Functional analysis of WASP and Las17p using *Saccharomyces cerevisiae* las17 Δ cells

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**Functional analysis of WASP and Las17p using
Saccharomyces cerevisiae las17Δ cells**

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ABBREVIATIONS

a.a	Amino acid
AD	DNA activation domain
Arp	Actin related protein
ATP	Adenosine 5'-triphosphate
BD	DNA binding domain
BSA	Bovine serum albumin
°C	Degree Celsius
CR16	Glucocorticoid regulated protein
DIC/Normarski	Differential interference contrast
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
DTT	Dithiothreitol
ECL	Enhanced chemiluminescence
<i>E.coli</i>	Escherichia coli
EB	Ethidium bromide
EDTA	Ethylenediamine tetra acetic acid
F-actin	Filamentous actin
FITC	Fluorescein isothiocyanate
G-actin	Monomeric globular actin
GFP	Green fluorescent protein
GST	Glutathione-S-transferase
HRP	Horseradish peroxidase
kDa	Kilo dalton
LB	Luria-Bertani
LiAc	Lithium acetate
LY	Lucifer Yellow dye
mRNA	Messenger ribonucleic acid
N-WASP	Neural WASP protein
OD	Optical density
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase Chain Reaction
PEG	Polyethylene glycol
PIP2	PtIns(4.5)P2, Phosphatidyl-inositol 4,5-diphosphate
PMSF	Phenylmethylsulfonyl fluoride
RFP	Red fluorescent protein
RT-PCR	Reverse transcription-polymerase chain reaction

SD	Synthetic Defined
SDS	Sodium dodecyl sulfate
SH3	Src homology 3
TEMED	N, N, N', N'-tetramethylethylenediamine
UV	Ultraviolet
WAS	Wiskott-Aldrich syndrome
WASP	Wiskott-Aldrich syndrome protein
WAVE	WASP family verprolin homologous protein
WH1	WASP homology domain I
WH2	WASP homology domain 2
WTCH	WIP and CR16 Homologous protein
WIP	WASP interacting protein
WIRE	WIP Related protein
WT	Wild type
XLN	X-linked neutropenia
XLT	X-linked thrombocytopenia
YPD	Yeast Extract/Peptone/Dextrose

One and three letter symbols of amino acid residues:

A	Ala	Alanine
C	Cys	Cysteine
D	Asp	Aspartic acid
E	Glu	Glutamic acid
F	Phe	Phenylalanine
G	Gly	Glycine
H	His	Histidine
I	Ile	Isoleucine
K	Lys	lysine
L	Leu	Leucine
M	Met	Methionine
N	Asn	Asparagine
P	Pro	Proline
Q	Gln	Glutamine
R	Arg	Arginine
S	Ser	Serine
T	Thr	Threonine
V	Val	Valine
W	Trp	Tryptophan
Y	Tyr	Tyrosine

SUMMARY

Wiskott Aldrich Syndrome (WAS) is a genetic disease caused by recessive mutations in the gene encoding the Wiskott Aldrich Syndrome protein (WASP). Patients suffering from WAS exhibit a variety of symptoms ranging from thrombocytopenia, eczema and immune disorders. WASP belongs to a family of proteins whose members include WASP, N-WASP, WAVE1-3 and Las17p. WASP has a WH1 domain at the N-terminus and the Arp2/3 complex activating VCA domain at the C-terminus. WASP is expressed predominantly in hematopoietic cells and is involved in integrating extracellular signals and regulating actin cytoskeleton. Las17p is the *S. cerevisiae* ortholog of mammalian WASP and has a similar domain structure as that of WASP. Deletion or mutation in the gene encoding Las17p makes the yeast cells inviable at restrictive temperatures and also causes defects in endocytosis and actin patch polarization at all temperatures.

In this study, a *S. cerevisiae las17Δ* strain was used to study the functional significance of WASP-WIP interaction and Las17p-Vrp1p interaction. The *las17Δ* mutant was used to show that both WASP and Las17p function as a complex with their respective WH1 domain binding partners WIP and Vrp1p respectively in suppressing the growth defects of the strain. The ability of the WASP-WIP complex to suppress the growth defects of *las17Δ* cells is dependent on the activity of the Arp2/3 complex activating VCA domain of WASP. In contrast to WASP, expression of the N-terminal region of Las17p was able to correct the defects of the *las17Δ* cells, suggesting that the VCA domain of Las17p is redundant with the Arp2/3 complex activation domain of type I myosins. Introducing mutations into the WH1 domain of WASP and Las17p, which abolishes interaction with

their respective binding partners WIP and Vrplp, compromised the activity of these proteins in suppressing the defects of the *las17Δ* cells. WIP regulates the activity of WASP possibly by mediating the localization of WASP and also by providing an additional actin binding V domain to the WASP-WIP complex. Functional studies were also carried out in order to examine the effects of 52 missense mutations causing the WAS disease on the activity of WASP in *las17Δ* cells. We found that missense mutations in the WH1 domain had a more pronounced effect on the activity of WASP in *las17Δ* cells, than the missense mutations in the other domains of the protein. Similar to the dependence on WIP for the activity of WASP in *las17Δ* cells, Vrplp also regulates Las17p albeit in a different manner. Mutation in Las17p which abolishes interaction with Vrplp has no effect on the localization of Las17p to the cortical patches of *las17Δ* cells but the mutant Las17p failed to correct the cytoskeletal defects of the *las17Δ* strain at 37°C. Studying the interaction between Las17p and Myo5p by pull down assays and Yeast Two Hybrid suggests that Vrplp promotes the formation of Las17p-Vrplp-Myo5p complex. The data presented in this study suggests that there are fundamental similarities and differences in the mechanism by which the relevant human and yeast proteins promote actin polymerization.

1 Introduction

The cell is the smallest functional unit of all known living organisms and is considered to be the building block of life. There are two kinds of organisms, namely prokaryotes and eukaryotes. Prokaryotes are unicellular organisms while eukaryotes are either unicellular (e.g. yeast) or multicellular like humans. A major difference between the prokaryotes and the eukaryotes is that eukaryotic cells contain membrane-bound compartments in which specific metabolic activities take place while prokaryotes lack the compartmentalization of cellular metabolic activities. Despite the morphological and physiological differences between them, prokaryotic cells and eukaryotic cells have certain similarities, both types of cells are membrane bound, use DNA as the genetic material and synthesize proteins by utilizing ribosomes. All cells are derived from pre-existing cells and irrespective of whether cells reproduce sexually or asexually, cells need to establish polarity, segregate the chromosomes and undergo cytokinesis to generate the daughter cells. All the cells maintain correct shape and interact with their environment and many cells also have the ability to change shape and migrate from place to place. All these spatial and mechanical functions depend, to a high degree in eukaryotes and to a lesser extent in prokaryotes, on a system of filaments collectively called as cytoskeleton. The entire focus of this dissertation would be on the proteins associated with eukaryotic cytoskeleton, particularly the actin filaments.

1.1 Cytoskeletal filaments

Eukaryotic cells contain three main types of cytoskeletal filaments namely microfilaments, intermediate filaments and microtubules (Fig. 1.1). Microfilaments/ actin

filaments are around 7 nm in diameter and are predominantly present at the plasma membrane. Microfilaments are involved in cytokinesis and also participate in cell-to-cell or cell-to-matrix junctions and in the transduction of signals. Microfilaments determine the shape of the cells surface and are essential for whole cell motility. Intermediate filaments are around 8-11 nm in diameter and are more stable than the actin filaments. Intermediate filaments support the fragile nuclear envelope and are involved in maintaining the three dimensional structure of the cell. Intermediate filaments provide mechanical strength to the cell and also provide resistance to shear stress. Microtubules are hollow cylinders of 25 nm in diameter and are composed of 13 protofilaments which are themselves assembled from smaller subunits. Microtubules are organized by the centrosome and are involved in intracellular transport and in the motility of the cilia and the flagella (Lodish et al., 2000).

The three kinds of filaments are formed by the assembly of their corresponding subunits which come together using a combination of end-to-end and end-to-side protein contacts. Among the three types of filaments, intermediate filaments are formed by the assembly of heterogeneous subunits while the other two filaments are assembled from homogeneous subunits namely, actin subunits for microfilaments and heterodimeric tubulin subunits (α and β) for microtubules (Lodish et al., 2000; Paustian et al., 2007).

Figure 1.1 The three cytoskeletal elements. (Adapted from Paustian et. al., 2007.)

1.2 Rate limiting step in the formation of the cytoskeletal filaments

The different cytoskeletal filaments are formed by the polymerization of their individual subunits. These subunits might be compact and globular like those of **actin** subunits for **actin** filaments and tubulin subunits for microtubules. In the case of **intermediate** filaments, the building blocks are themselves made up of smaller subunits which are elongated and fibrous (Lodish et al., 2000).

In order to assemble a new large filament, the subunits must first assemble into an initial complex which is stabilized by multiple subunit-subunit contacts and then the initial complex is elongated by the rapid addition of more subunits. The initial complex which is the precursor for the formation of the filament is called a nucleus and the process of nucleus assembly is called nucleation. The rate limiting step in the assembly of the filaments is the nucleation of the filament. This is primarily due to the instability of the smaller initial complex which creates a kinetic barrier to nucleation. During the polymerization of cytoskeletal filaments, there is **an** initial lag phase during which

filaments are not observed. However, during the lag phase, many nuclei are in the process of being assembled and so the lag phase is followed immediately by a rapid phase of polymerization during which the subunits add rapidly onto the end of the nucleus to form the filaments. The assembly of the cytoskeletal filaments is a dynamic process due to the addition of subunits to the elongating end and dissociation of the subunits from the other end. The system approaches a steady state when the rate of addition of the subunit to the filament is exactly balanced by the rate of dissociation of the subunit from the filament (Fig. 1.2) (Cooper et al., 1983; reviewed in Pollard, 1986).

Figure 1.2 Polymerization kinetics of cytoskeletal filaments.

The time course of polymerization of cytoskeletal filaments proceeds as follows, nucleation followed by growth and finally the steady state. (Adapted from Cooper et. al., 1983)

1.3 Microfilaments/Actin filaments

Actin is a highly conserved 42 kDa protein expressed in all eukaryotic cells from fungi to metazoans. Actin is one of the most abundant cellular proteins because actin structures cover most parts of the cell. In certain cells like muscle cells, actin comprises 10% by weight of the total cell protein and even in non-muscle cells, actin makes up to 5% of the cellular protein. Single cell organisms have one or two copies of actin gene while multicellular organisms have many genes coding for multiple isoforms of actin. For example, diploid yeast cells have two copies of the gene encoding Act1p, the yeast actin (Gallwitz and Seidel, 1980). In humans, there are two copies each of the genes encoding the different actin isoforms, α -actin, β -actin and γ -actin. α -actin is expressed in muscle

cells and is associated with muscle contraction, β -actin and γ -actin are expressed in non-muscle cells where β -actin is associated with actin polymerization in the cell leading edge and γ -actin is associated with stress fibers (Lodish et al., 2000). In any cell, the richest area of actin is found in the narrow region that lies underneath the plasma membrane, called cell cortex. In the cell cortex, the actin filaments are arranged in a network that excludes most organelles from the cortical cytoplasm (Lodish et al., 2000).

Actin exists in two forms, a globular monomer called G-actin and as a filamentous polymer F-actin which is a linear chain of G-actin subunits. Microfilaments/Actin filaments consist of two F-actin filaments which are interwoven with each other in a helical pattern. Each G-actin molecule has a binding site for a nucleotide (ATP or ADP) and a divalent cation (Mg^{2+}) (Carlier, 1991; Kinosian et al., 1993). The conformation of the G-actin molecule and the nature of the nucleotide bound to it depend on the status of the G-actin molecule, whether G-actin exists freely or whether it has already been incorporated into the F-actin filament (Belmont et al., 1999). F-actin filaments are polarized structures with a fast growing barbed end and a slow growing pointed end. The barbed end of the filament is favored for polymerization and ATP-G-actin monomers are preferentially added to this end. Once the G-actin subunit has been incorporated into the filament at the barbed end, the bound nucleotide undergoes hydrolysis to ADP, thus reducing the affinity of G-actin with the neighboring subunits and in turn leads to the depolymerization of the filament at the pointed end (Korn et al., 1987). This process of active addition and dissociation of G-actin to and from the F-actin filament is called treadmilling (Fig. 1.3).

Figure 1.3 Treadmilling of actin filaments. Treadmilling is caused by the hydrolysis of the ATP associated with G-actin. Filaments depolymerize at the pointed end and a steady concentration of ATP-G-actin is maintained which enables polymerization at the barbed end to balance depolymerization. (Adapted from Moseley et. al., 2006.)

The process of actin assembly can be blocked by the capping of the barbed end by capping proteins and small molecules. Actin assembly can then be restored either by uncapping the barbed end of the filament or by the severing of the filament to smaller filaments which would in turn generate new barbed end for actin polymerization (Welch et al., 2002). Actin polymerization is a tightly regulated event which is mediated by a cascade of proteins in order to prevent the reaction from going astray.

1.4 Arp2/3 complex

The two major classes of proteins which mediate the nucleation of actin filaments are the Formins (Evangelista et al., 2002; Sagot et al., 2002) and the Arp2/3 complex (Pantaloni et al., 2001; Winter et al., 1997). Formins are associated with the nucleation and elongation of unbranched actin filaments and they participate in cytokinesis and formation of actin filament bundles (Pruyne et al., 2002). The Arp2/3 complex is active at the leading edge of motile cells and it produces new actin filaments from the sides of the existing filaments (Mullins et al., 1998). Among the two families of actin nucleators, though Formins were identified earlier (Kleinebrecht et al., 1982; Machesky et al., 1994), the Arp2/3 complex has been the best characterized. The Arp2/3 complex consists of

seven subunits (Arp2, Arp3, ARPC1, ARPC2, ARPC3, ARPC4 and ARPC5) (Machesky et al., 1994; Winter et al., 1997) is present at a high concentration of up to 10 μM in the cytoplasm (Higgs et al., 1999). The Arp2/3 complex was first discovered in *Acanthamoeba castellanii* and has subsequently been isolated from several other organisms including vertebrates and yeast (Winter et al., 1997; Welch et al., 1997; Ma et al., 1998). The subunit composition of the Arp2/3 complex is well conserved in all these organisms. Algae, microsporidia and apicomplexa which do not express the Arp2/3 complex, have been found to express Formins (Pollard et al., 2002).

The Arp2/3 complex can bind to a preexisting actin filament at the tip or to the side. The Arp2/3 complex binds to the sides of a preexisting actin filament at an angle of 70° and would therefore nucleate the new F-actin filament in that orientation (Mullins et al., 1998). In motile cells, the Arp2/3 complex generates a network of branched actin filaments, whose tips push the cell membrane forward which in turn leads to the protrusion of the pseudopod (Welch et al., 1997). It has been proposed that the Arp2/3 complex nucleates actin assembly by bringing Arp2 and Arp3 subunits closer to each other in the complex and mimic the barbed end of an actin filament (Pollard et al., 2002). After the nucleation of the filament and during the elongation phase, the Arp2/3 complex remains bound to the pointed end of the nascent F-actin filament (Mullins et al., 1998).

1.5 Nucleation promoting factors (NPF)

In vitro studies have shown that purified Arp2/3 complex has minimum activity for the nucleation of actin filaments (Mullins et al., 1998; Welch et al., 1998). However, the

ability of the Arp2/3 complex to promote actin polymerization can be magnified many fold by the activation of the Arp2/3 complex by nucleation promoting factors (NPF) (reviewed in Pollard, 2007). Crystallographic studies on the bovine Arp2/3 complex has shown that in the inactive Arp2/3 complex, the Arp2 and the Arp3 subunits are not in close proximity with each other (Robinson et al., 2001). Recent studies employing techniques like electron microscopy and Fluorescence resonance energy transfer (FRET) analysis have shown that the binding of the NPF's with the Arp2/3 complex induces a large conformational change which in turn brings the Arp2 and Arp3 subunits closer together to nucleate actin (Goley et al., 2004; Rodal et al., 2005). Table 1.1 lists the known NPFs and the organisms in which they are expressed. The main feature that is conserved in all the NPFs is the presence of an Arp2/3-binding sequence called the CA domain. The CA domain consists of a short stretch of basic and acidic amino acids located at the C and A region respectively. It has been shown that though the CA region in the NPFs is sufficient for binding with the Arp2/3 complex, it is not capable of activating the complex *in vitro*. In order to activate the Arp2/3 complex, the NPFs must possess adjacent binding sites for either G-actin or F-actin. The NPFs have thus been classified into two categories based on the presence of binding sites for G-actin or F-actin. NPFs which possess one or two G-actin binding WH2/V domains are called Class I NPFs and include ActA, WASP, WAVE, Myosin I and CARMIL. Class II NPFs which include cortactin and Abplp, do not have any G-actin binding WH2/V domain but instead contain F-actin binding domains (FAB) (reviewed in Goley et al., 2006).

Table 1.1 Nucleation promoting factors (NPF)

NPF	Organism	References
ActA	<i>L. monocytogenes</i>	Welch et al., 1998
WASP	Metazoans, <i>S. cerevisiae</i> , <i>S. pombe</i>	Yarar et al., 1999; Winter et al., 1999 ; Rohatgi et al., 1999
WAVE	Metazoans, <i>D. discoidium</i>	Machesky et al., 1999
Myosin I	<i>S. cerevisiae</i> , <i>S. pombe</i>	Lechler et al., 2000; Evangelista et al., 2000; Lee et al., 2000; Sun et al., 2006
CARMIL	Metazoans, <i>D. discoidium</i> , <i>A. castellanii</i>	Jung et al., 2001
Cortactin	Metazoans	Weed et al., 2000; Uruno et al., 2001; Weaver et al., 2001
Abp1p	Metazoans, <i>S. cerevisiae</i>	Goode et al., 2001
Eps15	Metazoans, <i>S. cerevisiae</i>	Duncan et al., 2001

1.6 Activation of the Arp2/3 complex

Class I and Class II NPFs activate the Arp2/3 complex through slightly different mechanisms. Class I NPFs activate the Arp2/3 complex by first forming a ternary complex with Arp2/3 complex and G-actin (Fig. 1.4A). In this ternary complex, the interaction between the Arp2/3 complex and the CA domain of the NPF may cause a conformational change in the complex, resulting in its activation. Nucleation could then be initiated by the presentation of G-actin by the WH2 domain of the NPF (Robinson et al., 2001). Class II NPFs activate the Arp2/3 complex by a slightly different mechanism (Fig. 1.4B). It is hypothesized that the binding of the CA domain of the Class II NPFs to the Arp2/3 complex may induce a conformational change that enables the complex to bind to the side of the existing actin filament. The binding of the Arp2/3 complex to the

side of the mother filament might induce **further** conformational changes and subsequently leads to the activation of the complex and nucleation (Welch et al., 2002).

