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

Rajamuthiah Rajmohan¹, Ming Hwa Wong¹, Lei Meng¹, Alan L. Munn² and Thirumaran Thanabal¹*

¹. School of Biological Sciences, Nanyang Technological University, 60 Nanyang Drive, Singapore 637551, Republic of Singapore.
². School of Medical Science, Griffith University (Gold Coast Campus), Parklands Dr., Southport, Queensland 4222, Australia

Running title: Las17p interaction with Vrp1p and Arp2/3

* Corresponding author
E-mail address: thirumaran@ntu.edu.sg
Tel: 65-6316 2832
FAX: 65-6791 3856
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-Las17p1-368) 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.

Keywords: Actin filament, cortical actin patch, membrane traffic, Proline-rich motif, SH3 domain, type I myosin

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.
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 filaments 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].

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, Eg. 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 corticals 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 loss of Arp2/3 activation per se.

We show here that an N-terminal Las17p fragment comprising residues 1-368 (N-Las17p1-368) 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-Las17p1-368 that perturb Las17p-Vrp1p interaction and show they reduce the ability of N-Las17p1-368 and full-length Las17p to function at elevated temperature.
Materials and Methods

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 (MATa his3 leu2 ura3 trp1 mfa2Δ::FUS1::lacZ) and IDY223 (MATa his3 leu2 ura3 trp1 las17Δ::LEU2) have been described in Naqvi et al., [11]. AMY88 (MATa his4 leu2 ura3 lys2 vrp1Δ::KanMx bar1) has been described in Thanabalu et al., [21]. Yeast strain PJ69-4A (MATa his3 leu2 ura3 trp1 gal4Δ gal80Δ met2::GAL7-lacZ GAL2-ADE2 LYS2::GAL1-HIS3) [25] was used to test yeast two-hybrid interactions.

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 aa 368). It was used for constructing sub-clones and the mutant pN-LAS17TL construct by PCR mutagenesis (Fig. 1(a)).

Yeast two-hybrid assay - DNA encoding N-Las17p1-206 and N-Las17p207-368 were cloned in-frame with GAL4AD in vector pACT2 (activation domain, AD) (Clontech, Palo Alto, CA) DNA encoding C-Vrp1p364-817 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 + 2mM 3-AT to detect the expression of the \textit{HIS3} interaction reporter.

\textit{In vivo protein binding assays} – C-Vrp1p\textsubscript{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, Bartlesville, OK). The cell extract was clarified by high-speed centrifugation and the supernatant incubated with Ni\textsuperscript{2+}-NTA agarose beads for 1 hr at 24°C. The beads were subsequently washed twice with 1% Triton X-100 in PBS and twice with 10mM 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.

\textit{Western Blot} - Cell pellets (7 OD\textsubscript{600} unit each) were resuspended in 240 µl (1.85N NaOH/1.06 M β-mercaptoethanol) and incubated on ice for 10 min. The protein was precipitated by addition of an equal volume of 20% TCA (TriChloro Acetic Acid) and incubation on ice for 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 chemiluminence kit from Pierce.
(Rockford, Illinois). Bands were visualized by fluorography using X-ray film (Kodak Company, Rochester, NY).

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 hours before fixing. Fixed cells were washed once with PBS before being permeabilised using 1% Triton X-100 in PBS. The permeabilised cells were stained with Alexa-488-conjugated phalloidin (0.3 units) and analyzed by fluorescence microscopy [28].

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 hr before being incubated in fresh YPUAD containing LY (5mg/ml) for 1 hr at the same temperature. The cells were then washed 3 times with ice-cold PBS containing 10mM NaN₃, 10 mM NaF and visualized using fluorescence microscopy [29].

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).
Results

An N-terminal Las17p fragment (N-Las17p1-368) 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 \textit{LAS17} promoter) (Fig. 1A) to restore growth at 37°C to Las17p-deficient (\textit{las17}Δ) cells (Fig 1B, C).

