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Las17p–Vrp1p but not Las17p–Arp2/3 interaction is important for actin patch polarization in yeast

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ABSTRACT

The actin cytoskeleton plays a central role in many important cellular processes such as cell polarization, cell division and endocytosis. The dynamic changes to the actin cytoskeleton that accompany these processes are regulated by actin-associated proteins Wiskott–Aldrich Syndrome Protein (WASP) (known as Las17p in yeast) and WASP-Interacting Protein (WIP) (known as Vrp1p in yeast). Both yeast and human WASP bind to and stimulate the Arp2/3 complex which in turn nucleates assembly of actin monomers into filaments at polarized sites at the cortex. WASP–WIP interaction in yeast and humans are important for Arp2/3 complex stimulation in vitro. It has been proposed that these interactions are also important for polarized actin assembly *in vivo*. However, the redundancy of actin-associated proteins has made it difficult to test this hypothesis. We have identified two point mutations (L80T and H94L) in yeast WASP that in combination abolish WASP–WIP interaction in yeast. We also identify an N-terminal fragment of Las17p (N-Las17p$_{1-368}$) able to interact with Vrp1p but not Arp2/3. Using these mutant and truncated forms of yeast WASP we provide novel evidence that WASP interaction with WIP is more important than interaction with Arp2/3 for polarized actin assembly and endocytosis in yeast.

1. Introduction

The actin cytoskeleton plays a fundamental role in many cellular processes including polarized exocytosis, endocytosis, cell division and cell motility. Assembly of actin monomers into filaments (F-actin) may provide mechanical force on membranes driving these processes. In budding yeast (*Saccharomyces cerevisiae*), F-actin is organized into cortical actin patches, actin cables and the actomyosin ring [1–3] Actin patches are dynamic structures with a polarized distribution that cluster into small emerging buds, are randomly dispersed in the mother and large bud during mitosis, and cluster at the bud neck during cell division [1]. Cortical actin patches have been proposed to be sites of endocytosis [4]. Actin patches exhibit both short range motility at the cortex and long-range movement into the cytoplasm.

The branched actin filaments that comprise cortical actin patches [5] are nucleated by the Arp2/3 complex, a highly conserved seven subunit complex which nucleates actin filament assembly from actin monomers [6]. The long-range movement of actin patches is driven by Arp2/3-dependent F-actin assembly [7]. Like in vertebrates, in *S. cerevisiae* cells, Arp2/3 is activated by several nucleation promoting factors (NPFs) [8]. These include Las17p/Bee1p which is the yeast homologue of mammalian Wiskott–Aldrich Syndrome Protein (WASP) and the type I myosins Myo3p and Myo5p [9–16]. Vertebrate WASP is an actin-associated protein expressed in hematopoietic cells and mutations in WASP cause immune-deficiency due to actin cytoskeletal defects in lymphocytes [17]. WASP-related proteins (e.g. N-WASP and WAVEs) are also NPFs and are expressed in other cell types [18].

Both vertebrate WASP and yeast Las17p exhibit a modular structure with a WASP Homology 1 domain (WH1) in the N-terminal region that binds WIP (Vrp1p in yeast) and a C-terminal WH2 (WASP Homology 2) domain that binds actin monomers. Both proteins also have an acidic domain (A) at the C-terminal which binds to and activates Arp2/3 [18]. Las17p interacts with the C-terminal region of Vrp1p in a manner analogous to the interaction between WASP and WIP in vertebrates [11, 17, 19] and Las17p and type I myosins function redundantly in the activation of the Arp2/3 complex [13]. Given the existence of other NPFs, it is not yet clear whether the interaction of Las17p with Arp2/3 is essential *in vivo*. Indeed other NPFs have been shown to act redundantly with Las17p in activation of Arp2/3 [14–16].

Abbreviations: A, Acidic; F-actin, filamentous actin; GFP, green fluorescent protein; LY, Lucifer Yellow; NPF, Nucleation Promoting Factor; SH3, Src Homology 3; WA, WH2/A; WASP, Wiskott–Aldrich Syndrome Protein; WH1, WASP Homology 1; WH2, WASP Homology 2; WIP, WASP-Interacting Protein; WAVE, WASP and Verprolin Homologous Protein

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Unlike Las17p and type I myosins, Vrp1p and its vertebrate ortholog WIP do not interact with Arp2/3 but instead interact with NPFs and actin monomers, e.g. Las17p and type I myosins. The WH2 domains of Vrp1p and WIP bind monomeric actin and the Vrp1p WH2 domain is functionally redundant with the Las17p WH2 domain [13,16,20]. The WH2 domains are essential for Arp2/3 activation by Las17p and type I myosins and have been proposed to provide actin monomers competent for assembly into filaments by Arp2/3 [14,16]. The acidic domains (A) of either type I myosin (Myo3p or Myo5p) in combination with the WH2 domain of Vrp1p promote actin polymerization by activating the Arp2/3 complex analogous to Las17p [9, 13, 14, 16]. It has been shown recently that Vrp1p is recruited to cortical sites at least in part through interaction with Las17p and that Vrp1p in turn recruits type I myosins to the cortical sites prior to actin assembly at those sites [14–16, 21, 22]. This suggests that Vrp1p interaction with NPFs may play a role in recruitment of NPFs to cortical sites and determine the site of nascent actin patch formation.

Deletion of *LAS17* (*las17Δ*) results in Las17p-deficient cells with defects in bulk endocytosis (uptake of external materials by cells) and actin patch polarization at both permissive (24 °C) and restrictive temperature (37 °C) and a defect in growth at restrictive temperature [10, 11, 23]. These defects arise despite the presence of type I myosins, Myo3p and Myo5p each of which has an acidic domain (A) functionally redundant with that of Las17p [9, 13, 15]. This suggests that Las17p may possess important cellular roles in the actin cytoskeleton independent of Arp2/3 activation. In contrast to the phenotype observed upon deleting the entire *LAS17* gene, deleting only sequences encoding the WH2 or A domain of Las17p (Las17pΔWA) does not cause strong phenotypes at either 24 °C or 30 °C [12]. This suggests that either interaction with or stimulation of Arp2/3 is not essential *in vivo*, or that redundancy with other NPFs renders the specific role of Las17p in Arp2/3 activation non-essential. In addition to its role in binding and activating Arp2/3, Las17p plays a role in recruitment of Vrp1p to the cortex [14, 16, 21, 22]. It is possible that this role of Las17p (or other roles yet to be identified) is responsible for the defects observed in *las17Δ* cells rather than the loss of Arp2/3 activation per se.

We show here that an N-terminal Las17p fragment comprising of residues 1–368 (N-Las17p$_{1-368}$) that retains interaction with Vrp1p but not Arp2/3 functionally replaces full-length Las17p in growth; bulk endocytosis and actin patch polarization. We identify two mutations in the WH1 domain of N-Las17p$_{1-368}$ that perturb Las17p–Vrp1p interaction and show that they reduce the ability of N-Las17p$_{1-368}$ and full-length Las17p to function at elevated temperature.

2. Materials and methods

2.1. Strains, plasmids, media and reagents

SD minimal medium and YPUAD rich medium were prepared as described in Munn et al. [24]. Plasmid DNA was introduced into yeast cells using a modification of the lithium acetate protocol [24]. IDY19 (*MAT***a** *his3 leu2 ura3 trp1 mfa2::FUS1:lacZ*) and IDY223 (*MAT***a** *his3 leu2 ura3 trp1 las17::LEU2*) have been described in Naqvi et al. [11]. AMY88 (*MAT***a** *his4 leu2 ura3 lys2 vrp1::KanMx bar1*) has been described in Thanabalu et al. [21]. Yeast strain PJ69-4A (*MAT***a** *his3 leu2 ura3 trp1 gal4 gal80 met2::GAL7-lacZ GAL2-ADE2 LYS2::GAL1-HIS3*) [25] was used to test yeast two-hybrid interactions.

2.2. DNA techniques and plasmid construction

Standard DNA techniques were performed as described in Sambrook et al. [26]. The *LAS17* gene with the endogenous promoter [11] was digested with HindIII and BamHI and sub-cloned into the centromeric plasmid YCplac33 [27] to make pLAS17. pN-LAS17 (N-Las17p1–368 expressed from the *LAS17* promoter) was constructed by digesting pLAS17 with HindIII and EcoRI, filling the ends with Klenow enzyme and ligation to generate a stop codon (after a.a. 368). It was used for constructing sub-clones and the mutant pN-LAS17^{TL} construct by PCR mutagenesis (Fig. 1).

