Pal1-3xGFP, and co-localization with other markers was performed on an Olympus fluorescence BX61 upright microscope equipped with Nomarski differential interference contrast (DIC) optics, a Uplan Apo 100x objective (1.35 NA), a Roper CoollSnap HQ camera, and Sutter Lambda 10-2 excitation and emission filter wheels, and a 175 watt Xenon remote source with liquid light guide. Image capture was automated using Intelligent Imaging Innovations Slidebook 6 for Windows 7. Z-stacks of 0.25 μm of fields of cells were taken and a medial plane was selected for image analysis. Image analysis was carried out using Slidebook 6 software and later exported to TIF files. The images of 300 dpi were then cropped and arranged in Adobe Photoshop CS5 and Creative Cloud. Approximately 40 – 60 cells were considered for localization of Pal2-GFP and Pal1-3xGFP. For the quantification of Pal2-GFP patches containing Ede1-mCherry, Sla2-RFP, End3-mCherry and Abp1-RFP, 10 – 18 cells with distinct Pal2-GFP patches from a single medial plane were selected and examined for the presence/overlap of mCherry/RFP signal. The percentage of Pal2-GFP patches containing Ede1-mCherry, Sla2-RFP, End3-mCherry and Abp1-RFP was calculated by the ratio between the number of GFP/RFP (mCherry) overlapping patches to the total number of Pal2-GFP patches in the cells. The percentage overlap of Pal1-3xGFP patches with Pal2-mCherry patches was determined in a similar way. Statistics were performed using the GraphPad Prism 7 software. Two-tailed t-test was carried out for each pair to measure the significance of the data.

Cortical patch to cytosol fluorescence intensity ratio analysis was carried out on the Olympus fluorescence BX61 upright fluorescence microscope as described previously (Chi et al. 2012) using Slidebook 6.0 for Windows 7 for acquisition and analysis. Strains were grown at 30°C to early log phase and z-stack images (5 x 0.25 μm) were captured. Analysis was performed on a medial focal plane. The fluorescence intensity of the brightest cortical patch in a cell was divided by the fluorescence from the mother cell cytosol. A representative background intensity value (outside the cell) was also subtracted from both patch and mother cell cytosol intensities before calculating the patch/cytosol ratio (n ≥ 25 cells for each strain).

To prevent actin polymerization and inhibit internalization at endocytic sites, log phase cells were treated in synthetic medium supplemented with 200 μM Latrunculin-A (LAT-A; Enzo, BML-T119) for 1 h at 30°C. Control cells were treated in medium containing an equal volume of dimethyl sulfoxide (DMSO), the diluent for LAT-A.

**Protein purification and pull-down experiment**

His6-Ede1 (EH1-3) expressed from pET28c-EDE1(EH1-3) or His6-GFP expressed from pET22c-GFP were purified on Ni-NTA agarose beads (Cat No./ID: 30230, Qiagen) according to the manufacturer’s instructions. Yeast extracts were made from 8x10⁶ cells grown in YEPD to log phase. Cell lysates were prepared by resuspending each cell pellet in 1ml of lysis buffer (10 mM Tris pH 8.0, 140 mM NaCl, 0.1% Tween-20, 1 mM β-mercaptoethanol, 1mM PMSF and 1x protease inhibitor cocktail (Protease Inhibitor Cocktail (100X); Cell Signaling, Catalog no.5871)) and subjecting it to glass bead vortexing (4 times – 1 min vortex and 2 min pause on ice), followed by centrifugation at 15000 x g in an eppendorf 5415R centrifuge for 10 min at 4°C. An aliquot of supernatant was saved as input. For pull down experiments, 50 μl of Ni-NTA agarose slurry was equilibrated with lysis buffer and incubated with ∼30 μg of purified His6-Ede1 (EH1-3) or His6-GFP, at 4°C for 1.5 hr. Beads were washed three times with 1ml of wash buffer (lysis buffer + 20mM imidazole). Then 200μl of lyses was added to the bead alone or beads containing purified His6-Ede1 (EH1-3) or His6-GFP, followed by incubation at 4°C for 2 hr on a rotator. Beads were washed three times with wash buffer (lysis buffer without protease inhibitors + 10% glycerol) and resuspended in 30μl lysis buffer and 30μl of 4x SDS sample buffer. Samples were heated at 65°C for 5 min and stored at -20°C until used for immunoblot analysis. Twenty μl of samples from pull downs and 5μl of cell extracts for loading controls were run on Biorad precast 4–20% gradient mini gels and transferred to nitrocellulose membrane using Biorad Trans-blot turbo system (25V for 7 min). Pal2-GFP and Pal1-GFP were detected using anti-GFP antibody (1:1000, Roche; mouse monoclonal; catalog no. 11814660001), and His6-GFP and His6-Ede1 (EH1-3) were detected with 6x-His Tag monoclonal antibody (1:1000, Thermo Scientific; catalog no. 4A12E4). Goat anti-mouse conjugated with horseradish peroxidase (HRP) was used as the secondary antibody at 1:5000 dilution (Thermo Scientific). Chemiluminescence using SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Scientific; catalog no. 34095).