The results revealed that an N-terminal Las17p fragment (N-Las17p1-368) that interacts with Vrp1p (Fig. 1) restores the viability of \textit{las17}Δ 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 \textit{las17}Δ cells at both 24°C and 37°C (Fig. 2C, 3A, B). That N-Las17p1-368 is able to correct the actin cytoskeletal defects of \textit{las17}Δ cells was further supported by the observation that the sensitivity of \textit{las17}Δ cells to 1M salt was corrected by expression of N-Las17p1-368 (data not shown). Hence, the Vrp1p-interacting domain of Las17p is sufficient to functionally substitute for full-length Las17p \textit{in vivo}.

In order to determine the localization of Las17p and N-Las17p1-368, we fused DNA sequences encoding green fluorescent protein (GFP) to the 3’end of sequences encoding either full-length Las17p or N-Las17p1-368. Both Las17p-GFP and N-Las17p1-368-GFP fusion proteins expressed under the transcriptional control of \textit{LAS17} promoter rescued the growth defects of \textit{las17}Δ cells (data not shown). Live cell
imaging showed that, consistent with earlier reports, Las17p-GFP localized to cortical patches (Fig. 4A) and N-Las17p1-368-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-Las17p1-368-GFP is localised to cortical actin patches, we expressed Arc40-RFP (Red Fluorescent Protein) in cells also expressing Las17p-GFP or N-Las17p1-368-GFP. Arc40p is one subunit of Arp2/3 and is a known marker for cortical actin patches [30]. Both Las17p-GFP and N-Las17p1-368-GFP patches were found to partially (~30%) co-localize with Arc40-RFP/cortical actin patches (Fig. 4B).

**Isolation of a mutation in N-Las17p1-368 that abolishes function in growth**

In order to characterize the function of N-Las17p1-368, at 37°C, we sought mutations that compromised N-Las17p1-368 function at 37°C. We performed PCR mutagenesis on a DNA fragment encoding N-Las17p1-368, expressed the mutated sequences in las17Δ cells, and screened for loss of 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-Las17p1-368TL. N-Las17p1-368 with either single mutation (N-Las17p1-368T or N-Las17p1-368L) was able to rescue the growth defects of las17Δ (data not shown), thus both mutations are required to compromise the activity of N-Las17p1-368.

Although expression of N-Las17p1-368TL 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 the se defects was rescued at 37°C (Fig. 2C, 3A, B). Western blot analysis was performed to assess the level of expression of the wild type and mutated N-Las17p1-368 fragments in vivo. This analysis revealed that N-Las17p1-368TL is expressed at levels comparable to that of wild type N-Las17p1-368 at 24°C, however upon shifting the cells to 37°C for 2 hrs, N-Las17p1-368TL became undetectable (Fig. 5B). N-Las17p1-368TL-GFP localized to cortical actin patches at both 24°C and 37°C (Fig. 4A, B). However there were fewer N-Las17p1-368TL-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-Las17p1-368 and N-Las17p1-368TL from 2µ (high-copy number) plasmids. Under these conditions N-Las17p1-368TL levels approached those of N-Las17p1-368 even after 2hr at 37°C (Fig. 5B). Expression of N-Las17p1-368TL from a 2µ 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-Las17p1-368TL from a 2µ 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-Las17p1-368TL 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 2µ plasmid) such reduced expression cannot account for the observed loss of actin patch polarization at 37°C.