A. Class I NPFs

B. Class II NPFs

Figure 14 Activation of the Arp2/3 complex by Class I and Class II NPFs.

- A. Class I NPFs bind to inactive Arp2/3 complex and form a ternary complex consisting of the NPF, Arp2/3 complex and G-actin. The formation of the ternary complex induces a conformational change in the Arp2/3 complex resulting in its activation.
- B. Class II NPFs bind with inactive Arp2/3 complex to form a binary complex. The binary complex then binds to the side of the mother filament which results in the activation of the Arp2/3 complex.

(Adapted from Welch et al., 2002)

1.7 Wiskott Aldrich Syndrome (WAS)

The Wiskott Aldrich Syndrome protein (WASP) is one of the best characterized members of the Class I family of NPFs. Mutations in the gene encoding WASP cause the genetic disease Wiskott Aldrich Syndrome (WAS) (Derry et al., 1994). Wiskott Aldrich Syndrome was first described by Dr. Wiskott in 1937 and it was **further** characterized by Dr. Aldrich in 1954 and so the disease has **been** named after these individuals. Patients

suffering from WAS exhibit a variety of symptoms ranging from thrombocytopenia, eczema and bleeding disorders. However, only 27% of the patients suffering from WAS have all the above mentioned manifestation of WAS (Classic WAS). Almost 90% of the patients only have manifestation of eczema and thrombocytopenia at the onset of the disease. WAS patients also suffer from immune disorders characterized by recurrent bacterial and viral infections. The immune system dysfunction is probably due to reduced proliferation of T and B lymphocytes in response to antigens. Studies show that the patients also have a predisposition towards lymph node cancer (reviewed in Millard et al., 2004).

WAS is a rare X-linked inherited disorder that occurs at a frequency of 4/1,000,000 live male births. Though 90% of the affected patients are males, female carriers of the defective WASP gene are at risk due to inactivation of the functional WASP allele (Ariga et al., 1999). The disease can be treated by bone-marrow transplantation. Symptomatic treatment like splenectomy might also help by artificially raising the platelet count (reviewed in Krišukienė et al., 2003).

1.8 Wiskott Aldrich Syndrome protein (WASP)

WASP is a 502-residue proline rich protein that is predominantly expressed in hematopoietic cells (Derry et al., 1994). WASP belongs to a large family of proteins called the WASP family of proteins (Fig. 1.5). After the identification of WASP, a related ubiquitous protein, N-WASP, was isolated from the brain tissues (Miki et al., 1996). A protein related to WASP was identified in a genetic screen in *Dictyostelium* and named

suppressor of cAMP receptor (SCAR) (Bear et al., 1998). SCAR has also been isolated from mammalian cells, where three isoforms of SCAR are expressed. In mammalian cells, SCAR is also called as WASP family Verprolin homologous (WAVE) (Miki et al., 1998; Suetsugu et al., 1999). Yeast cells express only a single member of the WASP family, called Las17p in *S. cerevisiae* (Li, 1997) and Wsp1p in *S. pombe* (Lee et al., 2000). Thus the mammalian members of the WASP family include WASP, N-WASP, WAVE1, WAVE2 and WAVE3, all of which are implicated with cytoskeletal regulation (reviewed in Caron, 2002). The domain organization of WASP is remarkably conserved in the orthologous proteins across many species like humans, mouse, insects, nematodes and yeast (reviewed in Takenawa et al., 2001).

Figure 1.5 Domain structure of the members of the WASP family of proteins (adapted from Caron, 2002).

WH1- WASP homology domain 1, SH - Scar homology domain, BR- Basic region, GBD- GTPase binding domain, PRO- Proline rich region, V- Verprolin homology domain, C- Cofilin homology domain or connecting region, A- Acidic region.

WASP consists of a pleckstrin-homology (PH) domain at the N-terminal region which partially overlaps with the WASP homology (WH1) domain, followed by the GTPase binding domain (GBD), which is succeeded by a **proline** rich sequence and the verprolin homology-cofilin homology-acidic (VCA) region (Fig. 1.6). The PH domain has been shown to interact with phospholipids like Phosphatidylinositol (4,5)-biphosphate (Miki et al., 1996). The WH1 domain has been identified in many proteins which regulate cytoskeletal organization and has been shown to bind with **proline** rich proteins like WIP and WIRE (Ramesh et al., 1997; Aspenstrom, 2004). The GBD has been shown to interact with Cdc42 (Syrnons et al., 1996), a member of Rho family of GTPase which regulate **actin** cytoskeleton. The **proline** rich region has been shown to interact with SH3 domain containing proteins like Nck (Rohatgi et al., 2001) and Fyn (Banin et al., 1999). The VCA region has been shown to interact with G-actin and also bind and activate the Arp2/3 complex (Yamaguchi et al., 2000). The presence of many domains which interact with signaling proteins suggests an important role for WASP in the signaling cascades which regulate important physiological activities. Fifty percent of the mutations causing WAS are point mutations in the WH1 domain (Imai et al., 2003). Some of the mutations in the WH1 domain result in the synthesis of a truncated protein or an unstable protein and a few mutations have been shown to affect WASP-WIP interaction (Stewart et al., 1999).

Figure 1.6 Proteins that interact with WASP.

The Verprolin family members WIP, WIRE and CR16 interact with the WH1 domain. The GTPase Cdc42 interacts with the GBD domain. SH3 domain containing proteins like Nck and Fyn interact with the proline rich region. G-actin and the Arp2/3 complex bind to the VCA domain.

1.9 Physiological role of WASP

Patients suffering from WAS show a weak immune response to common antigens, due to T and B lymphocyte dysfunction and defects in transmembrane signaling in the immune cells (Simon et al., 1992; Huang et al., 1997). It has been shown that T and B lymphocytes from WAS patients and WASP null mice display a reduced proliferative response to antigens (Ochs et al., 1980; Zhang et al., 1999). Delayed phagocytosis and clearing of apoptotic cells by WASP deficient macrophages has also been reported (Leverrier et al., 2001). These manifestations were shown to be caused due to abnormality in cytoskeletal reorganization (Zhang et al., 1999). Over expression of WASP in mammalian cells has been shown to induce formation of actin clusters in the cytoplasm (Symons et al., 1996). These observations, coupled with the ability of WASP to interact with GTPases, G-actin and the Arp2/3 complex, imply a role for WASP in actin cytoskeletal organization (Miki et al., 1996; Machesky et al., 1998).

1.10 Regulation of WASP

WASP has been shown to adopt an inactive conformation *in vitro* through the binding of the VCA region with the GTPase binding domain (GBD), thereby masking the VCA region (Fig. 1.7A) (Kim et al., 2000). The masked VCA region is unable to activate the Arp2/3 complex. Under such an inactive state, the protein cannot promote actin polymerization. It has been shown that GTPases like Cdc42 and phospholipids like Phosphatidylinositol (4,5)-biphosphate can cooperatively stimulate WASP to promote actin polymerization (Higgs and Pollard, 2000). The binding of Cdc42 and phospholipids with WASP disrupts the inactive conformation of WASP and exposes the VCA region (Fig. 1.7B). The exposed VCA region is therefore free to bind and activate the Arp2/3 complex. The activated Arp2/3 complex serves a very important role as an actin nucleating factor and promotes the formation of F-actin (reviewed in Takenawa et al., 2001). Recently, a slightly different model for the activation of N-WASP has been suggested. According to this model, WASP/N-WASP exists as an inactive complex with WIP. The activation of WASP/N-WASP by Cdc42 requires the presence of an adaptor protein Toca-1 which is capable of binding simultaneously with both WASP and Cdc42 (Ho et al., 2004) (Fig. 1.7C). The exact role played by Toca-1 in the activation of WASP/N-WASP needs further investigation.

Figure 1.7 The putative mechanism of activation of WASP. WASP is in an inactive state due to interaction between the VCA region and the GTPase binding domain (GBD), which is disrupted when Cdc42 binds with the GBD. The VCA region is subsequently unmasked and is free to promote actin polymerization by activating the Arp2/3 complex. It is suggested that the binding of Cdc42 with WASP could be mediated by adaptor proteins like Toca-1.

1.11 WASP interacting protein (WIP)

It has been reported that most of the WASP in mammalian cells exists as a complex with WIP (Sasahara et al., 2002). WIP was discovered in a yeast two-hybrid screen using WASP as bait and it has been shown that the C-terminal region of WIP (consisting of the WASP binding domain) interacts with the WH1 domain of WASP (Fig. 1.8) (Ramesh et al., 1997). WIP is a member of a family of proteins, called the Verprolin family. The Verprolin family of proteins is a family of proline rich proteins and it includes the mammalian proteins WIP (Ramesh et al., 1997), WIRE/WICH (Aspenstrom, 2002, Kato et al., 2002) and CR16 (Ho et al., 2001) and the yeast protein Vrp1p (Donnelly et al., 1993). Among the mammalian members of the Verprolin family, WIP and WIRE are expressed in a ubiquitous manner but the expression of CR16 is restricted to the brain, heart, lungs and colon (Ramesh et al., 1997; Weiler et al., 1996; Aspenstrom, 2002; kato et al., 2002). WIP is a 503 residue protein which is expressed at high levels in lymphoid tissues and it shows 90% sequence similarity with its rat homologue. WIP has the actin-binding verprolin homology (V) domain which contains the KLKK motif and shows significant similarity to the N-terminal region of the yeast protein Verprolin (Vrp1p). Apart from these two domains, WIP also has a number of proline-rich regions containing putative SH3-binding motifs that have the consensus sequence PPPfXP where “f” represents a hydrophobic residue. In addition to the KLKK motif, the V domain of WIP also contains three XPPPPP sequences (Actin-Based Motility 2 sequence, ABM2 motif, X can be represented by A, L, S or G), which bind to profilin, a G-actin binding protein (Ramesh et al., 1997; Gertler et al., 1996; Purich et al., 1997; Reinhard et al., 1995).

Figure 1.8 Domain organization of WIP.

WIP has the G-actin binding V domain at the N-terminal region and the WASP binding domain (WBD) at the C-terminal region. WIP also contains a number of PPPfXP motifs which bind with SH3 domain containing proteins.

S. cerevisiae strains deficient for Vrp1p have several cellular defects (Donnelly et al., 1993; Munn et al., 1995). Expression of human WIP is able to complement the growth defects in *vrp1* null and *vrp1-1* mutant strains and also restore endocytosis and actin patch polarization in the *vrp1-1* mutant strain (Vaduva et al., 1999). Thus Vrp1p is the yeast homologue of mammalian WIP. In mammalian cells, over expression of WIP in transfected mammalian cells induces actin polymerization and formation of membrane projections (Ramesh et al., 1997), suggesting a role for WIP in cytoskeletal organization. A WASP-WIP complex is also readily detected in resting cells (Ramesh et al., 1997). Some of the mutations causing Wiskott Aldrich Syndrome have been shown to affect WASP-WIP binding (Stewart et al., 1999) suggesting that such interaction might be crucial for WASP's function. The role of WIP in N-WASP's function was investigated using *in vitro* actin polymerization assay and showed that WIP inhibits Cdc42 mediated activation of N-WASP, suggesting that WIP's role is to stabilize N-WASP in its inactive conformation (Martinez-Quiles et al., 2001). The contribution of WIP to WASP's activity needs further investigation.

1.12 Actin cytoskeleton in *Saccharomyces cerevisiae*

In *S. cerevisiae*, the filamentous actin (F-actin) is organized into cortical actin patches, actin cables and the actomyosin ring (Fig. 1.9) (Adams et al., 1984; Bi et al., 1998; Lippincott et al., 1998). Actin patches are complex structures composed of around 30 proteins (Pruyne et al., 2000). Actin cables are cross linked bundles of actin filaments. Among these actin structures, the cortical actin patches take up most of the filamentous actin in the cell. Actin patches are clustered in regions of active secretion with the actin cables oriented towards the patch clusters (Kilmartin et al., 1984).

Actin patches are concentrated at sites of polarized growth. It was shown that mutations in the actin patch components renders the cells defective in endocytosis, suggesting that the actin patches play a crucial role in the endocytic machinery of the cell (Riezman, 1985). Moreover, treatment of cells with latrunculin-A (a drug that binds irreversibly with G-actin) arrests endocytosis in the cells (Ayscough et al., 1997), reinforcing the link between actin patches and endocytosis. Actin patches might also play a role in exocytosis as mutating some of the patch components results in the accumulation of post-Golgi vesicles (Mulholland et al., 1997). The primary role of actin cables is in the transport of cargo from the mother cell to the bud in a budding cell (Hill et al., 1996; Pruyne et al., 1998; Yin et al., 2000). During the early stages of cell cycle in *S. cerevisiae*, polarity factors accumulate at the location of the nascent bud site. The assembly of the actin cables is initiated from this nascent bud site and the reorientation of the cables takes place in order to enable the growth and secretion to the future bud tip. These cables then serve

as tracks for the delivery of the cargo to the nascent bud, using the motor activity of the myosin proteins (Hill et al., 1996; Pruyne et al., 1998; Yin et al., 2000).

1.13 Dynamics of actin patches and cables

Actin patches and the actin cables are dynamic structures which undergo remarkable changes during the life cycle of an *S. cerevisiae* cell. Actin patches have a life span of around 10-20 seconds. Actin patches are also capable of rapid movement, their motility rate ranges from 0.1-0.5 $\mu\text{m/s}$ (Carlsson et al., 2002; Waddle et al., 1996). In budding cells, the formation and enlargement of the bud takes place due to the polarization of the cytoskeleton and subsequent transport of cargo to the bud. Polarization of the actin patches and actin cables is essential for establishing cell polarity in dividing cells. At the start of the cell cycle when the nascent bud site is marked, the actin patches and the actin cables in the mother cell converge at the nascent bud site (Fig. 1.9A). During the growth of the bud, the actin patches redistribute over the bud surface and the actin cables extend from the mother cell to the bud (Fig. 1.9B). Finally, at cytokinesis, the patches and the cables reorient to the mother-bud junction (Fig. 1.9C). When the actin cytoskeleton is depolarized, the patches are randomly distributed in the cortex and the cables form a meshwork in the cytoplasm (Amberg, 1998).

Figure 1.9 Dynamics of actin patches during cell division in *S. cerevisiae* (Adapted from Moseley et al., 2006).

- A. Actin patches and cables converge at the putative bud site on the mother cell.
- B. During the enlargement of the bud, the actin patches are distributed over the bud and the cable are oriented along the mother-bud axis.
- C. During cytokinesis, the patches and the cables reorient to the mother-bud junction.

1.14 Regulators of the *S. cerevisiae* Arp2/3 complex

One of the components of the cortical actin patches in yeast cells is the Arp2/3 complex. In *S. cerevisiae*, Arp2/3 complex consists of the subunits Arp2p, Arp3p, Arc15p, Arc18p, Arc19p, Arc35p and Arc40p (Winter et al., 1997; Winter et al., 1999). The *S. cerevisiae* Arp2/3 complex is regulated by five NPFs namely Las17p, Myo3p, Myo5p, Pan1p and Abp1p. Just like the NPFs in mammalian cells, the NPFs in *S. cerevisiae* also have the Arp2/3 complex binding CA domain. Among the five NPFs, Myo3p, Myo5p and Las17p belong to the Class I category of NPFs. The other two NPFs belong to the Class II category. The five NPFs are involved in the maturation of the cortical actin patch and they co-localize with the Arp2/3 complex at the cortical actin patches. However, the NPFs arrive at the cortical patches at different stages in the maturation of the actin patch (Sun et al., 2006), suggesting that the NPFs possibly perform both distinct and overlapping functions.

1.15 Actin cytoskeleton and endocytosis in yeast

The earliest indication that actin is involved in endocytosis in yeast was provided by the observation that temperature-sensitive actin mutants were unable to carry out fluid phase endocytosis (Kubler et al., 1993). The endocytic process can be divided into the following steps, coated pit formation, membrane invagination and sequestration, detachment of the newly formed vesicle and the movement of the vesicle away from the plasma membrane and into the cytoplasm (Qualmann et al., 2000). Each of these steps could involve the motor activity of myosins using the actin cytoskeleton as tracks (Riezman et al., 1996). The specific role played by actin in endocytosis is not clear. It is

suggested that the actin cytoskeleton could invaginate the plasma membrane by inducing a curvature on the cytosolic side of the membrane which would in turn enable the nascent vesicle to pinch off from the plasma membrane (Schmidt et al., 1997). It is also possible that the cortical cytoskeleton needs to be removed from the plasma membrane in order to make the plasma membrane less rigid and thus assist in endocytosis (Trifaro et al., 1993). It has been reported that treatment of cells with jasplakinolide (a drug which stabilizes F-actin) inhibits endocytosis by accumulating putative vesicles at the plasma membrane (Ayscough, 2000), suggesting that the actin cytoskeleton is involved in the release of nascent vesicles from the plasma membrane. It was also shown recently that FM4-64 labeled endosomes in *S. cerevisiae* co-localize with actin patches labeled with Abp1p-GFP both during the assembly of the actin patches at the bud cortex and also during the movement of the patch away from the bud and towards the mother cell (Huckaba et al., 2004; Toshima et al., 2006). Therefore, cortical actin patches could be putative sites of internalization during endocytosis in yeast cells.