**Data availability**

Strains and plasmids are available upon request. Figure S1 shows the sequence alignment of the “Pal domain” of Pal2 and Pal1. Figure S2 shows the growth phenotype of GAL11:CHC1 strains with the indicated genotypes streaked on YEP+glucose at elevated temperatures. Figure S3 shows the patch to cytosol fluorescence intensity ratio for Pal2-GFP and Pal1-3xGFP in wild type and different endocytic mutants. Figure S4 shows the patch to cytosol fluorescence intensity ratio for Sla1-GFP in wild type and palΔ mutants. Table S1 contains the yeast strains used for pooled linkage analysis and whole genome sequencing. Table S2 and Table S3 show the list of yeast strains and plasmids used in this study, respectively. Table S4 shows the tetrad data demonstrating that palΔ is not synthetic lethal with chc1Δ. Supplemental material available at Figshare: https://doi.org/10.25387/g3.7582868.

**RESULTS**

**Identification of the SCD1 locus using pooled linkage analysis and next generation whole genome sequencing**

To discover functional mutations in yeast among a large excess of polymorphisms and incidental mutations, a strategy was developed based on next-generation sequencing, called pooled linkage analysis (Birkeland et al. 2010, Song et al. 2014, Lang et al. 2015, Linder et al. 2016). We employed the same strategy for the identification of the SCD1 locus. In the original studies where this polymorphism was
et al. (Carroll has been reported to be involved in clathrin-mediated endocytosis, a whole genome duplication (Byrne and Wolfe 2005). Intriguingly, Pal1 (Figure 1B). junction, where codon 41 for tyrosine is mutated to a stop codon.

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seen as dark gray boxes. The red arrows indicate the splicing junction. 9

scd1-i allele just upstream of the 5’ splice junction. Kakonen and co-workers used this gene name in a prior study (Brach et al. 2014).

Pal2Δ, but not Pal1Δ, is synthetically lethal with clathrin heavy chain deficiency

We previously showed that CHC1 expressed via the GAL1 promoter confers regulated control of clathrin HC synthesis (Nelson and Lemmon 1993). On galactose medium GAL1:CHC1 cells express clathrin HC and grow normally, but on glucose medium CHC1 expression is repressed and cells either grow slowly (scd1-v) or are inviable (scd1-i) (Figure 2A). We then deleted PAL2 in these two strains (Figure 2A).

The strains grew normally on galactose, but deletion of PAL2 in the GAL1:CHC1 scd1-v background now led to inviability on glucose, similar to the GAL1:CHC1 scd1-i strain. In contrast, deletion of PAL1 did not cause inviability nor did it further impair growth of the original GAL1:CHC1 scd1-v strain on galactose or glucose (Figure 2A), even at elevated temperature of 34°C (Figure S2). All of the strains were inviable on glucose at 37°C, as expected for cells lacking clathrin HC (Figure S2). These results indicate that pal2Δ, but not pal1Δ, is synthetically lethal with clathrin HC-deficiency.