**L80T H94L Mutations in N-Las17p1-368 affect binding to Vrp1p**

The reduced expression of N-Las17p1-368TL 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-Las17p1-368 and N-Las17p1-368 TL (each tagged with GFP) with the C-terminal Las17p binding domain of Vrp1p (C-Vrp1p364-817) (tagged with 6His) in vivo using co-precipitation at 24°C (Fig. 6A). While N-Las17p1-368-GFP interacted with C-Vrp1p364-817-6His, N-Las17p1-368 TL did not interact with C-Vrp1p364-817-6His (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-Las17p1-368 with C-Vrp1p364-817 was also tested in a yeast two-hybrid assay and the L80T H94L mutations in N-Las17p1-368 abolished interaction with C-Vrp1p364-817 (data not shown). We have previously shown that C-Vrp1p364-817-GFP does not localize to cortical patches in las17Δ strain [21]. We used this observation to test the interaction between C-Vrp1p364-817-GFP and N-Las17p1-368 in las17Δ strain. C-Vrp1p364-817-GFP expressed from a CEN plasmid localized to cortical patches at both 24°C and 37°C in the presence of N-Las17p1-368 expressed from either CEN (data not shown) or 2µ plasmid (Fig. S2) but not in the presence of N-Las17p1-368 TL expressed from CEN (data not shown) or 2µ plasmid at both 24°C and 37°C (Fig. S2). Together, these data suggest that the interaction between N-Las17p1-368 and C-Vrp1p364-817 is abolished by the L80T H94L mutations. The L80T H94L mutations did not perturb interaction of N-Las17p1-368 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-Las17p1-368 TL loss of function and reduced state level is most likely due to specific loss of interaction with Vrp1p.

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-Las17p1-368-GFP and N-Las17p1-368
tL-GFP with the SH3 domain of Myo5p (a.a.1085-1219) (tagged with 6His). Both N-Las17p1-368-GFP and N-Las17p1-368TL-GFP were pulled down from yeast cell lysates with Myo5p-SH3-6His (Fig. 6B). However, the N-Las17p1-368-GFP band was more intense than the N-Las17p1-368TL-GFP band, suggesting that more molecules of N-Las17p1-368-GFP are in complex with the Myo5p-SH3 domain than the mutant N-Las17p1-368TL-GFP. This could be due to association of N-Las17p1-368-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-Las17p1-206-GFP which includes the Vrp1p binding region but lacks the Myo5p-SH3 binding motifs and N-Las17p207-368-GFP which conversely includes the region rich in Myo5p-SH3 binding motifs but lacks the Vrp1p binding region. The interaction of N-Las17p1-206 (tagged with GFP) with C-Vrp1p364-817 or Myo5p-SH3 (each tagged with 6His) in vivo was examined in a pull down assay (Fig. 6A, B). N-Las17p1-206-GFP interacted strongly with both C-Vrp1p364-817 and Myo5p-SH3 in the pull down assay consistent with the possibility that N-Las17p1-206 pulls down with Myo5p-SH3 due to formation of a complex with Vrp1p and that the N-Las17p1-206-Myo5p-SH3 interaction is indirect and mediated by Vrp1p. The N-Las17p207-368-GFP was very unstable thus we could not carry out the pull down assay (data not shown). The interactions of N-Las17p1-206 and N-Las17p207-368 with C-Vrp1p364-817 and Myo5p-SH3, was also examined in a yeast two-hybrid assay. The yeast two-hybrid assay showed that N-Las17p207-368 interacts with Myo5p-SH3 but not C-Vrp1p364-817 (Fig. 6C). Conversely, N-Las17p1-206 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.

**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, B, C). Las17p_{TL} was also able to restore actin 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 2μ plasmid to rescue actin patch polarization at 37°C (Fig. 8A, B). The Las17p_{TL} expressed from 2μ 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 Las17pTL affect Las17p interaction with Vrp1p. This was assessed by testing the binding of Las17p or Las17pTL (each tagged with GFP) in yeast cell lysate with C-Vrp1p364-817 (tagged with 6His) in a pull down assay. Las17p-GFP interacts with C-Vrp1p364-817, 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-Las17p1-206 in las17Δ cells under the transcriptional regulation of LAS17 promoter from a cen plasmid. The results revealed that an N-terminal Las17p fragment (N-Las17p1-206) that interacts with Vrp1p but lacks both the proline rich sequences and WA domain required for interaction with Arp2/3 (Fig. 1A) 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).