2.3. Yeast two-hybrid assay

DNA encoding N-Las17p$_{1-206}$ and N-Las17p$_{207-368}$ were cloned in-frame with GAL4AD in vector pACT2 (activation domain, AD) (Clontech, Palo Alto, CA) DNA encoding C-Vrp1p$_{364-817}$ and Myo5p (SH3) (a.a. 1085–1219) were cloned in-frame with GAL4BD in vector pAS2-1 (binding domain, BD) (Clontech, Palo Alto, CA). The AD and BD plasmids were sequentially transformed into PJ69-4A and the transformants were selected on solid SD minimal medium lacking tryptophan and leucine. The two-hybrid interactions were then investigated by testing the growth of the transformants on the same medium lacking histidine + 2 mM 3-AT to detect the expression of the *HIS3* interaction reporter.

2.4. In vivo protein binding assays

C-Vrp1p$_{364-817}$-6His or Myo5p(SH3)-6His were expressed in IDY223 or AMY88 cells also expressing GFP-tagged Las17p fragments. The yeast strains were grown to exponential phase at 24 °C and harvested. The cell pellet was resuspended in PBS (Phosphate Buffered Saline) and lysed using a mini Bead Beater 8 (Biospec Products,

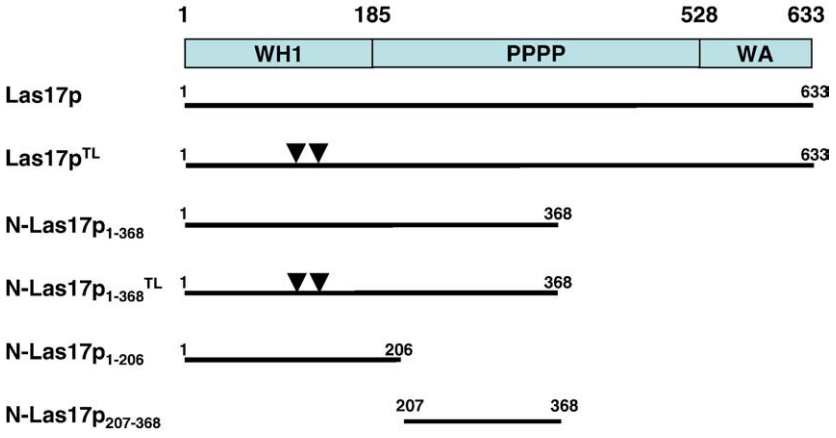


Fig. 1. Las17p domain structure and the constructs used. Schematics of Las17p and its deletion constructs. Las17p contains an N-terminal WH1 domain (WH1, 1–184), a proline-rich region (PPPP, 185–527) and C-terminal WH2 and A domains (WA, 528–633). Truncated proteins used for functional studies or for pull-down assays are shown as black bars.

Bartlesville, OK). The cell extract was clarified by high-speed centrifugation and the supernatant incubated with Ni^{2+}-NTA agarose beads for 1 h at 24 °C. The beads were subsequently washed twice with 1% Triton X-100 in PBS and twice with 10 mM imidazole in PBS. The bound proteins were eluted by boiling the beads in SDS-PAGE loading buffer and analyzed by SDS-PAGE and western blot.

2.5. Western blot

Cell pellets (7 OD_{600} unit each) were resuspended in 240 µl (1.85 N NaOH/1.06 M β-mercaptoethanol) and incubated on ice for 10 min. The protein was precipitated by the addition of an equal volume of 20% TCA (TriChloro Acetic Acid) and incubation on ice for

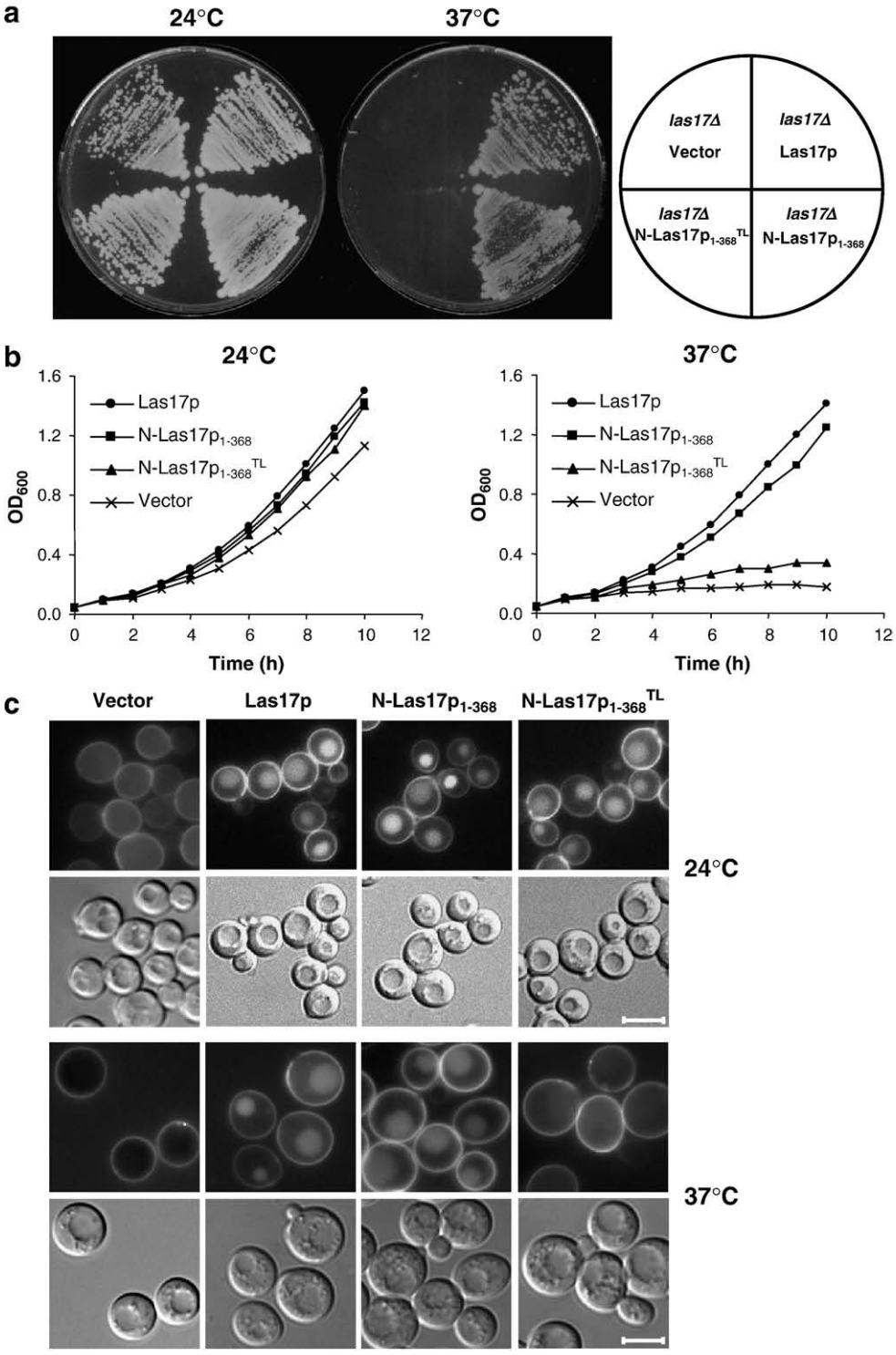


Fig. 2. N-Las17p_{1-368} functionally substitutes for full-length Las17p in growth and fluid-phase endocytosis. (a) Growth at 24 °C and 37 °C of *las17Δ* cells harboring empty vector or CEN plasmids expressing Las17p, N-Las17p_{1-368} or N-Las17p^{TL}_{1-368}. Each strain was streaked for single colonies on YPUAD agar, incubated at either 24 °C or 37 °C, and photographed after 3 days. (b) Growth curve of *las17Δ* cells carrying empty vector or CEN plasmids expressing Las17p, N-Las17p_{1-368} or N-Las17p^{TL}_{1-368}. Overnight YPUAD cultures of each strain were diluted to an OD_{600} of 0.05 in fresh YPUAD medium and incubated at either 24 °C or 37 °C. OD_{600} was monitored at 1 h intervals. (c) *las17Δ* cells harboring empty vector, or CEN plasmids expressing Las17p, N-Las17p_{1-368} or N-Las17p^{TL}_{1-368} were grown in YPUAD to exponential phase at 24 °C, either left at 24 °C or shifted to 37 °C for 1 h, before carrying out LY uptake assay for 1 h at either 24 °C or 37 °C. Upper panels: FITC-fluorescence optics to visualize LY. Lower panels: DIC optics to visualize cell profiles. Bar, 5 µm.

10 min and the pellet was collected by centrifugation. The pellet was resuspended in 100 µl of SDS-PAGE loading buffer. The proteins were resolved on a 10% SDS-PAGE gel, electroblotted onto nitrocellulose membranes and probed with appropriate primary antibodies and secondary antibodies conjugated to horseradish peroxidase (HRP) and detected using a chemiluminescence kit from Pierce (Rockford, Illinois). Bands were visualized by fluorography using X-ray film (Kodak Company, Rochester, NY).