We next tested whether PAL2 could complement GAL1:CHC1 scd1-i in glucose medium, and found that PAL2 on a CEN plasmid indeed rescued the growth defect of GAL1:CHC1 scd1-i strains (with or without pal2Δ) (Figure 2B, upper panels). In an attempt to identify SC1 earlier, we performed a multi-copy suppressor screen on GAL1:CHC1 scd1-i yeast. SCD2-SCD6 were identified in this screen, but none were allelic to the SCD1 locus ([Nelson and Lemmon 1993, Gelperin et al. 1995, Nelson et al. 1996, Huang et al. 1997], unpublished observations). We might have expected to identify PAL2 in this screen, so we considered the possibility that overexpression of PAL2 might cause impaired growth of wild type yeast or Chc- cells. But overexpression of PAL2 (pPAL2(2μ))
were recovered (Table S4). YCp CHC1 plasmid (Leu+Ura-) (Table 1). This con-

Our pooled linkage/sequence analysis indicates that YHR097C (PAL2) is SCD1 locus.

Our classical genetic approach is required. To this end, we also rescued GAL1:CHC1 scd1-i strains (with or without pal2Δ) on glucose and had no effect on cell growth when CHC1 was expressed on galactose medium (Figure 2B, lower panels).

**Localization of Pal2-GFP and Pal1-GFP**

The clathrin endocytic pathway in yeast initiates with an immobile phase where the early endocytic factors, such as Syp1, Edel and clathrin, are first recruited to cortical patches, followed by the assembly of mid-late coat factors (e.g., Slal, Slal, Ent/H2). Edel and Syp1 leave the cortex just as the rapidly mobile actin-driven invagination phase commences, which is followed by scission of the coated vesicle. After release of the nascent vesicle, it is rapidly uncoated and moves inwards and coat factors are recycled for the next round of endocytosis (Boettner et al. 2011, Weinberg and Drubin 2012, Goode et al. 2015). Previous studies have shown that Pal1-GFP forms patches at the cell cortex that are characteristic of endocytic coat proteins and it is considered an early arriving endocytic factor (Carroll et al. 2012). Global analysis of protein localization reported that GFP-tagged Pal2 localizes to the cytoplasm and nucleus (Huh et al. 2003). However, due to the homology with Pal1, we speculated that Pal2 might also localize to endocytic sites. We generated a strain expressing Pal2 with a C-terminal GFP tag. GFP-tagged Pal2 is functional as we obtained viable spores in genetic crosses with chc1Δ (not shown). Since Pal1-GFP was difficult to visualize in prior studies (Carroll et al. 2012), we generated a strain that expresses Pal1 with a C-terminal 3xGFP tag. However, pal1Δ has no phenotype even in combination with chc1Δ or pal2Δ (see below), so it was not possible to confirm Pal1-3xGFP function. But it behaved much like Pal1-GFP studied previously (Carroll et al. 2012). Since Pal2-GFP was observed at the bud neck (32% of cells; n = 60), similar to Pal1-3xGFP (38% of cells; n = 60; Figure 3A, 3B).

Since pal2Δ shows synthetic lethality with chc1Δ and its paralog Pal1 is an early endocytic factor, we next tested growth phenotypes of pal2Δ with deletions of PAL1 and other early endocytic factor genes,

![Figure 2](https://example.com/figure2.png)