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-Las17p₁-₃₆₈) is sufficient to restore fluid-phase endocytosis, actin patch polarization and growth at 37°C to \( \text{las17}^{-} \) cells. We screened for and isolated an N-Las17p₁-₃₆₈ mutant, (N-Las17p₁-₃₆₈\(_{\text{TL}}\)) which abolished the interaction between Las17p and Vrp1p. N-Las17p₁-₃₆₈ with the mutation (N-Las17p₁-₃₆₈\(_{\text{TL}}\)) retains the ability to rescue the fluid phase endocytosis and actin patch polarization defects of \( \text{las17}^{-} \) cells at 24°C, but loses this ability at 37°C. Expression of N-Las17p₁-₃₆₈\(_{\text{TL}}\) using 2\( \mu \) plasmid rescued the growth and fluid phase endocytosis defect of \( \text{las17}^{-} \) at 37°C but not the actin patch polarization defect of \( \text{las17}^{-} \) 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\( \mu \) 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-Las17p₁-₃₆₈\(_{\text{TL}}\) 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-Las17p1-368 without the Arp2/3 activating domain (WA) rescue all the defects of las17Δ cells? This may be because N-Las17p1-368 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-Las17p1-368 and N-Las17p1-368 TL form complexes with Myo5p, the wild type N-Las17p1-368 was found to be in a complex with more molecules of Myo5p than the mutant N-Las17p1-368 TL (Fig. 6) consistent with the ability of N-Las17p1-368 but not N-Las17p1-368 TL to interact with Vrp1p. Thus, N-Las17p1-368 TL forms a complex with Myo5p only through direct binding. In contrast, the wild type N-Las17p1-368 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-Las17p1-368 complex may enhance N-Las17p1-368 stability and its ability to rescue the growth defects as well as bulk endocytosis and actin patch polarization at 37°C compared to N-Las17p1-368 TL. We tested the possibility that N-Las17p1-368 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-Las17p1-368 compared to N-Las17p1-368 TL even at 37°C (data
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-Las17p1-368 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.

**Acknowledgements**

We thank S. Oliferenko for critical reading of the manuscript and Y. Ivan for expert technical help. This work was supported by Agency for Science, and Technology and Research (A*STAR), Biomedical Research Council grant A*STAR 05/1/22/19/392. Alan Munn acknowledges funding from National Health and Medical Research Council (Australia) Project grant 252750 and core funding from the Queensland State Government.
References


Figure legends

**Figure 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.

**Figure 2**: N-Las17p₁-368 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-Las17p₁-368 or N-Las17p₁-368TL. 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-Las17p₁-368 or N-Las17p₁-368TL. Overnight YPUAD cultures of each strain were diluted to an OD₆₀₀ of 0.05 in fresh YPUAD medium and incubated at either 24°C or 37°C. OD₆₀₀ was monitored at 1 hr intervals.

(c) las17Δ cells harboring empty vector, or CEN plasmids expressing Las17p, N-Las17p₁-368 or N-Las17p₁-368TL were grown in YPUAD to exponential phase at 24°C, either left at 24°C or shifted to 37°C for 1 hr, before carrying out LY uptake assay for
1 hr 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.

**Figure 3:** N-Las17p1-368 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-Las17p1-368 or N-Las17p1-368\(^{\text{TL}}\) or 2μ plasmids expressing either N-Las17p1-368 or N-Las17p1-368\(^{\text{TL}}\) 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-Las17p1-368 or N-Las17p1-368\(^{\text{TL}}\) or 2μ plasmid expressing either N-Las17p1-368 or N-Las17p1-368\(^{\text{TL}}\) 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.

