Clathrin light chain directs endocytosis by influencing the binding of the yeast Hip1R homologue, Sla2, to F-actin

Douglas R. Boettnera, Helena Friesena, Brenda Andrewsb, and Sandra K. Lemmona

aDepartment of Molecular and Cellular Pharmacology, University of Miami, Miami, FL 33101; bDepartment of Molecular and Medical Genetics, University of Toronto, Toronto, ON M5S 1A8, Canada

ABSTRACT The role of clathrin light chain (CLC) in clathrin-mediated endocytosis is not completely understood. Previous studies showed that the CLC N-terminus (CLC-NT) binds the Hip1/Hip1R/Sla2 family of membrane/actin-binding factors and that overexpression of the CLC-NT in yeast suppresses endocytic defects of clathrin heavy-chain mutants. To elucidate the mechanistic basis for this suppression, we performed synthetic genetic array analysis with a clathrin CLC-NT deletion mutation (clc1Δ19-76). clc1Δ19-76 suppressed the internalization defects of null mutations in three late endocytic factors: amphiphysins (rvs161 and rvs167) and verprolin (vpr1). In actin sedimentation assays, CLC binding to Sla2 inhibited Sla2 interaction with F-actin. Furthermore, clc1Δ19-76 suppression of the rvs and vpr phenotypes required the Sla2 actin-binding talin-Hip1/R/Sla2 actin-tethering C-terminal homology domain, suggesting that clc1Δ19-76 promotes internalization by prolonging actin engagement by Sla2. We propose that CLC directs endocytic progression by pruning the Sla2-actin attachments in the clathrin lattice, providing direction for membrane internalization.

INTRODUCTION

Clathrin is a major coat protein involved in vesicle formation during receptor-mediated endocytosis and sorting in the trans-Golgi network (TGN)/endosomal system (Traub, 2005). Clathrin is found as a triskelion containing three extended heavy chains (CHCs) trimerized at their C-termini and three light chains (CLCs). These assemble into a characteristic polyhedral coat on the cytosolic face of the membrane to facilitate clathrin-coated vesicle (CCV) formation. CHC interacts with adaptor proteins, which are involved in recruitment of clathrin to the membrane, as well as the binding to and sorting of cargo. Although the role of CHC in CCV formation is well established, the roles of CLC are not completely understood.

Previous work showed that CLCs bind along the vertex-proximal region of the CHC leg and are found on the outer surface of the clathrin lattice, where they are positioned to interact with other cytosolic and regulatory factors (Fotin et al., 2004). They are important for stabilization of the mammalian triskelion hub fragments in vitro (Ybe et al., 2003, 2007) and are essential for CHC trimerization in yeast (Chu et al., 1996; Huang et al., 1997). Biochemical studies also show that CLC inhibits spontaneous triskelion self-assembly (Ungewickell and Ungewickell, 1991; Liu et al., 1995), and this activity depends on the conserved N-terminal acidic region of CLC (Ybe et al., 1999). Recent work suggested that interaction of CLC or the CLC amino terminus with CHC might prevent the bending of the CHC knee that is required for lattice assembly (Wilbur et al., 2010). However, depletion of CLC or overexpression of CLC lacking the N-terminal regulatory domain in animal cells has little effect on endocytosis, although some effect on TGN/endosomal sorting has been observed (Huang et al., 2004; Chen and Brodsky, 2005; Poupon et al., 2008; Wilbur et al., 2008).

The CLC N-terminal acidic region also interacts with the central coiled-coiled dimerization domain of the Hip1/Hip1R/Sla2 family of proteins (Chen and Brodsky, 2005; Legendre-Guillemin et al., 2005; Newpher et al., 2006). Some evidence suggests that Hip1/R binding to CLC promotes clathrin assembly by releasing the CLC
N-terminal negative regulation on CHC (Chen and Brodsky, 2005; Legendre-Guillemin et al., 2005). Because Hip1-related proteins contain both an AP180 N-terminal homology domain (ANTH) that binds membrane-associated phosphatidylinositol and a talin/Hip1/R/Sla2 actin-tethering C-terminal homology domain (THATCH) that binds F-actin, they may provide a critical link between the membrane and the actin cytoskeleton or regulate actin assembly during clathrin-mediated transport (McCann and Craig, 1997; Yang et al., 1999; Legendre-Guillemin et al., 2002; Hyun et al., 2004; Senetar et al., 2004; Sun et al., 2005; Brett et al., 2006; Wilbur et al., 2008). Depletion of Hip1 family proteins from cells leads to aberrant actin assemblies at clathrin-coated membranes in both yeast and animal cells (Kaksonen et al., 2003; Engqvist-Goldstein et al., 2004; Newpher et al., 2005; Le Clainche et al., 2007; Poupon et al., 2008), and similarly treatments that affect the ability of CLC to interact with Hip1R in animal cells perturb actin structures at clathrin-coated membranes (Chen and Brodsky, 2005; Poupon et al., 2008; Wilbur et al., 2008; Saffarian et al., 2009). Still, the role of CLC regulation of Hip1 family proteins remains unclear.

Yeast provides elegant ways to address these questions by combining live-cell imaging with powerful molecular genetic tools. This model system has established a spatiotemporal map from recruitment to disassembly and is now elucidating details about the molecular functions of a large number of endocytic factors. Clathrin-mediated endocytosis in yeast takes place at cortical patches and involves many factors that have animal cell counterparts (Engqvist-Goldstein and Drubin, 2003; Robertson et al., 2009; Galletta et al., 2010). Generally the process has an immobile stage for establishing the endocytic site and a rapid, mobile phase when invagination and vesicle formation occur. During the mobile phase (1–2 min) several endocytic coat factors and adaptors collect at a cortical patch, including the F-BAR domain protein Syp1 (FCHO1/FCHO2 homologue), Ede1 (an Eps15 homology [EH] domain protein), and clathrin (Newpher et al., 2005; Boettner et al., 2009; Reider et al., 2009; Stimpson et al., 2009). Later in the mobile phase Sla2 (Hip1/Hip1R) appears, followed by Sla1 (an SH3 domain–containing protein), the two EH domain factors Pan1 and End3, and the WASp homologue, Las17 (Kaksonen et al., 2003, 2005; Newpher and Lemmon, 2006). Many actin-binding or -remodeling proteins, such as Abp1, capping protein, and Arp2/3 complex, appear or are activated during the mobile phase, which lasts only 10–15 s (Kaksonen et al., 2003, 2005). At the onset of the mobile phase, the WIP homologue, verprolin (Vrp1), localizes to the cortical site, where it recruits and activates the type-I myosins (Myo3/5), which are potent Arp2/3 activators believed to produce a final burst of actin needed to drive invagination (Kaksonen et al., 2003, 2005; Jonsdottir and Li, 2004; Sun et al., 2006; Galletta et al., 2008). When the patch has invaginated 200–300 nm, the vesicle pinches off, facilitated by the N-BAR domain amphiphysin homologues Rvs161 and Rvs167, uncoating occurs, and the vesicle associated with actin moves deeper into the cell (Kaksonen et al., 2003, 2005).