**Figure 2** pal2Δ is synthetic lethal with loss of clathrin heavy chain. (A) GAL1:CHC1 strains with the indicated SCD1 and PAL1 genotypes were streaked on galactose and glucose medium and grown for 4 days. Strains used are: scd1-i (SL214); scd1-v (SL350); scd1-v pal2Δ (SL7249); scd1-v pal1Δ (SL7261); scd1-i pal2Δ (SL7251). (B) GAL1:CHC1 strains with the indicated SCD1 and PAL1 genotypes were transformed with vector control (pCEN or p2μ) or plasmids expressing Pal2 (pCEN-PAL2 or pPAL2 2μ). The strains were grown to log phase in YEP-galactose and then transferred to YEP-glucose for ~14 hr. Then 10-fold serial dilutions were spotted on galactose and glucose plates and grown for 4 days. Strains used were: scd1-i +pCEN (SL7444); scd1-i +pCEN-PAL2 (SL7464); scd1-i pal2Δ +pCEN (SL7447); scd1-i pal2Δ +pCEN-PAL2 (SL7448); scd1-i +p2μ (SL7278); scd1-i +pPAL2 (2μ) (SL7279); scd1-i pal2Δ +p2μ (SL7280); scd1-i pal2Δ +pPAL2 (2μ) (SL7281).

| GAL1: CHC1 | Galactose | Glucose |
|------------|-----------|---------|
| scd1-i + pCEN |          |         |
| scd1-i + pCEN-PAL2 |          |         |
| scd1-i pal2Δ + pCEN |          |         |
| scd1-i pal2Δ + pCEN-PAL2 |          |         |
| scd1-i + p2μ |          |         |
| scd1-i + pPAL2 (2μ) |          |         |
| scd1-i pal2Δ + p2μ |          |         |
| scd1-i pal2Δ + pPAL2 (2μ) |          |         |

YHR097C (PAL2) is SCD1 locus

Our pooled linkage/sequence analysis indicates that PAL2 is SCD1. Supporting this we so far have shown that pal2Δ causes synthetic lethality in clathrin HC deficient yeast and PAL2 complements the sde1-1 mutation. However to prove that PAL2 is the SCD1 locus, a classical genetic approach is required. To this end, we also deleted PAL2 in CHC1 sde1-1 cells and crossed this (CHC1 pal2Δ:pATMx6; SL7098) with a chc1ΔLEU2 sde1-1 strain where the spore segregant came out of tetrads protected by YCP50-CHC1 (SL98). The plasmid was dropped from the chc1Δ/CHC1 heterozygous diploid and subjected to tetrad analysis. If PAL2 is SCD1, then the diploid strain would be homozygous at the SCD1 locus (pal2Δ::pATMx6;sde1-1) and we expect 2 viable CHC1 and 2 dead spores (chc1Δ::LEU2 with either sde1-1 or pal2Δ::pATMx6) in all tetrads. If PAL2 is not the SCD1 locus, then some of the chc1Δ spores would survive by receiving the sde1-1 allele from the CHC1 sde1-1 parent. Twenty dissected tetrads segregated 2 viable:2 dead with all viable spores being CHC1 Leu+ (Table 1). When we performed the same experiment in the presence of YCPCHC1 (URA3) the plasmid rescued the growth of chc1Δ::LEU2 spores (Leu+ Ura+), but we did not recover any Chc1 spores growing in the absence of the plasmid (Leu- Ura-) (Table 1). This confirms that PAL2/YHR097C is the SCD1 locus, and sde1-1 is a truncation mutant of PAL2.

We also tested whether pal1Δ is synthetic lethal with chc1Δ, but as shown in the GAL1:CHC1 shut down experiments (Figure 2A), pal1Δ does not cause inviability with clathrin HC deficiency. This was confirmed in tetrads from crosses of pal1Δ sde1-1 and chc1Δ YCPCHC1 sde1-1, where viable chc1Δ::LEU2 pal1Δ::pATMx6 spores were recovered (Table S4).
Table 1  pal2Δ is synthetic lethal with chc1Δ

| Diploid genotype SL98 X SL7098 | No. of tetrads with ratio of viable to dead spores | No. of spores with phenotype |
|-------------------------------|---------------------------------|-------------------------------|
| chc1Δ;LEU2/CHC1 scd1-1/pal2Δ;NatMX6  | 4:0 | Leu+Ura+  |
| chc1Δ;LEU2/CHC1 scd1-1/pal2Δ;NatMX6 (YCP50-CHC1) | 3:1 | Leu+Ura+ |
| chc1Δ;LEU2/CHC1 scd1-1/pal2Δ;NatMX6 (YCP50-CHC1) | 2:2 | Leu+Ura+  |