1.16 The *Saccharomyces cerevisiae* WASP homologue, Las17p

Las17p, the *S. cerevisiae* homologue of WASP (Li, 1997) is one of the first proteins to be recruited to the cortical actin patches in yeast (Sun et al., 2006). The domain organization of Las17p is similar to that of mammalian WASP. Las17p exhibits a modular structure with a WASP Homology 1 domain (WH1) at the amino terminal, the WH2 (WASP Homology 2 domain) and an acidic domain (A) at the carboxyl terminal part of the protein (Fig. 1.10). A key difference between WASP and Las17p is the lack of a GTPase binding domain (GBD) in Las17p. Cdc42p regulates the localization of Las17p to regions

of polarized cell growth (Lechler et al., 2001), although unlike WASP, Las17p does not bind directly to Cdc42p.

The WH1 domain of Las17p interacts with the C-terminal region of the yeast WIP, Vrp1p in a manner analogous with the interaction between WASP and the WASP Interacting protein (WIP) (Naqvi et al., 1998; Ramesh et al., 1997). The proline rich regions of Las17p interact with the SH3 domains of Bzz1p, Hof1p, Myo3p, Myo5p and Rvs167p (Soulard et al., 2002; Naqvi et al., 2001; Evangelista et al., 2000; Lechler et al., 2000; Colwill et al., 1999). The VCA domain of Las17p interacts with G-actin and also binds and activates the Arp2/3 complex (Rodal et al., 2003).

Figure 1.10 Domain organization of Las17p.

Vrp1p interacts with the WH1 domain of Las17p. SH3 domain containing proteins like Myo3p, Myo5p and Rvs167p interact with the proline rich motifs. G-actin and the Arp2/3 complex interact with the VCA domain of Las17p.

A purified fragment encompassing only the VCA domain of Las17p is able to activate the *S. cerevisiae* Arp2/3 complex albeit to a lesser degree compared to the full-length Las17p (Rodal et al., 2003). Moreover, genetic studies showed that complete deletion of LAS17 causes severe defects than the deletion of only the VCA domain of Las17p (Winter et al., 1999). These results suggest that the activity of full length Las17p in activating the

Arp2/3 complex is not only due to its VCA domain but also due to additional regions of Las17p. It is possible that the N-terminal region of Las17p contributes to the activation of the Arp2/3 complex through the activity of other cytoskeletal proteins (Vrp1p, Myo3p, Myo5p) that bind to the N-terminal region of Las17p (Naqvi et al., 1998; Evangelista et al. 2000; Lechler et al., 2000). Deletion of the C-terminal region of Las17p encompassing the acidic domain results in fragmentation of the vacuoles in the strain (Eitzen et al., 2002). The motility of vacuoles or endosomes during endocytosis also requires actin polymerization induced by Las17p (Chang et al., 2003 and 2005). The involvement of Las17p in endocytosis was further substantiated by the finding that the fusion of vacuoles was inhibited in presence of antibodies raised against Las17p (Eitzen et al., 2002). It was shown recently that Vrp1p is recruited to the cortical patches through Las17p and Vrp1p in turn recruits type I myosins to the cortical patches (Sun et al., 2006). Type I myosins like Myo3p and Myo5p are un-conventional myosins possessing a C-terminal Arp2/3 complex activating CA domain (Evangelista et al. 2000; Lechler et al., 2000). Both Myo3p and Myo5p are functionally redundant and are involved in endocytosis and vesicle transport (Mooseker et al., 1995). The acidic domain (CA) of Las17p, Myo3p and Myo5p are functionally redundant as deleting the acidic domain of any two of the three proteins has no effects on the yeast cells but deleting the acidic domain of all the three proteins arrests the growth of the cells at 37°C (Evangelista et al., 2000; Lechler et al., 2000). It has been shown recently that Myo5p and Vrp1p function as a complex in activating the Arp2/3 complex (Sun et al., 2006).

The lack of a GBD domain in Las17p and the observation that purified full-length Las17p is able to promote **actin** polymerization in *vitro* even in the absence of Cdc42p (Rodal et al., 2003), suggests that Las17p is constitutively active. It was shown that the activity of Las17p was inhibited but not abolished by Las17p ligands like Bbc1p (Rodal et al., 2003), suggesting that Las17p is regulated by trans-inhibition rather than by intra-molecular inhibition as is observed in mammalian WASP.

1.17 Vrplp

Vrplp is a proline rich protein related to mammalian WIP (Ramesh et al., 1997). Vrplp colocalizes with **actin** patches and plays a similar role as Las17p in the distribution of cortical **actin** patches. Additionally, Vrplp is also involved in regulating endocytosis in *S. cerevisiae* (Donnelly et al., 1993; Munn et al., 1995; Vaduva et al., 1997). Deletion or mutation in the gene encoding Vrplp leads to depolarization of the cortical **actin** patches and loss of endocytosis and also abolishes cell growth at 37°C (Donnelly et al., 1993; Munn et al., 1995; Vaduva et al., 1997). Interestingly, although Las17p is involved in recruiting Vrplp to the cortical **actin** patches, cortical patches containing Las17p are depolarized in *vrp1*Δ cells (Lechler et al., 2001). It is possible that the **actin** patches are depolarized in *vrp1*Δ cells due to the endocytosis defect in the strain (Valdez-Taubaz and Pelham, 2003). Vrplp is also required for proper vacuolar morphology and is also involved in the fusion of vacuoles (Eitzen et al., 2002). Vrplp has a domain organization similar to that of mammalian WIP. Vrplp interacts with G-**actin** through the V domain located at the N-terminal region of the protein and also through two additional **actin** binding domains located at the regions 270-364 and 460-490 in the protein (Fig. 1.11)

(Thanabalu et al., 2007). The N-terminal region of Vrplp also has the Hoflp trap domain (HOT) which interacts with SH3 domain containing protein Hoflp (Ren et al., 2005). The C-terminal region of Vrplp interacts with Las17p through the Las17p Binding Domain (LBD) (Naqvi et al., 1998).

Both the N-terminal and the C-terminal halves of Vrplp show some redundancy in activity. Expression of either the N-terminal or the C-terminal fragment is able to rescue the growth defects of the *vrplΔ* mutant cells. However, only the C-terminal fragment of Vrplp is capable of restoring the polarization of the **actin** patches in the *vrplΔ* cells (Thanabalu et al., 2001). Both the N-terminal and the C-terminal fragments of Vrplp are capable of independently binding with type I myosins (Evangelista et al., 2000). It has been shown that Vrplp is essential for the polarized distribution of Las17p to the cortical patches (Anderson et al., 1998; Lechler et al., 2001), suggesting that Vrplp plays an important role in Las17p-mediated activation of the Arp2/3 complex.

Figure 1.11 Domain organization of Vrplp.

Vrplp has the G-actin binding V domain and the Hoflp binding HOT domain (H) at the N-terminus.

Two additional actin binding domains (VH2N and VH2C) are also located one each at the N-terminus and the C-terminus, respectively. The C-terminal fragment of Vrplp also has the Las17p binding domain (LBD).

1.18 Myosins in *S. cerevisiae*

The myosins are a super-family of motor proteins that generate directed movement along actin filaments in an ATP-dependent manner (Mooseker et al., 1993; Williams et al., 1994). Based on sequence similarity, the myosin family can be divided into 11 classes (Mooseker et al., 1995). Of the 11 classes of myosins, *S. cerevisiae* has 3 classes of myosins: type I myosins (**Myo3p, Myo5p**), type II myosins (**Myo1p**) and type V myosins (**Myo2p, Myo4p**) (Watts et al., 1987; Johnston et al., 1991; Haarer et al., 1994; **Goodson** et al., 1995; **Goodson** et al., 1996). The type I myosins localize to the cortical actin patches and are involved in the activation of the **Arp2/3** complex and regulate endocytosis (**Geli** and Riezman, 1996; Sun et al., 2006). The type II myosins localize to the bud neck and play a role in cytokinesis (Bi et al., 1998; Lippincott et al., 1998) similar to type II myosins in fission yeast (**Naqvi** et al., 1999). Lastly, the type V myosins accumulate at the bud tip and are involved in cargo transport along actin cables (Lillie et al., 1994; Schott et al., 2002; **Hwang** et al., 2003). Among the two type I myosins in yeast, **Myo3p** was the first to be identified (**Goodson** et al., 1995; **Geli** and Riezman, 1996).

Figure 1.12 Domain organization of Myo3p/Myo5p (The numbers indicate location of domains in Myo5p).

The N-terminus of type I myosins regulates the motor activity through the motor head domain. At the C-terminus, the TH1 and TH2 domain bind with cellular membrane and F-actin respectively. The SH3 domain binds with proline rich proteins like Las17p and Vrp1p. The CA domain binds and activates the Arp2/3 complex.

The type I myosins contain a motor head domain at the N-terminus whereas the C-terminal region contains the neck region (N) followed by the TH1 domain, TH2 domain, SH3 domain and the acidic domain (CA) (Fig. 1.12). It has been shown that the motor head domain of myosins interacts with **actin** and is capable of molecular motion through the hydrolysis of ATP (Adams and Pollard, 1986, Sun et al., 2006). The motor domain of the type I myosins also contains a phosphorylation site (TEDS) which regulates the motor activity of the protein as the phosphorylation of the TEDS site leads to the activation of the ATPase activity of the motor domain (Barylko et al., 2000). The physiological significance of the TEDS site was indicated by the finding that only the expression of wild type Myo3p but not the TEDS mutant Myo3p^{S357A} was able to rescue the budding defects in *myo3Δ myo5Δ* cells (Wu et al., 1997). The neck region (N) is made up of α -helical structures and binds with Calmodulin (Geli et al., 1998; Sun et al., 2006). The motor activity of myosins can be inhibited by Ca²⁺ mediated release of Calmodulin from the neck region (N) (Collins et al., 1990). The TH1 domain of type I myosins has been shown to mediate the binding of the protein to cellular membranes (Lee et al., 2000). The TH2 domain of type I myosins has been shown to bind with F-actin (Rosenfeld and Rener, 1994) while the SH3 domain has been shown to bind directly with proline rich proteins like Las17p and Vrp1p (Evangelista et al., 2000; Lechler et al., 2000). The C-terminal region of Myo5p (encompassing the CA domain) is able to promote **actin** polymerization in the presence of yeast cytosol (Geli et al., 2000) and the deletion of the CA domain prevents Myo5p induced Arp2/3 dependent actin polymerization *in vitro* (Sun et al., 2006).

Myo3p and Myo5p share 86% identity in the amino acid sequence at motor domain and 62% identity in the rest of the molecule (Brown, 1997). Deletion of either MYO3 or MYO5 does not cause any obvious phenotype for growth in *S. cerevisiae* cells but deletion of both the type II myosins is lethal to the cells (Geli et al., 1996; Goodson et al., 1996), suggesting redundancy of function. Even though the type I myosins are classified under the Class I category of NPFs, they lack a G-actin binding domain (Mooseker et al., 1995). The SH3 domains of type I myosins interact with proline rich proteins like Vrplp and Las17p (Geli et al, 2000; Evangelista et al., 2000). It was shown that a C-terminal fragment of Myo5p encompassing the SH3 domain is capable of promoting actin polymerization *in vitro* (Geli et al., 2000). Introducing mutations in the C-terminal fragment of Myo5p that abolish interaction with Vrplp also disrupt the ability of the fragment to promote actin polymerization *in vitro* (Geli et al., 2000). Therefore, though the type I myosins lack a G-actin binding domain, they could possibly promote actin polymerization through their binding with other proteins such as Las17p which possess a G-actin binding domain. These findings suggest that the VCA domain at the C-terminal region of Las17p possibly has a similar role with that of the WH2 domain of Vrplp and the acidic domain (A) of Myo3p and Myo5p. Based on Yeast Two Hybrid data for interaction between proline rich peptides and the C-terminal region of Myo3p and Myo5p, it has been suggested that Las17p has 4 potential Myo3p/Myo5p binding sites while Vrplp has 22 Myo3p/Myo5p binding sites. The presence of three actin binding sites in Vrplp as compared to one actin binding site in Las17p (Thanabalu et al., 2007) and the possibility that Vrplp has 5 times as many Myo3p/Myo5p binding sites as compared to

Las17p (Evangelista et al., 2000) suggests that Vrp1p is possibly more efficient than Las17p in promoting actin polymerization mediated by type I myosins.

1.19 Aims of the project

Wiskott Aldrich Syndrome (WAS) is a rare but fatal disease and the patients suffering from WAS have a high mortality rate unless they receive bone marrow transplantation. Recent studies have shown that the average age of survival for WAS patients is around 15 years. Since the disease is caused by mutation in the Wiskott Aldrich Syndrome protein (WASP) which is predominantly of hematopoietic origin, most of the research on WASP was done by employing hematopoietic cells. However, performing large scale deletion or mutational analysis in mammalian systems is an expensive and time consuming process. Many mammalian proteins have orthologs in yeast cells which makes *S. cerevisiae* an attractive model system to study mammalian proteins, primarily because of the ease with which yeast cells can be propagated and the availability of powerful molecular biology tools to manipulate the cells. In contrast to five known members of the WASP family in mammals, *S. cerevisiae* expresses only one homologue of WASP, Las17p. Deletion of LAS17 renders the cells defective in growth and other cellular processes dependent on the actin cytoskeleton (Li, 1997; Madania et al., 1999). Therefore, human WASP can be expressed in the *las17Δ* cells to examine the suppression of the phenotype of the mutant yeast strain. However, there are also potential limitations of using a unicellular organism like *S. cerevisiae* as a model organism especially because it lacks some important features of multicellular organisms like immune system, tissues and organs. *S. cerevisiae* undergo a form of development unique to fungi and only require

cell-to-cell communication for mating. Additionally, yeast cells have closed mitosis and divide asymmetrically by budding unlike human cells.

The first aim of the present project was to develop a system to study the activity of WASP in *S. cerevisiae*. Using this system, we want to address the following questions: 1) Does expression of human WASP rescue the defects of *S. cerevisiae las17Δ* cells? 2) Determine if additional proteins (mammalian or yeast) are necessary for the activity of WASP in suppressing defects of the *las17Δ* cells. 3) Determine the role of the WASP interacting protein (WIP) in regulating WASP activity. 4) Characterize the missense mutations causing Wiskott Aldrich Syndrome (WAS), X-linked thrombocytopenia (XLT) and X-linked neutropenia (XLN) using *las17Δ* cells. Establishing such a model would enable us to characterize the domains of WASP and facilitate screening for potential drugs in the treatment of WAS. The second aim of this study was to examine the activity of the N-terminal and the C-terminal regions of Las17p in complementing the deficiency of the full length protein in *las17Δ* cells. There is an abundance of literature which describes the function of WASP in hematopoietic cells and the activity of its domains. In contrast to the studies done on WASP, there has been no systematic functional analysis on the domains of Las17p. In this project, systematic deletions were carried out on the domains of Las17p and the activity of the recombinants was studied in *las17Δ* cells. The interaction of Las17p with other cytoskeletal proteins and the importance of these interactions for the activity of Las17p were also studied. The findings from these experiments could be extended to human WASP and would thus enable a better understanding of the functions of the domains of human WASP.

2 Materials and Methods

2.1 Materials

2.1.1 *Saccharomyces cerevisiae* strains

The *Saccharomyces cerevisiae* strains used in this study are listed below.

Strain	Genotype	Reference
IDY19	<i>MATa his3 leu2 ura3 trp1 mfa2Δ::FUS1:lacZ</i>	Naqvi <i>et al.</i> , 1998
IDY166	<i>MATa his3 leu2 ura3 trp1 las17Δ::URA3</i>	Naqvi <i>et al.</i> , 1998
IDY223	<i>MATa his3 leu2 ura3 trp1 las17Δ::LEU2</i>	Naqvi <i>et al.</i> , 1998
AMY88	<i>MATa his4 leu2 ura3 lys2 vrp1Δ::KanMx bar1</i>	Thanabalu. T. <i>et al.</i> , 2001
PJ69-4A	<i>MATa his3 leu2 ura3 trp1 gal4Δ gal80Δ met2::GAL7-lacZ GAL2-ADE2 LYS2::GAL1-HIS3</i>	James <i>et al.</i> , 1996

2.1.2 *Escherichia coli* strain

The *Escherichia coli* strain DH5α was used for amplifying plasmids and in DNA subcloning experiments.

DH5α	F'/endA1 hsdR17(r _k -m _k +) supE44 thi-1 recA1 gyrA (Nal _r)relA1 !(lacZYA-argF) _{U169} (Φ80lacZ!M15)	(Hanahan, 1983)
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2.1.3 Commercial Vectors

The vector pUC19 was used in DNA subcloning experiments. The vectors pACT2 and pAS2-1 which express the Gal4p Activation domain and the Gal4p Binding domain respectively were used in Yeast Two Hybrid assays. The YCplac and YEplac (Gietz *et al.*, 1988) series of plasmids were used for protein expression in *Saccharomyces cerevisiae* strains for functional studies.

Vector	Resistance	Selective marker	Expression Host
pUC19	Amp	-	<i>E. coli</i>
pACT2	Amp	Leu	<i>S. cerevisiae/E. coli</i>
pAS2-1	Amp	Trp	<i>S. cerevisiae/E. coli</i>
YCplac22	Amp	Trp	<i>S. cerevisiae/E. coli</i>
YCplac33	Amp	Ura	<i>S. cerevisiae/E. coli</i>
YCplac111	Amp	Leu	<i>S. cerevisiae/E. coli</i>
YEplac112	Amp	Trp	<i>S. cerevisiae/E. coli</i>
YEplac195	Amp	Ura	<i>S. cerevisiae/E. coli</i>
YEplac181	Amp	Leu	<i>S. cerevisiae/E. coli</i>

2.1.4 Plasmids

The plasmid constructs used in this study are listed in Appendix 1. Human WASP and Las17p were placed under the transcriptional regulation of the VRP1 promoter and the LAS17 promoter respectively, for expression in yeast cells.

2.1.5 Antibodies

The antibodies used in this study are listed below and the dilutions used for the Western blot experiments are specified. The secondary antibodies are conjugated with Horseradish peroxidase (HRP).