Here we explore how the CLC N-terminus (CLC-NT) regulates the yeast Hip1R homologue, Sla2. Sla2 arrives at endocytic sites well after clathrin, so it is not required for assembly of clathrin (Newpher et al., 2006; Newpher and Lemmon, 2006). As in mammalian cells, deletion of SLA2 leads to accumulation of early coat factors at the cortex and unproductive actin comet tails emanating from these sites (Kaksonen et al., 2003; Newpher et al., 2005). Our previous work discovered that CLC1, which encodes CLC, is a high-copy suppressor of clathrin HC–deficient (chl1Δ) yeast, indicating that CLC possesses regulatory functions during endocytosis (Huang et al., 1997). Furthermore, we isolated Sla2 as a CLC-interacting protein (Henry et al., 2002); as in animal cells, the yeast CLC-NT interacts with the coiled-coiled domain of Sla2 (Newpher et al., 2006; Newpher and Lemmon, 2006). Of interest, overexpression of the N-terminal Sla2-binding region of CLC suppresses the endocytic phenotypes of clathrin-null mutants, including progression of stalled Sla2-containing endocytic patches (Newpher et al., 2006). However, deletion of the CLC-NT alone has minimal effect on its own, likely due to redundancy of the endocytic machinery.

To further investigate the regulatory role of CLC during endocytic progression we took advantage of the genome-wide synthetic genetic array (SGA) approach (Tong and Boone, 2006), using a clc1 mutant encoding CLC lacking the Sla2-binding region (clc1Δ19-76) as the query strain. To our surprise, the clc1Δ19-76 allele alleviated growth defects of null mutants for three late endocytic factors: verprolin (Vrp1) and the amphiphysins (Rvs161 and Rvs167). Our analysis of this suppression by live-cell imaging, combined with biochemical evidence, suggests that CLC negatively regulates Sla2 binding to the actin cytoskeleton to restrict the number and/or location of Sla2′s actin attachments to promote internalization.

RESULTS

Synthetic genetic array analysis with clc1Δ19-76

We present the first SGA screen performed on a clathrin mutant. Genetic analysis of clathrin mutants previously were limited to small-scale screens (Bensen et al., 2000, 2001), and large-scale SGA analysis was not done because clathrin-null yeast (clc1A or chc1A) exhibit massive polyploidy (Lemmom et al., 1990; Huang et al., 1997), which renders them unreliable participants in these screens. To explore the mechanism of CLC regulation of endocytosis, we generated a strain (SL5677) for SGA analysis carrying an integrated copy of CLC1 to create a CLC1-deleted strain (clc1Δ19-76). This clc1Δ19-76 yeast was similar to wild-type yeast in all tests performed (see later discussion).

For SGA analysis clc1Δ19-76 was first systematically crossed to a collection of 177 temperature-sensitive (ts) alleles enriched for genes encoding proteins involved in membrane trafficking, endocytosis, and actin function (listed in Supplemental Table S1B). This screen identified 11 genes whose mutations caused synthetic growth defects in the presence of the clc1Δ1 mutation, including the endocytic coat factor Pan1 and the Arp2/3 complex subunit Arp3 (Figure 1G), although the latter showed major differences, depending on the arp3 allele, and might be complicated by additional functions of the Arp2/3 complex, for example, mitochondrial movement (Boldogh et al., 2001). We confirmed the pan1Δ genetic interaction with an additional allele, pan1Δ20, which was in fact synthetic lethal with clc1Δ19-76 (Supplemental Figure S1A). A larger-scale SGA screen involved crossing the clc1Δ19-76 mutant to 3885 strains from the viable deletion collection. This screen identified 336 aggravating mutations (synthetic growth phenotypes; Supplemental Table S3B) and 58 mutants whose growth phenotypes were alleviated by clc1Δ19-76 (synthetic rescue phenotypes; Table 1A and Supplemental Table S3A).

Gene ontology categories were used to sort the data set by biochemical processes (Supplemental Table S2). Fifteen percent of genes identified by the deletion screen were associated with the Gene Ontology term “vesicle transport,” in vast excess of the expected 5% found in the genome (p ≤ 7 × 10⁻¹⁰). Several protein complexes showed statistically significant representation in our data
FIGURE 1: Synthetic genetic array analysis with the clc1-Δ19-76 allele. (A) Schematics of Clc1 and Clc1-Δ19-76, highlighting regions that bind Sla2 (blue), CHC (gray), or calmodulin (yellow) and the region comprising an EF hand motif (green). (B) Immunoblot of protein extracts from wild type (SL1462), clc1-Δ19-76 (SL5677), and clc1Δ (SL1620) probed with anti-Clc1 and anti-Pgk1 (loading control). (C) Same as B, but probed with anti-Chc1 monoclonal antibodies. (D–G) Edges represent published physical protein–protein interactions; nodes are white if they were not in the screen and gray if no interaction was identified. If double mutants produced a synthetic growth defect relationship, they are labeled in red, and nodes demonstrating synthetic rescue with clc1-Δ19-76 are labeled in green. (D) clc1-Δ19-76 demonstrated a relationship with three AP-1–complex subunits (p ≤ 0.01). (E) Four of the five core retromer complex proteins were identified (p ≤ 0.01). (F) Three of the five components of the GET complex were identified (p ≤ 0.01). (G) Endocytic network showing hits from SGA analysis with clc1-Δ19-76.
<table>
<thead>
<tr>
<th>Gene</th>
<th>Description (homologue)</th>
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<tr>
<td><strong>A. Null mutations showing synthetic rescue with clc1-Δ19-76</strong></td>
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</tr>
<tr>
<td>VRP1</td>
<td>WASp-interacting protein 1, regulates myosin I (WIP)</td>
</tr>
<tr>
<td>VPH1</td>
<td>V-type proton ATPase 116-kDa subunit A</td>
</tr>
<tr>
<td>YMR166C</td>
<td>Mitochondrial ATP-Mg/P carrier</td>
</tr>
<tr>
<td>ELM1</td>
<td>Ser/Thr protein kinase, regulates morphogenesis and cytokinesis</td>
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<tr>
<td>ROD1</td>
<td>Art4, α-arrestin</td>
</tr>
<tr>
<td>CBF1</td>
<td>Centromere-binding factor</td>
</tr>
<tr>
<td>FIS1</td>
<td>Mitochondrial fission protein (Fis1)</td>
</tr>
<tr>
<td>EST1</td>
<td>Telomere elongation (hEST1A, hEST1B)</td>
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<tr>
<td>RPL14A</td>
<td>Ribosomal protein L14 (RPL14)</td>
</tr>
<tr>
<td>RVS167</td>
<td>BIN1/amphyphisin/endophilin</td>
</tr>
<tr>
<td>SIN3</td>
<td>Histone deacetylase complex (SIN3A)</td>
</tr>
<tr>
<td>LSM6</td>
<td>Like SM-protein involved in mRNA decay, U6 snRNP component</td>
</tr>
<tr>
<td>CEX1</td>
<td>tRNA export from nucleus</td>
</tr>
<tr>
<td>SHE4</td>
<td>Binds unconventional myosins</td>
</tr>
<tr>
<td>VAC14</td>
<td>Regulation of phosphatidylinositol 3,5-bisphosphate synthesis (hVAC14)</td>
</tr>
<tr>
<td>ARL1</td>
<td>ADP-ribosylation factor–like; TGN–endosomal transport (ARL)</td>
</tr>
<tr>
<td>CSE2</td>
<td>RNA polymerase II transcription mediator complex</td>
</tr>
<tr>
<td>GAP1</td>
<td>General amino acid permease</td>
</tr>
<tr>
<td>EM15</td>
<td>Succinate dehydrogenase subunit</td>
</tr>
<tr>
<td>BRR1</td>
<td>Component of spliceosomal snRNPs</td>
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<tr>
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<td>Unknown</td>
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<tr>
<td>FMP23</td>
<td>Unknown</td>
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<tr>
<td>PMA2</td>
<td>Plasma membrane H⁺ ATPase</td>
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<td>MAP2</td>
<td>Methionine aminopeptidase (METAP2)</td>
</tr>
<tr>
<td>HOM6</td>
<td>Homoserine dehydrogenase</td>
</tr>
<tr>
<td>CPR7</td>
<td>Peptidyl-prolyl cis–trans isomerase</td>
</tr>
<tr>
<td>PEX27</td>
<td>Peripheral peroxisomal membrane protein</td>
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<tr>
<td>UBP6</td>
<td>Ubiquitin-specific protease of proteosome (USP14)</td>
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<td>BER1</td>
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<td>SNU66</td>
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<tr>
<td>PAT1</td>
<td>Deadenylation-dependent mRNA-decapping factor</td>
</tr>
<tr>
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<td>BIN1/amphyphisin/endophilin</td>
</tr>
<tr>
<td>SYS1</td>
<td>Golgi membrane protein</td>
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<tr>
<td><strong>B. ts mutants showing synthetic growth defects with clc1-Δ19-76</strong></td>
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<tr>
<td>ALG2</td>
<td>Mannosyltransferase (ALG2)</td>
</tr>
<tr>
<td>ARP3</td>
<td>Component of the Arp2/3 complex (ACTR3B)</td>
</tr>
<tr>
<td>COP1</td>
<td>α subunit of COPI coatomer complex (COPA)</td>
</tr>
<tr>
<td>LST8</td>
<td>Torc1/2 component</td>
</tr>
<tr>
<td>MCD4</td>
<td>Involved in GPI anchor synthesis (PIGN)</td>
</tr>
<tr>
<td>PAN1</td>
<td>EH domain containing endocytic factor</td>
</tr>
<tr>
<td>PIK1</td>
<td>Phosphatidylinositol 4-kinase</td>
</tr>
<tr>
<td>SEC2</td>
<td>Post-Golgi GEF for Sec4</td>
</tr>
<tr>
<td>SEC21</td>
<td>γ subunit of coatomer (COPG2)</td>
</tr>
<tr>
<td>YIF1</td>
<td>Fusion of ER-derived COPII vesicles (YIF1A)</td>
</tr>
<tr>
<td>YIP1</td>
<td>Fusion of ER-derived COPII vesicles (YIPF5)</td>
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snRNP, small nuclear ribonucleoprotein.

