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The Actin-Regulating Kinase Prk1p Negatively Regulates Scd5p, a Suppressor of Clathrin Deficiency, in Actin Organization and Endocytosis

Kenneth R. Henry,[1,2,6] Kathleen D'Hondt,[3] Ji Suk Chang,[2] David A. Nix,[4] M. Jamie T.V. Cope,[4,7] Clarence S.M. Chan,[5] David G. Drubin,[4] and Sandra K. Lemmon[2,8,*]

[1]Department of Genetics
[2]Department of Molecular Biology and Microbiology
Case Western Reserve University
Cleveland, Ohio 44106
[3]Department of Biochemistry
Ghent University and Medical Protein Research Flanders Interuniversity Institute for Biotechnology
B-9000 Ghent
Belgium
[4]Department of Molecular and Cell Biology
University of California, Berkeley
Berkeley, California 94720
[5]Section of Molecular Genetics and Microbiology and Institute for Cellular and Molecular Biology
University of Texas
Austin, Texas 78712

Summary

Endocytosis is a dynamic process requiring a network of interacting proteins that assemble and disassemble during cargo capture and vesicle formation. A major mechanism for regulation of this process involves the reversible phosphorylation of endocytic factors [1–3]. Recently, members of a new kinase family, the Ark/Prk kinases, which include mammalian AAK1 and GAK as well as yeast Prk1p, Ark1p, and Akl1p, were shown to regulate components of the endocytic machinery [4]. These include animal AP-1/AP-2 μ chains and yeast Pan1p (Eps15-like), Sla1p, and epsins [2, 5–10], but other potential targets are likely. *SCD5,* an essential yeast gene, was identified as a suppressor of clathrin deficiency [11, 12]. We also showed that Scd5p is required for normal cortical actin organization and endocytosis, possibly as a targeting subunit for protein phosphatase type 1 (PP1) [13, 14]. Scd5p contains a central triple repeat (3R) motif related to a known Prk1p consensus phosphorylation site L/IxxQxTG [8, 10], except that Q is replaced by T. In this study we demonstrate that the Scd5p 3R sequence is phosphorylated by Prk1p to negatively regulate Scd5p. Furthermore, we show that Prk1p, Ark1p, and Akl1p have different substrate specificities and play distinct roles in actin organization and endocytosis.

*Correspondence: slemmon@newssun.med.miami.edu
[6]Present address: Laboratory of Biochemistry, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20892.
[7]Present address: Symyx Therapeutics, Inc., Santa Clara, California 95051.
[8]Present address: Department of Molecular and Cellular Pharmacology, University of Miami School of Medicine, Miami, Florida 33136.

Results and Discussion

The 3R Region Is Important for Negative Regulation of Scd5p

Scd5p contains a binding site for protein phosphatase type 1 (PP1), a central region of 20 amino acids repeated three times (3R) followed by a region of 9 repeats of 12 amino acids (9R) and a glutamine-rich carboxy-terminal domain (Figure 1A). In previous studies, we analyzed an *scd5* mutant, *scd5-Δ338*, which expresses a C-terminal truncation of 338 amino acids [13]. This mutation removes the Scd5p 9R and Gln-rich region and causes temperature-sensitive growth, endocytic, and actin defects. To better understand the role of the Scd5p domains, additional truncation mutants were analyzed (Figure 1A).

Scd5-Δ140p lacks most of the C-terminal Gln-rich region. In *scd5-Δ338*, *scd5-Δ425*, and *scd5-Δ448* (3R class mutations), the Gln-rich and 9R regions are removed but three, two, or one repeat of the 3R region, respectively, remain. *scd5-Δ467* and *scd5-Δ523*, N-terminal (N-T) class mutations, completely eliminate the 3R, 9R, and Gln-rich regions (Figure 1A). An isogenic set of strains expressing the mutant proteins as the sole source of Scd5p was generated. Immunoblotting showed that the mutant proteins are stable and expressed well (not shown). Most of the truncation mutants are temperature sensitive for growth, although *scd5-Δ140* grows similar to wild-type *SCD5* (see Supplemental Figure S1A at http://www.current-biology.com/cgi/content/full/13/17/1564/DC1).