2.1.5.1 Primary antibodies

Antibody	Dilution	Company
Rabbit polyclonal anti-GFP	1:5000	Molecular Probes (Eugene, OR, USA)
Mouse polyclonal anti-GFP	1:1000	This study
Rabbit polyclonal anti-GST	1:1000	Molecular Probes(Eugene, OR, USA)
Mouse polyclonal anti-GST	1:1000	This study

Rabbit polyclonal anti-HisT	1:1000	Molecular Probes(Eugene, OR, USA)
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2.1.5.2 Secondary antibodies

Antibody	Dilution	Company
Mouse anti-rabbit IgG	1:5000	Sigma (St. Louis, Mo, USA)
Rabbit anti-mouse IgG	1:5000	Sigma (St. Louis, Mo, USA)

2.1.6 Enzymes and kits used for molecular biology

The restriction endonucleases used in subcloning experiments were purchased from New England Biolabs (NEB) (Ipswich, MA, USA), Fermentas (Hanover, MD, USA) or Roche (Indianapolis, IN, USA). The ~~pfu~~ DNA polymerase from Stratagene (La Jolla, CA, USA) and the DNA polymerase from BIOTOOLS (Madrid, Spain) were used for PCR amplification. The T4 DNA ligase was from Roche (Indianapolis, IN, USA). Site directed mutagenesis kit was purchased from Stratagene (La Jolla, CA, USA). Plasmid DNA was isolated from E. coli cells by using QIAprep Spin Miniprep Kit (Qiagen) (Valencia, CA, USA) and the QIAquick Gel extraction Kit (Qiagen) (Valencia, CA, USA) was used for DNA extraction from Agarose gels. The QIAquick PCR Purification Kit (Qiagen) (Valencia, CA, USA) was used for the purification of PCR fragments.

2.1.7 Chemicals and reagents

Unless otherwise stated, all the chemicals were of analytical grade and purchased from Sigma Aldrich Chemical Company (St. Louis, MO, USA). DNA marker ladder was from NEB (Ipswich, MA, USA). The dNTP (dTTP, dATP, dGTP and dCTP) mixtures were

purchased from Invitrogen (Carlsbad, CA, USA). Protein molecular weight marker was from Bio-Rad Laboratories (Hercules, CA, USA). Phenylmethylsulfonyl fluoride (PMSF), 1, 4-Dithiothreitol (DTT) and protease inhibitors complete-mini protease tablets were from Roche (Indianapolis, IN, USA). The X-gal (1-Bromo-4-Chloro-3-indolyl-b-D-galactopyranoside) was from BioRad (Hercules, CA, USA). Isopropyl-Thio-B-D-galactopyranoside (IPTG) was from Promega (Madison, WI, USA). Ampicillin was purchased from Sigma-Aldrich (St. Louis, MO, USA). AbI PRISM DNA sequencing kit (Big Dye) was purchased from Applied Biosystems (Foster City, CA, USA). Ni^{2+} -NTA resin and GST sepharose were purchased from Amersham Biosciences (Buckinghamshire, UK). The Alexa-488-conjugated phalloidin was from Molecular Probes (Eugene, OR, USA). Lucifer Yellow dye was from Fluka. Nitrocellulose membrane was from Bio-Rad (Hercules, CA, USA).

2.1.8 Buffers and solutions

2.1.8.1 TAE (Tris-acetate-EDTA, 50X)

A 50X stock solution was prepared by dissolving 242 g of Tris base, 57.1 ml of glacial acetic acid and 100ml of 0.5 M EDTA (pH 8.0) in 1000 ml of dd H_2O .

2.1.8.2 Agarose gel

One percent agarose was made by suspending 10 g of agarose in 1000 ml of 1X TAE buffer and microwaved for 10 min. The agarose solution was maintained in liquid form at 60°C by storing in an oven.

2.1.8.3 DNA loading buffer

DNA loading buffer (6X) was made by adding bromophenol blue (0.25%), xylene cyanol FF (0.25%) and sucrose (40%) in dd H₂O. The solution was stored at 4°C.

2.1.8.4 10% SDS

Ten grams of SDS was dissolved in 100 ml of dd H₂O. Do not cool the solution.

2.1.8.5 5X Tris-glycine electrophoresis buffer

Ninety four grams of glycine, 15.1 g of Tris and 50 ml of 10% SDS stock solution were dissolved in 1000 ml of dd H₂O, the pH of the solution was adjusted to 8.3.

2.1.8.6 2X SDS-PAGE gel-loading buffer

SDS-PAGE gel-loading buffer (2X) had 100 mM Tris-Cl (pH 6.8), 200 mM dithiothreitol, 4% SDS, 0.2% Bromophenol blue and 20% glycerol. The solution was stored at 4°C.

2.1.8.7 10% Ammonium persulphate

One gram of ammonium persulphate was dissolved in 10 ml of dd H₂O and the solution was stored at 4°C.

2.1.8.8 SDS PAGE gel destaining solution

Methanol, dd H₂O and glacial acetic acid were mixed in the ratio of 450:450:100 to make the destaining solution.

2.1.8.9 SDS PAGE gel staining solution

Staining solution was made by dissolving 0.25 g of Brilliant Blue R-250 in 100 ml of destaining solution.

2.1.8.10 Membrane transfer buffer

3.03 g of Tris, 14.4 g of glycine and 200 ml of methanol were dissolved in 800 ml of dd H₂O.

2.1.8.11 1X Tris buffered saline (TBS)

TBS had 20 mM Tris and 500 mM NaCl, the pH was adjusted to 7.5.

2.1.8.12 Wash solution

5 ml of 10% Triton X-100 solution was added to 995 ml of 1X Phosphate buffered saline.

2.1.8.13 Blocking solution

0.5 g of non-fat dry milk was dissolved in 10 ml of 1X Phosphate buffered saline and stored at 4 °C until further use.

2.1.8.14 1M Tris buffer

121.12 g of Tris was dissolved in 800 ml of dd H₂O and the pH of the solution was adjusted to 7.5 or 8.0 using HCl and then the volume was made up to 1000 ml with dd H₂O.

2.1.8.15 5M NaCl

NaCl (5M) was prepared by dissolving 292 g of NaCl in 1000 ml of dd H₂O and the solution was autoclaved.

2.1.8.16 10X TE buffer

TE buffer had 0.1 M Tris-HCl and 10 mM EDTA, the pH was adjusted to 7.5 and autoclaved.

2.1.8.17 50% PEG 3350

Fifty grams of PEG 3350 was dissolved in 100 ml of sterile deionized H₂O. The solution was warmed in a microwave to enable the PEG 3350 to dissolve faster.

2.1.8.18 10X LiAc

One molar lithium acetate solution was prepared in dd H₂O, the pH adjusted to 7.5 and autoclaved.

2.1.8.19 Washing buffer for GST beads

One percent Triton X-100 in PBS.

2.1.8.20 Elution buffer for GST beads

Ten millimolar reduced glutathione in PBS.

2.1.8.21 Washing buffer for Ni-NTA beads

Twenty millimolar phosphate buffer (pH 7.2), 1M NaCl, 20mM imidazole and 5mM 2-mercaptoethanol.

2.1.8.22 Elution buffer for Ni-NTA beads

Twenty millimolar phosphate buffer (pH 6.0), 0.5M NaCl, 200mM imidazole and 5mM 2-mercaptoethanol.

2.1.8.23 Bacterial and Yeast culture media

2.1.8.23.1 Antibiotics stock

Ampicillin (1000X) was 100 mg/ml of Ampicillin in dd H₂O (100 µgm/ml in working solution).

2.1.8.23.2 Luria-Bertani (LB) medium

Ten grams of Bacto-Tryptone, 5g of Bacto-Yeast extract and 5 g of NaCl dissolved in 1000 ml of dd H₂O and autoclaved. To make LB agar, 20 g of Bacto-Agar was added to 1000 ml of liquid LB medium and autoclaved. Plates were then poured when the temperature of the medium had cooled to below 60°C.

2.1.8.23.3 YPUAD medium

Twenty grams of Bacto-Peptone, 10 g of Bacto-Yeast extract, 0.08 g of adenine hemisulphate and 0.04 g of uracil were dissolved in 1000 ml of dd H₂O and autoclaved. To make YPUAD agar, 20 g of Bacto-Agar was added to 1000 ml of liquid YPUAD

medium and autoclaved. Plates were then poured when the temperature of the medium had cooled down below 60°C.

2.1.8.23.4 SD medium

To make SD medium, 6.7 g of Yeast Nitrogen base, 20 g of Glucose and the different D/O supplements were dissolved in 1000 ml of dd H₂O and autoclaved. To make the SD media without Histidine for Yeast Two Hybrid strains, filter sterilized 3-AT was added to a final concentration of 2 mM. To make SD agar, 20 g of Bacto-Agar was added to 1000 ml of liquid SD medium and autoclaved. Plates were then poured when the temperature of the medium had cooled down below 60°C.

2.1.8.23.5 3-AT stock solution

One molar stock solution of 3-AT was prepared in dd H₂O and filter sterilized. The solution was stored at 4°C.

2.2 Methods

2.2.1 Growth and maintenance of *E. coli*

E. coli was cultured in LB broth or grown on LB agar plates and maintained at 37°C. *E. coli* strains harboring plasmids encoding an ampicillin selection marker were cultured in LB broth or grown on LB agar plates supplemented with ampicillin (100 µg/ml final concentration).

2.2.2 *E. coli* glycerol stock

E. coli strains intended for long term preservation were maintained in glycerol stock as described. Five ml LB media was inoculated with a single colony of the *E. coli* strain and cultured until mid log phase. An 800 µl aliquot of the bacterial culture was mixed with 200 µl of 80% glycerol and then transferred to a cryo-vial and stored at -80°C.

2.2.3 Preparation of *E. coli* competent cells (CaCl₂ method)

A bacterial colony was picked from LB plate and inoculated in 2 ml of LB medium and grown overnight at 37°C. The culture was diluted to an OD₆₀₀ of 0.05 in 50 ml of LB and incubated at 37°C to an OD₆₀₀ of 0.8. The culture was then transferred to sterile centrifuge Falcon tubes and stored in ice until further use. The cells were spun at 5500 rpm at 4°C for 5 minutes. The supernatant was discarded and the pellet was resuspended in 25 ml of ice cold sterile 100 mM MgCl₂. The cells were then spun at 5500 rpm for 10 minutes at 4°C. The supernatant was discarded and the cell pellet was resuspended in 5 ml of sterile ice cold 100 mM CaCl₂ for 2 hours to become transformation competent. 2 ml of sterile ice cold 50% glycerol was added to the cell suspension. The cells were later stored at -80 °C as 200µl aliquots in eppendorf tubes. The cells and the reagents were always maintained at 4 °C.

2.2.4 Transformation of DNA into *E. coli*

Twenty µl of competent cells were added into an eppendorf tube. The plasmid DNA (1 µl) was added to the competent cells and incubated on ice for 30 minutes. The tube was then incubated at 42°C in a water bath for 90 seconds and then immediately put on ice for 5

minutes. The cells were then plated on LB agar containing antibiotic and incubated at 37°C.

2.2.5 Isolation of plasmid DNA from *E. coli*

A single bacterial colony was inoculated in 3 ml of LB containing antibiotic and grown overnight at 37°C. The cells were spun at 5500 rpm at 4°C for 5 minutes. The plasmid DNA was isolated from the pellet according to the manual provided in the plasmid purification kit.

2.2.6 Growth and maintenance of *S. cerevisiae*

S. cerevisiae strains were cultured in YPUAD liquid culture or streaked on YPUAD agar plates maintained at 24°C. When necessary, strains harboring plasmids encoding a nutrient selection marker were grown in Synthetic Defined (SD) liquid media or SD agar plates. For the functional studies on proteins expressed in *S. cerevisiae*, the strains were streaked on two identical YPUAD agar plates, one plate was maintained at 24°C and the other plate was maintained at 37°C to examine the growth of the strain at restrictive temperatures.

2.2.7 Cryopreservation of *S. cerevisiae* strains

S. cerevisiae strains intended for long term preservation were maintained in glycerol stock. A single colony of the *S. cerevisiae* strain was streaked on YPUAD agar plate and incubated at room temperature for 3-4 days. Then using a plastic loop, a thick patch of

yeast cells was scooped from the culture plate and resuspended in 500 µl of YPUAD + 20% glycerol contained in a cryo-vial and stored at -80°C.

2.2.8 Transformation of yeast cells

Several colonies of yeast cells were inoculated in 25 ml of YPUAD and grown overnight at 24°C. The overnight culture was diluted to an OD₆₀₀ of 0.2 and the cells were grown until they had reached an OD₆₀₀ of 0.8. The culture was then transferred to 50 ml centrifuge tubes and spun at 1000X g for 5 minutes. The supernatant was discarded and the pellet was washed with 30 ml of dd H₂O and spun again at 1000X g for 5 minutes. The supernatant was discarded and the pellet was resuspended in 1 ml of dd H₂O and transferred to a 1.5 ml eppendorf tube. The cell suspension was spun at maximum speed in a micro-centrifuge for 15 seconds. The supernatant was discarded and the pellet was resuspended in 250 µl of 100 mM LiAc.

Fifty µl aliquots of cells was taken in eppendorf tubes and used for each transformation. The cells were spun at maximum speed in a micro-centrifuge for 15 seconds and the supernatant was discarded. To the pellet was added 240 µl of 50 % PEG, 36 µl of 1 M LiAc, 73 µl of dd H₂O, 5 µl of sonicated salmon sperm DNA and 1 µl of plasmid DNA. The transformation tubes were vortexed for 1 minute, incubated at 24°C for 30 minutes and heat shocked at 42°C for 20 minutes. The cells were then centrifuged at 7000 rpm for 15 seconds, the supernatant was discarded and the pellet was resuspended in 400 µl of dd H₂O. Hundred µl of cell suspension was plated on selective media plates and incubated at 24°C until the colonies grew large enough for analysis.

2.2.9 Yeast Two hybrid assay

S. cerevisiae strain PJ69-4A was grown in YPUAD liquid culture and prepared for transformation of bait plasmid pAS2-1 and prey plasmid pACT2 in which the proteins whose interactions are being checked are expressed as fusion constructs. The transformed cells were then grown on SD minimal media lacking Trp and Leu. Once the colonies had grown large enough, they were analyzed for growth on SD medium lacking Trp, Leu and His to check for interaction between the proteins.

2.2.10 Fluid-phase endocytosis assay

Yeast colonies were inoculated in 25 ml of YPUAD and incubated at 24°C in a shaking flask incubator. The next day, the overnight culture was diluted to an OD₆₀₀ of 0.1-0.2 and the cells were allowed to grow at 24°C until they had reached an OD₆₀₀ of 0.6-0.8. If the assay was to be done to examine the ability of the cells to carry out fluid phase endocytosis at 37°C, the cells were shifted to 37°C for 2 hours prior to proceeding to the next step of the protocol. A 1000 µl aliquot of the cell culture was transferred to a micro-centrifuge and the cells were centrifuged at 3000 rpm for 2 minutes. The supernatant was discarded and the cells were resuspended in 90 µl of fresh YPUAD liquid. 10 µl of Lucifer Yellow stock solution (50 mg/ml Lucifer yellow carbohydrazide, dilithium salt in H₂O) was added to the tube and gently flicked to mix the contents of the tube. The tube was aerated by puncturing holes on the lid of the eppendorf tube with the aid of a 19 gauge needle. Cells were incubated at 24°C or 37°C for 1 hour with occasionally flicking to aerate the sample. Endocytosis was stopped by adding 1 ml of ice cold wash buffer (50 mM Na₂H₂PO₄, 10 mM NaF, 10 mM NaN₃, pH 7.0) and the tube was immediately placed

on ice. Cells were then spun down at 3000 rpm for 2 minutes at room temperature. Supernatant was removed gently by pipetting and cells were resuspended in 1 ml of fresh ice cold wash buffer. The washing process was repeated 3 times or more to reduce the background of Lucifer yellow dye. Cells were finally suspended in 10 μ l of ice cold washing buffer and left on ice. One μ l of cell suspension was pipetted onto a clean microscope slide. A cover slip was immediately placed on the cell suspension and pressed gently and evenly. Cells on slides were observed using microscope under E4 filter (Lucifer yellow) to visualize Lucifer yellow staining and under Nomarski (differential interference contrast) filter to visualize the vacuole morphology.

During the entire course of the assay, due care was taken to handle the cells as gently as possible, especially while centrifuging the cells. Centrifuging the cells at speeds higher than the recommended speed of 3000 rpm would generate enormous shear forces capable of destroying the vacuolar morphology which would in turn affect the quality of the microscopic images obtained from the assay.

2.2.11 Quick F-actin staining of actin patches

Yeast colonies were inoculated in 25 ml of YPUAD and incubated at 24°C in a shaking flask incubator. The overnight culture was diluted to an OD₆₀₀ of 0.1-0.2 and the cells were allowed to grow at 24°C until they had reached an OD₆₀₀ of 0.6-0.8. If the assay was to be done to examine the ability of the cells to carry out fluid phase endocytosis at 37°C, the cells were shifted to 37°C for 2 hours prior to proceeding to the next step of the protocol. A 900 μ l aliquot of the cell culture was transferred to an eppendorf tube, the

cells were then fixed by the addition of 100 μ l of 37% formaldehyde and incubated for 5 minutes at room temperature. After fixation, the cells were spun at 5000 rpm for 2 minutes. The supernatant was discarded and the cells were washed with 1000 μ l of PBS. The cells were again spun at 5000 rpm for 2 minutes, the supernatant was discarded and the cells were permeabilized by the addition of 1000 μ l of 1% Triton X-100 and incubated for 15 minutes. After permeabilizing the cells, the cells were spun at 5000 rpm for 2 minutes and then washed thrice with PBS. After the washing process, the cells were suspended in 50 μ l of PBS containing 0.1 mg/ml of Alexa-488-conjugated phalloidin. After incubating the cells at room temperature for 30 minutes, the cells were washed thrice with PBS and then finally resuspended in 10 μ l of PBS. In order to prepare the sample for visualization under the microscope, 1 μ l of the sample was placed onto a glass slide and a cover slip was placed on top of the cells such that the sample was spread out evenly. The cells were then visualized in a microscope under the appropriate filter.