*Genes whose null mutation caused synthetic rescue phenotypes in combination with clc1-Δ19-76 with an ε > 0.180 and p < 0.015. Results are ranked by ε score. Verprolin (VRP1) and both amphiphysins (RVS167 and RVS161) are highlighted in red.

*Genes with an adjusted calibrated p between 0.1 and 1, ranked by this value.

**TABLE 1:** Selected genes identified by SGA screens.
(i.e., greater than expected for the size of the data set). Included was the clathrin-associated TGN/endosomal AP-1 adaptor complex (p < 0.01; Figure 1D). These negative genetic interactions are consistent with previous work combining clathrin and AP-1 adaptor mutations (Stepp et al., 1995; Yeung et al., 1999). Negative genetic interactions were also observed with a number of other factors involved in retrograde transport from the endosome to the TGN (Supplemental Table S2), including most components of the retromer complex (Figure 1E; p < 0.01). We hypothesize that this exacerbated growth phenotype was caused by impairment of two parallel retrograde pathways from the endosome, one involving clathrin and another involving retromer. In addition, we identified several subunits of the GET complex, which is required for insertion of tail-anchored proteins into the endoplasmic reticulum (ER) (Simpson et al., 2010) (Figure 1F; p < 0.01). GET complex–requiring soluble N-ethylmaleimide–sensitive factor attachment protein receptors, such as SNc2 and VAM3, were also identified, suggesting that GET complex interactions may just phenocopy the loss of these downstream targets.

In accordance with the previously described clathrin synthetic lethality screen using a ts chc1 allele, chc1Δ521 (Bensen et al., 2000, 2001), we identified four mutants (rc1Δ, gg1Δ, vps21Δ, and inp53Δ) with synthetic growth defects in combination with clc1Δ19-76. We suspect that some of these negative genetic interactions with the clc1Δ mutant may be related to the reduced levels of Chc1; however, we cannot rule out that the CLC-NT has some TGN/endosomal–specific function. Genetic interactions involving mutations affecting other stages of the secretory/endosomal transport pathways and a number of membrane transporters were identified (Supplemental Table S2). Others were observed with genes important for cell wall biosynthesis, glycosylation, lipid/sphingolipid and phosphatidyl inositol synthesis, and components affecting ubiquitin modification. The last-named observation is consistent with previous studies showing that clathrin-deficient yeast accumulate ubiquitinated conjugates and deplete cellular ubiquitin stores (Nelson and Lemmon, 1993).

In addition to pan1Δ and arp3Δ found in the ts screen, a null allele of EDE1, which encodes an EH-domain early endocytic adaptor, caused synthetic growth defects in combination with clc1Δ19-76 (Figure 1G). Most interesting was that clc1Δ19-76 suppressed the growth defects of null mutations for three late-stage endocytic factors: Vrp1 (verprolin, the type I myosin activator related to WIP) and Rvs161 and Rvs167 (amphiphysins involved in vesicle scission; Figure 1G and Table 1). These unusual positive genetic interactions seemed more likely to yield mechanistic insight into the role of the CLC-NT. Thus we focused primarily on vrp1Δ and vps16Δ since their t scores were high, but vps16Δ gave similar, although more subtle effects, as shown for vps16Δ.

The clc1Δ19-76 allele suppresses growth and endocytic defects of vrp1Δ and vps16Δ.

To ensure the validity of these genetic interactions, the clc1Δ19-76 mutation was recapitulated in our SL1462 lab strain and crossed to either vrp1Δ or vps16ΔΔ. Whereas clc1Δ19-76 yeast grew well at 30 or 37°C, vrp1Δ (Figure 2A) and vps16ΔΔ (Figure 2B) cells were ts at 37°C. Consistent with the SGA screen, clc1Δ19-76 suppressed the ts growth of vrp1Δ (Figure 2A) and vps16ΔΔ (Figure 2B) at 37°C. Actin polarity defects are often associated with endocytic mutations. clc1Δ19-76 cells were polarized like wild type, and the clc1Δ allele substantially suppressed the polarity defects caused by vps16ΔΔ as well (Figure 2C, C and D).

Lucifer yellow (LY) uptake assays were used to test bulk fluid-phase endocytosis (Figure 2, E and F). LY uptake in the clc1Δ19-76 mutant was similar to wild type. As expected, both vrp1Δ and vps16ΔΔ yeast had minimal uptake, with only 5–8% of cells labeled vacuoles. However, when combined with the clc1Δ19-76 allele there was significant restoration of LY internalization compared with either null allele alone (Figure 2, E and F). Similarly the LY uptake defect in vps16ΔΔ was suppressed by clc1Δ19-76 (Supplemental Figure S1B). Because we saw no defect in growth at elevated temperature, polarity, or LY uptake in the clc1Δ19-76 mutant alone, we conclude that this mutation suppresses the defects caused by the verprolin or amphiphysin gene deletions.

clc1Δ19-76 rescues vrp1Δ defects in endocytic dynamics and inward vesicle movement

Previous reports showed that verprolin arrives at the endocytic patch just prior to the actin phase, where it plays a role in the recruitment and activation of the potent Arp2/3–activating type I myosins (Myo3 and Myo5), which are important for actin-dependent membrane invagination (Anderson et al., 1998; Evangelista et al., 2000; Gelli et al., 2000; Sirotkin et al., 2005; Sun et al., 2006). Thus, inward movement of endocytic patches is defective in vrp1Δ yeast because of reduced Myo3/5 at the patch and impaired activation of residual myosin present (Sun et al., 2006). To visualize whether clc1Δ19-76 suppressed the defects of vrp1Δ, we combined these mutations with early endocytic markers (either Sla2–green fluorescent protein [GFP] or Sla1–GFP) and a mobile phase/actin marker (Abp1–red fluorescent protein [RFP]), and collected time-lapse movies of their dynamics in live cells. Unlike growth defects, which were best observed at elevated temperatures, the endocytic defects of vrp1Δ and vps1ΔΔ, see later discussion) were highly penetrant, so movies were taken at 25°C.