Receptor-mediated endocytosis of ^{35}S-labeled α factor by the Ste2p receptor at 25°C was relatively normal or slightly slowed in the *scd5* C-terminal truncation mutants (see Supplemental Figure S1B). After shifting to 37°C, endocytosis in *scd5-Δ140* was still relatively efficient, while the 3R class truncations (Δ338, Δ425, Δ448) caused a rapid block of α factor internalization. Surprisingly, endocytosis was partially restored in the N-T class mutants (Δ467, Δ523) where the 3R region was also deleted (Figure 1B).

Similar results were observed for fluid phase endocytosis, which was analyzed by uptake of Lucifer yellow (LY) into the vacuole (see representative mutants at 37°C in Figure 1C and not shown). All mutants internalized LY well at 25°C. At 37°C, 3R class mutants were defective, while *scd5-Δ140* and NT-class mutants exhibited LY internalization.

Actin organization, particularly cortical actin, is affected in many endocytic mutants [15], including *scd5-Δ338* [13]. At 34°C, F-actin staining with Alexa 568-phalloidin was normal in *scd5-Δ140*, while *scd5-Δ425*, a 3R mutant, had depolarized actin patches and actin cables were thin and barely visible (Figure 1C). Similar results were observed in *scd5-Δ448* and *scd5-Δ338*, the other 3R mutants (not shown and [13]). In contrast, mutants expressing only the N-terminal domain, *scd5-Δ523* (Figure 1C) and *scd5-Δ467* (not shown), had relatively normal actin patch polarization and actin cables. Therefore,