2.2.12 Genomic DNA extraction from yeast cells

Yeast colonies were inoculated in 25 ml of YPUAD and incubated at 24°C in a shaking flask incubator. The next day, the overnight culture was diluted to an OD₆₀₀ of 0.1-0.2 and the cells were allowed to grow at 24°C until they had reached an OD₆₀₀ of 0.6-0.8. A 50 ml culture of exponentially growing cells was transferred to a 50 ml falcon and the cells were spun at 5000 rpm for 5 minutes. The supernatant was discarded and the pellet was resuspended in 500 μ l of 1 M sorbitol and 100 μ l of 0.5 M EDTA. The cells suspension was transferred to an eppendorf tube, 50 μ l of 5-10 mg/ml yeast lytic enzyme was then added into the eppendorf tube and the tube was incubated at 37°C for 1 hour.

The tube was then spun at 14000 rpm for 30 seconds, the supernatant was discarded and the pellet was resuspended in 500 μ l of Tris-EDTA buffer (50 mM Tris, 20 mM EDTA, pH 7.5). 50 μ l of 10% SDS was then added to the cell suspension and the contents of the tube were thoroughly mixed. The tube was then incubated at 65°C for 30 minutes and after this, 300 μ l of 5 M potassium acetate was added into the same tube and incubated on ice for 1 hour. The tube was spun at 14000 rpm for 5 minutes, the supernatant was transferred to a new eppendorf tube and 1000 μ l of isopropanol was added and mixed. The tube was left on the bench for 5 minutes and then spun briefly. The supernatant was discarded and the pellet was washed with 1 ml of 70% ethanol. Pellet was collected by another spin for 5 minutes and air dried for half an hour (maximum). The air dried pellet was resuspended in 100 μ l of TE buffer (pH 7.5). 5 μ l of 10 mg/ml RNase was then added to the tube and the tube was incubated at 37°C for 1 hour and extracted with phenol twice. 30 μ l of DNA precipitation buffer (3 M Potassium, 5M Acetate, pH 5.8) was added to the tube and mixed, followed by the addition of 200 μ l of 100% ethanol. The tube was incubated on ice for 5 minutes and spun for 10 minutes at top speed. The supernatant was discarded and the pellet was washed with 1 ml of 70% ethanol, vortexed and spun at top speed for 5 minutes. The supernatant was discarded and pellet was briefly air dried before resuspending it in 100-300 μ l of TE buffer.

2.2.13 Extraction of total protein from yeast for western blot

Yeast colonies were inoculated in 25 ml of YPUAD and incubated at 24°C in a shaking flask incubator. The next day, the overnight culture was diluted to an OD₆₀₀ of 0.1-0.2 and the cells were allowed to grow at 24°C until they had reached an OD₆₀₀ of 0.6-0.8.

Cells corresponding to a total cell culture OD₆₀₀ of 7 units were harvested by spinning the cell culture at 5000 rpm for 5 minutes. The supernatant was discarded and the pellet was washed with 30 ml of sterile water. The cell suspension was then spun at 5000 rpm for 5 minutes and the supernatant was discarded. The pellet was resuspended in 1000 µl of ice cold 1X TE buffer and transferred to an eppendorf tube. The tube was then spun at 14000 rpm for 30 seconds and the supernatant was discarded. The pellet was resuspended in 240 µl mixture containing 1.85 M NaOH and 1.06 M β-mercaptoethanol. The tube was incubated on ice for 10 minutes and then 240 µl of 20% TCA was added to the contents of the tube and incubated on ice for 10 minutes. After the incubation period, the tube was centrifuged at 14000 rpm for 10 minutes and the supernatant was discarded. The pellet was washed with 1000 µl of ice cold acetone and the pellet was collected by spinning the tube at 14000 rpm for 10 minutes. The pellet was resuspended in 100 µl of Urea buffer (8 M Urea, 50 mM Tris, pH 6.8 and 2% SDS).

2.2.14 Lysis of yeast cells (Bead beater)

A 50 ml culture of exponentially growing yeast cells was transferred to a 50 ml falcon and then centrifuged at 5000 rpm for 5 minutes. The supernatant was discarded and the pellet was washed with 30 ml of sterile water. The cells were centrifuged again at 5000 rpm for 5 minutes and the supernatant was discarded. The pellet was resuspended in 1000 µl of sterile water and transferred to an eppendorf tube. Cells were pelleted at 14000 rpm for 30 seconds and the supernatant was discarded. The cell pellet was weighed to ensure that the wet weight never exceeded beyond 200 pg. The cell pellet was resuspended in 300 µl of lysis buffer (PBS) and Protease Inhibitor Cocktail (Sigma) was added to the cell

suspension at the ratio of 5 μ l of the cocktail for 100 μ g wet weight of the cell pellet. The contents of the eppendorf tube were briefly vortexed. Then the glass beads for cell lysis were added to the tube such that the volume of the glass beads never exceeded 500 μ l. The eppendorf tube was placed in the bead beater equipment and subjected to bead beating at maximum power for 4 times at 30 seconds intervals and in between each bead beating cycle, the tube was incubated in ice for 30 seconds. After lysing the cells, a small hole was made at the bottom of the eppendorf tube with the aid of a 19 gauge needle. The eppendorf tube was then placed on a fresh eppendorf tube and spun at 7000 rpm for 45 seconds to collect the cell lysate in the fresh eppendorf tube. The cell lysate was then spun at 14000 rpm for 45 minutes to pellet the cell debris and the supernatant was transferred to another eppendorf tube for analysis.

2.2.15 GST Pull down assay

In order to study the *in vivo* interaction between two proteins expressed in yeast, we used GST and GFP tagging strategy. The GFP fusion protein and the GST fusion protein of interest were expressed in the *S. cerevisiae* strain. The cells expressing the fusion proteins were inoculated in 25 ml of YPUAD and incubated at 24°C in a shaking flask incubator. The next day, the overnight culture was diluted to an OD₆₀₀ of 0.1-0.2 and the cells were allowed to grow at 24°C until they had reached an OD₆₀₀ of 0.6-0.8. Yeast cells were harvested from 100 ml of the cell culture by spinning the cells in a 50 ml falcon at 5000 rpm for 5 minutes. The supernatant was discarded and the pellet was washed with sterile water and the cell suspension was spun again at 5000 rpm for 5 minutes. The supernatant was discarded and the pellet was subjected to glass bead lysis to extract total protein

lysate (section 2.2.14). The GST beads were then prepared for the pull down assay by washing the GST beads once with sterile water and then followed by washing with PBS twice. The equilibrated GST beads were then added to the total protein lysate and the pull down was carried out by incubating the beads at 4°C for 2 hours. The beads were then spun at 14000 rpm for 5 minutes and the supernatant was discarded. The beads were subsequently washed 3 times with PBS containing 1% Triton X-100. Finally the proteins bound to the beads were eluted out by treating the beads with 100 µl of Urea buffer (8 M Urea, 50 mM Tris, pH 6.8 and 2% SDS). 10 µl of the eluted protein was analyzed by SDS PAGE electrophoresis and then subjected to western blotting.

2.2.16 His tag pull down assay

His tag pull down assay was also employed to examine the interaction between two proteins expressed in yeast cells. The two proteins of interest were tagged alternatively, either with a GFP tag or a His-tag and the tagged proteins were co-expressed in the *S. cerevisiae* strain. The yeast transformants were inoculated in 25 ml of YPUAD and incubated at 24°C in a shaking flask incubator. The overnight culture was diluted to an OD₆₀₀ of 0.1-0.2 and the cells were allowed to grow at 24°C until they had reached an OD₆₀₀ of 0.6-0.8. Yeast cells were harvested from 100 ml of the cell culture by spinning the cells in a 50 ml centrifuge tube at 5000 rpm for 5 minutes. The supernatant was discarded and the pellet was washed with sterile water and the cell suspension was spun again at 5000 rpm for 5 minutes. The supernatant was discarded and the pellet was subjected to glass bead lysis to obtain the total protein lysate (section 2.2.14). The Ni-NTA agarose beads were then prepared for the pull down assay by washing the beads

thrice with PBS. The pull down was then carried out by adding the beads to the total protein lysate and the beads were incubated at 4°C for 2 hours. The beads were then spun at 14000 rpm for 5 minutes and the supernatant was discarded. The beads were subsequently washed 3 times with wash buffer and finally the proteins bound to the beads were eluted out by treating the beads with 100 µl of Urea buffer (8 M Urea, 50 mM Tris, pH 6.8 and 2% SDS). Ten µl of the eluted protein was analyzed by SDS PAGE electrophoresis and then subjected to western blotting.

2.2.17 Agarose gel electrophoresis (DNA)

One percent agarose gel containing 0.5 µg/ml of EB was cast. After the gel had set, it was soaked in 1X TAE buffer and the samples were loaded in the different lanes. The samples were resolved by subjecting the gel to electrophoresis at 100 V. The process was stopped when the dye front had traveled halfway through the gel. The resolved DNA samples were then visualized under UV light.

2.2.18 Extraction of DNA from the agarose gel

The DNA sample resolved in the agarose gel was first visualized under UV light and the band of interest was excised out with a scalpel and placed in an eppendorf tube. The DNA was extracted according to the manual provided in the plasmid purification kit.

2.2.19 Construction of recombinant plasmids

Purified DNA fragments were digested with restriction enzymes and the reaction tube was prepared according to the instructions supplied by the manufacturer. All the

digestions were performed at 37°C for 2-3 hours and in the case of restriction enzymes having star activity like *Bam*HI and *Eco*RI, the restriction enzyme digestion was carried out for a maximum duration of 2 hours. The products of the restriction enzyme digestion were resolved by agarose gel electrophoresis and the DNA fragments of interest were excised from the agarose gel and purified. Around 2 µl of each of the purified DNA fragment was subjected to agarose gel electrophoresis in order to verify the presence of sufficient quantities of DNA fragment enough for the ligation reaction.

Purified insert DNA was ligated with linearized vector DNA by using T4 DNA ligase and the ligation reaction mixture was made according to the instructions supplied by the manufacturer. The ligation reaction mixture was incubated at 24°C for 4 hours and then introduced into competent *E. coli* cells by following the transformation protocol for *E. coli*.

2.2.20 Verification of recombinant plasmid constructs

2.2.20.1 Restriction digestion

The presence of the recombinant plasmid clone was determined through restriction digestion by employing restriction enzymes that target unique sites on the plasmid at regions flanking the cloned DNA insert. The products of the restriction digestion reaction were analyzed by agarose gel electrophoresis to confirm the size of the expected DNA fragments.

2.2.20.2 Colony screening PCR

Colony screening PCR is a convenient method for examining the presence of the recombinant clone without having to first extract the plasmids from the bacterial strains. Single colonies of bacterial cells growing on LB (ampicillin) agar plates were inoculated in 5 ml of LB (ampicillin) liquid and incubated overnight at 37°C. 5 µl of the bacterial cell culture was used as a template in a PCR amplification reaction by employing primers that are able to hybridize only to regions on the cloned DNA insert and not on the vector backbone. The products of the PCR amplification reaction were then subjected to agarose gel electrophoresis and a PCR band of the correct size can be visualized only for those bacterial colony templates harboring recombinant plasmids.

2.2.21 Polymerase chain reaction (PCR)

The PCR reaction mixture contained the following, 5 µl of 10X reaction buffer, 1 µl of 10 µM forward primer, 1 µl of 10 µM reverse primer, 0.5 µl of template DNA, 0.5 µl of 1 U Taq DNA polymerase and dd H₂O to make up the volume of the mixture to 50 µl. The reaction mixture was made in thin-wall 0.2 ml PCR tube and the reaction was performed as follows: 95 °C for 3 min, 95 °C for 50 seconds, 50 °C for 50 seconds, 72 °C for duration depending on the length of the fragment being amplified (1 min/ 1 k bases). The PCR reaction was cycled 25 times for optimal amplification of the target fragment. The primers used in this study are listed in Appendix 2A and 2B.

2.2.22 Site-directed mutagenesis (SDM)

Mutations of interest were generated at specific locations in the target DNA sequence by employing the QuikChange Site-Directed Mutagenesis kit from Stratagene (La Jolla, CA, USA) or by employing the Overlap Extension technique.

2.2.22.1 SDM kit

Site-directed mutagenesis (SDM) was performed according to the instructions provided in the manual of the SDM kit. After performing the PCR for mutagenesis, the PCR product was transformed into competent cells and plated onto LB plate containing ampicillin. The plasmid DNA from different clones was extracted and sequenced to identify the clone bearing the mutation of interest.

2.2.22.2 Overlap extension PCR

This technique was carried out as described (Higuchi et al., 1988; Fig. 2.1). In this method of mutagenesis, the DNA fragment which encompasses the target region that needs to be mutagenised was amplified by two separate PCR amplification reactions. Each PCR reaction was carried out using one flanking primer (A or D) that hybridizes at one end of the target DNA fragment and one internal primer (B or C) that hybridizes at the site of mutation and contains the mismatched bases. The DNA fragments amplified by the two PCR reactions, AB and CD were purified. The third PCR reaction was then carried out by using the DNA fragments AB and CD as template and by employing the flanking primers A and D. The DNA fragment amplified by the third PCR reaction would bear the mutation of interest and was subsequently subcloned and sequenced.

Figure 2.1 Schematic representation of site-directed mutagenesis by overlap extension PCR method.
A,D: Flanking primers; B,C: Internal mutagenesis primers

2.2.23 DNA quantification

Purified DNA was diluted with TE buffer or water and the OD_{260} was measured with the spectrophotometer. DNA concentration was calculated using the following formula:

$$\text{DNA concentration } (\mu\text{g}/\mu\text{l}) = OD_{260} \times \text{Dilution factor} \times A/1000;$$

A=50 $\mu\text{g}/\text{ml}$ if double stranded DNA or plasmid,

A=33 $\mu\text{g}/\text{ml}$ if single stranded DNA,

A=40 $\mu\text{g}/\text{ml}$ if single stranded RNA.

The ratio OD_{260}/OD_{280} was used to assess the purity of samples.

OD_{260}/OD_{280} between 1.8 and 2.0 indicates the absorption due to nucleic acids.

OD_{260}/OD_{280} less than 1.8 indicates impurity caused by proteins and/or other matter capable of absorbing UV light.

OD_{260}/OD_{280} higher than 2.0 indicates possibility of RNA contamination in the DNA sample.

2.2.24 DNA sequencing

The fidelity of the DNA fragments cloned into vectors after PCR amplification was verified by DNA sequencing. The sequencing reaction was carried out in a 200 μ l PCR tube as follows. 2.5 μ l of purified plasmid was added to the sequencing reaction mixture containing 8 μ l of BigDye terminator, 0.7 μ l of 10 mM sequencing primer, 0.5 μ l of DMSO and 8.3 μ l of water. The contents of the PCR tube were gently vortexed, spun down and the PCR reaction was carried out as follows.

95°C for 5 minutes,

96°C for 10 seconds, 50°C for 5 seconds, 60°C for 4 minutes, 30 cycles.

The products of the sequencing PCR reaction were analyzed on ABI Genetic Analyzer 3100.

2.2.25 SDS-PAGE electrophoresis

The protein samples were resolved by SDS-PAGE electrophoresis. 10-12% separation gel was used depending on the size of the protein sample. The sample was first mixed with 2X loading buffer and heated at 90-100 °C for about 2-3 min. Around 10 μ l of the

protein sample was loaded in each lane and the electrophoresis was carried out at constant current of 30 mA until the tracking dye front had migrated to the end of the gel. The gel was further processed either for staining of the proteins or for Western Blot analysis

2.2.26 Western Blot analysis

Western Blot analysis was used for the detection of specific proteins expressed as GFP fusion constructs. The SDS-PAGE gel in which the proteins had been resolved was directly used for electroblotting. The nitrocellulose membrane, gel and filter papers were soaked in the transfer buffer for about 5 minutes. The nitrocellulose membrane and the protein gel were overlaid on each other and sandwiched between 4 filter papers on each side and placed in the transfer cassette. The transfer cassette was then placed inside the transfer cell in the correct orientation such that the transfer of the proteins would take place in the direction from the protein gel and on to the nitrocellulose membrane. The transfer was carried out at a constant voltage of 85 V for 2 hrs. After transfer, the membrane was incubated in 5% milk solution in PBS for 30 minutes. Then the membrane was incubated in primary antibody (1:1000 dilution of the appropriate antibody in 5% milk solution) for overnight at 4°C. The membrane was then washed in Wash solution for 3 times, 15 minutes per wash. The membrane was incubated in the secondary antibody (1:10000 dilution of the appropriate secondary antibody conjugated to Horseradish peroxidase (HRP) in 5 % milk solution) for 1 hour at 24°C. The membrane was then washed and the protein bands were detected with PICO (Pierce).

3 Results

3.1 WASP suppresses the growth defect of *S. cerevisiae las17Δ* strain in the presence of WIP

3.1.1 Introduction

S. cerevisiae Las17p is a proline rich protein which is homologous to the mammalian WASP (Li, 1997). *S. cerevisiae las17Δ* strain is unable to grow at 37°C and has defects in endocytosis and actin patch polarization (Li, 1997; Madania, 1999). It has been shown earlier that the cytoskeletal defects of yeast mutant strains can be rescued by the expression of mammalian cytoskeletal proteins. Expression of isoforms of human fimbrin (T- and L-fimbrins) under the transcriptional regulation of a *GALI* promoter complements the *sac6* null mutant and restores the polarization of the actin cytoskeleton in the mutant strain (Adams et al. 1995). Similarly, expression of human WIP under the transcriptional regulation of a *GAL1* promoter complements the growth defects of *vrpl* null and *vrpl-1* mutant strains and also restores endocytosis and actin patch polarization in the *vrpl-1* mutant strain (Vaduva et al., 1999). Therefore, the ability of human WASP to rescue the defects of *S. cerevisiae las17Δ* strain was examined. The aim of this study was to establish a system in which human WASP is able to suppress the defects of yeast *las17Δ* strain and use the system to characterize the function of the different domains of WASP. This will lead to a better understanding of the molecular defects in Wiskott Aldrich Syndrome.

Figure 3.1 Schematic map of WASP constructs.

Figure 3.2 Schematic map of **WIP** constructs.