**Figure 4:** N-Las17p1-368 retains the ability to localize to cortical actin patches

(a) *las17Δ* cells expressing green fluorescent protein (GFP), Las17p-GFP, N-Las17p1-368-GFP or N-Las17p1-368\(^{\text{TL}}\)-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 hr. 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-Las17p1-368-GFP or N-Las17p1-368\textsuperscript{TL}-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.

**Figure 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-Las17p1-368 or N-Las17p1-368\textsuperscript{TL} or a 2μ plasmid expressing N-Las17p1-368\textsuperscript{TL}. 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-Las17p1-368-GFP, N-Las17p1-368\textsuperscript{TL}-GFP, Las17p-GFP or Las17p\textsuperscript{TL}-GFP from low (left, CEN plasmid) or high (right, 2μ 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 hr. Protein extract was isolated and analyzed using anti-GFP (α-GFP) and anti-hexokinase (α-Hex) serum as described in Materials and Methods.
**Figure 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-Vrp1p364-817-6His in combination with either GFP only, N-Las17p1-368-GFP, N-Las17p1-368<sup>TL</sup>-GFP or N-Las17p1-206-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-Las17p1-368-GFP, N-Las17p1-368<sup>TL</sup>-GFP or N-Las17p1-206-GFP. (I) Western blot of the proteins eluted from Ni<sup>2+</sup>-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-Las17p1-206, N-Las17p<sub>207-368</sub> or empty vector were tested for two-hybrid interaction with pBD (DNA binding domain)
plasmids expressing C-Vrp1\textsubscript{364-817} (C-Vrp1p) or Myo5p(SH3) or empty vector in two-hybrid reporter strain PJ69-4A. Two-hybrid interaction activates expression of the \textit{HIS3} reporter gene and was scored by growth on selective media lacking histidine (+2 mM 3-aminotriazole).

\textbf{Figure 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 \textit{las17}Δ cells expressing Las17p or Las17p\textsuperscript{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 \textit{las17}Δ cells harboring low-copy-number (CEN) plasmids expressing Las17p or Las17p\textsuperscript{TL} from LAS17 promoter or empty vector.

(c) The L80T H94L mutations do not abolish the endocytic function of full-length Las17p. \textit{las17}Δ cells expressing wild type Las17p or mutant Las17p\textsuperscript{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. \textit{Upper panels}: LY. \textit{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-Vrp1p364-817-6His 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.

**Figure 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 a low-copy-number (CEN) plasmids or 2μ plasmid or 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 hr (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 a low-copy-number (CEN) plasmids or 2μ plasmid were grown in YPUAD to exponential phase at 24°C and either left at 24°C or shifted to 37°C for 2 hr and analysed as described in Fig. 3B.

**Figure 9:** N-Las17p1-206 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-Las17p1-368 or N-Las17p1-206. 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-Las17p1-368 or N-Las17p1-206 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-Las17p1-368 or N-Las17p1-206 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.
Fig 1

Las17p

Las17p_{TL}

N-Las17p_{1-368}

N-Las17p_{1-368}_{TL}

N-Las17p_{1-206}

N-Las17p_{207-368}
Fig 2

a

24°C  37°C

![Bacterial growth at 24°C and 37°C](image)

![Diagram showing growth at different conditions](image)

b

24°C  37°C

![Graph showing growth under different conditions](image)

C

![Images showing bacterial growth](image)

![Images showing growth under different conditions](image)
**Fig 3**

**a**

<table>
<thead>
<tr>
<th>Vector</th>
<th>Las17p</th>
<th>N-Las17p&lt;sub&gt;1-368&lt;/sub&gt;</th>
<th>N-Las17p&lt;sub&gt;1-368&lt;/sub&gt;&lt;sup&gt;TL&lt;/sup&gt;</th>
<th>Vector</th>
<th>Las17p</th>
<th>N-Las17p&lt;sub&gt;1-368&lt;/sub&gt;</th>
<th>N-Las17p&lt;sub&gt;1-368&lt;/sub&gt;&lt;sup&gt;TL&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