2.6. Visualization of F-actin

Yeast cells grown to exponential phase in YPUAD at 24 °C were fixed by direct addition of 3.7% formaldehyde (final concentration) to the culture. For actin patch polarization at 37 °C the cells were shifted to 37 °C for 2 h before fixing. Fixed cells were washed once with PBS before being permeabilized using 1% Triton X-100 in PBS. The permeabilized cells were stained with Alexa-488-conjugated phalloidin (0.3 U) and analyzed by fluorescence microscopy [28].

2.7. Fluid-phase endocytosis

To visualize fluid-phase endocytosis, yeast cells growing exponentially in YPUAD at 24 °C. The cells were then harvested and preincubated at 24 °C or 37 °C for 1 h before being incubated in fresh YPUAD containing LY (5 mg/ml) for 1 h at the same temperature. The cells were then washed 3 times with ice-cold PBS containing 10 mM NaN_3, 10 mM NaF and visualized using fluorescence microscopy [29].

2.8. Fluorescence microscopy

Cells growing exponentially at 24 °C in YPUAD were harvested, washed once with PBS and the cell suspension was applied to a microscope slide. Fluorescence was visualized by fluorescence microscopy using a Leica DMRA2 microscope (Leica, Singapore) and images captured using a CoolSNAP^{HQ} camera (Roper Scientific, Trenton, NJ).

3. Results

3.1. An N-terminal Las17p fragment (N-Las17p$_{1-368}$) functionally replaces full-length Las17p in growth, actin cytoskeleton polarization and bulk endocytosis

It is not known which of the various Las17p domains (Fig. 1) is important for growth at elevated temperature. To determine the minimal sequences of Las17p sufficient to support growth at elevated temperature, we tested the ability of centromeric plasmids expressing truncated Las17p proteins (under the transcriptional control of the *LAS17* promoter) (Fig. 1) to restore growth at 37 °C to Las17p-deficient (*las17Δ*) cells (Fig. 2).

The results revealed that an N-terminal Las17p fragment (N-Las17p$_{1-368}$) that interacts with Vrp1p (Fig. 1) restores the viability of *las17Δ* cells at 37 °C on solid and in liquid media (Fig. 2a, B). This Las17p fragment was also able to restore fluid-phase endocytosis and a polarized distribution of actin patches to *las17Δ* cells at both 24 °C and 37 °C (Fig. 2c, 3a, b). That N-Las17p$_{1-368}$ is able to correct the actin cytoskeletal defects of *las17Δ* cells was further supported by the observation that the sensitivity of *las17Δ* cells to 1 M salt was corrected by expression of N-Las17p$_{1-368}$ (data not shown). Hence, the Vrp1p-interacting domain of Las17p is sufficient to functionally substitute for full-length Las17p *in vivo*.

In order to determine the localization of Las17p and N-Las17p$_{1-368}$, we fused DNA sequences encoding green fluorescent protein (GFP) to the 3′ end of sequences encoding either full-length Las17p or N-Las17p$_{1-368}$. Both Las17p-GFP and N-Las17p$_{1-368}$-GFP fusion proteins expressed under the transcriptional control of *LAS17* promoter rescued the growth defects of *las17Δ* cells (data not shown). Live

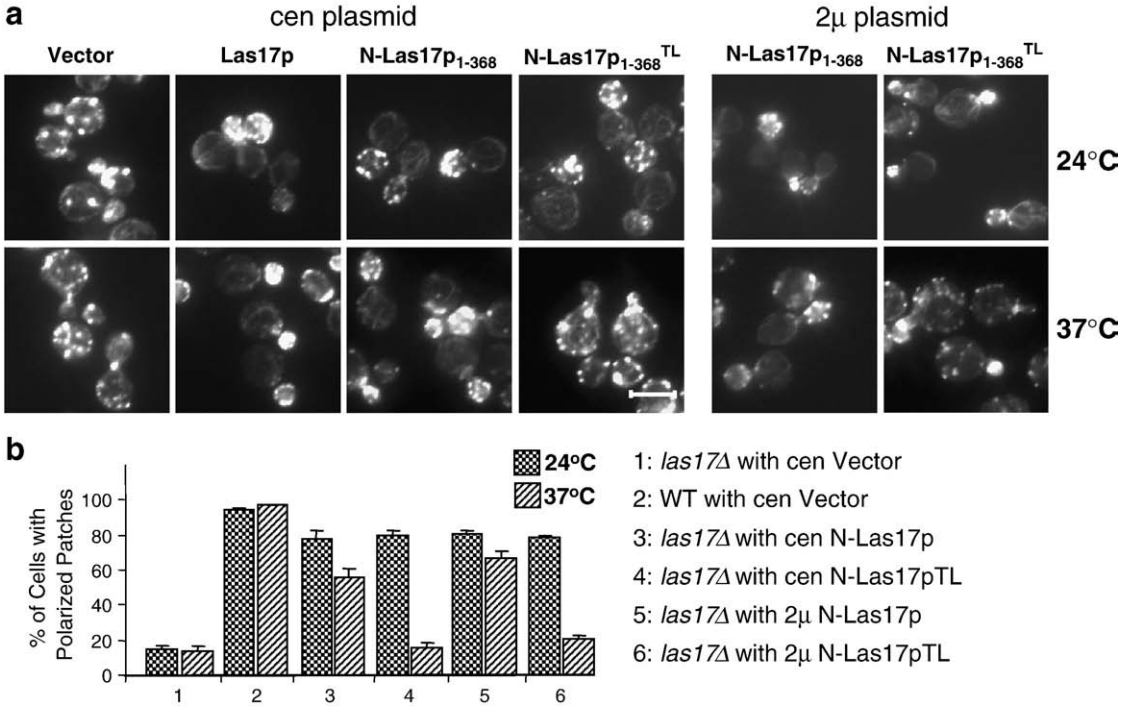


Fig. 3. N-Las17p$_{1-368}$ functionally substitutes for full-length Las17p in actin patch polarization. (a) *las17Δ* cells harboring empty vector, or *las17Δ* cells harboring CEN plasmids expressing Las17p, N-Las17p$_{1-368}$ or N-Las17p$^{TL}_{1-368}$ or 2 µm plasmids expressing either N-Las17p$_{1-368}$ or N-Las17p$^{TL}_{1-368}$ were grown in YPUAD and the actin patches at 24 °C and 37 °C were visualized as described in Materials and methods. Bar, 5 µm. (b) Wild type (WT) or *las17Δ* cells harboring vector alone or *las17Δ* cells harboring centromeric (CEN) plasmids expressing Las17p, N-Las17p$_{1-368}$ or N-Las17p$^{TL}_{1-368}$ or 2 µm plasmid expressing either N-Las17p$_{1-368}$ or N-Las17p$^{TL}_{1-368}$ were grown in YPUAD and the actin patches at 24 °C and 37 °C were visualized as described in Materials and methods. 100 small budded cells were analyzed and scored as polarized if the mother has less than 5 patches and scored as depolarized otherwise. The experiment was repeated three times with similar results.

cell imaging showed that, consistent with earlier reports, Las17p-GFP localized to cortical patches (Fig. 4a) and N-Las17p$_{1-368}$-GFP also localized to cortical patches in *las17Δ* cells at both 24 °C and 37 °C (Fig. 4a). In contrast, GFP alone showed a diffuse cytoplasmic distribution. In order to determine whether N-Las17p$_{1-368}$-GFP is localised to cortical actin patches, we expressed Arc40-RFP (Red Fluorescent Protein) in cells also expressing Las17p-GFP or N-Las17p$_{1-368}$-GFP. Arc40p is one subunit of Arp2/3 and is a known marker for cortical actin patches [30]. Both Las17p-GFP and N-Las17p$_{1-368}$-GFP patches were found to partially (~30%) co-localize with Arc40-RFP/cortical actin patches (Fig. 4b).

3.2. Isolation of a mutation in N-Las17p$_{1-368}$ that abolishes function in growth

In order to characterize the function of N-Las17p$_{1-368}$, at 37 °C, we sought mutations that compromised N-Las17p$_{1-368}$ function at 37 °C. We performed PCR mutagenesis on a DNA fragment encoding N-Las17p$_{1-368}$, expressed the mutated sequences in *las17Δ* cells, and screened for the loss of the ability to restore growth at 37 °C. We subsequently screened the mutants' ability to restore fluid-phase endocytosis and to correct the actin patch polarization defect of *las17Δ* cells at 24 °C to ensure that the mutation does not abolish function at 24 °C. We identified one mutant that fulfilled this criteria and it had two amino acid substitutions L80T and H94L, thus labeled as N-Las17p$^{TL}_{1-368}$. N-Las17p$_{1-368}$ with either single mutation (N-Las17p$^{T}_{1-368}$ or N-Las17p$^{L}_{1-368}$) was able to rescue the growth defects of *las17Δ* (data not shown), thus both mutations are required to compromise the activity of N-Las17p$_{1-368}$.