The heterozygous diploid was then sporulated (in the absence and presence of plasmid YCP50-CHC1), followed by tetrad dissection. Data in the table represent the number of tetrads with ratio of viable to dead spores and number of spores with different phenotypes. If pal2Δ is integrated at the SCD1 locus, we expect no viable Leu+ Ura+ (chc1Δ) spores in the absence of the CHC1, URA3 plasmid, YCP50-CHC1.

EDE1, SYP1 and YAP1801/2 at normal (30º) and elevated temperatures (34º and 37º), but saw no effect either in tetrads or by direct gene deletions (data not shown). We then examined whether early endocytic proteins, Ede1, Syp1, Yap1801/2, and clathrin HC (Kaksonen et al. 2005, Newpher et al. 2005, Toshima et al. 2006, Boettner et al. 2009, Carroll et al. 2009, Reider et al. 2009, Stimpson et al. 2009, Carroll et al. 2012), are required for the recruitment of Pal2-GFP or Pal1-3xGFP to cortical sites. However, both Pal2-GFP and Pal1-3xGFP localization to cortical patches and the bud neck in null mutants of each of these genes was similar to the wildtype (Figures 4A, 4B and data not shown). We also performed cortical patch to cytosol patch fluorescence intensity ratio analysis to assess whether there was any defect in localization of Pal proteins in these endocytic mutants. However, there was no significant difference in patch to cytosol fluorescence intensity ratio of the Pal proteins in the mutants compared to the wildtype strain (Figure S3A & S3B). These results suggest that the early endocytic factors tested are not required cortical recruitment factors for the Pal proteins. We also verified that Pal1 and Pal2 do not affect each other’s localization (≥78% of cells had cortical patches; n = 45, upon deletion of the paralog; Figure 4A & 4B), and thus their recruitment to the cell surface is not interdependent.

To determine whether Pal2 and Pal1 have a role in the recruitment of other endocytic factors, we examined the localization of GFP tagged Ede1, Syp1, Sla2 and Sla1 in pal2Δ or pal1Δ pal2Δ cells. However, no significant difference in the localization of these endocytic factors was observed compared to wild type (Figure 5). We also noted that there was more cytosolic Sla1 in pal2Δ or pal1Δ pal2Δ cells (Figure 5 and see cortical patch to cytosol fluorescence intensity ratio in Figure S4). Altogether, our data suggest that Pal2 and Pal1 localization at endocytic sites is independent of early endocytic factors and both proteins are not required for recruitment of Ede1, Syp1 and Sla2, but they affect recruitment of Sla1 to the cortex during the immobile phase of internalization.

Pal2 localizes to cortical patches containing other endocytic factors

To determine whether Pal2 is actually in endocytic sites at the cortex, we tested for co-localization of Pal2-GFP with endocytic factors that mark different phases of clathrin-mediated endocytosis. These include Ede1 – early immobile phase, Sla2 and End3 – mid/late immobile phase, and Abp1 – actin/mobile phase (Boettner et al. 2011). To enhance any co-localization signal, we treated cells with Latrunculin-A (LAT-A), a drug that inhibits actin polymerization by sequestering monomers of actin and thereby stalling the endocytosis process (Ayscough 1998). The Pal2-GFP signal overlapped significantly with Ede1-mCherry, Sla2-mCherry and End3-mCherry, even in the absence of LAT-A (DMSO control cells) (Figure 6A – 6C). However, Pal2-GFP appeared to be largely at the surface more like Ede1 and not in invaginating structures. Consistent with this Pal2-GFP did not show significant overlap with Abp1-RFP (Figure 6D), which marks the mobile invagination phase of internalization and arrives at or just after Syp1 and Ede1 leave the cell surface (Boettner et al. 2009, Stimpson et al. 2009).