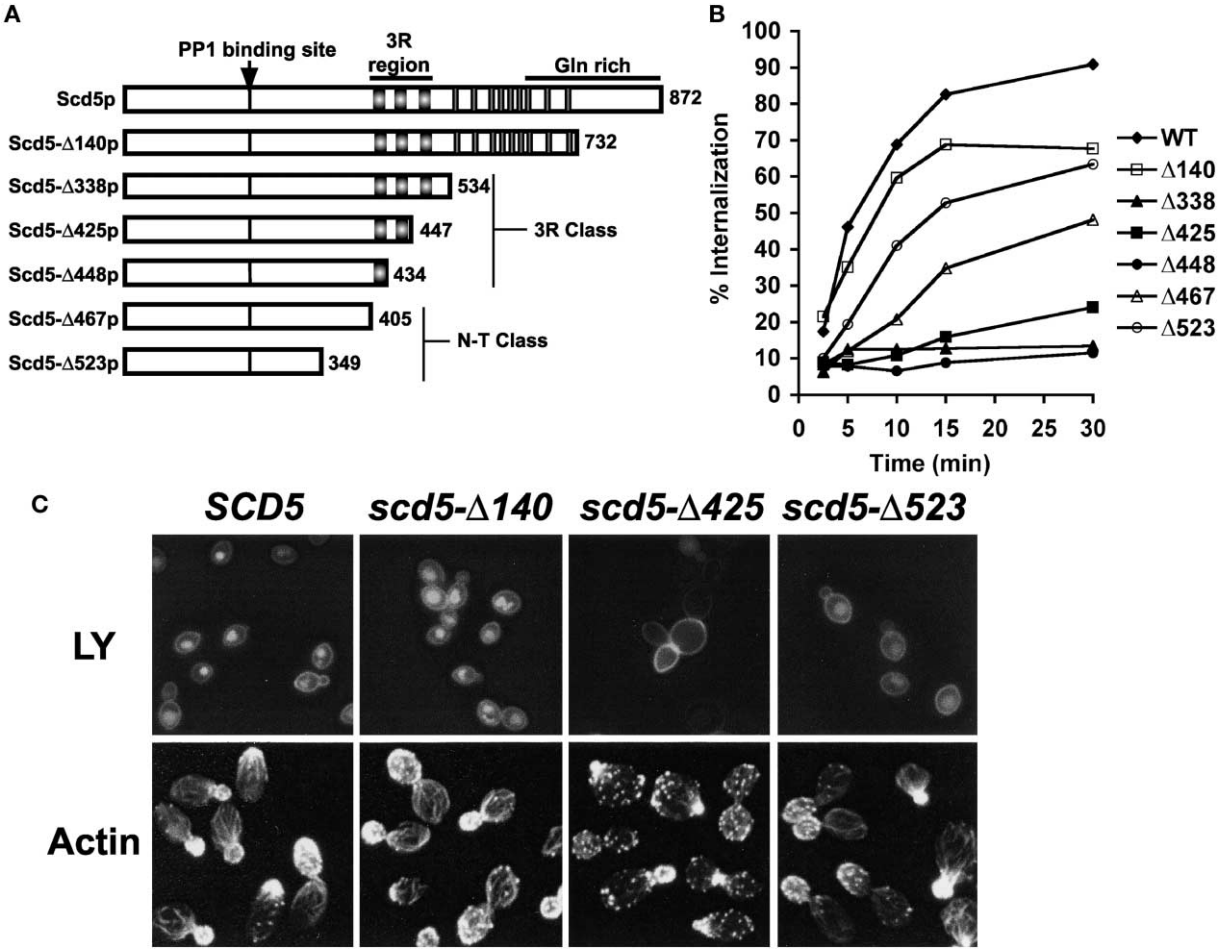

Figure 1. Endocytosis and Actin Organization Are Defective in *scd5* 3R Class Mutants, but Not in N-T Class Mutants at 37°C

(A) Scd5p domains and truncation mutants. Scd5p is shown with its PP1 binding site (black bar), three repeats of 20 amino acids (3R; bars with highlight), nine repeats of 12 amino acids (9R; gray), and Gln-rich tail. 3R and N-T class mutants are indicated.

(B) Receptor-mediated endocytosis: cells were shifted to 37°C for 15 min and then pulsed with radio-labeled α factor. Samples taken at the indicated times were processed to determine percent α factor internalized. Strains are: wild-type (SL3921; solid diamond); *scd5-Δ140* (SL4031; open square); *scd5-Δ338* (SL3993; solid triangle); *scd5-Δ425* (SL3926; solid square); *scd5-Δ448* (SL3924; solid circle); *scd5-Δ467* (SL3923; open triangle); and *scd5-Δ523* (SL3927; open circle).

(C) Upper panels (LY): cells were incubated at 25°C or preshifted to 37°C for 15 min. Incubation was continued in the presence of LY for 60 min and cells examined for uptake into the vacuole. DIC imaging (not shown) showed vacuoles were visible in all strains. Lower panels (Actin): cells were shifted to 34°C for 2.5 hr before fixation and staining with Alexa-568-phalloidin to visualize filamentous actin. Strains are: wild-type (SL4395), *scd5-Δ140* (SL4396), *scd5-Δ425* (SL4398), and *scd5-Δ523* (SL4401).

the 3R class truncation mutants, which retain the triple repeat region, have severe endocytic and actin defects, while further deletion of the 3R region partially restores actin and endocytic functions.

Deletion of Prk1p Suppresses *scd5-Δ338*

The 3R region of Scd5p contains LxxTxTG repeats resembling the Pan1p, Sla1p, and Ent1/2p L/IxxQxTG sequences that are phosphorylated by Prk1p. In fact, Pan1p and Ent1/2p also have LxxTxTG motifs. Prk1p phosphorylation of L/IxxQxTG repeats negatively regulates Pan1p and causes dissociation of a Pan1p-End3p-Sla1p complex [8, 9]. Also, a mutation in *PRK1* was identified as an extragenic suppressor of *pan1-4* [8]. The Scd5p truncation results suggested that the 3R region might be a target for negative regulation by Prk1p or one of the other related kinases, Ark1p or Akl1p. If this is the case, reduction in a regulating kinase might suppress *scd5-Δ338* phenotypes. Consistent with this, while *scd5-Δ338* grew poorly at 34°C, cells containing *akl1Δ*, *prk1Δ*, or *ark1Δ* in combination with *scd5-Δ338* grew at this temperature, although *prk1Δ* suppression was strongest and was even observed at 37°C (Figure 2A). *prk1-Δ* also suppressed the endocytic defect of *scd5-Δ338* (Figure 2B). In contrast, *ark1Δ* exacerbated the *scd5-Δ338* internalization phenotype, even at 25°C. *akl1Δ* had no obvious effect on endocytosis of *scd5-Δ338* at either temperature. On their own, *prk1Δ* and *akl1Δ* took up LY well at both temperatures; however, *ark1Δ* had a noticeably weaker LY signal at 25°C (not shown), indicating that *ark1Δ* reduces endocytosis on its own.