Although expression of N-Las17p$^{TL}_{1-368}$ does not rescue the growth defect of *las17Δ* cell, it did restore fluid-phase endocytosis and polarization of actin patches at 24 °C. However, neither of these defects was rescued at 37 °C (Figs. 2c, 3a, b). Western blot analysis was performed to assess the level of expression of the wild type and

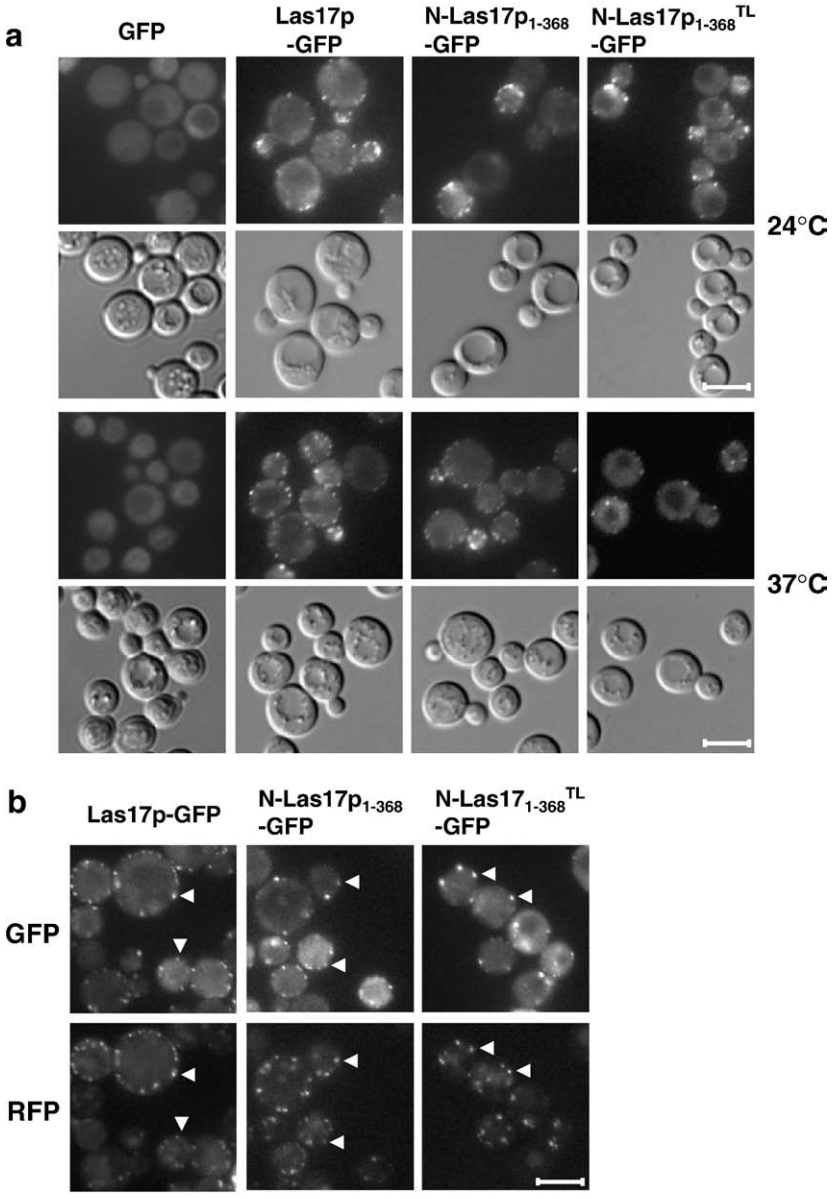


Fig. 4. N-Las17p$_{1-368}$ retains the ability to localize to cortical actin patches. (a) *las17Δ* cells expressing green fluorescent protein (GFP), Las17p-GFP, N-Las17p$_{1-368}$-GFP or N-Las17p$^{TL}_{1-368}$-GFP from CEN plasmids were grown in YPUAD to exponential phase at 24 °C and either left at 24 °C or shifted to 37 °C for 2 h. GFP was visualized in living cells by fluorescence microscopy. Upper panels: GFP. Lower panels: DIC optics Bar, 5 μm. (b) *las17Δ* cells co-expressing Arc40-RFP and either Las17p-GFP, N-Las17p$_{1-368}$-GFP or N-Las17p$^{TL}_{1-368}$-GFP were grown in YPUAD to exponential phase at 24 °C. Both GFP and RFP were visualized in living cells by fluorescence microscopy. Arrows point to patches where Arc40p-RFP and Las17p-GFP co-localize. Upper panels: GFP. Lower panels: RFP. Bar, 5 μm.

mutated N-Las17p$_{1-368}$ fragments *in vivo*. This analysis revealed that N-Las17p$^{TL}_{1-368}$ is expressed at levels comparable to that of wild type N-Las17p$_{1-368}$ at 24 °C, however upon shifting the cells to 37 °C for 2 h, N-Las17p$^{TL}_{1-368}$ became undetectable (Fig. 5b). N-Las17p$^{TL}_{1-368}$-GFP localized to cortical actin patches at both 24 °C and 37 °C (Fig. 4a, b). However there were fewer N-Las17p$^{TL}_{1-368}$-GFP patches at 37 °C compared to 24 °C, suggesting that the loss of activity at 37 °C could be due to reduced level of the mutant protein. In order to test whether the loss of activity at 37 °C is due to poor expression of the mutant protein at 37 °C we expressed both N-Las17p$_{1-368}$ and N-Las17p$^{TL}_{1-368}$ from 2 μm (high-copy number) plasmids. Under these conditions N-Las17p$^{TL}_{1-368}$ levels approached those of N-Las17p$_{1-368}$ even after 2 h at 37 °C (Fig. 5b). Expression of N-Las17p$^{TL}_{1-368}$ from a 2 μm plasmid rescued the growth defect of *las17Δ* at 37 °C (Fig. 5a) without correcting the actin patch polarization defect at 37 °C (Fig. 3a, b). Over-expression of N-Las17p$^{TL}_{1-368}$ from a 2 μm plasmid does not perturb the actin cytoskeleton in wild type yeast cells (Fig. S1a, b). Hence, while loss of function (growth and endocytosis) of N-Las17p$^{TL}_{1-368}$ at 37 °C when expressed from CEN plasmid may be due to reduced protein level (as evidenced by rescue when the mutated fragment is over-expressed from a 2 μm plasmid) such reduced expression cannot account for the observed loss of actin patch polarization at 37 °C.

3.3. L80T H94L mutations in N-Las17p$_{1-368}$ affect binding to Vrp1p

The reduced expression of N-Las17p$^{TL}_{1-368}$ at 37 °C may be due to protein instability caused by loss of interaction with other key cytoskeletal regulators. Las17p has been shown to interact with many cytoskeletal proteins including actin, Vrp1p, Bzz1p, Myo3p, Myo5p and Rvs167p [11, 13, 15, 31–33]. Residues 20–122 of Las17p mediate interaction with Vrp1p [13]. Since the L80T H94L mutations fall in this region of the protein, we first examined the interaction of N-Las17p$_{1-368}$ and N-Las17p$^{TL}_{1-368}$ (each tagged with GFP) with the C-terminal Las17p binding domain of Vrp1p (C-Vrp1p$_{364-817}$) (tagged with 6His) *in vivo* using co-precipitation at 24 °C (Fig. 6a). While N-Las17p$_{1-368}$-GFP interacted with C-Vrp1p$_{364-817}$-6His, N-Las17p$^{TL}_{1-368}$ did not interact with C-Vrp1p$_{364-817}$-6His (Fig. 6a). We were unable to determine the interaction at 37 °C due to degradation of the mutant proteins at 37 °C (data not shown). Interaction of N-Las17p$_{1-368}$ with C-Vrp1p$_{364-817}$ was also tested in a yeast two-hybrid assay and the L80T H94L mutations in N-Las17p$_{1-368}$ abolished interaction with C-Vrp1p$_{364-817}$ (data not shown). We have previously shown that C-Vrp1p$_{364-817}$-GFP does not localize to cortical patches in *las17Δ* strain [21]. We used this observation to test the interaction between C-Vrp1p$_{364-817}$-GFP and N-Las17p$_{1-368}$ in *las17Δ* strain. C-Vrp1p$_{364-817}$-GFP expressed from a CEN plasmid localized to cortical patches at both 24 °C and 37 °C in the presence of N-Las17p$_{1-368}$ expressed from either CEN (data not shown) or 2 μm plasmid (Fig. S2) but not in the presence of N-Las17p$^{TL}_{1-368}$ expressed from CEN (data not shown) or 2 μm plasmid at both 24 °C and 37 °C (Fig. S2). Together, these data suggest that the interaction between N-Las17p$_{1-368}$ and C-Vrp1p$_{364-817}$ is abolished by the L80T H94L mutations. The L80T H94L mutations did not perturb interaction of N-Las17p$_{1-368}$ with a panel of other actin cytoskeletal proteins including Hof1p, Bzz1p and Rvs167p based on yeast two-hybrid assay (data not shown). This suggests that N-Las17p$^{TL}_{1-368}$ loss of function and reduced state level is most likely due to specific loss of interaction with Vrp1p.