The clc1Δ19-76 mutation alone caused no significant change in the lifetime of any endocytic marker we tested, and invagination was normal (Figure 3, A–C, and unpublished data). In contrast, vrp1Δ yeast demonstrated dramatic lifetime elongation of both Sla1–GFP and Sla2–GFP (Figure 3, A–C). Sla1–GFP lifetimes were prolonged from 31 ± 6 s in wild type to 69 ± 17 s in vrp1Δ (p < 0.0001; Figure 3, A and C). Sla2–GFP was more severely affected, with lifetimes extended from 49 ± 15 s in wild type to 165 ± 57 s in the verprolin mutant (p ≤ 0.0001; Figure 3, B and C). Combining clc1Δ19-76 with vrp1Δ significantly reduced the lifetimes of both Sla1–GFP (to 56 ± 14 s; p ≤ 0.001) and Sla2–GFP (to 73 ± 25 s; p ≤ 0.0001; Figure 3, A–C). Of note, there was also an elongation of the actin stage of endocytosis in vrp1Δ, as marked by Abp1–RFP, but this was not suppressed by the clc1Δ19-76 mutation.

The invagination defect caused by vrp1Δ was also suppressed by clc1Δ19-76. In vrp1Δ 80% of Sla2 patches accumulated some degree of Abp1–RFP (Figure 3, D–F), but only 20% of these showed any movement inward from the cortex. This percentage increased to 47% in vrp1Δ clc1Δ19-76 (Figure 3, D–E). This was also seen in single-particle tracking of movement of Sla2–GFP, which normally invaginates 200–300 nm with the forming vesicle and disassembles shortly after vesicle scission. The depth of Sla2–GFP inward movement was impeded in vrp1Δ (65 ± 13 nm vs. 250 ± 20 nm in wild type, n = 10), but this was restored to near wild-type distances (190 ± 43 nm, n = 10) in vrp1Δ clc1Δ19-76 (Figure 3F).

clc1Δ19-76 rescues rvs167Δ defects in endocytic dynamics

Rvs161 and Rvs167 form a heterodimer through their N-BAR domain and are recruited late to endocytic sites (Kaksonen et al., 2005; Sun et al., 2006) (Figures 3, A and C). Rvs161 and Rvs167 are required for the formation and de novo synthesis of the retromer clathrin coat, which is required for insertion of tail-anchored proteins at the TGN (Etienne-Mane et al., 2005). Rvs161 and Rvs167 form a heterodimer through their N-BAR domain and are recruited late to endocytic sites (Kaksonen et al., 2005; Sun et al., 2006). Rvs161 and Rvs167 are required for the formation and de novo synthesis of the retromer clathrin coat, which is required for insertion of tail-anchored proteins at the TGN (Etienne-Mane et al., 2005). Rvs161 and Rvs167 form a heterodimer through their N-BAR domain and are recruited late to endocytic sites (Kaksonen et al., 2005; Sun et al., 2006). Rvs161 and Rvs167 are required for the formation and de novo synthesis of the retromer clathrin coat, which is required for insertion of tail-anchored proteins at the TGN (Etienne-Mane et al., 2005). Rvs161 and Rvs167 form a heterodimer through their N-BAR domain and are recruited late to endocytic sites (Kaksonen et al., 2005; Sun et al., 2006). Rvs161 and Rvs167 are required for the formation and de novo synthesis of the retromer clathrin coat, which is required for insertion of tail-anchored proteins at the TGN (Etienne-Mane et al., 2005).
Figure 2: clc1-Δ19-76 suppresses the growth, actin polarity, and fluid-phase endocytosis defects of verprolin and amphiphysin null mutants. (A) Growth with vrp1Δ: wild type (SL1462), clc1-Δ19-76 (SL6044), and vrp1Δ clc1-Δ19-76 (SL6049) were fivefold serially diluted, plated on YEPD, and grown at 30 or 37°C for 60 h. (B) Growth with rvs167Δ: wild type (SL1462), clc1-Δ19-76 (SL6044), rvs167Δ (RH2951), and rvs167Δ clc1-Δ19-76 (SL6052) were plated and grown as in A. (C) Actin polarization: strains shown in A and B were grown at 25°C, fixed, and stained with Alexa Fluor 568–phalloidin. Each image is a max-Z projection of 12 (0.2 μm) optical sections after nearest-neighbor deconvolution (bar, 5 μm). (D) Quantification of actin polarization reported as percentage of small and medium budded cells with >50% of phalloidin stain in the bud (n = 75). (E) Example micrographs of strains indicated in A and B after Lucifer yellow (LY) uptake for 1 h at 25°C (bar, 5 μm). (F) Quantification of LY uptake reported as percentage of cells with vacuolar LY (n = 90).
retraction behavior, in which endocytic patches begin to internalize but then return to the cell cortex and dissipate, consistent with a role in vesicle scission (Kaksonen et al., 2005; Yoon et al., 2010).

By time-lapse imaging we found that Sla2-GFP lifetimes were lengthened by twofold in \(\text{rvs167} \Delta\), but the clc1-\(\Delta19-76\) allele restored Sla2 lifetimes to near wild type (99 ± 33 vs. 54 ± 21 s, \(p \leq 0.0001\), \(\text{rvs167} \Delta\) vs. \(\text{rvs167} \Delta\) clc1-\(\Delta19-76\), respectively) (Figure 4, B and C). The clc1-\(\Delta19-76\) mutation also significantly reduced the lifetimes of Sla1-GFP (51 ± 14 vs. 44 ± 13 s, \(p \leq 0.03\), \(\text{rvs167} \Delta\) vs. \(\text{rvs167} \Delta\) clc1-\(\Delta19-76\), respectively; Figure 4, A and C). Similar although more subtle results were obtained in \(\text{rvs161} \Delta\) yeast (Supplemental Figure S1C). In addition, combining the clc1-\(\Delta19-76\) allele with either \(\text{rvs167} \Delta\) or \(\text{rvs161} \Delta\) increased the number of internalizing patches and reduced the retraction of endocytic markers back to the cortex as compared with either rvs mutant alone (Figure 4D and Supplemental Figure S1D).

**Neither vrp1\(\Delta\) nor \(\text{rvs167} \Delta\) is suppressed by clc1\(\Delta\)**

To test whether the suppression by clc1-\(\Delta19-76\) was unique to this clc1 allele and not due to general loss of CLC function or the slightly reduced levels of clathrin HC, we tested the ability of the null clc1\(\Delta\) to suppress vrp1\(\Delta\) or \(\text{rvs167} \Delta\) growth and endocytosis. In either case neither temperature sensitivity nor endocytic dynamics was rescued (Figure 5). The endocytic profiles seen in vrp1\(\Delta\) clc1\(\Delta\) yeast were more characteristic of a clathrin-null than a verprolin-null mutant (Newpher and Lemmon, 2006) since nearly 77% of Sla2-GFP patches persisted longer than 360 s (Figure 5B). Of interest, the characteristic actin comet tails caused by clc1\(\Delta\) were missing in vrp1\(\Delta\) clc1\(\Delta\) cells, probably due to impaired recruitment of the type I myosins in vrp1 and, as a consequence, reduced actin assembly. \(\text{rvs167} \Delta\) clc1\(\Delta\) cells also had severe endocytic defects (Figure 5D). Again, 77% of all Sla2-GFP patches were arrested, and in these cells dramatic actin comet tails were evident in 23% of patches.