3.1.2 Expression of WASP does not suppress the growth defects of *las17Δ* cells

In order to express human WASP in *las17Δ* strain and study its activity, the cDNA encoding human WASP was amplified from a human cDNA library using appropriate primers (Appendix 2B), cloned into a yeast shuttle vector YEplac112 and placed under the transcriptional regulation of the VRP1 promoter. The plasmid bearing the WASP gene was introduced into *las17Δ* strain (IDY166) by Lithium Acetate transformation. The yeast transformants expressing WASP were streaked on YPUAD plates to examine the growth at 37°C and a growth curve at 37°C was plotted by measuring the OD₆₀₀ of the

liquid culture at 1 hour intervals. The *las17Δ* cells expressing human WASP were unable to grow at 37°C in both the plate assay and the liquid culture assay (Fig. 3.3).

Figure 3.3 Expression of WASP does not suppress the growth defects of *las17Δ* cells.

- A.** Empty vector or plasmid expressing WASP was introduced into *las17Δ* cells (IDY166). The transformants and wild type strain IDY19 were streaked on YPUAD plates to examine growth at 24°C and 37°C.
- B.** A growth curve was plotted by measuring the OD₆₀₀ at 1 hour intervals for liquid culture of the above mentioned strains grown at 24°C and 37°C.

3.1.3 WASP-GFP does not localize to cortical patches in *las17Δ* cells

It has been shown that Las17p localizes to the cortical patches in *S. cerevisiae* cells (Li, 1997). To characterize the observed lack of complementation by expression of WASP in *las17Δ* cells, the sub-cellular localization of WASP in *las17Δ* cells was examined. A translation fusion construct of WASP and GFP was made by fusing the GFP coding sequence to WASP open reading frame at the C-terminus. GFP, Las17p-GFP and WASP-GFP were expressed in *las17Δ* cells and the GFP signal was analyzed using live cell

imaging. **Las17p-GFP** localized to the cortical patches (Fig. 3.4A) but cells expressing WASP-GFP showed a diffused cytoplasmic staining very similar to that observed in cells expressing only GFP (Fig. 3.4A). In order to determine if WASP was unable to complement due to lack of expression in the *las17Δ* cells, total protein extract from *las17Δ* strains expressing GFP or WASP-GFP were analyzed by western blotting with Anti-GFP antibody. Immunoblotting of protein extracts showed that WASP-GFP was expressed in these cells (Fig. 3.4B).

Figure 3.4 WASP-GFP does not localize to the cortical patches.

- A. *las17Δ* cells (**IDY166**) expressing GFP, **Las17p-GFP** or WASP-GFP were grown in YPUAD liquid to exponential phase at 24°C. GFP signal was visualized in living cells by fluorescence microscopy. Upper panels: FITC-fluorescence optics. Lower panels: DIC optics. Bar, 5μm.
- B. Total protein extract from **IDY166 transformants** growing exponentially at 24°C, expressing GFP or WASP-GFP were separated by SDS-PAGE and Western blotted. The GFP fusion proteins were detected using anti-GFP serum and Hexokinase (loading control) was detected using anti-Hexokinase serum.

3.1.4 Expression of WASP rescues the growth defects of *las17Δ* strain in the presence of WIP

In mammalian cells, WASP forms a complex with WIP (WASP interacting protein) similar to the complex formed by **Las17p** and **Vrp1p** in yeast cells (Naqvi et al., 1998). In *S. cerevisiae* cells, deletion of the **LAS17** or **VRP1** gene results in mutant strains having

similar phenotypes (Li, 1997; Naqvi et al., 1998; Madania et al., 1999) and it has also been shown that Las17p and Vrp1p function as a complex (Thanabalu et al., 2007). Deletion of WIP in mammalian cells results in a phenotype similar to that of the deletion of WASP in mammalian cells (Anton et al., 2002). WIP is the human homologue of yeast Vrp1p and has been shown to complement *vrp1-1* and *vrp1Δ* mutant as well as localize to cortical patches in *S. cerevisiae* (Vaduva et al., 1999). Thus the possibility that WIP may be necessary for the function of WASP in *las17Δ* cells was examined by expressing both human WIP and WASP in this strain. *S. cerevisiae las17Δ* cells expressing WASP and WIP were able to grow at 37°C in both the plate assay and the liquid culture assay (Fig. 3.5).

3.1.5 Co-expression of WASP and WIP in *las17Δ* cells does not rescue the defects in endocytosis and actin patch polarization

Co-expression of WASP and WIP in *las17Δ* cells restored growth of the strain at 37°C. Next, the ability of the *las17Δ* cells co-expressing WASP and WIP, to carry out fluid phase endocytosis was examined. As compared to the wild type cells, the mutant cells co-expressing WASP and WIP were unable to take up Lucifer Yellow (LY) into the vacuoles as indicated by the lack of fluorescence in the vacuoles (Fig. 3.6A). The actin patches in *las17Δ* strain have an aberrant structure and distribution. The distribution of actin patches in the *las17Δ* cells co-expressing WASP and WIP was therefore examined and found that the actin patches showed an aberrant and depolarized distribution (Fig. 3.6B). It has been shown that it is possible to rescue the growth defects of yeast mutant strains without simultaneously rescuing the other defects in the strain as shown by the

expression of N-Vrp1p in *vrp1Δ* cells, which rescues the growth defect of the strain at 37°C without rescuing the **actin** patch polarization defect of this strain (Thanabalu et al., 2001).

Figure 3.5 Co-expression of **WASP** and **WIP** suppresses the growth defects of *las17Δ* cells.

- A. *las17Δ* cells (IDY166) were transformed with plasmids in the following combinations: empty vector, vector expressing **WASP** or vectors expressing **WASP** and **WIP**. The transformants and wild type strain IDY19 were streaked on YPUAD plates to examine growth at 24°C and 37°C.
- B. A growth curve was plotted by measuring the OD₆₀₀ at 1 hour intervals for liquid culture of the above mentioned strains grown at 24°C and 37°C.

Figure 3.6 Co-expression of WASP and WIP does not suppress the defects in endocytosis and **actin** patch polarization of IDY166.

- A. Exponentially growing cells of the strain IDY19 and the IDY166 transformants harboring empty vector, vector expressing WASP or vectors expressing WASP and WIP, incubated with LY at 24°C for 1 hr. The cells were washed thoroughly and LY signal was visualized by fluorescence microscopy. Upper panels: fluorescence optics. Lower panels: DIC optics. Bar, 5μm.
- B. Exponentially growing cells at 24°C of the above mentioned strains were fixed, permeabilized and F-actin stained with Alexa-488-conjugated phalloidin. The actin patches were visualized by fluorescence microscopy. Bar, 5μm.

3.1.6 Expression of WIP alone in *las17Δ* cells does not rescue growth defect of the strain

Expression of both WASP and WIP in *las17Δ* cells rescues the growth defects of the strain but expression of WASP alone in the strain did not rescue the growth defects of the strain. This in turn raises the possibility that the ability of the strain expressing WASP and WIP to grow at 37°C could also be due to the activity of WIP. This was examined by introducing plasmid expressing WIP in *las17Δ* cells and the transformants were streaked

on YPUAD plates to examine the growth of the strain at 37°C. The mutant cells expressing WIP were unable to grow at 37°C (Fig. 3.7).

Figure 3.7 Expression of WIP in IDY166 does not suppress the growth defects of the strain.

las17Δ cells expressing WIP or WASP + WIP were streaked on YPUAD plates to examine growth at 24°C and 37°C.

3.1.7 Human WASP does not interact with *S. cerevisiae* Vrplp

Las17p localizes to the cortical patches in the absence of Vrplp but the cortical patches are depolarized (Lechler et al., 2001) suggesting a possible role for Vrplp in the polarized distribution of Las17p. It has also been shown that human WASP interacts with WIP in a manner similar to the interaction between Las17p and Vrplp (Ramesh et al. 1997). The ability of WASP to interact with Vrplp was examined in a Yeast Two Hybrid assay. The DNA encoding WASP or Las17p (a.a 1-234) were cloned into the vector pAS2-1 (pBD) and the DNA encoding human WIP or Vrplp (a.a 364-817) was cloned into the vector pACT2 (pAD). pAD-WIP and pAD-Vrplp₃₆₄₋₈₁₇ were introduced into the *S. cerevisiae* strain PJ69-4A along with pBD vector expressing WASP or Las17p₁₋₂₃₄ and the transformants were screened for growth on SD minimal media plates lacking histidine. The PJ69-4A strain expressing BD-WASP and AD-WIP was able to grow on SD minimal media plates lacking histidine but the strain expressing BD-WASP and AD-Vrplp₃₆₄₋₈₁₇ was unable to grow on SD minimal media plates lacking histidine (Table

3.1), suggesting that WASP does not interact with Vrp1p. On the other hand, the PJ69-4A strain expressing BD-Las17p₁₋₂₃₄ and AD-Vrp1p₃₆₄₋₈₁₇ was able to grow on SD minimal media plates lacking histidine but the strain expressing BD-Las17p₁₋₂₃₄ and AD-WIP was unable to grow on SD minimal media plates lacking histidine (Table 3.1). This suggests that only Las17p interacts with Vrp1p but the human homologue of Las17p, WASP doesn't interact with Vrp1p.

Table 3.1 Human WASP does not interact with Vrp1p.

	BD-Las17p ₁₋₂₃₄	BD-WASP	BD-Vector
AD-Vrp1p ₃₆₄₋₈₁₇	+		
AD-WIP		+	
AD-Vector			

S. cerevisiae strain PJ69-4A was co-transformed with plasmids expressing the BD fusion proteins and the AD fusion proteins in combinations as indicated in the table. The transformants were streaked on SD minimal media plates lacking histidine and incubated at 30°C for 5 days. The growth of the strains on the plates (+) indicated an interaction between the AD and BD fusion proteins expressed by the strain. “-” indicates lack of interaction.

3.1.8 WIP mediates cortical patch localization of WASP

Las17p is recruited to the cortical patches by Vrp1p during polarized cell growth (Lechler et al., 2001). Human WASP does not interact with *S. cerevisiae* Vrp1p, suggesting a possible explanation for WASP's inability to localize to cortical patches and possibly the lack of complementation. To determine the localization of WASP in the presence of WIP in *S. cerevisiae las17Δ* cells, WASP-GFP and WIP were co-expressed in this strain. In the presence of WIP, WASP-GFP was found to localize to cortical patches of yeast cells at both 24°C and 37°C (Fig. 3.8). Since WASP-GFP was able to localize to cortical patches only in the presence of WIP, it raised the possibility that WIP was mediating the localization of WASP. Therefore, the localization of WIP-GFP was then examined in

las17Δ cells. WIP-GFP was able to localize to cortical patches in the *las17Δ* strain at both 24°C and 37°C, even in the absence of WASP (Fig. 3.8). Since WASP is able to interact with WIP (Ramesh et al., 1997), it suggests that WIP mediates WASP localization to the cortical actin patches.

Figure 3.8 WASP-GFP localizes to the cortical patches of *las17Δ* cells in the presence of WIP.

las17Δ cells expressing GFP, WASP-GFP, WIP-GFP or WASP-GFP + WIP were grown to an exponential phase at 24°C. The cells were immediately processed for microscopic visualization or they were shifted to 37°C for 2 hours prior to the processing. GFP signal was visualized in living cells by fluorescence microscopy. Upper panels: FITC-fluorescence optics. Lower panels: DIC optics. Bar, 5μm.

3.1.9 WIP mediates the localization of WASP to cortical actin patches

In order to determine whether WASP-GFP is localized to cortical actin patches, the ability of WASP-GFP to co-localize with patches containing Arc40p-RFP was examined in cells expressing WASP-GFP and WIP. Arc40p is a component of the yeast Arp2/3 complex and localizes to the cortical actin patches in yeast cells (Winter et al., 1997). Cells expressing WASP-GFP, WIP and Arc40p-RFP were then subjected to epifluorescence microscopy. Some of the WASP-GFP patches were found to co-localize

with Arc40p-RFP patches at both 24°C and 37°C (Fig. 3.9), suggesting that WASP-WIP complex is localized to cortical actin patches.

Figure 3.9 WASP-GFP localizes to actin patches of *las17Δ* cells, in the presence of WIP.

las17Δ cells co-expressing WASP-GFP, Arc40p-RFP and WIP were grown to an exponential phase at 24°C. The cells were processed immediately for microscopic visualization or they were shifted to 37°C for 2 hours prior to the processing. GFP and RFP were visualized by fluorescence microscopy. Arrows point to co-localizing patches. Bar, 5μm.

3.1.10 Expression of WIP enhances the stability of WASP in *las17Δ* cells

In order to study the expression of WASP in the presence of WIP, total protein extract was prepared from *las17Δ* strain co-expressing WASP-GFP and WIP. The proteins were resolved in a SDS-PAGE gel and subsequently subjected to Western analysis using anti-GFP antibody. Immunoblotting of protein extracts showed that WASP-GFP was more abundant in cells expressing both WASP-GFP and WIP than in cells expressing only WASP-GFP (Fig. 3.10A), suggesting that the stability of WASP is enhanced in the presence of WIP.

Next, we determined whether the expression of WASP has any effect on the expression of WIP. Total protein extracts from *las17Δ* expressing WIP-GFP in the presence and

absence of WASP was subjected to Western analysis using anti-GFP antibody. Immunoblotting of protein extracts showed that the expression of WIP-GFP was similar, both in the presence or absence of WASP (Fig. 3.10B), suggesting that expression of WASP does not enhance the stability of WIP.

Figure 3.10 Expression of WIP enhances the stability of WASP in *las17Δ* cells.

Total protein extract was prepared from IDY166 transformants expressing GFP, WASP-GFP, WASP-GFP + WIP, WIP-GFP or WIP-GFP + WASP growing at exponential phase at 24°C. The extracted proteins were separated by SDS-PAGE and Western blotted. The GFP fusion proteins were detected using anti-GFP serum and Hexokinase (loading control) was detected using anti-Hexokinase serum.

3.1.11 Fusion of CAAX box to C-terminus of WASP does not improve the activity of WASP

The presence of WIP mediates localization of WASP to actin patches and suppression of growth defect of *las17Δ* strain. This in turn raises the question as to whether WASP would be able to suppress the growth defect of the mutant strain if WASP localized to plasma membrane by other localization signal. Fusion of a CAAX box motif (GCCIIIC) at the carboxyl terminal of proteins confers them with strong cortical localization (Golsteyn et al., 1997). Fusing Raslp's CAAX box to GFP localizes GFP to the plasma membrane of *S. cerevisiae* and fusing CAAX to the N-terminal region of Vrp1p (N-Vrp1p)

enhanced the activity of N-Vrplp-CAAX (Thanabalu et al., 2001). Therefore, the CAAX box of *S. cerevisiae* Raslp was fused to the carboxyl terminal of WASP and WASP-GFP (Fig. 3.1). *Las17p* deficient yeast cells expressing WASP-CAAX were streaked on YPUAD to examine growth at 37°C. The mutant cells expressing WASP-CAAX were unable to grow at 37°C (Fig. 3.11A). Visualization of the mutant cells expressing WASP-GFP-CAAX under a fluorescence microscope showed that WASP-GFP-CAAX localized to the cortical membrane (Fig. 3.11B). Therefore, localization of WASP to plasma membrane alone may not be sufficient for the activity of WASP in yeast cells, suggesting that WIP has additional roles besides mediating WASP localization. Another possibility is that, rather than general localization to the plasma membrane, WASP needs to localize specifically to certain membrane sub-domains through its interaction with WIP in order to rescue the growth defect of the mutant strain.

Figure 3.11 Expression of WASP-CAAX does not suppress the growth defects of *las17Δ* cells.

A. *las17Δ* cells expressing WASP-CAAX or WASP + WIP were streaked on YPUAD plates to examine growth at 24°C and 37°C.

B. *las17Δ* cells expressing GFP, GFP-CAAX, WASP-GFP or WASP-GFP-CAAX were grown to exponential phase at 24°C. GFP signal was visualized in living cells by fluorescence microscopy. Upper panels: FITC-fluorescence optics. Lower panels: DIC optics. Bar, 3 μm.

3.1.12 Human WASP interacts with *S. cerevisiae* actin and SH3 domain containing proteins in a Yeast Two Hybrid assay

Las17p has been shown to interact with many cytoskeletal proteins like actin, Vrp1p, Bzz1p, Hof1p, Myo3p, Myo5p and Rvs167p (Naqvi et al., 1998; Soulard et al., 2002; Naqvi et al., 2001; Evangelista et al., 2000; Lechler et al., 2000; Colwill et al., 1999). Most of these proteins have been shown to be essential for the survival of yeast cells at high temperature as well as for endocytosis and actin patch polarization. SH3 domain containing proteins like Bzz1p, Hof1p, Myo3p, Myo5p and Rvs167p bind with Las17p through the interaction between the SH3 domain and the proline rich motifs of Las17p. The interaction of WASP with actin and the SH3 domains of Bzz1p, Hof1p, Myo3p, Myo5p and Rvs167p were examined in a Yeast Two Hybrid assay. The DNA encoding *S. cerevisiae* actin and the SH3 domains of the 5 SH3 domain containing proteins were cloned individually into the vector pAS2-1 (pBD). pAD-WASP was introduced into the *S. cerevisiae* strain PJ69-4A along with each of the pBD vectors expressing actin or the SH3 domains of the 5 SH3 domain containing proteins. The transformants were then screened for growth on SD minimal media plates lacking histidine. Only the PJ69-4A strains co-expressing BD-WASP and the AD domain fused with Act1p, Myo5p₁₀₈₅₋₁₂₁₉, Hof1p₅₅₁₋₆₆₉ or Bzz1p₄₉₄₋₆₆₃ were able to grow on SD minimal media plates lacking histidine (Table 3.2), suggesting that WASP is able to interact with actin and the SH3 domains of Myo5p, Hof1p and Bzz1p. Therefore, it is possible that WASP is able to suppress the growth defect of *las17Δ* cells possibly through its interaction with other *S. cerevisiae* cytoskeletal proteins like actin, Myo5p, Hof1p and Bzz1p.

Table 3.2 Human WASP interacts with *S.cerevisiae* actin and cytoskeletal proteins.