3.4. Vrp1p promotes the formation of a Las17p/Vrp1p/Myo5p complex

Type I myosins, Myo3p and Myo5p can interact with Las17p via direct binding of their SH3 domains with proline-rich motifs in Las17p or indirectly via other proteins [13, 15]. Therefore, we examined the interaction of N-Las17p$_{1-368}$-GFP and N-Las17p$^{TL}_{1-368}$-GFP with the SH3 domain of Myo5p (a.a. 1085–1219) (tagged with

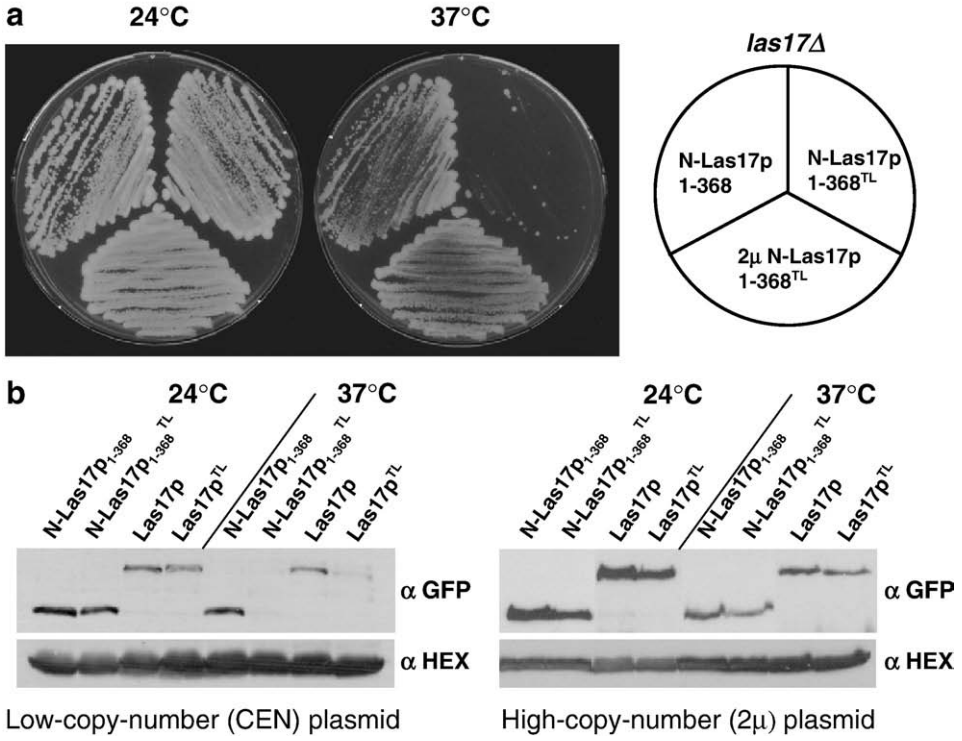
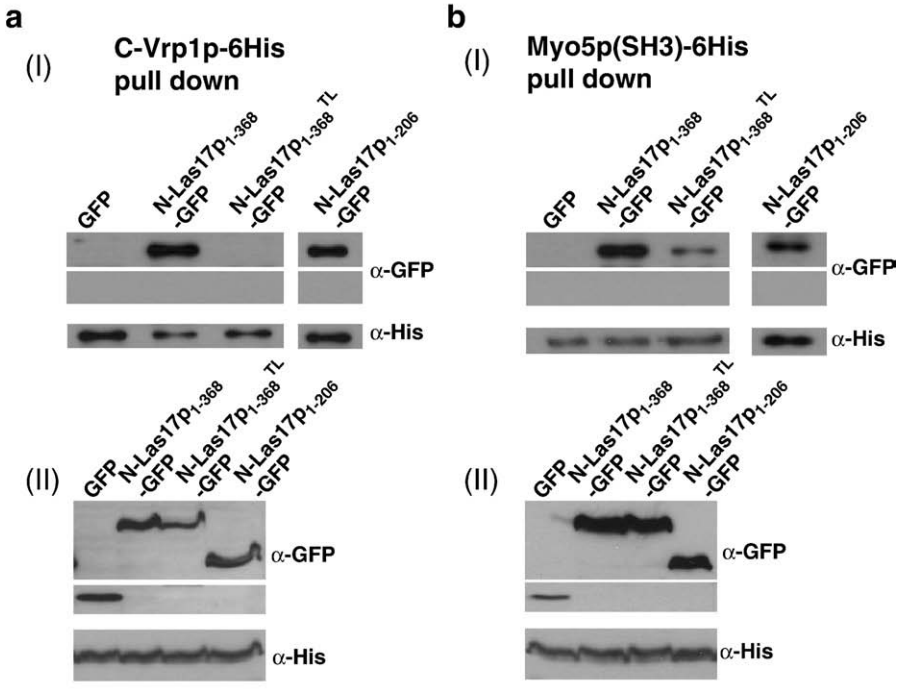


Fig. 5. Loss of function in growth due to the L80T H94L mutations is due to reduced steady-state protein expression. (a) Growth at 24 °C and 37 °C of *las17Δ* cells harboring centromeric (CEN) plasmids expressing N-Las17p$_{1-368}$ or N-Las17p$^{TL}_{1-368}$ or a 2 μm plasmid expressing N-Las17p$^{TL}_{1-368}$. Each strain was streaked for single colonies on YPUAD agar, incubated at either 24 °C or 37 °C, and photographed after 3 days. (b) Steady-state expression levels of wild type and mutant Las17p proteins. *las17Δ* cells harboring plasmids expressing N-Las17p$_{1-368}$-GFP, N-Las17p$^{TL}_{1-368}$-GFP, Las17p-GFP or Las17p^{TL}-GFP from low (left, CEN plasmid) or high (right, 2 μm plasmid) copy number plasmids were grown in YPUAD to exponential phase at 24 °C and either left at 24 °C or shifted to 37 °C for 2 h. Protein extract was isolated and analyzed using anti-GFP (α-GFP) and anti-hexokinase (α-Hex) serum as described in Materials and methods.



c Yeast two-hybrid protein interaction assay

pAD	pBD		
	C-Vrp1p	Myo5p(SH3)	Vector
N-Las17p$_{1-206}$	+	-	-
N-Las17p$_{207-368}$	-	+	-
Vector	-	-	-

Fig. 6. The L80T and H94L mutations in Las17p abolish interaction with Vrp1p. The Las17p-GFP fusion proteins and Vrp1p-6His-tagged proteins (as indicated below) were co-expressed from the endogenous promoters on low-copy number (CEN) plasmids in either *vrp1Δ* cells (a) or *las17Δ* cells (b) and the 6His-tag was used to pull down the 6His-tagged protein and its associated proteins from total cell lysates at 24 °C with beads as described in Materials and methods. Bound proteins were eluted from the beads and resolved by SDS-PAGE and analyzed by Western blot using anti-GFP (α-GFP) serum and anti-His (α-His) serum. (a) Analysis of Vrp1p–Las17p interaction by pull-down assays. *vrp1Δ* cells expressing C-Vrp1p$_{364-817}$-6His in combination with either GFP only, N-Las17p$_{1-368}$-GFP, N-Las17p$^{TL}_{1-368}$-GFP or N-Las17p$_{1-206}$-GFP. (I) Western blot of the proteins eluted from beads following incubation of beads with cleared lysate. (II) Western blot of total cleared lysate before incubation with beads. (b) Analysis of Las17p–Myo5p interactions by pull-down assays. *las17Δ* cells expressing Myo5p(SH3)-6His in combination with either GFP only, N-Las17p$_{1-368}$-GFP, N-Las17p$^{TL}_{1-368}$-GFP or N-Las17p$_{1-206}$-GFP. (I) Western blot of the proteins eluted from Ni^{2+}-NTA-Agarose beads following incubation of beads with cleared lysate. (II) Western blot of total lysate cleared before incubation with beads. (c) Yeast two-hybrid analysis of Vrp1p–Las17p and Las17p–Myo5p interactions. pACT2 (activation domain) plasmids expressing N-Las17p$_{1-206}$, N-Las17p$_{207-368}$ or empty vector were tested for two-hybrid interaction with pBD (DNA binding domain) plasmids expressing C-Vrp1p$_{364-817}$ (C-Vrp1p) or Myo5p(SH3) or empty vector in two-hybrid reporter strain PJ69-4A. Two-hybrid interaction activates expression of the *HIS3* reporter gene and was scored by growth on selective media lacking histidine (+2 mM 3-aminotriazole).