We also quantified the number of Pal2 patches that contained the early (Ede1) and middle/late endocytic coat factors (Sla2/End3), as well as actin (Abp1). Consistent with Pal2 arriving early at endocytic sites, 68% of Pal2 patches contained Ede1 (n = 111 from 18 cells), 31% contained Sla2 (n = 67 from 12 cells), 35% contained End3 (n = 56 from 13 cells), and only 8% contained Abp1 (n = 77 from 11 cells) (Figure 6E). As predicted, Pal1-3xGFP and Pal2 tagged with mCherry also colocalized at cortical sites (67%; n = 78 from 11 cells; Figure 6F). This supports the idea that Pal2 arrives before Sla2, End3 and Abp1. Overall these results suggest that Pal2 is an early endocytic factor that may leave the cortex without internalization, possibly similar to Ede1 and Syp1 (Boettner et al. 2009, Reider et al. 2009, Stimpson et al. 2009).

Eps15 homology (EH)-domain containing protein, Ede1, interacts with Pal2 and Pal1

Ede1, the homolog of mammalian Eps15 (Gagny et al. 2000), contains 3 N-terminal EPS15 homology (EH) domains (EH1: 7-102; EH2: 128-221; EH3: 270-365) which recognize the Asn-Pro-Phe (NPF) motifs (Figure 7A) in target ligands (de Beer et al. 2000, 2001). Another major feature of Ede1 is the presence of different EH domains that bind to ligands at different states of internalization (Simpson et al. 2000, Carroll et al. 2009, Stimpson et al. 2009). The EH1 domain specifically binds to ligands at the late endosome, with the EH3 domain binding to ligands at the plasma membrane and the EH2 domain binding to ligands at early endosomes (Manser et al. 2002). Finally, we performed a co-localization experiment to test the idea that Pal2 arrives before Sla2, End3 and Abp1. Overall these results suggest that Pal2 is an early endocytic factor that may leave the cortex without internalization, possibly similar to Ede1 and Syp1 (Boettner et al. 2009, Reider et al. 2009, Stimpson et al. 2009).

Figure 3 Pal2 and Pal1 localize to cortical patches. (A) Pal2-GFP (SL7455); (B) Pal1-3xGFP (SL7335) were imaged by fluorescence microscopy. Shown are images from a single medial plane of a z-stack. Scale bar: 5μm.
DISCUSSION

It has been over 30 years since the identification of a polymorphism in the gene referred to as *SCD1*, suppressor of clathrin deficiency, where one allele (scd1-i) resulted in inviability of clathrin HC-deficient yeast, but the other (scd1-v) allowed survival of cells lacking clathrin HC (Lenmon and Jones 1987). At the time this was surprising because the concept of synthetic lethality in yeast was relatively novel and there were limited examples in the literature (Huffaker et al. 1987, Kaiser and Schekman 1990). It was also unanticipated to discover this random polymorphism in what were considered to be relatively isogenic yeast strains (Mortimer and Johnston 1986). Controversy over this finding ensued because it was unexpected that yeast cells should even survive without clathrin (Payne and Schekman 1985, Lenmon and Jones 1987, Payne et al. 1987, Schekman and Payne 1988, Munn et al. 1991, Robinson 2015). Indeed, viable chc1Δ yeast exhibit very severe growth defects and a number of trafficking and other cell phenotypes, so their survival was considered tenuous at best. In fact, it was argued that mutations in many additional genes might cause a very sick strain to be inviable, and thus the possibility that the *SCD1* locus would have anything to do with clathrin function seemed remote (Payne et al. 1987, Schekman and Payne 1988, Munn et al. 1991). Moreover, use of the term “supressor” for this polymorphism, though genetically valid, may have implied to some that the “mutant allele” was a viability conferring “suppressing” allele (scd1-v) (Lenmon and Jones 1987, Schekman and Payne 1988) (see response by Lenmon & Jones to (Schekman and Payne 1988)). However, genetic analysis demonstrated that none of these multicity suppressors are allelic to the *SCD1* locus (Nelson and Lenmon 1993, Gelperin et al. 1995, Nelson et al. 1996, Huang et al. 1997, Chang et al. 2002, Henry et al. 2002, Henry et al. 2003, Newpher et al. 2006, Chi et al. 2012). However, genetic mapping strategies were thwarted by the propensity of clathrin-deficient yeast to become polyploid and their poor transformation efficiency (Lenmon and Jones 1987, Lemmon et al. 1990).