	AD-WASP	AD-Vector
BD-Act1p	+	-
BD-Bzz1p₄₉₄₋₆₆₃	+	-
BD-Hof1p₅₅₁₋₆₆₉	+	-
BD-Myo3p₁₁₁₈₋₁₁₈₈	-	-
BD-Myo5p₁₀₈₅₋₁₂₁₉	+	-
BD-Rvs167p₄₂₃₋₄₈₂	-	-
BD-Vector	-	-

PJ69-4A transformants co-expressing the indicated BD and AD fusion proteins were streaked on **SD** minimal media plates lacking histidine and incubated at 30°C for 5 days. The growth of the strains on the plates (+) indicated an interaction between the **AD** and BD fusion proteins expressed by the strain. “-” indicates lack of interaction.

3.1.13 A constitutively active variant of WASP, WASP^{L270P} is able to rescue growth defects of *las17Δ* strain but only in the presence of WIP

It has been shown that substitution of Leucine270 to Proline (L270P) in WASP makes it constitutively active because the mutation allows actin polymerization even in the absence of Cdc42 (Devriendt et al., 2001). Therefore, the ability of WASP bearing the L270P mutation, to suppress growth defects of *las17Δ* cells was examined. The L270P mutation was engineered in the DNA encoding WASP through site directed mutagenesis to generate the mutant construct WASP^{L270P} (Fig. 3.1). Plasmid expressing WASP^{L270P} was introduced into *las17Δ* cells and the transformants were streaked on YPUAD plates to examine for growth at 37°C. The mutant cells expressing WASP^{L270P} were unable to grow at 37°C (Fig. 3.12). In order to determine whether WASP^{L270P} requires the presence of WIP to rescue the growth defects of *las17Δ* cells, plasmids expressing WASP^{L270P} and WIP were introduced into *las17Δ* cells and the transformants were streaked on YPUAD plates to assess growth at 37°C. Mutant cells co-expressing WASP^{L270P} and WIP were able to grow at 37°C (Fig. 3.12), suggesting that even though WASP^{L270P} is constitutively active, it still requires the presence of WIP to rescue the growth defects of *las17Δ* cells.

3.1.14 WASP^{L270P} localizes to the cortical patches of *las17Δ* strain only in the presence of WIP

In order to determine whether being constitutively active would enable WASP^{L270P} to localize to cortical patches of *las17Δ* cells, a WASP^{L270P}-GFP fusion construct was generated. WASP^{L270P}-GFP was expressed in *las17Δ* cells and the transformants were subjected to fluorescence microscopy. WASP^{L270P}-GFP was unable to localize to cortical patches and showed a diffused cytoplasmic staining pattern (Fig. 3.13). When WIP was also expressed in the *las17Δ* cells expressing WASP^{L270P}-GFP, then WASP^{L270P}-GFP was able to localize to the cortical patches (Fig. 3.13). Therefore, expression of WIP is essential for the localization and activity of WASP^{L270P} in *las17Δ* cells.

Figure 3.12 WASP^{L270P} suppresses the growth defects of *las17Δ* cells only in the presence of WIP.

- A. IDY166 transformants harboring empty vector or vectors expressing WASP^{L270P}, WASP + WIP or WASP^{L270P} + WIP were streaked on YPUAD plates to examine growth at 24°C and 37°C.
- B. A growth curve was plotted by measuring the OD₆₀₀ at 1 hour intervals for liquid culture of the indicated strains grown at 37°C.

Figure 3.13 WASP^{L270P}-GFP localizes to the cortical patches of *las17Δ* cells in the presence of WIP. Exponentially growing *las17Δ* cells expressing GFP, WASP-GFP + WIP, WASP^{L270P}-GFP or WASP^{L270P}-GFP + WIP were processed for visualizing protein localization at 24°C or 37°C. GFP signal was visualized in living cells by fluorescence microscopy. Upper panels: FITC-fluorescence optics. Lower panels: DIC optics. Bar, 5μm.

3.1.15 WASP^{L270P}-CAAX is unable to rescue the growth defects of *las17Δ* cells

WIP mediates WASP localization to the cortical actin patches in *las17Δ* cells. Though WASP^{L270P} has been shown to be constitutively active, WASP^{L270P} is able to rescue the growth defects of *las17Δ* cells only if WIP is also expressed in the strain. In order to determine if WASP^{L270P} is able to rescue the growth defects of *las17Δ* cells if it was localized to the plasma membrane through the localization activity of the CAAX motif, the CAAX box of *S. cerevisiae* Ras1p was fused to the carboxyl terminal of WASP^{L270P} (Fig. 3.1) and the fusion protein was expressed in the *las17Δ* strain. Las17p deficient

yeast cells expressing WASP^{L270P}-CAAX were unable to grow at 37°C (Fig. 3.14), suggesting that localization of WASP^{L270P} to the plasma membrane alone may not be sufficient for the activity of WASP in yeast.

Figure 3.14 Expression of WASP^{L270P}-CAAX does not suppress the growth defects of *las17Δ* cells. *las17Δ* cells transformed with plasmids expressing proteins in the following combinations: empty vector, WASP^{L270P}-CAAX or WASP + WIP. The transformants were streaked on YPUAD plates to examine growth at 24°C and 37°C.

3.1.16 WH1 domain of WASP and the WASP Binding Domain of WIP are essential for WASP-WIP interaction

The WH1 domain (a.a 1-138) of WASP and the WBD domain (a.a 404-503) of WIP are critical for the formation of WASP-WIP complex (Ramesh et al., 1997) and the interaction between the domains was verified here through a Yeast Two Hybrid Assay. DNA encoding WASP₁₃₉₋₅₀₂ (Fig. 3.1) and DNA encoding WIP₁₋₄₀₃ (Fig. 3.2) were cloned into the Yeast Two Hybrid vectors, pAS2-1 (pBD) and pACT2 ('AD) respectively. WASP₁₃₉₋₅₀₂ had a deletion of the WH1 domain (1-138) and WIP₁₋₄₀₃ had a deletion of the WASP Binding Domain (404-503). The yeast strain PJ69-4A was then transformed with the following vector combinations pBD-WASP + pAD-WIP, pBD-WASP₁₃₉₋₅₀₂ + pAD-WIP and pBD-WASP + pAD-WIP₁₋₄₀₃, the transformants were analyzed for growth on SD minimal media plates lacking histidine. Unlike PJ69-4A cells

expressing WASP + WIP, the transformants expressing BD-WASP₁₃₉₋₅₀₂ + AD-WIP or BD-WASP + AD-WIP₁₋₄₀₃ were unable to grow on SD minimal media plates lacking histidine (Table 3.3), suggesting that deleting the WH1 domain of WASP or the WBD domain of WIP abolishes the interaction between WASP and WIP.

Table 3.3 The WH1 domain of WASP and the WASP binding domain of WIP are essential for WASP-WIP interaction.

	BD-WASP	BD-WASP ₁₃₉₋₅₀₂	BD-Vector
AD-WIP	+		-
AD-WIP ₁₋₄₀₃		N.D	-
AD-Vector	-	-	-

S. cerevisiae strain PJ69-4A was co-transformed with plasmids expressing the BD fusion proteins and the AD fusion proteins in combinations as indicated in the table. The transformants were streaked on SD minimal media plates lacking histidine and incubated at 30°C for 5 days. The growth of the strains on the plates (+) indicated an interaction between the AD and BD fusion proteins expressed by the strain. “-” indicates lack of interaction. “N.D” indicates assay not done.

3.1.17 WASP-WIP interaction is essential for rescuing growth defects of *S. cerevisiae las17Δ* cells

Both the WASP-WH1 domain and the WIP-WBD domain are essential for the interaction between WASP and WIP. In order to address the importance of interaction between WASP and WIP for the function of WASP, *las17Δ* cells were transformed with plasmids expressing the proteins in the following combinations, WASP₁₃₉₋₅₀₂ + WIP or WASP + WIP₁₋₄₀₃. The transformants were assessed for their growth at 24°C and 37°C. The mutant cells expressing WASP₁₃₉₋₅₀₂ + WIP or WASP + WIP₁₋₄₀₃ were unable to grow at 37°C in either the plate assay or the liquid culture assay (Fig. 3.15), suggesting that WASP and WIP need to interact with each other in order to suppress the growth defect of *las17Δ*

cells. The interaction is probably required for the localization of WASP to cortical patches or for stabilizing WASP in yeast cells.

Figure 3.15 WASP-WIP interaction is essential for suppression of growth defects of *las17Δ* cells.

- A.** *las17Δ* cells were transformed with plasmids expressing proteins in the following combinations: empty vector, WASP + WIP, WASP₁₃₉₋₅₀₂ + WIP or WASP + WIP₁₋₄₀₃. The transformants were streaked on YPUAD plates to examine growth at 24°C and 37°C.
- B.** A growth curve was plotted by measuring the OD₆₀₀ at 1 hour intervals for liquid culture of the above mentioned strains grown at 24°C and 37°C.

3.1.18 WASP-WIP interaction is critical for localization of WASP to cortical actin patches

WASP₁₃₉₋₅₀₂ does not suppress the growth defect of *las17Δ* cells even in the presence of WIP. Since WASP₁₃₉₋₅₀₂ does not interact with WIP, it raises the possibility that WASP₁₃₉₋₅₀₂ is unable to localize to the cortical patches. The localization of WASP₁₃₉₋₅₀₂

was thus examined. A GFP fusion construct, WASP₁₃₉₋₅₀₂-GFP and WIP were co-expressed in *las17Δ* cells and the transformants were examined under a fluorescence microscope. WASP₁₃₉₋₅₀₂-GFP did not localize to the cortical **actin** patches, instead showed a **diffused** cytoplasmic staining pattern (Fig. 3.16), suggesting that WASP₁₃₉₋₅₀₂ was unable to localize to the cortical patches due to its inability to interact with WIP.

Figure 3.16 WASP-WIP interaction is critical for localization of WASP to the cortical patches of *las17Δ* cell. *las17Δ* cells expressing GFP, WASP-GFP + WIP, WASP₁₃₉₋₅₀₂-GFP + WIP or WASP-GFP + WIP₁₋₄₀₃ were grown in YPUAD liquid to exponential phase at 24°C. GFP signal was visualized in living cells by fluorescence microscopy. Upper panels: FITC-fluorescence optics. Lower panels: DIC optics. Bar, 5μm.

Immunoblotting of cell extracts from *las17Δ* cells expressing WASP₁₃₉₋₅₀₂-GFP and WIP showed that WASP₁₃₉₋₅₀₂-GFP was stable (Fig. 3.17), even though WASP₁₃₉₋₅₀₂-GFP was unable to interact with WIP and this was reflected even in the GFP signal as the cells expressing this construct were very bright but showed **diffused** cytoplasmic staining (Fig. 3.16). Then the expression of WASP₁₃₉₋₅₀₂-GFP was examined in *las17Δ* cells which had been transformed with only **plasmid** expressing WASP₁₃₉₋₅₀₂-GFP and not WIP. Immunoblotting of cell extracts from *las17Δ* cells expressing only WASP₁₃₉₋₅₀₂-GFP showed that WASP₁₃₉₋₅₀₂-GFP was very stable, even in the absence of WIP (Fig. 3.17).

Thus deleting the WH1 domain enhanced the stability of WASP, however this does not lead to the suppression of the growth defects of the mutant strain.

Figure 3.17 WASP₁₃₉₋₅₀₂ is stable even in the absence of interaction with WIP.

Total protein extract was prepared from IDY166 transformants expressing WASP-GFP + WIP, WASP-GFP + WIP₁₋₄₀₃, WASP₁₃₉₋₅₀₂-GFP + WIP or WASP₁₃₉₋₅₀₂-GFP growing at exponential phase at 24°C. The extracted proteins were separated by SDS-PAGE and Western blotted. The GFP fusion proteins were detected using anti-GFP serum and Hexokinase (loading control) was detected using anti-Hexokinase serum.

We localized WASP-GFP in the presence of WIP₁₋₄₀₃ was examined in *las17Δ* cells by epifluorescence microscopy. WASP-GFP was unable to localize to the cortical actin patches in the presence of WIP₁₋₄₀₃ (Fig. 3.16). Next, we determined whether deletion of the WASP Binding Domain of WIP has any effect on the localization of WIP₁₋₄₀₃ to cortical actin patches. DNA encoding GFP was fused to the C-terminus of the DNA encoding WIP₁₋₄₀₃. WIP₁₋₄₀₃-GFP was expressed in *las17Δ* cells and the transformants were subjected to epifluorescence microscopy. WIP₁₋₄₀₃-GFP localized to the cortical patches of *las17Δ* cells at both 24°C and 37°C (Fig. 3.18), suggesting that the WASP Binding Domain of WIP was not needed for the localization of WIP to cortical patches in yeast cells. Therefore, WIP₁₋₄₀₃ was not able to mediate WASP-GFP localization to cortical patches due to lack of interaction between WASP and WIP₁₋₄₀₃. Immunoblotting of cell extracts showed that WASP-GFP was present at lower levels in the extracts from *las17Δ* cells expressing WASP-GFP and WIP₁₋₄₀₃ as compared with that from the transformants co-expressing full length WIP and WASP-GFP (Fig. 3.17).

Figure 3.18 WASP binding domain of WIP is not required for localization to the cortical patches of *las17Δ* cells.

las17Δ cells expressing GFP, WIP-GFP or WIP₁₋₄₀₃-GFP were grown in YPUAD liquid to exponential phase at 24°C. The cells were immediately processed for microscopic visualization or they were shifted to 37°C for 2 hours prior to the processing. GFP signal was visualized in living cells by fluorescence microscopy. Upper panels: FITC-fluorescence optics. Lower panels: DIC optics. Bar, 5μm.

3.1.19 Expression of WASP₁₃₉₋₅₀₂-CAAX does not suppress the growth defects of *las17Δ* cells

Expression of WASP suppresses the growth defect of *las17Δ* strain in the presence of WIP. It was shown earlier that one of the possible roles played by WIP in regulating the activity of WASP was to improve the stability of WASP in *las17Δ* cells. Deleting the WH1 domain of WASP enhanced the stability of the protein which was seen in both a Western blot of protein extracts and also by live cell imaging. This raises the question as to whether this construct would be able to suppress the growth defect of *las17Δ* cells, if it was targeted to the plasma membrane using CAAX motif. Fusion of a CAAX box motif (GCCIIIC) at the carboxyl terminal of proteins confers them with strong cortical localization (Golsteyn et al., 1997). The CAAX box of *S. cerevisiae* Ras1p was fused to the carboxyl terminal of WASP₁₃₉₋₅₀₂, the fusion protein WASP₁₃₉₋₅₀₂-CAAX (Fig. 3.1)

was expressed in the *las17Δ* mutant strain both in the presence and absence of WIP. The transformants were streaked on YPUAD plates to examine for growth at 37°C. The mutant cells expressing WASP₁₃₉₋₅₀₂-CAAX were unable to grow at 37°C (Fig. 3.19A, B), even in the presence of WIP. Western blotting of protein extracts showed that the expression of WASP₁₃₉₋₅₀₂-CAAX was comparable to that of WASP₁₃₉₋₅₀₂ (Fig. 3.19C). Thus, enhanced stability and CAAX mediated localization did not abolish the need for interaction with WIP for suppressing the growth defect of the mutant strain.

Figure 3.19 WASP₁₃₉₋₅₀₂-CAAX does not suppress the growth defects of *las17Δ* cells.

- A. *las17Δ* cells (IDY166) were transformed with plasmids expressing proteins in the following combinations: empty vector, WASP₁₃₉₋₅₀₂-CAAX or WASP + WIP. The transformants were streaked on YPUAD plates to examine growth at 24°C and 37°C.
- B. *las17Δ* cells (IDY166) were transformed with plasmids expressing proteins in the following combinations: empty vector, WASP + WIP or WASP₁₃₉₋₅₀₂-CAAX + WIP. The transformants were streaked on YPUAD plates to examine growth at 24°C and 37°C.
- C. Total cell lysate from *las17Δ* cells (IDY166) expressing WASP₁₃₉₋₅₀₂ or WASP₁₃₉₋₅₀₂-CAAX was western blotted and probed with anti-WASP serum and anti-Hexokinase serum.

3.1.20 The VCA domain at the C-terminal region of WASP is essential for the activity of the protein

The VCA domain of WASP is essential for *in vitro* stimulation of the nucleation activity of Arp2/3 complex (Rohatgi et al., 1999). Deleting the VCA domain of WASP inhibits the ability of WASP to activate the Arp2/3 complex which in turn affects the rate of actin polymerization. In order to determine the role of the VCA domain in the activity of WASP in yeast cells, plasmid encoding WASP₁₋₄₂₄ (Fig. 3.1) was generated. Plasmids expressing WASP₁₋₄₂₄ and WIP were introduced into *las17Δ* cells and the transformants were examined for growth on YPUAD plates at 37°C and a growth curve at 37°C was plotted by measuring the OD₆₀₀ of the liquid culture at 1 hour intervals. The mutant cells expressing WIP and WASP₁₋₄₂₄ were unable to grow at 37°C in both the plate assay and liquid culture (Fig. 3.20) in yeast cells, suggesting that the VCA domain of WASP was essential for the activity of WASP.

Figure 3.20 The VCA domain essential for the activity of WASP in *las17Δ* cells.

- A. *las17Δ* cells (IDY166) were transformed with plasmids expressing proteins in the following combinations: empty vector, WASP + WIP or WASP₁₋₄₂₄ + WIP. The transformants were streaked on YPUAD plates to examine growth at 24°C and 37°C.
- B. A growth curve was plotted by measuring the OD₆₀₀ at 1 hour intervals for liquid culture of the above mentioned strains grown at 24°C and 37°C.

3.1.21 WASP₁₋₄₂₄-GFP is able to localize to the cortical patches of *las17Δ* cells in the presence of WIP

Expression of WASP₁₋₄₂₄ and WIP in *las17Δ* cells does not suppress the growth defects of the strain. A fusion construct WASP₁₋₄₂₄-GFP was created and co-expressed with WIP in *las17Δ* cells and the transformants were analyzed by fluorescence microscopy. WASP₁₋₄₂₄-GFP localized to the cortical patches in *las17Δ* cells at both 24°C and 37°C (Fig. 3.21).