6His). Both N-Las17p$_{1-368}$-GFP and N-Las17p$^{TL}_{1-368}$-GFP were pulled down from yeast cell lysates with Myo5p-SH3-6His (Fig. 6b). However, the N-Las17p$_{1-368}$-GFP band was more intense than the N-Las17p$^{TL}_{1-368}$-GFP band, suggesting that more molecules of N-Las17p$_{1-368}$-GFP are in complex with the Myo5p-SH3 domain than the mutant N-Las17p$^{TL}_{1-368}$-GFP. This could be due to association of N-Las17p$_{1-368}$-GFP with Vrp1p as Vrp1p has multiple predicted Myo5p SH3 binding sites [15]. In order to test whether direct interaction with Myo5p was affected by the L80T H94L mutations, we made two constructs, N-Las17p$_{1-206}$-GFP which includes the Vrp1p binding region but lacks the Myo5p-SH3 binding motifs and N-Las17p$_{207-368}$-GFP which conversely includes the region rich in Myo5p-SH3 binding motifs but lacks the Vrp1p binding region. The interaction of N-Las17p$_{1-206}$ (tagged with GFP) with C-Vrp1p$_{364-817}$ or Myo5p-SH3 (each tagged with 6His) *in vivo* was examined in a pull-down assay (Fig. 6a, b). N-Las17p$_{1-206}$-GFP interacted strongly with both C-Vrp1p$_{364-817}$ and Myo5p-SH3 in the pull-down assay consistent with the possibility that N-Las17p$_{1-206}$ pulls down with Myo5p-SH3 due to formation of a complex with Vrp1p and that the N-Las17p$_{1-206}$–Myo5p-SH3 interaction is indirect and mediated by Vrp1p. The N-Las17p$_{207-368}$-GFP was very unstable thus we could not carry out the pull-down assay (data not shown). The interactions of N-Las17p$_{1-206}$ and N-Las17p$_{207-368}$ with C-Vrp1p$_{364-817}$ and Myo5p-SH3, was also examined in a yeast two-hybrid assay. The yeast two-hybrid assay showed that N-Las17p$_{207-368}$ interacts with Myo5p-SH3 but not C-Vrp1p$_{364-817}$ (Fig. 6c). Conversely, N-Las17p$_{1-206}$ interacts with C-Vrp1p$_{364-817}$, but not Myo5p-SH3 (Fig. 6c). Thus, the Las17p region affected by the L80T H94L mutations appears to specifically mediate binding to Vrp1p.

3.5. The L80T H94L mutations do not abolish the function of full-length Las17p

We next analyzed how the L80T H94L mutations affect the function of full-length Las17p which has the Arp2/3 activating WA domains. Expression of Las17p^{TL} in *las17Δ* cells restored fluid-phase endocytosis at both 24 °C and 37 °C and also corrected the growth defects at 37 °C (Fig. 7a–c). Las17p^{TL} was also able to restore actin

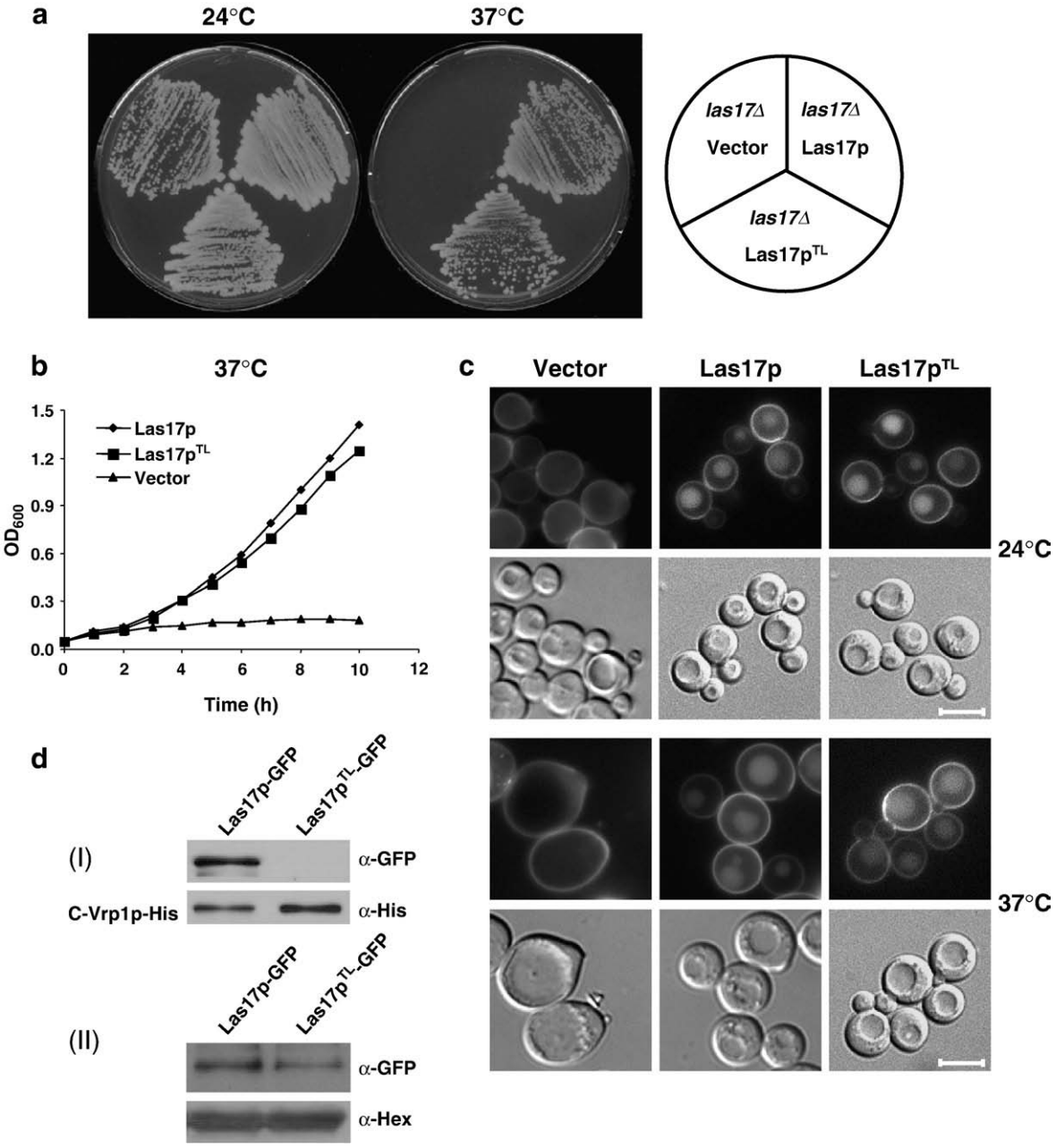


Fig. 7. Vrp1p–Las17p interaction is functionally redundant with Las17p–Arp2/3 interaction for growth at elevated temperature and endocytosis. (a) The Las17p L80T H94L mutations do not affect growth or colony formation on solid media. Growth at 24 °C and 37 °C of *las17Δ* cells expressing Las17p or Las17p^{TL} from the endogenous *LAS17* promoter on low-copy-number (CEN) plasmids or harboring empty vector. Each strain was streaked for single colonies on YPUAD agar, incubated at 24 °C or 37 °C and photographed after 3 days. (b) The Las17p L80T H94L mutations do not affect doubling time in liquid culture. Growth curve of *las17Δ* cells harboring low-copy-number (CEN) plasmids expressing Las17p or Las17p^{TL} from *LAS17* promoter or empty vector. (c) The L80T H94L mutations do not abolish the endocytic function of full-length Las17p. *las17Δ* cells expressing wild type Las17p or mutant Las17p^{TL} from the *LAS17* promoter on low-copy-number (CEN) plasmids or harboring empty vector were grown in YPUAD were assayed for LY uptake as described in Materials and methods. Upper panels: LY. Lower panels: DIC optics. Bar, 5 μm. (d) The L80T and H94L mutations in Las17p abolish interaction with Vrp1p. Total (cleared) extracts from *las17Δ* cells co-expressing C-Vrp1p$_{364–817}$-6His from the *VRP1* promoter and either expressing Las17p-GFP or Las17p^{TL}-GFP from the *LAS17* promoter on low-copy-number (CEN) plasmids were incubated with Ni^{2+}-NTA agarose beads, processed and analysed as described in Fig. 6a. (I) Western blot of the proteins eluted from beads following incubation of beads with cleared lysate. (II) Western blot of total (cleared) lysate before incubation with beads.

patch polarization to *las17Δ* cells at 24 °C (Fig. 8a, b). Las17p^{TL} was not able to fully restore actin patch polarization at 37 °C (Fig. 8a, b).