The advent of powerful pooled linkage analysis and whole genome sequencing (Birkeland et al. 2010, Song et al. 2014, Lang et al. 2015, Linder et al. 2016) allowed us to finally identify the *SCD1* locus, solving this long unresolved question. We found that *scd1-i* is a mutation in *YHR097c*, also referred to as *PAL2* due to the existence of a paralogue, *PAL1* (Carroll et al. 2012, Brach et al. 2014). The *scd1-i* allele has a stop used yeast strains to make them more amenable to genetic manipulation, so this still left the nature of the *SCD1* locus and the *scd1-i* allele unclear (see response of Lemmon & Jones to (Schekman and Payne 1988)).

The search for the *SCD1* locus proved to be highly challenging. We initially used a multicity suppression strategy, seeking genes whose overexpression could suppress the lethality of *scd1-i* clathrin HC-deficient cells in which *CHC1* was under control of the repressible GAL1 promoter (Nelson and Lenmon 1993). We reasoned that overexpression might overcome the need to know which allele of *SCD1* was dominant or recessive. In the process we identified *SCD2-SCD6* and uncovered very interesting biology, including genes encoding proteins that linked directly to membrane trafficking and endocytosis (Nelson and Lenmon 1993, Gelperin et al. 1995, Nelson et al. 1996, Huang et al. 1997, Chang et al. 2002, Henry et al. 2002, Henry et al. 2003, Newpher et al. 2006, Chi et al. 2012). However, genetic analysis demonstrated that none of these multicity suppressors are allelic to the *SCD1* locus (Nelson and Lenmon 1993, Gelperin et al. 1995, Nelson et al. 1996, Huang et al. 1997); Gelperin and S. Lemmon (unpublished). A caveat to this approach was we did not know the nature of the *SCD1* locus in existing plasmid libraries. In addition, other genetic mapping strategies were thwarted by the propensity of clathrin-deficient yeast to become polyploid and their poor transformation efficiency (Lenmon and Jones 1987, Lemmon et al. 1990).
codon that results in a truncated protein, and the scd1-v allele is the wild type SCD1/PAL2 gene. This confirms that it is a loss of function allele that is synthetic lethal with clathrin HC deficiency. Of particular interest, though, our data indicate that the Pal2/Scd1 protein plays a role in clathrin-mediated endocytosis, arguing against the concept that this mutation was not likely relevant to clathrin.

The first Pal1 protein was characterized in S. pombe cells, where it localizes to the cell tips during interphase and at the cell division plane during mitosis and cytokinesis (Ge et al. 2005). A null mutation causes morphological and polarity phenotypes, including pear shaped and spherical cells, thus the name Pal1 for pears and lemons. A possible role in endocytosis was suggested by an association with Sla2, related to HIP1/R in mammalian cells (Ge et al. 2005). More recently Pal1 was studied in S. cerevisiae where it was found to localize to sites of clathrin-mediated endocytosis and behave like an early endocytic coat factor (Carroll et al. 2012). Our studies here indicate its paralog, Pal2, also may play an endocytic role. It localizes to cortical patches containing other endocytic coat factors, including Pal1, and it interacts with the early coat factor Ede1, like Pal1. For technical reasons, we have not been able to study Pal2’s dynamics by live cell imaging. However, it appears to localize more like early endocytic factors that do not internalize, such as Ede1 and Syp1. There was no obvious displacement from the cortex, and limited colocalization with actin as seen in factors found in invaginating vesicles. This would be a distinction from Pal1 which seems to internalize with endocytic vesicles (Carroll et al. 2012).