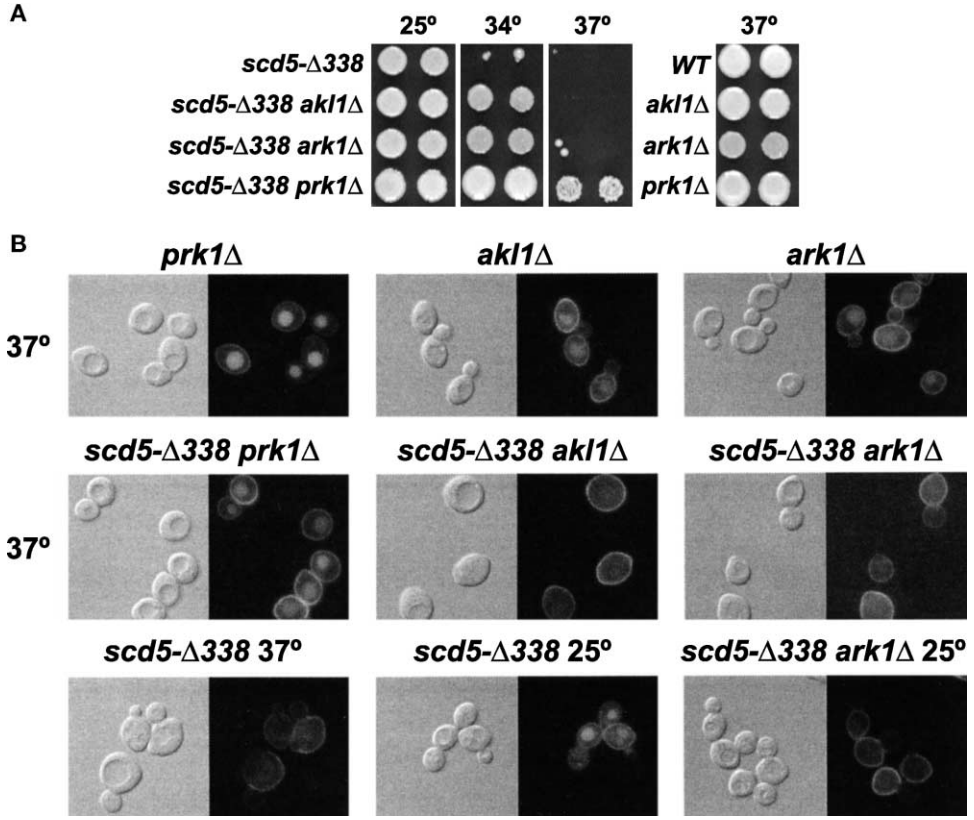

Figure 2. Effect of Deletion of Ark/Prk Kinase Genes on Growth and Endocytosis of *scd5-Δ338*

(A) Growth: cultures were grown in YEPD to log phase. Cells at 1×10^7/ml or a 1/4 dilution were spotted on YEPD plates and grown for 3 days at the indicated temperatures.

(B) LY uptake: cells were incubated at 25°C or preshifted to 37°C for 15 min. Then, LY was added and incubation was continued for 60 min. Cells were photographed using DIC (to visualize the vacuole) and fluorescence microscopy. Strains are: wild-type (SL1528), *scd5-Δ338* (SL3920), *akl1Δ* (SL4578), *ark1Δ* (SL4580), *prk1Δ* (SL4579), *scd5-Δ338 akl1Δ* (SL4581), *scd5-Δ338 ark1Δ* (SL4582), and *scd5-Δ338 prk1Δ* (SL4583).

Combining *prk1Δ* with *scd5-Δ338* also suppressed the actin patch depolarization defect normally detected in *scd5-Δ338* at 34°C (Supplemental Figure S2). In addition, there seemed to be slightly less actin depolarization in the *akl1Δ scd5-Δ338* double mutant at 34°C (not shown), but this suppression was weaker than that conferred by *prk1Δ*. *ark1Δ* resembled *scd5-Δ338* at 34°C (not shown), indicating *ark1Δ* could not suppress either the endocytic or actin defects of *scd5-Δ338*. These data suggest that Prk1p phosphorylates the 3R region to negatively regulate Scd5p function in endocytosis and actin organization.