We analyzed the localization and expression of Las17p^{TL} using Las17p^{TL}-GFP. The presence of the L80T H94L mutations did not affect the localization of Las17p^{TL}-GFP to cortical patches in *las17Δ* cells at 24 °C or 37 °C (Fig. S3). Western blotting of cell extracts from *las17Δ* cells expressing Las17p-GFP or Las17p^{TL}-GFP showed that the expression of Las17p^{TL} was similar to that of Las17p at 24 °C but the expression of Las17p^{TL} at 37 °C was reduced compared to Las17p (Fig. 5b). Thus we also analyzed the ability of Las17p^{TL} over-expressed from a 2 μm plasmid to rescue actin patch polarization at 37 °C (Fig. 8a, b). The Las17p^{TL} expressed from 2 μm plasmid did not rescue the actin patch defect at 37 °C. Hence the L80T H94L mutations abolish actin patch polarization at 37 °C but not at 24 °C and do not abolish growth at 37 °C. Over-expression of Las17p and Las17p^{TL} does not perturb the actin cytoskeleton in wild type yeast cells (Fig. S1a, b).

We next examined if these mutations in Las17p^{TL} affect Las17p interaction with Vrp1p. This was assessed by testing the binding of Las17p or Las17p^{TL} (each tagged with GFP) in yeast cell lysate with C-Vrp1p$_{364–817}$ (tagged with 6His) in a pull-down assay. Las17p-GFP

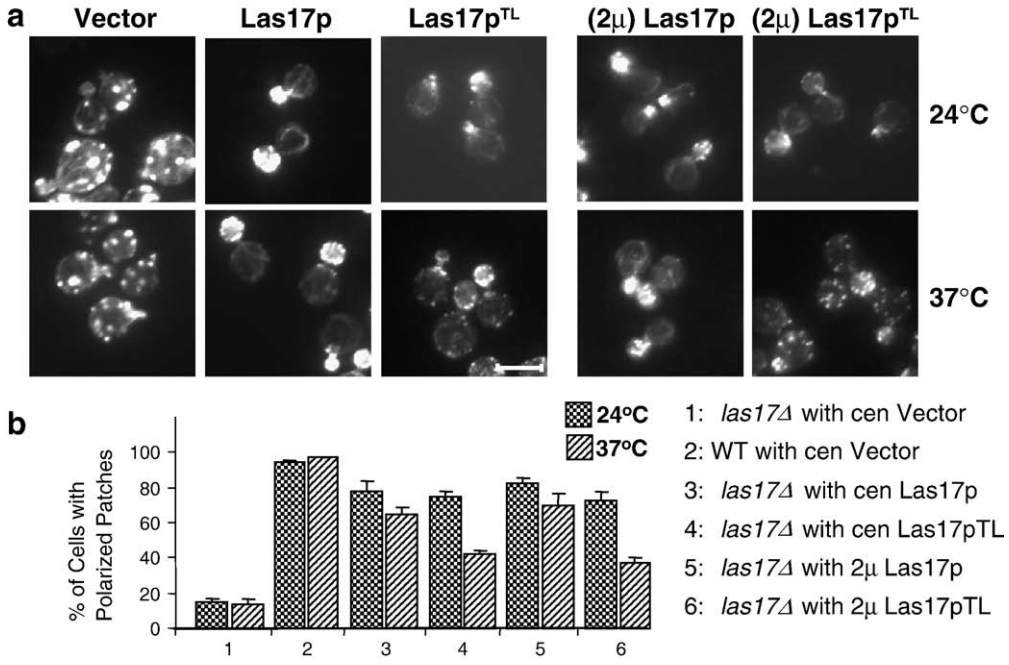


Fig. 8. Vrp1p–Las17p interaction is critical for actin patch polarization at 37 °C. (a) The Las17p L80T H94L mutations perturb actin patch polarization only at 37 °C. *las17Δ* cells expressing Las17p or Las17p^{TL} from the *LAS17* promoter from either low-copy-number (CEN) plasmids or a 2 μm plasmid or a harboring empty vector were grown in YPUAD to exponential phase at 24 °C and either left at 24 °C (upper panel) or shifted to 37 °C for 2 h (lower panel). Bar, 5 μm. (b) Wild type (WT) or *las17Δ* cells harboring vector alone or *las17Δ* cells expressing Las17p or Las17p^{TL} from the *LAS17* promoter from either low-copy-number (CEN) plasmids or a 2 μm plasmid were grown in YPUAD to exponential phase at 24 °C and either left at 24 °C or shifted to 37 °C for 2 h and analysed as described in Fig. 3b.

interacts with C-Vrp1p$_{364–817}$, but the L80T H94L mutations abolished this interaction (Fig. 7d). Loss of interaction of Las17p with Vrp1p maybe a possible reason why the L80T H94L mutations result in lowered Las17p stability and abolish actin patch polarization at 37 °C. Thus, the binding of Las17p to Vrp1p is not necessary for function at 24 °C but becomes essential at 37 °C for protein stability and actin patch polarization.

In order to address the role of the proline sequences (a.a. 207–368) in N-Las17p we expressed the N-Las17p$_{1–206}$ in *las17Δ* cells under the transcriptional regulation of *LAS17* promoter from a cen plasmid. The results revealed that an N-terminal Las17p fragment (N-Las17p$_{1–206}$) that interacts with Vrp1p but lacks both the proline-rich sequences and WA domain required for interaction with Arp2/3 (Fig. 1) restores the viability of *las17Δ* cells at 37 °C (Fig. 9a). This Las17p fragment was also able to localize to cortical patches (Fig. S3), restore fluid-phase endocytosis and a polarized distribution of actin patches to *las17Δ* cells at 24 °C but not 37 °C (Fig. 9b, c).

4. Discussion

In this study, we have shown that expression of an N-terminal fragment of Las17p that interacts with Vrp1p but not Arp2/3 (N-Las17p$_{1–368}$) is sufficient to restore fluid-phase endocytosis, actin patch polarization and growth at 37 °C to *las17Δ* cells. We screened for and isolated an N-Las17p$_{1–368}$ mutant, (N-Las17p$^{TL}_{1–368}$) which abolished the interaction between Las17p and Vrp1p. N-Las17p$_{1–368}$ with the mutation (N-Las17p$^{TL}_{1–368}$) retains the ability to rescue the fluid-phase endocytosis and actin patch polarization defects of *las17Δ* cells at 24 °C, but loses this ability at 37 °C. Expression of N-Las17p$^{TL}_{1–368}$ using 2 μm plasmid rescued the growth and fluid-phase endocytosis defect of *las17Δ* at 37 °C but not the actin patch polarization defect of *las17Δ* at 37 °C. Similarly the mutation abolished the ability of full-length Las17p to rescue the actin patch polarization defect at 37 °C even when expressed from a 2 μm plasmid. Thus Las17p–Vrp1p interaction is not critical for growth at elevated temperature or fluid-phase endocytosis or actin patch polarization at 24 °C. However Las17p–Vrp1p interaction is essential for polarization of actin patches at 37 °C.

The amino acid residues L80 and H94 mutated in N-Las17p$^{TL}_{1–368}$ are located within the WH1 domain of Las17p (Fig. 1). These amino acid residues are conserved in the WH1 domain of mammalian WASP, the corresponding residues in human WASP being L101 and H115 which lies in the region of WASP 101–150 which mediates interaction with WIP [17]. Mutation of H115 to Y in human WASP leads to a severe form of Wiskott–Aldrich Syndrome [34]. Several of the mutations in WASP which cause the disease have been shown to abolish WASP–WIP interaction [17], suggesting that mutations in the WH1 domain of WASP affect function by abolishing the interaction with WIP, the human homologue of yeast Vrp1p. Consistent with an important role for WASP–WIP interaction we have previously found that expression of human haematopoietic WASP is able to correct the growth defects of *las17Δ* cells if human WIP is co-expressed. Mutations in human WASP that abolish WASP–WIP interaction also abolished the ability of human WASP to correct growth defects of *las17Δ* cells in the presence of human WIP [35].