**clc1-\(\Delta19-76\) suppression of vrp1\(\Delta\) and \(\text{rvs167} \Delta\) requires actin binding by Sla2**

As shown earlier, deletion of the N-terminus of CLC suppressed the loss of Vrp1 and Rvs proteins, three components of the actin phase of internalization. Because CLC-N terminus binds directly to the Sla2 coiled-coil region and Sla2 binds directly to F-actin through its THATCH domain, we considered the possibility that the clc1-\(\Delta19-76\) mutant effects were mediated through altered ability to regulate Sla2 actin binding. Therefore we examined the effect of CLC on Sla2 in vitro using F-actin binding.

**FIGURE 3:** Defects in endocytic dynamics of vrp1\(\Delta\) are suppressed when combined with clc1-\(\Delta19-76\). (A) Representative kymographs of Sla1-GFP/Abp1-RFP patches in wild type (SL5311), clc1-\(\Delta19-76\) (SL6065), and vrp1\(\Delta\) (SL6056). (B) Representative kymographs of Sla2-GFP/Abp1-RFP patches in wild type (SL5927), clc1-\(\Delta19-76\) (SL6084), and vrp1\(\Delta\) (SL6081). (C) Fluorescence lifetimes of Sla2-GFP, Sla1-GFP, and Abp1-RFP in strains shown in A and B. Data are reported as average ± SD (\(n \geq 50\)). \(\uparrow p \leq 0.0001\) vs. wild type; \(\uparrow \downarrow p \leq 0.0001\) vs. vrp1\(\Delta\); \(\uparrow \downarrow p \leq 0.001\) vs. vrp1\(\Delta\). (D) Tangential kymographs illustrating inward movement of Sla2-GFP/Abp1-RFP endocytic patches in wild type, vrp1\(\Delta\), and vrp1\(\Delta\) clc1-\(\Delta19-76\). (E) Percentage of Sla2-GFP/Abp1-RFP patches that demonstrate “normal” inward movement (\(n = 90\)). (F) Example plots of Sla2-GFP trajectories comparing inward movement from wild type, vrp1\(\Delta\), and vrp1\(\Delta\) clc1-\(\Delta19-76\). Each point represents a 2-s frame, with the length of the lines between frames indicating distance moved. Initial coordinates are highlighted in green, and the final time points are in red.
Percentage of patches that show “normal” inward movement in wild type; ‡p ≤ 0.0001 vs. wild type. Data are reported as average lifetimes of Sla2-GFP, Sla1-GFP, and Abp1-RFP in strains shown in A and B. Representative kymographs of Sla2-GFP/Abp1-RFP patches in wild type (SL5927), (SL6191). (B) Representative kymographs of Sla1-GFP/Abp1-RFP patches in wild type (SL5311), suppressed when combined with clc1-Δ19-76. (C) Fluorescence lifetimes of Sla2, and Abp1 for fluorescence lifetimes and movement (Figure 7, C, D, and G). In yeast, five endocytic NPFs have been identified: Pan1, Las17, Myo3, Myo5, and Abp1 (Winter et al., 2000; Duncan et al., 2001; Goode et al., 2001), although Pan1 and Abp1 are considered weak NPFs compared with the strong activities of Las17 or the type I myosins (Sun et al., 2001). In yeast, five endocytic NPFs have been identified: Pan1, Las17, Myo3, Myo5, and Abp1 (Winter et al., 2000; Duncan et al., 2001; Goode et al., 2001), although Pan1 and Abp1 are considered weak NPFs compared with the strong activities of Las17 or the type I myosins (Sun et al., 2001). The WASp homologue, Las17, aids in recruitment of Myo3 and Myo5 (Anderson et al., 2000; Sirotkin et al., 2005; Sun et al., 2006; Wong et al., 2010). Because verprolin binds these three potent NPFs, we tested whether the Vrp1, which in turn recruits and activates the redundant type I myosins (Sun et al., 2006). The WASp homologue, Las17, aids in recruitment of Vrp1, which in turn recruits and activates the redundant type I myosins Myo3 and Myo5 (Anderson et al., 1998; Evangelista et al., 2000; Geli et al., 2000; Sirotkin et al., 2005; Sun et al., 2006; Wong et al., 2010). Because verprolin binds these three potent NPFs, we tested whether the clc1-Δ19-76 would suppress null mutations in these genes. The clc1-Δ19-76 mutation was crossed to a myo3Δ myo5Δ strain, and resultant segregants were tested for growth. myo3Δ myo5Δ mutants showed extremely impaired growth at both 30 and 37°C, but combining the double null with clc1-Δ19-76 suppressed this defect (Figure 7A). Again using time-lapse imaging, we examined Sla1, Sla2, and Abp1 for fluorescence lifetimes and movement (Figure 7, C, D, and G). In myo3Δ myo5Δ cells the lifetimes of Sla2 patches were extremely delayed compared with wild type, and only 8% of patches internalized, consistent with previous work (Sun et al., 2006). However, these phenotypes were rescued by combination with the

**FIGURE 4:** Defects in endocytic dynamics of rvs167Δ are suppressed when combined with clc1-Δ19-76. (A) Representative kymographs of Sla1-GFP/Abp1-RFP patches in wild type (SL5311), clc1-Δ19-76 (SL6191). (B) Representative kymographs of Sla2-GFP/Abp1-RFP patches in wild type (SL5927), clc1-Δ19-76 (SL6084), rvs167Δ (SL6108), and rvs167Δ clc1-Δ19-76 (SL6197). (C) Percentage of patches that show “normal” inward movement following Abp1 accumulation (n = 90). Similar results were obtained for rvs167Δ (see Supplemental Figure S1).
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The **Δ** allele. Sla2-GFP lifetimes decreased from 226 ± 102 to 56 ± 36 s (p ≤ 0.0001, myo3Δ myo5Δ vs. myo3Δ myo5Δ clc1-Δ19-76, respectively; Figure 7, D and G), and the percentage of internalizing patches rose to 58% when clc1-Δ19-76 was present in the myosin mutant. We also saw a mild effect of clc1-Δ19-76 in myo3Δ (unpublished data) or myo5Δ alone (Figure 7, A and C–G), although generally the single myosin mutants were only slightly defective on their own, which explains why they did not appear in the SGA screen.

Deletion of LAS17 caused temperature-sensitive growth at 37°C, which was also suppressed by clc1-Δ19-76 (Figure 7B). las17Δ greatly increased both Sla1-GFP (80 ± 38 s), as shown previously (Sun et al., 2006), and Sla2-GFP (124 ± 62 s) lifetimes compared with wild type (Figure 7, E–G). When las17Δ was combined with clc1-Δ19-76 the lifetime of Sla1 was restored to normal (33 ± 7 s, p ≤ 0.0001 vs. las17Δ), and there was significant improvement of the lifetime of Sla2 (78 ± 36 s, p ≤ 0.0001 vs. las17Δ; Figure 7, E–G). Of interest, clc1-Δ19-76 also suppressed the extended lifetime of Abp1-RFP in las17Δ or myo3Δ myo5Δ (Figure 7, C–G), which was not observed for vrp1Δ and rvs167Δ (Figures 3C and 4C). Overall these results show that clc1-Δ19-76 also suppresses endocytic defects caused by loss of the major NPFs.