The 3R Region LxxTxTG Motif Is Thr Phosphorylated by Prk1p and Akl1p

To determine whether the Scd5p LxxTxTG motif is phosphorylated on threonine residues, the 3R region was expressed in wild-type yeast as a GST fusion under control of the *GAL1* promoter. GST-3R was affinity purified on glutathione beads, treated with or without calf intestinal phosphatase (CIP), and analyzed by immunoblotting (Figure 3B). In the absence of CIP, a doublet was observed when blotting with anti-Scd5p or anti-phospho-Thr antibodies. In the presence of CIP, a single band was seen with anti-Scd5p, while no signal was detected with anti-phospho-Thr antibodies. Thus, the 3R region of Scd5p is Thr phosphorylated in vivo.

To examine whether the Ark/Prk kinases phosphorylate the triple repeat region in vivo, the galactose-inducible GST-3R was coexpressed with galactose-inducible kinases or kinase dead (KD) versions. After induction in galactose medium, cell extracts were generated and analyzed by immunoblotting with anti-GST antibodies (Figure 3C). In all cases the doublet of GST-3R was detected. In each of the KD mutants, the faster migrating band was much more intense than the band of slower mobility. Wild-type Ark1p also did not significantly increase phosphorylation of the 3R region. However, Akl1p and Prk1p overexpression produced an increase in the intensity of the upper bands relative to KD mutants or Ark1p. CIP treatment caused the upper bands to collapse into a single lower band, verifying the band shift is due to phosphorylation (not shown). Therefore, Akl1p and Prk1p, but not Ark1p, can phosphorylate Scd5p's 3R region in vivo.

To determine whether any of the Ark/Prk kinases directly phosphorylate the 3R LxxTxTG motif, we assayed the kinases, affinity purified from yeast, against peptide

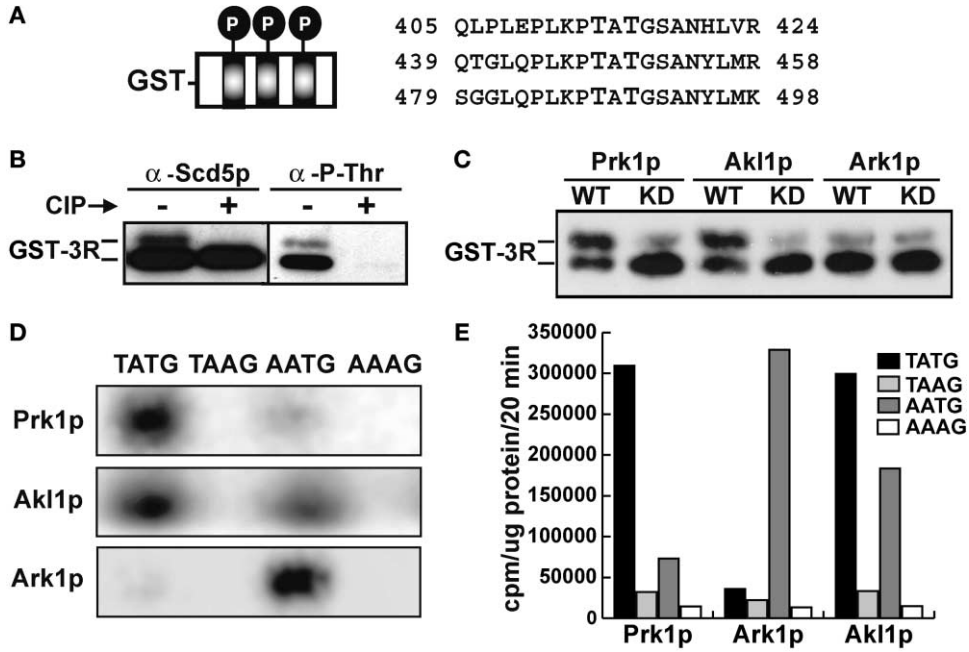

Figure 3. The 3R Tegion of Scd5p Is Phosphorylated by Prk1p and Akl1p

(A) GST-3R fusion region and sequence of the 20 amino acid repeats with the LxxTxTG motif.

(B) The 3R region is phosphorylated on Thr in vivo. A protease-deficient strain (BJ2168) transformed with pJSC14 (*CEN*, *LEU2*, P_{GAL1}*GST-3R*) was induced for expression, and the GST fusion was affinity purified from protein extracts using glutathione-Sepharose 4B beads. GST-3R samples, treated with or without CIP, were subjected to SDS-PAGE, immunoblotting, and probing with anti-Scd5p or anti-phospho-Thr polyclonal antibodies.