24°C | 37°C

**b**

- 1: *las17Δ* with cen Vector
- 2: WT with cen Vector
- 3: *las17Δ* with cen N-Las17p
- 4: *las17Δ* with cen N-Las17p<sup>TL</sup>
- 5: *las17Δ* with 2μ N-Las17p
- 6: *las17Δ* with 2μ N-Las17p<sup>TL</sup>

% of Cells with Polarized Patches

1 2 3 4 5 6

24°C | 37°C
Fig 5

24°C  37°C

las17Δ

N-Las17p 1-368
N-Las17p 1-368<sup>TL</sup>

2μ N-Las17p 1-368<sup>TL</sup>

Low-copy-number (CEN) plasmid

High-copy-number (2μ) plasmid
Fig 6

a) C-Vrp1p-6His pull down

b) Myo5p(SH3)-6His pull down

C) Yeast two-hybrid protein interaction assay

<table>
<thead>
<tr>
<th>pAD</th>
<th>pBD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C-Vrp1p</td>
</tr>
<tr>
<td>N-Las17p1-206</td>
<td>+</td>
</tr>
<tr>
<td>N-Las17p207-368</td>
<td>-</td>
</tr>
<tr>
<td>Vector</td>
<td>-</td>
</tr>
</tbody>
</table>
Fig 7

(a) Petri dish images showing the effect of temperature on the growth of strains expressing Las17p, Las17pTL, and a vector control.

(b) Graph showing the OD_{600} over time at 37°C for strains expressing Las17p, Las17pTL, and a vector control.

(c) Microscopic images of cells expressing Las17p, Las17pTL, and a vector control at 24°C and 37°C.

(d) Western blot analyses showing the expression of α-GFP and α-His proteins in cells expressing Las17p-GFP, Las17pTL-GFP, C-Vrp1p-His, and a vector control at 37°C.
Fig 8

(a) 

1: las17Δ with cen Vector 
2: WT with cen Vector 
3: las17Δ with cen Las17p 
4: las17Δ with cen Las17pTL 
5: las17Δ with 2µ Las17p 
6: las17Δ with 2µ Las17pTL 

(b) 

% of Cells with Polarized Patches

24°C

37°C
Fig 9

(a) 24°C  37°C

(b) 24°C  37°C

(c) 24°C  37°C
Figure S1

a

\[las17\Delta\]

\begin{tabular}{|c|c|c|c|c|c|}
\hline
 & \text{Vector} & \text{Vector} & \text{Las17p} & \text{Las17p}^{\text{TL}} & \text{N-Las17p}_{1-368} & \text{N-Las17p}_{1-368}^{\text{TL}} \\
(2\mu) & (2\mu) & (2\mu) & (2\mu) & (2\mu) & (2\mu) & (2\mu) \\
\hline
\end{tabular}

\begin{tabular}{|c|c|}
\hline
 & \text{24°C} \\
\hline
\end{tabular}

\begin{tabular}{|c|c|}
\hline
 & \text{37°C} \\
\hline
\end{tabular}

b

\begin{itemize}
\item 1: \textit{las17}\Delta \text{ with } 2\mu \text{ Vector}
\item 2: WT with 2\mu Vector
\item 3: WT with 2\mu Las17p
\item 4: WT with 2\mu Las17p^{TL}
\item 5: WT with 2\mu N-Las17p
\item 6: WT with 2\mu N-Las17p^{TL}
\end{itemize}
Figure S2

N-Las17p1-368  N-Las17p1-368

24°C

C-Vrp1p364-817-GFP

37°C
Figure S3

**las17Δ**

<table>
<thead>
<tr>
<th>GFP</th>
<th>Las17p-GFP</th>
<th>Las17p&lt;sup&gt;T&lt;/sup&gt;-GFP</th>
<th>N-Las17p&lt;sub&gt;1-206&lt;/sub&gt;-GFP</th>
</tr>
</thead>
</table>

24°C

37°C