**DISCUSSION**

In this study we performed SGA with yeast containing a deletion of the Sla2-binding region of CLC in order to elucidate the functional significance of this interaction in vivo. This unbiased approach identified a large number of negative genetic interactions with clc1-Δ19-76, including many with components involved in clathrin-mediated and TGN/endosomal trafficking. We focused on three endocytic factors, Vrp1, Rvs167, and Rvs161, whose mutations were suppressed by clc1-Δ19-76, as positive genetic interactions were likely to be specific to the clc1-Δ19-76 allele and provide information on the regulatory role of the CLC–Sla2 interaction.

On the basis of our results, we propose the model shown in Figure 8, where CLC–Sla2 interaction prevents Sla2 binding to F-actin. This CLC inhibition would thereby restrict the number of attachments between Sla2 and actin, possibly to an area near the neck or edge of the clathrin coat (Figure 8A). Consistent with this,
immuno–electron microscopy (immuno-EM) of unroofed cells showed Hip1R associated with actin filaments primarily at the edge of clathrin lattices and pits (Engqvist-Goldstein et al., 2001). In yeast Sla2-actin attachments at the edge of the pit may provide directionality to the force produced by actin assembly or even assist in vesicle scission. CLC is likely to achieve this regulation by causing conformational changes of Sla2 that regulate THATCH accessibility to actin. Self-interaction of the THATCH and coiled-coil regions of Sla2 were first suggested by two-hybrid analysis (Yang et al., 1999). Electron microscopy of purified Hip1R or Hip1 showed the existence of both compact and extended profiles of the proteins (Engqvist-Goldstein et al., 2001; Wilbur et al., 2008), but the more compact structures are more prevalent in the presence of a 22–amino acid clathrin LCA N-terminal peptide containing the CLC-Hip1/R binding site (Wilbur et al., 2008). Moreover, in surface plasmon resonance analysis the CLC NT peptide decreased the affinity of Hip1 or Hip1R binding to F-actin, suggesting that CLC binding in vitro negatively regulates Hip1/R attachments to actin by promoting Hip1/R self-interaction (Wilbur et al., 2008).

Our work provides the first in vivo studies to support this regulatory model by showing that clc1Δ-1976, a clc1 mutant lacking its Sla2 interaction domain, can overcome several mutations leading to reduced late-stage actin assembly (vpr1Δ, myo3Δ, las1Δ, las2Δ) or failure to adequately narrow the neck of a vesicle (rvs167Δ/rvs161Δ). Recent studies suggested that CLC-NT prevents bending of the CHC knee, which impairs clathrin assembly (Wilbur et al., 2010). Thus, deletion of the CLC-NT in our studies could be more favorable for formation of a curved lattice and promote membrane deformation. However, clc1Δ-1976 suppression of vpr1Δ and rvs167Δ depended on the actin-binding THATCH region of Sla2, which is more consistent with a mechanism of suppression involving Sla2 interaction with the actin cytoskeleton.

Immuno-EM analysis showed that the N-BAR proteins Rvs161 and Rvs167 are situated along the tubular invagination above the clathrin coat. This is consistent with their association with the narrowing neck of the invaginating vesicle and function in constriction of the membrane, leading to vesicle scission (Kaksonen et al., 2005; Idriissi et al., 2008). New studies suggest that the amphiphysins may work in concert with the yeast dynamin Vps1, although the endocytic defects of vps1 mutants are much less severe and went unnoticed for some time (Nannapaneni et al., 2010; Smaczynska-de et al., 2010; our unpublished data). Although actin assembly would be robust in the amphiphysin mutants, the lack of constriction on the neck would impair scission, which is observed as retraction events by live-cell imaging (Kaksonen et al., 2005; Smaczynska-de et al., 2010; Youn et al., 2010; Figure 8B). At the end of retraction, usually the coats and actin dissipate. We suggest that increased binding to actin by Sla2 allowed by the clc1Δ-1976 mutation might overcome this by stabilizing the coat and actin network (Figure 8D). This could drive the invaginations deeper into the cell, thus narrowing the neck of the invagination, or the new attachments could promote scission directly. Supporting this, in some cases we observed multiple retraction events and then internalization, as if the stabilization of the patch allowed added coat and actin assembly and new attempts at vesicle scission (D.R.B., unpublished observations).

The role of verprolin is to recruit and activate the type I myosins and is thus critical for the actin assembly burst that drives invagination (Anderson et al., 1998; Gell et al., 2000; Sirokin et al., 2005; Sun et al., 2006). In yeast lacking Vrp1 (or myosin I function), there is no obvious inward movement of the endocytic coat (Figure 8C), despite the fact that the actin marker Abp1 is recruited (Sun et al., 2006). Residual actin assembly is likely provided by other endocytic NPFs, including Las17 (WASP); however, these may not be sufficient or properly positioned to drive invagination when the myosin function is impaired. We suggest that this defect is overcome by clc1Δ-1976 since it would increase the duration of Sla2 binding to actin, as well as affect the location and number of attachments (Figure 8E). In addition, this may stabilize the actin network to overcome the reduced NPF activity caused by vpr1Δ effects on Myo3/5 activity.

Because clc1Δ-1976 could suppress vpr1Δ, one might predict that the clc1Δ-1976 allele could suppress other NPF mutations. Abp1 is a weak NPF, and abp1Δ has no phenotype on its own, so we did not expect to see suppression by the clc1 mutation. However, our genetic screens found that clc1Δ-1976 exacerbated the growth defect of pan1Δ-4 (discussed later). We directly examined the three most potent NPF activities at the endocytic patch, Las17, and type I myosins (Winter et al., 1999; Evangelista et al., 2000; Lechler et al., 2000; Duncan et al., 2001; Goode et al., 2001), which were not identified in the SGA screen. We found that clc1Δ-1976 also suppressed the growth and endocytic defects of las17Δ and myo3Δ. Thus the model for verprolin (Figure 8) applies to these NPFs as well. Although las17Δ was not tested, three las17Δ alleles were analyzed by SGA, but their phenotypes may have been either too weak or too severe to observe suppression in a large-scale screen. Redundancy of the type-I myosins likely prevented their identification in the SGA.

We note that there was a difference in the suppression of vpr1Δ as compared with las17Δ or myo3Δ, in that clc1Δ-1976 suppressed the slowed Abp1 lifetime of these NPF mutations but not that of vpr1Δ. The reason for this distinction is not clear. Because Vrp1 binds both Las17 and the myosins, we suggest that Vrp1 also
regulates the activity of the WASp. Thus in vrp1Δ the stimulatory activity of each of the major NPFs might be impaired, extending the time needed to produce a competent F-actin network for internalization. In las17Δ or myo3Δ myo5Δ, at least one of the major NPFs would still be active.