A few questions still need to be answered about the Pal proteins in yeast. First, are Pal1 and Pal2 redundant? Arguing against this idea is pal2Δ is synthetic lethal with chc1Δ, while pal1Δ is not. However, it could be that Pal2 is just more abundant than Pal1, so depletion of Pal2 has more of an effect in the absence of clathrin HC. However, proteomic quantification of the number of Pal proteins per cell showed little difference in abundance (Saccharomyces Genome Database, (Kulak et al. 2014)).

Furthermore, we still don’t know what are the exact function(s) of the Pal proteins. There is no obvious phenotype of the null mutations alone or in combination, or combined with other early endocytic factors tested so far, except for the genetic interaction of PAL2 with CHC1. Although, further supporting an endocytic role for the Pal proteins, pal2Δ and pal1Δpal2Δ did lead to an increase in the cytoplasmic level of Sla1 compared to the wild type strain. This is similar to the effects seen on Sla1 when genes for other early endocytic factors are deleted (Kaksonen et al. 2005, Brach et al. 2014). Also, both proteins have NPF motifs and bind to the EH region of Ede1. Prior studies suggested that Ede1 is needed to recruit Pal1 to the cell cortex (Carroll et al. 2012), but our results did not confirm these findings, as both Pal1 and Pal2 were in cortical patches without Ede1.

It is possible that the Pal proteins have an endocytic function that is only critical under certain conditions, such as cell stress or during morphogenesis events like mating. Alternatively, they are selective cargo adaptors for membrane proteins that have yet to be identified. Deletions
containing recombinant His6-Ede1(EH1-3). Both Pal2 and Pal1 co-purify or Pal1-GFP (SL7442) were incubated with Ni-NTA agarose beads with Ede1 and are detected by anti-GFP antibody. Inputs containing recombinant His6-GFP (His6-GFP) as a control. Neither Pal2 nor Pal1 or Pal1-GFP were incubated with Ni-NTA agarose beads containing recombinant His6-Ede1(EH1-3). (A) Schematic representation of Pal2, Pal1 and Ede1 protein-cargo motifs. Further studies will be needed to investigate these possibilities and to understand the roles of the Pal proteins.

Figure 7 Interaction between Pal2-GFP and Pal1-GFP with His6-Ede1 (EH1-3). (A) Schematic representation of Pal2, Pal1 and Ede1 proteins showing the Pal homology domain and NPF motifs in Pal2 and Pal1; and 3 EH-domains in Ede1. The double arrow under the Ede1 EH domain is the region used in the pulldown experiments in panel B. (B) Protein lysates from yeast cells expressing Pal2-GFP (SL7455) or Pal1-GFP (SL7442) were incubated with Ni-NTA agarose beads containing recombinant His6-Ede1(EH1-3). Both Pal2 and Pal1 co-purify with Ede1 and are detected by anti-GFP antibody. Inputs containing Pal2-GFP or Pal1-GFP are shown at the right; ‘**’ shows non-specific bands. (C) Protein lysates as in (B) from yeast cells expressing Pal2-GFP or Pal1-GFP were incubated with Ni-NTA agarose beads containing recombinant His6-GFP (His6-GFP) as a control. Neither Pal2 nor Pal1 co-purifies with His6-GFP.

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ACKNOWLEDGEMENTS

We thank Dr. Beverly Wendland and Dr. Derek Prosser for the pET28c-EDE1(EH1-3) construct. We are grateful to Dr. Patel and Dr. Zhang for the gift of HIS6-GFP. This work was supported by National Institutes of Health grants R01 GM120767 to T.E.W. and R01-GM055796 to S. K. L.
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Communicating editor: N. Rhind