(C) Phosphorylation of Scd5p's 3R region in strains overexpressing Ark/Prk kinases. Cells (SL1463) cotransformed with pJSC14 (GST-3R) and a kinase expression vector (pJC203, Akl1p; pKRH28, Akl1p-KD; pSKL1, Ark1p; pSKL3, Ark1p-KD; pDD554, Prk1p; or pDD561, Prk1p-KD) were induced for expression in galactose. Extracts prepared from equal amounts of cells were subjected to SDS-PAGE, immunoblotting, and probing with anti-GST antibodies to detect the GST-3R fusion.

(D) Prk1p and Akl1p, but not Ark1p, directly phosphorylate the LxxTxTG motif in vitro. Immunoprecipitated GFP-tagged Prk1p or Akl1p or purified Ark1p were added to reactions containing 70 μg of oligopeptides (QPLKPTATGSANYL, QPLKPTAAGSANYL, QPLKPAATGSANYL, or QPLKPAAAGSANYL). Gels were silver stained to confirm equal amounts of oligopeptides in each reaction (data not shown). Phosphorylation was detected by a Hewlett Packard Instant Imager system.

(E) Quantification of in vitro phosphorylation shown in (D).

substrates (Figures 3D and 3E). The 3R region peptide QPLKP**TATG**SANYL was strongly phosphorylated by Prk1p and Akl1p. Mutating TATG to AATG greatly reduced phosphorylation by Prk1p, similar to previous observations [8]. However, the AATG peptide was still strongly phosphorylated by Akl1p, but not to the same extent as TATG. TATG was a poor substrate for Ark1p, but, surprisingly, the AATG mutant was robustly phosphorylated. Mutations of TATG to TAAG and TATG to AAAG nearly eliminated phosphorylation of the peptides by all three kinases (Figures 3D and 3E). KD versions were also inactive (not shown). Additionally, the AAAG mutant data indicate that the serine in the peptide is not phosphorylated by these kinases. Therefore, supporting the in vivo kinase analysis, Akl1p and Prk1p phosphorylate the LxxTxTG motif, while Ark1p prefers different sequences.

scd5-3TT>3AA-Δ338 Suppresses *scd5-Δ338* Phenotypes

If phosphorylation on the threonine residues causes defects in growth and endocytosis of *scd5-Δ338*, mutating the 3R region threonines to alanine should mimic dephosphorylation and rescue *scd5-Δ338*. Indeed, cells expressing Scd5-3TT>3AA-Δ338p, which changes the 3R region repeats from LKPTATG to LKPAAAG, grew well at both 25°C and 37°C, unlike *scd5-Δ338*, which is temperature sensitive (Figure 4A). The 3TT to 3AA mutation in *scd5-Δ338* also restored endocytosis of LY at 37°C (Figure 4B) and actin organization was also partially suppressed (not shown). These data further indicate that phosphorylation of the Scd5p 3R region by Prk1p negatively regulates Scd5p.

Based upon our data, we propose that Scd5p exists in an "on" or "off" state where the 3R region serves as a "switch." In *scd5-Δ338*, *scd5-Δ425*, or *scd5-Δ448*, the 3R region could be more accessible to phosphorylation by Prk1p, leading to severe defects in endocytosis and actin. In contrast, N-T class truncations lacking the 3R region or *scd5-3TT>3AA-Δ338* would not be subject to Prk1p regulation and would be constitutively "on," allowing partial suppression of these phenotypes. The phosphorylation could affect Scd5p's interaction with other binding partners or it could affect the localization of Scd5p, similar to what has been shown for other clathrin accessory factors in animal cells or for the