PAN1 is one of the few essential endocytic genes. It encodes a scaffolding coat factor that binds several other endocytic coat proteins. Pan1 arrives at endocytic patches ∼20–30 s before the actin phase and has weak NPF activity (Kaksonen et al., 2003; Sun et al., 2006; Huang and Cai, 2007). In addition, the central domain of Sla2 (including the coiled-coil region) binds Pan1 and negatively regulates Pan1 NPF activity in vitro. Because the pan1-4 product lacks its NPF activation domain (Li et al., 2011), we initially surmised that the genetic interaction with clc1-Δ19-76 might be due to Pan1’s importance in priming actin assembly at the coat to initiate curvature. We tested this using pan1-20, which results in a truncation downstream of the NPF activation region but deletes the C-terminal proline-rich domain (PRD; Barker et al., 2007). This mutant also caused synthetic lethality with clc1-Δ19-76, consistent with the pan1-4 SGA results. These data suggest that the CLC-NT shares redundancy with functions of the Pan1-PRD, which may include the role of PRDs in binding to SH3-domain proteins (Barker et al., 2007). However, we also cannot rule out that one role of CLC-NT binding to Sla2 may be to release Sla2’s reported negative regulation of Pan1 NPF activity (Toshima et al., 2007).

The loss of CLC–Sla2 interaction might lead to prolonged Sla2 inhibition, which in the context of an impaired Pan1 could be
severely detrimental. Further studies are needed to uncover the basis for this synthetic lethality.

In mammals, Hip1R binds F-actin barbed ends in association with cortactin, which inhibits actin filament depolymerization in vitro (Le Clainche et al., 2007). This was suggested to channel new actin assembly and to stabilize F-actin. Despite a lack of a cortactin homologue in yeast, suppression by clc1-Δ19-76 may involve a broader distribution of anchoring surrounding the invagination and increased stability of the network by barbed end binding. We note that capping protein also binds to barbed ends and terminates filament elongation, but Sla2 is unique in that its ANTH region would also serve to tether the membrane to actin. In fact, complete absence of Sla2 leads to impaired invagination and unproductive actin comet tails emanating from the cell surface (Kaksonen et al., 2003). Thus, localized Sla2 contact with F-actin performs a unique function in directing elongation of the membrane protrusions and/or scission.

The invaginating sites in yeast have an extended tubular morphology with a clathrin coat at the tip and actin assembled surrounding the tubule (Idrissi et al., 2008). This architecture of such tubular assemblies with associated actin and clathrin has also been observed in mammalian cells. For example, internalizing vesicular stomatitis virus was found in deep invagination profiles with a clathrin cap at its base and an extensive actin network along the membrane surrounding the virus extending up to the cell surface, suggesting that actin is required for clathrin-mediated endocytosis of large particles (Cureton et al., 2009). In addition, long actin-dependent tubules with clathrin pits at the tip are generated in dynamin 1 and 2 double-knockout cells (Ferguson et al., 2009). These endocytic structures have been recapitulated in vitro from plasma membrane sheets incubated in the presence of cytosol and GTPγS, which blocks scission (Wu et al., 2010). Although the manner in which these structures are formed is unknown, they share remarkable

**FIGURE 8:** Model for CLC regulation of Sla2 binding to F-actin at endocytic patches. (A–C) Model of endocytic patches in wild type (A), rvs167Δ (B), and vrp1Δ (C). (D, E) Models for how preventing CLC regulation of the Sla2–actin interaction helps to overcome amphiphysin (D) and verprolin (E) mutants by increasing attachments to F-actin. Suppression of myo3/5Δ and las17Δ is similar to that of vrp1Δ.
similarities with yeast tubular invaginations, suggesting a role for Hip1 and Hip1R in their formation.

In summary, we believe that our results support the idea that a major role of CLC is to control endocytic progression by pruning the Sla2–actin attachments in the endocytic coat so that anchoring is restricted, possibly to the edge or neck of the invaginating vesicle. Releasing attachments in the coat could contribute to development of the endocytic vesicle and may be needed to promote directional membrane internalization. Restricting attachments to near the neck might also promote vesicle scission.

MATERIALS AND METHODS

Yeast strains and growth assays

Saccharomyces cerevisiae strains used in this study are listed in Supplemental Table S1A. Standard methods and media were used for genetic manipulations, growth, and transformation of yeast (Guthrie and Fink, 1991). To perform growth-plating assays, overnight log-phase liquid cultures were diluted to a starting concentration of 5 × 10⁶ cells/ml and then fivefold serially diluted in 96-well plates. Diluted cells were pinned with a multiprong frog onto yeast extract/peptone/dextrose (YPD) plates and grown at indicated temperatures for 48–60 h.

Generation of the integrated clathrin light-chain allele (clc1-Δ19-76:NatMX6) was done as follows. First a HisMX6 PCR fragment, flanked by Nco1 sites with CLC1 ends to excise codons 19–76, was cotransformed into yeast with pRS424 containing CLC1. Recombinant plasmids were shuttled into bacteria from Trp+ His+ colonies, cut with Nco1, and ligated to excise the HisMX6, yielding pTM45 with the clc1-Δ19-76 allele. Note that the recombination changed codon 19 from GAC (Asp) to GAA (Glu) and inserted two codons representing the Nco1 site: CCA (Pro) and TGG (Trp). Next the NatMX6 marker was PCR amplified from a pFA6a-NatMX6 template and integrated via homologous recombination downstream of the clc1-Δ19-76 coding sequence in pTM45. The resultant plasmid (pDRB1) was used as a template for two PCR amplifications. The first contained the 5′ flanking CLC1 DNA, the mutant allele, and 500 nucleotides of the NatMX6 marker. The second contained the full NatMX6 marker followed by 3′ sequence flanking CLC1. These products were cotransformed into the SGA tester strain y7092 (Tong et al., 2007), as well as our laboratory wild-type strain (SL1462) for recombination as previously described (Wach et al., 1997; Longtine et al., 1997). Diploids were selected, grown at 30°C, and tetrad dissected. Isolates that segregated from tetrads with pKH2 (Longtine et al., 1997) were used in crosses. Diploids were selected, grown at 30°C, and tetrad dissected. Isolates that segregated from tetrads with pKH2 (Longtine et al., 1997) were used in crosses.

SGA screens

SGA screens were conducted as described (Tong and Boone, 2006). In one screen the clc1-Δ19-76:NatMX6 query strain (SL5677) was crossed to a miniarray consisting of 177 strains with ts mutations in genes annotated with roles in actin, endocytosis, and vesicle trafficking (www.yeastgenome.org; Supplemental Table S1B). Construction of the ts mutant strains and methodology for screening them have been described (Li et al., 2011). The second screen was performed against an array of 3885 nonessential null mutants. Genetic interactions from the SGA screens were processed and identified as previously described (Tong et al., 2004). The results from the knock-out screen were normalized and the interactions measured as deviations from the null mutant alone. These relative fitness measurements were used to assign genetic interaction scores ε as described in Costanzo et al. (2010). Results here are reported as synthetic rescue if they had ε > 0.16 and p < 0.05. Results are reported as synthetic growth defects if they had ε < −0.12 and p < 0.05.

Assignment of screen results to biological process (Supplemental Table S2) was performed based on Gene Ontology assignments using Slim-Go enrichment at the Saccharomyces Genome Database (www.yeastgenome.org) and then manually refined. To perform network analysis, each screen hit was used as a node, and additional node/edge attributes (from the Saccharomyces Genome Database) were layered onto the data set using Cytoscape (Kohl et al., 2010). Known physical interactions were used to identify protein complexes (Figures 1, D–G) using the MCODE plug-in for Cytoscape (Bader and Hogue, 2003).