How does N-Las17p$_{1–368}$ without the Arp2/3 activating domain (WA) rescue all the defects of *las17Δ* cells? This may be because N-Las17p$_{1–368}$ is present in a complex with other proteins that have an Arp2/3 activating domain of their own (e.g. type I myosins, Myo3 and Myo5). Las17p has 4 predicted type I myosin SH3 domain binding motifs compared to 17 such motifs in Vrp1p [15]. Both N-Las17p$_{1–368}$ and N-Las17p$^{TL}_{1–368}$ form complexes with Myo5p, the wild type N-Las17p$_{1–368}$ was found to be in a complex with more molecules of Myo5p than the mutant N-Las17p$^{TL}_{1–368}$ (Fig. 6) consistent with the ability of N-Las17p$_{1–368}$ but not N-Las17p$^{TL}_{1–368}$ to interact with Vrp1p. Thus, N-Las17p$^{TL}_{1–368}$ forms a complex with Myo5p only through direct binding. In contrast, the wild type N-Las17p$_{1–368}$ can form a complex with Myo5p both through direct binding as well as indirectly through its interaction with Vrp1p. The increased amount of type I myosin in the N-Las17p$_{1–368}$ complex may enhance N-Las17p$_{1–368}$ stability and its ability to rescue the growth defects as well as bulk endocytosis and actin patch polarization at 37 °C compared to N-Las17p$^{TL}_{1–368}$. We

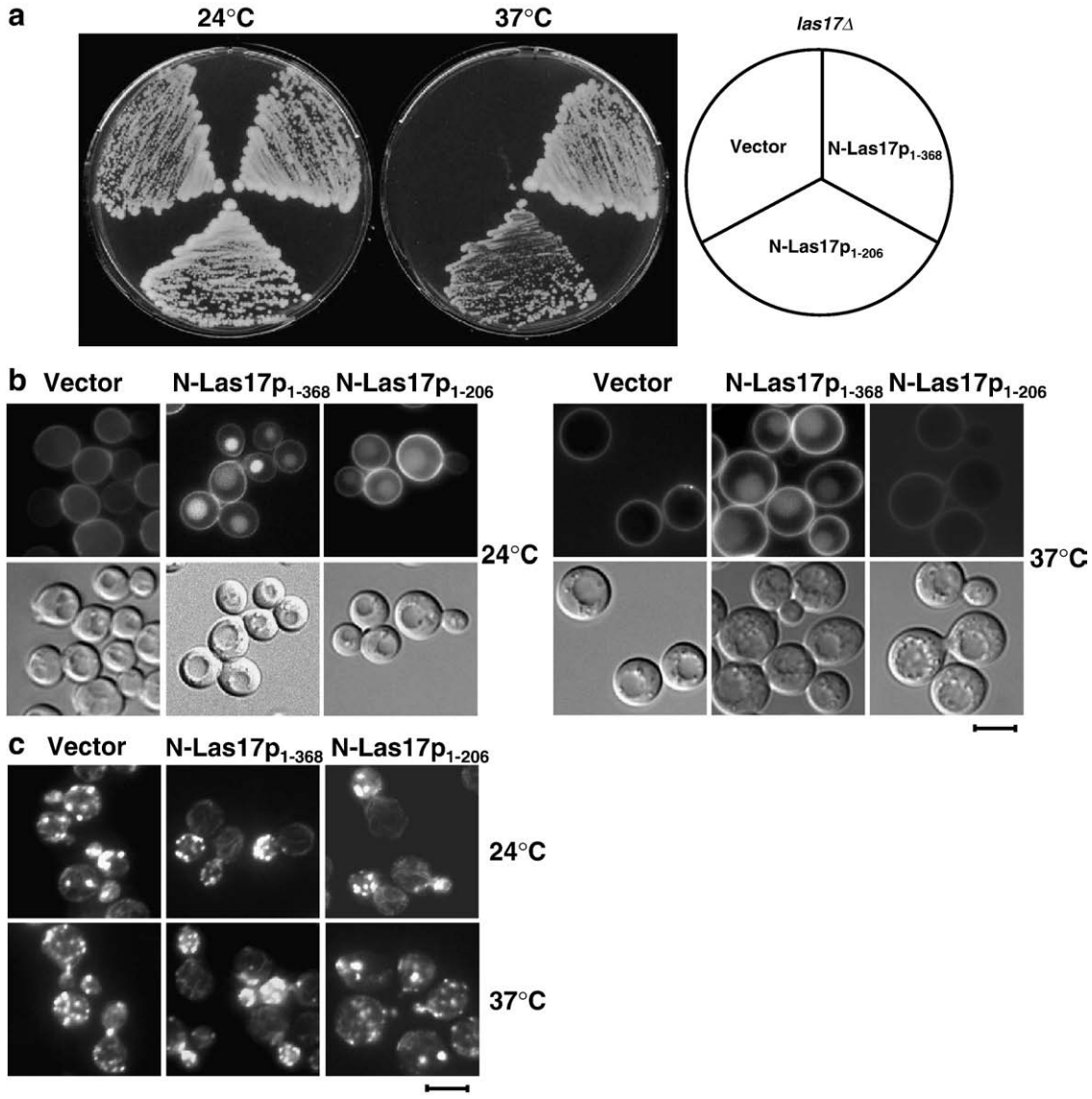


Fig. 9. N-Las17p$_{1-206}$ functionally substitutes for full-length Las17p in growth at 37 °C; fluid-phase endocytosis and actin patch polarization at 24 °C. (a) Growth at 24 °C and 37 °C of *las17Δ* cells harboring empty vector or CEN plasmids expressing N-Las17p$_{1-368}$ or N-Las17p$_{1-206}$. Each strain was streaked for single colonies on YPUAD agar, incubated at either 24 °C or 37 °C, and photographed after 3 days. (b) *las17Δ* cells harboring empty vector, or CEN plasmids expressing N-Las17p$_{1-368}$ or N-Las17p$_{1-206}$ were grown in YPUAD were used to carry out LY uptake assay as described in Materials and methods. Upper panels: FITC-fluorescence optics to visualize LY. Lower panels: DIC optics to visualize cell profiles. Bar, 5 μm. (c) *las17Δ* cells harboring empty vector, or CEN plasmids expressing N-Las17p$_{1-368}$ or N-Las17p$_{1-206}$ were grown in YPUAD and the actin patches at 24 °C and 37 °C were visualized as described in Materials and methods. Bar, 5 μm.

tested the possibility that N-Las17p$_{1-368}$ rescues the defects of *las17Δ* cells by stabilizing Vrp1p protein levels but could not detect any enhanced stability of Vrp1p-GFP in the presence of N-Las17p$_{1-368}$ compared to N-Las17p$^{TL}_{1-368}$ even at 37 °C (data not shown). Hence, we believe that it is the physical interaction itself that is important for Las17p, Vrp1p and type I myosin function.

The differential requirement for Vrp1p interaction at 37 °C compared to 24 °C suggests that a complex containing N-Las17p$_{1-368}$ and type I myosin may have enough NPF activity to restore bulk endocytosis and actin patch polarization at 24 °C but to perform these functions at 37 °C, the complex needs to also contain Vrp1p. The proline-rich sequences between 207–368 in Las17p are essential for actin patch polarization and endocytosis at 37 °C. Inclusion of Vrp1p in the complex is predicted to increase the amount of type I myosins as well as provide an additional WH2 domain and two other actin binding domains to supply assembly-competent actin monomers [21, 22] for nucleation of filament assembly by the Arp2/3. While advantageous even at 24 °C, the extra NPF and actin-monomer-binding activities may become necessary for function at 37 °C. The reduced steady-state level of Las17p mutant proteins unable to bind Vrp1p at 37 °C suggests that Vrp1p binding to the WH1 domain of Las17p may stabilize Las17p in *S. cerevisiae*. Consistent with this, WIP binding to the WH1 domain of WASP stabilizes WASP in mammalian cells as well as in *S. cerevisiae* [35, 36]. Vrp1p may act as chaperone for Las17p and this role becomes essential under the stress of high temperature.

Our results highlight the functional conservation of the WASP–WIP complex in humans and *S. cerevisiae* (Las17p–Vrp1p). In both organisms the WIP (WIP or Vrp1p) stabilizes the respective WASP (WASP or Las17p). In yeast Las17p has built in redundancy such that loss of the WA domain does not lead to severe phenotypes probably due to complex formation with type I myosins. In mammals the WASP–WIP complex includes cortactin, a protein with an N-terminal acidic domain and C-terminal SH3 domain but without a WH2 domain [37,38]. Thus the interaction of cortactin with the WASP–WIP complex in humans may play a similar role to myosin I interaction with the

Las17p–Vrp1p complex in *S. cerevisiae*. In humans mutations that abolish WASP–WIP interaction lead to Wiskott–Aldrich Syndrome, a disease with cytoskeletal abnormalities analogous to those that result from loss of Las17p–Vrp1p interaction in *S. cerevisiae*. This suggests that the interaction between WASP and WIP is crucial for WASP functions and/or stability in yeast and humans.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbamcr.2009.02.012.

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