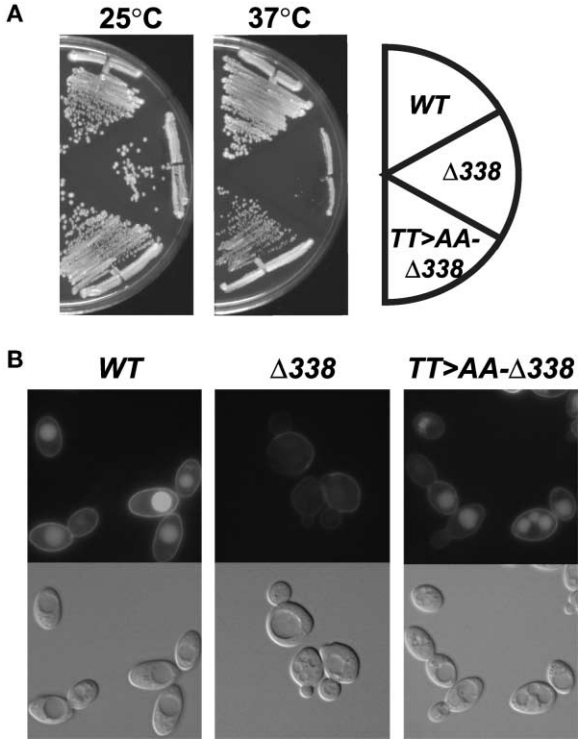

Figure 4. Growth and Endocytic Defects of *scd5-Δ338* Are Suppressed by Mutating the Triple Repeat Threonines to Alanines

(A) Wild-type (SL4395), *scd5-Δ338* (SL4397), and *scd5-3TT>3AA-Δ338* (SL4676) strains were streaked onto YEPD and incubated for 3 days at 25°C or 37°C.

(B) Cells as indicated in (A) were preshifted to 37°C for 15 min. Then, LY was added, and incubation was continued for 60 min. Cells were photographed using fluorescence microscopy (upper panels) and DIC (lower panels).

Pan1p-End3p-Sla1p complex in yeast (e.g., see [3, 8, 9, 16–18]).

Our studies also provide evidence that Prk1p and Ark1p are not functionally redundant. Previous studies suggested overlapping functions, since only a *prk1Δ ark1Δ* double mutant, but not the single mutants, exhibits an endocytic and dramatic actin patch aggregation defect [10, 19]. Additionally, Sla1p and Pan1p phosphorylation shows dependency on both Ark1p and Prk1p [9], although there is no data confirming that Ark1p directly phosphorylates the Sla1p/Pan1p/Ent1/2p L/IxxQxTG motifs or that this affects assembly of the Pan1p-Sla1p-End3p complex. In fact, Ent1/2p phosphorylation appears to be reliant on Prk1p, and weakly Akl1p, but not on Ark1p [10]. Furthermore, we found that Ark1p does not phosphorylate the Scd5p 3R domain, but shows strong preference for LxxAxTG rather than LxxTxTG, which is phosphorylated by both Prk1p and Akl1p. Moreover, combining *scd5-Δ338* with *ark1Δ* has adverse consequences for endocytosis, but *prk1Δ* suppresses the *scd5-Δ338* phenotypes. Together, these data suggest that both Ark1p and Prk1p regulate endocytosis and actin organization, but they phosphorylate different targets or different sites on the same target.

The role of Akl1p is less clear. It could be partially redundant with Prk1p, but expressed at lower levels, since *akl1Δ* very weakly suppressed the actin defects of *scd5-Δ338* and had a slight effect on Ent1/2p phosphorylation, while *ark1Δ* did not [10]. Alternatively, Akl1p could be partially redundant with both Prk1p and Ark1p, since it recognizes both LxxTxTG and LxxAxTG. Thus, *akl1Δ*'s weak effects on *scd5-Δ338* might reflect combined positive (*prk1Δ*-like) and negative (*ark1Δ*-like) genetic interactions.

Previously we showed that binding of PP1 to Scd5p is crucial for endocytosis and actin organization, and Scd5p partially colocalizes with cortical actin patches [13, 14]. PP1 is a broad specificity phosphatase that is directed to specific substrates by regulatory subunits [20]. Scd5p could target PP1 to dephosphorylate actin patch proteins allowing actin reassembly, thereby opposing the Ark/Prk kinases, which seem to trigger actin patch disassembly [8, 9, 19]. Phosphorylation of the 3R region by Prk1p could, thus, attenuate Scd5p-PP1 to ensure actin patch/endocytic factor disassembly.

Supplemental Data

Supplemental Data including all Experimental Procedures and the table of strains used in this study (Table S1) can be found at http://www.current-biology.com/cgi/content/full/13/17/1564/DC1/.

Acknowledgments

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