Microscopy and image analysis

Live-cell imaging of endocytosis was carried out essentially as described in Boettner et al. (2009). Cells were grown to log phase at 25°C in synthetic medium, concentrated, immobilized on polylysine-coated coverslips, mounted on slides in 1.6% agarose, and then imaged at 25°C. All fluorescence lifetimes were calculated from movies acquired on an Olympus (Center Valley, PA) BX71 inverted microscope equipped with differential interference contrast (DIC) optics, an UPlan Apo 150× total internal reflection fluorescence objective (numerical aperture [NA], 1.45), Hamamatsu (Hamamatsu, Japan) ImagEM C910013 512 × 512 bit EM charge-coupled device camera, and a Sutter Instrument (Novato, CA) Lambda DG4 rapid wavelength switcher with a 300-W xenon lamp. Images were captured using SlideBook 4.2 for PC platform (Intelligent Imaging Innovations, Denver, CO). Following capture, all movies were photo-bleach corrected in SlideBook using the exponential correction function. Average patch lifetimes and standard deviations were determined from 30–40 patches for each strain. Student’s t test was used to calculate p values. All kymographs, projection images, and example micrographs were generated in SlideBook and then exported to Adobe Photoshop (San Jose, CA) for figure assembly.

Single-particle tracks were generated from movies exported from SlideBook into ImageJ (National Institutes of Health, Bethesda, MD) using the plug-in ParticleTracker (Sbalzarini and Koumoutsakos, 2005). Particle trajectories that aligned 90 deg from the cell cortex were converted from pixel to metric distances and graphed using SigmaPlot.

All other microscopy was carried out on an Olympus fluorescence BX61 upright microscope equipped with Nomarski DIC optics, a UPlan 5 Apo 100× objective (NA 1.4), a CoolSnap HQ camera (Roper Scientific Germany, Ottobrunn, Germany), Sutter Instrument Lambda 10-2 excitation and emission filter wheels, and a 175-W xenon remote source with liquid light guide. Image capture was automated using SlideBook 4.01 for the Mac.

Analysis of actin polarization in phalloidin stained cells was done as follows. Cells were grown in YEPD to a concentration of 5 × 10⁶ cells/ml at 30°C. Formaldehyde was added directly to growth medium to a final concentration of 4%, and cultures were continued for
10 min. Cells were collected and resuspended in phosphate-buffered saline (PBS; 140 mM NaCl, 2.7 mM KCl, 10 mM Na$_2$HPO$_4$, 1.8 mM KH$_2$PO$_4$) containing 4% formaldehyde and incubated at room temperature for an additional 1 h. After two washes in PBS, cells were stained overnight at 4°C with 6.6 μM Alexa 568-phalloidin (Molecular Probes, Invitrogen, Carlsbad, CA). Cells were washed five times in PBS and immobilized on polylysine-coated coverslips for imaging. A series of optical Z-sections (0.2 μm) were captured and then deconvolved by the nearest-neighbor algorithm and projected into a single plane using SlideBook. Polarization of actin was quantified on cells with small or medium-size buds as described by Bi et al. (1998). Total fluorescence measurements were made for the whole cell and the mother cell in SlideBook. Cells with more than 50% of the total fluorescence in the mother cell were scored as non-polarized (n = 50).

Lucifer yellow (Molecular Probes) uptake was performed at 30°C for 1 h as described in Dulic et al. (1991).

Biochemical methods

Bacterial expression plasmids for GST-Sla2 (pTMNS), GST-Sla2-(292-968), and 6xHis-Clc1 (pTMN3) are described in Newpher and Lemmon (2006). GST-Crn1 was expressed in bacteria from pGAT2-CRN(1–651) (Goode et al., 1999). The vector for expression of GST-Sla2-(717-968) (pDRB7) was generated by ligation of a Sla2 PCR fragment encoding amino acids 717–968 (THATCH domain) with BamHI/SalI ends into pGEX-4t.

GST fusions were expressed in Rosetta Escherichia coli (Agilent Technologies, Santa Clara, CA). Cultures were grown to log phase at 37°C, then induced with 0.5 mM isopropyl-β-D-thiogalactoside for 6 h at 25°C. After pelleting, cells were resuspended in lysis buffer (140 mM NaCl, 2.7 mM KCl, 10 mM Na$_2$HPO$_4$, 1.8 mM KH$_2$PO$_4$, pH 7.3) containing 0.5% Triton X-100 (v/v), 1 mM dithiothreitol (DTT), a protease inhibitor cocktail (Stepp et al., 1995), and lysozyme (0.5 mg/ml) and incubated on ice for 15 min. Cells were lysed by sonication, and the lysate was cleared by centrifugation at 16,000 × g for 20 min. GST fusions were absorbed from the cleared supernatant onto glutathione–agarose beads (GE Healthcare, Piscataway, NJ) for 1.5 h on a rocker at 4°C. Beads were loaded into a poly-prep column and washed with 10 column volumes of lysis buffer and then GST fusions were eluted in 50 mM Tris-HCl, pH 8.0, 10 mM reduced glutathione, and 1 mM DTT. Peak fractions were exchanged into actin assembly buffer (see later discussion), concentrated in a Centricon-10 size exclusion filter (Millipore, Billerica, MA), brought up to 10% glycerol, snap frozen, and stored at −80°C. The 6xHis-Clc1 (pTMN3) was expressed and purified from BL-21 E. coli (DE3) as described in Newpher and Lemmon (2006).

Actin cosedimentation assays were performed as described by Gohla et al. (2005). Prior to use, all protein samples proteins were precleared by centrifugation at 100,000 × g in a Beckman Airfuge equipped with an A-100 rotor. Nonmuscle actin (Cytoskeleton, Denver, CO) was assembled for 1 h at room temperature in actin assembly buffer (4.5 mM Tris-HCl, 20 μM CaCl$_2$, 50 mM KCl, 2 mM MgCl$_2$, 1 mM ATP, pH 8.0). Assembled F-actin was added at a final concentration of 10 μM to either input proteins alone or input proteins preincubated for 1 h with a fivefold molar excess of 6xHis-Clc1. Input proteins were tested at the following concentrations: 3 μM GST-Sla2-292-958, 3 μM GST-Sla2-717-968, 1 μM GST-Sla2, and 1 μM GST-Crn1. Binding reactions were performed at 25°C for 1 h (typically in 50 μl) in actin assembly buffer, and then actin and actin-associated proteins were pelleted by centrifugation in the Airfuge at 100,000 × g for 1 h at room temperature. The supernatant was removed and the pellet was resuspended in water. The supernatant and pellet were brought to equal volumes in SDS–PAGE loading buffer, boiled for 3 min, and separated by SDS–PAGE on 8–20% gradient polyacrylamide gels (Invitrogen). Gels were stained with Coomassie brilliant blue, and high-resolution images were captured for densitometry analysis using ImageJ. Trapping of soluble proteins was accounted for by sedimentation of bovine serum albumin (2 μM) in the presence of 10 μM F-actin.

For immunoblots of clathrin LC and HC, cultures were grown to log phase (5 × 10$^8$ cells/ml) at 30°C, and 10 ml were concentrated and subjected to glass bead lysis in 250 μl of 0.1 M Tris, pH 7.5, containing 1 mM phenylmethylsulfonyl fluoride and a protease inhibitor cocktail (Stepp et al., 1995). Lysates were centrifuged for 20 min at 10,000 × g, and equivalent volumes of the supernatants were analyzed by SDS–PAGE and immunoblotted using anti-Chc1 mouse monoclonal antibodies (Lemmon et al., 1988), anti-Clc1 rabbit polyclonal antiserum (a gift from Greg Payne), or anti-PGK1 mouse monoclonal antibodies (Molecular Probes) as a loading control. Antibody decoration was detected by an Odyssey Infrared Imaging System (LiCor, Lincoln, NE) using IRDye700- or IRDye800-conjugated secondary antisera (LiCor).

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