Figure 3.21 The VCA domain of WASP is not essential for the localization of WASP to the cortical patches.

las17Δ cells (IDY166) were transformed with plasmids expressing proteins in the following combinations: GFP, WASP-GFP + WIP or WASP₁₋₄₂₄-GFP + WIP. The transformants were grown to exponential phase at 24°C. The cells were immediately processed for microscopic visualization or they were shifted to 37°C for 2 hours prior to the processing. GFP signal was visualized in living cells by fluorescence microscopy. Upper panels: FITC-fluorescence optics. Lower panels: DIC optics. Bar, 5 μm.

The ability of WASP₁₋₄₂₄-GFP to localize to the cortical actin patches was examined by expressing Arc40p-RFP in the cells expressing WASP₁₋₄₂₄-GFP and WIP, the yeast cells were then visualized under a fluorescence microscope to detect localization of WASP₁₋₄₂₄-GFP and Arc40p-RFP. Some of the WASP₁₋₄₂₄-GFP patches were found to co-localize with Arc40p-RFP patches at both 24°C and 37°C (Fig. 3.22), suggesting that the WASP₁₋

WASP₁₋₄₂₄-WIP complex is localized to cortical actin patches. Analysis of the protein expression from *las17Δ* cells expressing WASP₁₋₄₂₄-GFP and WIP also showed that the deletion mutant is expressed at levels comparable to that of the full length protein (Fig. 3.23). It has been shown that deletion at the C-terminus of WASP does not affect the interaction between WASP and WIP (Ramesh et al., 1997). Therefore, deleting the VCA domain of WASP does not affect its localization to the cortical patches or its stabilization in the presence of WIP, although it is unable to rescue the growth defects of *las17Δ* cells.

Figure 332 WASP₁₋₄₂₄-GFP co-localizes with the actin patches of *las17Δ* cells, in the presence of WIP. *las17Δ* cells co-expressing WASP₁₋₄₂₄-GFP, Arc40p-RFP and WIP were grown in YPUAD liquid medium in exponential phase at 24°C. The cells were processed immediately for microscopic visualization or they were shifted to 37°C for 2 hours prior to the processing. GFP and RFP were visualized by fluorescence microscopy. Arrows point to co-localizing patches. Bar, 5μm.

Figure 3.23 Expression of WASP₁₋₄₂₄-GFP in *las17Δ* cells. Total protein extract was prepared from IDY166 transformant expressing WASP-GFP + WIP and WASP₁₋₄₂₄-GFP + WIP growing at exponential phase at 24°C. The extracted protein was separated by SDS-PAGE and Western blotted. The GFP fusion proteins were detected using anti-GFP serum and Hexokinase (loading control) was detected using anti-Hexokinase serum.

3.1.22 The DDW motif is essential for the activity of WASP

All the members of the WASP family of proteins have a conserved DDW motif which encompasses a conserved tryptophan residue at the carboxyl terminal of the protein. In WASP, the DDW motif is represented by the residues DEW (498-500). It has been shown by *in vitro* studies that the Arp2/3 complex binds to the VCA region of WASP through the DDW motif (Marchand et al., 2001; Weaver et al., 2002; Goode et al., 2001). Such studies also showed that mutating the conserved tryptophan residue in the DDW motif affects the affinity of the VCA region towards the Arp2/3 complex (Marchand et al., 2001; Weaver et al., 2002; Goode et al., 2001). In order to determine the role of the DDW motif in the activity of WASP in *las17Δ* cells, the DDW motif was mutated by replacing the residues DEW (498-500) with AAA by PCR mutagenesis to generate plasmid expressing the WASP^{AAA} mutant (Fig. 3.1). Plasmids expressing WASP^{AAA} and WIP were introduced into the *S. cerevisiae las17Δ* strain and the transformants were examined for growth at 37°C. The transformants expressing WASP^{AAA} and WIP were unable to grow at 37°C in both the plate assay and the liquid culture assay (Fig. 3.24).

Figure 3.24 The DDW motif in the VCA domain is essential for the activity of WASP

- A. *las17Δ* cells (IDY166) were transformed with plasmids expressing proteins in the following combinations: empty vector, WASP + WIP or WASP^{AAA} + WIP. The transformants were streaked on YPUAD plates to examine growth at 24°C and 37°C.
- B. A growth curve was plotted by measuring the OD₆₀₀ at 1 hour intervals for liquid culture of the above mentioned strains grown at 24°C and 37°C.

3.1.23 WASP^{AAA}-GFP localizes to the cortical patches of *las17Δ* cells in the presence of WIP

Expression of WASP^{AAA} and WIP in *las17Δ* cells did not rescue the growth defect of the strain. A fusion construct, WASP^{AAA}-GFP was constructed to determine its localization. WASP^{AAA}-GFP and WIP were then expressed in *las17Δ* cells and the transformants were analyzed by fluorescence microscopy. WASP^{AAA}-GFP localized to cortical patches at both 24°C and 37°C (Fig 3.25) in the presence of WIP.

The ability of WASP^{AAA}-GFP to localize to the cortical actin patches was examined by expressing **Arc40p-RFP** in the cells expressing **WASP^{AAA}-GFP** and **WIP**, the yeast cells were then visualized under a fluorescence microscope. Some of the WASP-GFP patches co-localized with **Arc40p-RFP** patches at both 24°C and 37°C (Fig. 3.26), suggesting that the **WASP^{AAA}-WIP** complex is localized to cortical **actin** patches. **Immunoblotting** of protein extracts from *las17Δ* cells expressing **WASP^{AAA}-GFP** and **WIP** showed that the expression of **WASP^{AAA}-GFP** was comparable to the expression of wild type WASP (Fig. 3.27). This shows that the VCA domain and the DDW motif within the VCA domain are essential for the activity of WASP in suppressing the growth defects of *las17Δ* cells which in turn implies that WASP's ability to activate the **Arp2/3** complex is essential for rescuing the growth defects of *las17Δ* cells.

Figure 3.25 WASP^{AAA} localizes to the cortical patches in *las17Δ* cells.

las17Δ cells (**IDY166**) were transformed with **plasmids** expressing proteins in the following combinations: **GFP**, **WASP-GFP + WIP** or **WASP^{AAA}-GFP + WIP**. The **transformants** were grown in YPUAD liquid to exponential phase at 24°C. The cells were immediately processed for microscopic visualization or they were shifted to 37°C for 2 hours prior to the processing. GFP signal was visualized in living cells by fluorescence microscopy. Upper panels: FITC-fluorescence optics. Lower panels: DIC optics. Bar, 5μm.

Figure 3.36 WASP^{AAA}-GFP localizes to cortical actin patches in *las17Δ* cells, in the presence of WIP. *las17Δ* cells (IDY166) were co-transformed with vectors expressing WASP^{AAA}-GFP, Arc40p-RFP and WIP. The transformants were grown in YPUAD liquid to exponential phase at 24°C. The cells were processed immediately for microscopic visualization or they were shifted to 37°C for 2 hours prior to the processing. GFP and RFP were visualized by fluorescence microscopy. Arrows point to co-localizing patches. Bar, 5 μm.

Figure 3.27 Expression of WASP^{AAA}-GFP in *las17A* cells. Total protein extract was prepared from IDY166 transformants expressing WASP-GFP + WIP and WASP^{AAA}-GFP + WIP growing at exponential phase at 24°C. The extracted proteins were separated by SDS-PAGE and Western blotted. The GFP fusion proteins were detected using anti-GFP serum and Hexokinase (loading control) was detected using anti-Hexokinase serum.

3.1.24 Mutation in the VCA domain of WASP does not affect the interaction of the mutant WASP with WIP, *S. cerevisiae* actin and the SH3 domains of Myo5p, Hoflp and Bzz1p

Deletion of the VCA domain of WASP or mutating just the DDW motif in the VCA domain of WASP abolished the ability of WASP to rescue the growth defects of *las17Δ* cells, in the presence of WIP. In order to determine whether mutation in the C-terminal

region of WASP has any effects on the interaction of WASP with WIP and the yeast cytoskeletal proteins, a Yeast Two Hybrid assay was performed. The strain PJ69-4A was co-transformed with plasmid expressing BD domain fused with WIP, Act1p or SH3 domains of Bzz1p, Hof1p and Myo5p along with plasmid expressing AD domain fused with WASP, WASP₁₋₄₂₄ or WASP^{AAA}. The transformants were then streaked on SD minimal media plates lacking histidine to examine the interaction between the proteins. The transformants co-expressing BD-WIP, BD-Act1p, BD-Bzz1p₄₉₄₋₆₆₃, BD-Hof1p₅₅₁₋₆₆₉ or BD-Myo5p₁₀₈₅₋₁₂₁₉ with all the three combinations of AD-WASP constructs were able to grow on SD minimal media plates lacking histidine (Table 3.4), suggesting that deletion of the entire VCA domain or mutating just the DDW motif in the VCA domain of WASP has no effect on the interaction of WASP with WIP or the *S. cerevisiae* cytoskeletal proteins..

Table 3.4 Mutation in the VCA domain does not affect WASP interaction with WIP, Act1p or the **SH3** domains of Bzz1p, Hof1p and Myo5p.

	AD-WASP	AD-WASP ₁₋₄₂₄	AD-WASP ^{AAA}	Vector
BD-WIP	+	+	+	
BD-Act1p	+	+	+	
BD-Bzz1p₄₉₄₋₆₆₃	+	+	+	
BD-Hof1p₅₅₁₋₆₆₉	+	+	+	
BD-Myo5p₁₀₈₅₋₁₂₁₉	+	+	+	
BD-Vector		-		

S. cerevisiae strain PJ69-4A was co-transformed with plasmids expressing the BD fusion proteins and the AD fusion proteins in combinations as indicated in the table. The transformants were streaked on SD minimal media plates lacking histidine and incubated at 30°C for 5 days. The growth of the strains on the plates (+) indicated an interaction between the AD and BD fusion proteins expressed by the strain. “-“ indicates lack of interaction.

3.1.25 WH2/V domain of WIP is essential for the activity of WASP-WIP complex

The WH2/V domain with the KLKK motif in WIP (Ramesh et al., 1997) is essential for complementation of *vrp1* mutant (Vaduva et al., 1999). In order to test the role of this domain for the activity of the WASP-WIP complex, DNA encoding WIP₅₁₋₅₀₃ (Fig. 3.2) which has a deletion of the V domain was generated by PCR and cloned into the yeast expression vector. Plasmids expressing WASP and WIP₅₁₋₅₀₃ were introduced into *las17Δ* cells and the transformants were examined for growth at 37°C. The *las17Δ* cells co-expressing WASP and WIP₅₁₋₅₀₃ were unable to grow at 37°C in both the plate assay and the liquid culture assay (Fig. 3.28).

Figure 3.28 The WH2/V domain of WIP is essential for the activity of the WASP-WIP complex .

- las17Δ* cells (IDY166) were transformed with plasmids expressing proteins in the following combinations: empty vector, WASP + WIP or WASP + WIP₅₁₋₅₀₃. The transformants were streaked on YPUAD plates to examine growth at 24°C and 37°C.
- A growth curve was plotted by measuring the OD₆₀₀ at 1 hour intervals for liquid culture of the above mentioned strains grown at 24°C and 37°C.

3.1.26 WIP₅₁₋₅₀₃ is able to localize to cortical patches in *las17Δ* cells

WASP was unable to rescue the growth defect of *las17Δ* cells in the presence of WIP₅₁₋₅₀₃. The localization of WIP₅₁₋₅₀₃ was therefore examined in *las17Δ* cells by generating a fusion construct WIP₅₁₋₅₀₃-GFP. Plasmid expressing WIP₅₁₋₅₀₃-GFP was introduced into *las17Δ* cells and the transformants were analyzed by fluorescence microscopy. WIP₅₁₋₅₀₃-GFP localized to cortical patches in *las17Δ* cells at both 24°C and 37°C (Fig. 3.29) suggesting that the V domain of WIP is not essential for the localization of WIP in *las17Δ* cells.

Figure 3.29 WIP₅₁₋₅₀₃ localizes to the cortical **actin** patches of *las17Δ* cells.

las17Δ cells (IDY166) were transformed with plasmids expressing proteins in the following combinations: GFP, WIP-GFP or WIP₅₁₋₅₀₃-GFP. The transformants were grown in YPUAD liquid to exponential phase at 24°C. The cells were immediately processed for microscopic visualization or they were shifted to 37°C for 2 hours prior to the processing. GFP signal was visualized in living cells by fluorescence microscopy. Upper panels: FITC-fluorescence optics. Lower panels: DIC optics. Bar, 5µm..

3.1.27 WIP₅₁₋₅₀₃ is unable to interact with actin but is able to interact with WASP

The V domain of WIP has the **actin** binding KLKK motif (Ramesh et al., 1997). In order to determine whether the deletion of the V domain has any effect on the interaction of WIP with yeast **actin** and WASP, a Yeast Two Hybrid assay was performed. The strain

PJ69-4A was transformed with plasmid expressing AD-WIP₅₁₋₅₀₃ along with plasmid expressing BD-Actlp or BD-WASP. The transformants were then streaked on SD media lacking histidine to examine the interaction between the proteins. Unlike the transformants expressing BD-Actlp and AD-WIP, the transformants expressing BD-Actlp and AD-WIP₅₁₋₅₀₃ were unable to grow on SD media lacking histidine (Table 3.5). However, transformants expressing BD-WASP and either of the two AD-WIP variants were able to grow on SD minimal media plates lacking histidine (Table 3.5). Therefore, deleting the V domain of WIP abolishes the interaction between WIP and actin but does not have any effect on the interaction between WASP and WIP.

Table 3.5 WIP₅₁₋₅₀₃ does not interact with actin but interacts with WASP

	BD-WASP	BD-Actlp	BD-Vector
AD-WIP	+	+	-
AD-WIP ₅₁₋₅₀₃	+	-	-
AD-Vector		-	-

PJ69-4A transformants expressing the indicated combination of **BD** and **AD** fusion proteins were streaked on SD minimal media plates lacking histidine and incubated at 30°C for 5 days. The growth of the strains on the plates (+) indicated an interaction between the AD and **BD** fusion proteins expressed by the strain. “-“ indicates lack of interaction.

3.1.28 WIP₅₁₋₅₀₃ is able to mediate localization of WASP to cortical actin patches and also stabilizes WASP-GFP in *las17Δ* cells

The localization of WASP-GFP in *las17Δ* cells in the presence of WIP₅₁₋₅₀₃ was examined by introducing plasmids expressing WASP-GFP and WIP₅₁₋₅₀₃ in *las17Δ* cells and the transformants were analyzed by fluorescence microscopy. WIP₅₁₋₅₀₃ was able to mediate localization of WASP-GFP to cortical patches in *las17Δ* cells at both 24°C and 37°C (Fig. 3.30). Western blotting of protein extracts from *las17Δ* cells expressing

WASP-GFP and WIP₅₁₋₅₀₃ showed that the expression of WASP-GFP in the strain was comparable to the expression of WASP-GFP in cells expressing full length WIP (Fig. 3.31).

Figure 3.30 WASP-GFP localizes to the cortical patches of *las17Δ* cells in the presence of WIP₅₁₋₅₀₃.

las17Δ cells (IDY166) were transformed with plasmids expressing proteins in the following combinations: GFP, WASP-GFP + WIP or WASP-GFP + WIP₅₁₋₅₀₃. The transformants were grown in YPUAD liquid to exponential phase at 24°C. The cells were immediately processed for microscopic visualization or they were shifted to 37°C for 2 hours prior to the processing. GFP signal was visualized in living cells by fluorescence microscopy. Upper panels: FITC-fluorescence optics. Lower panels: DIC optics. Bar, 5μm..

The ability of WASP-GFP to localize to cortical actin patches in the presence of WIP₅₁₋₅₀₃ was then examined in *las17Δ* cells expressing Arc40p-RFP using epifluorescence microscopy. Some of the WASP-GFP patches were found to co-localize with Arc40p-RFP patches (Fig. 3.32), suggesting that the WASP-WIP₅₁₋₅₀₃ complex is localized to cortical actin patches. Thus the inability of WASP and WIP₅₁₋₅₀₃ to suppress the growth defect of *las17Δ* cells is not due to inefficient localization or instability of WASP. This suggests that the V domain of WIP is essential for WASP's activity, possibly by providing an additional actin binding site to the WASP-WIP complex.

Figure 3.31 Expression of WASP-GFP in the presence of WIP₅₁₋₅₀₃, in *las17Δ* cells.

Total protein extract was prepared from IDY166 transformants expressing WASP-GFP + WIP and WASP-GFP + WIP₅₁₋₅₀₃ growing at exponential phase at 24°C. The extracted proteins were separated by SDS-PAGE and Western blotted. The GFP fusion proteins were detected using anti-GFP serum and Hexokinase (loading control) was detected using anti-Hexokinase serum.

Figure 3.32 WASP-GFP localizes to cortical actin patches in the presence of WIP₅₁₋₅₀₃.

las17Δ cells (IDY166) were co-transformed with vectors expressing WASP-GFP, Arc40p-RFP and WIP₅₁₋₅₀₃. The transformants were grown in YPUAD liquid to exponential phase at 24°C. The cells were processed immediately for microscopic visualization or they were shifted to 37°C for 2 hours prior to the processing. GFP and RFP were visualized by fluorescence microscopy. Arrows point to co-localizing signals. Bar, 5 μm.

WASP Constructs	Growth (37°C)	Localization	
		24°C	37°C
WASP			
WASP + WIP	+	+	+
WASP-CAAX		N	N
WASP ^{L270P}			
..... + WIP	+	+	+
WASP ^{L270P} -CAAX		N	N
WASP ₁₃₉₋₅₀₂ + WIP			
WASP + WIP ₁₋₄₀₃			
WASP ₁₃₉₋₅₀₂ -CAAX		N	N
WASP ₁₃₉₋₅₀₂ -CAAX + WIP		N	N
WASP ₁₋₄₂₄ + WIP		+	+
WASP ^{AAA} + WIP		+	+
WASP + WIP ₅₁₋₅₀₃	-	+	+

Table 3.6 Summary of functional studies on the expression of human WASP in *S. cerevisiae las17Δ* cells.

+: indicates ability to suppress growth defect or the localization to the cortical patches of *las17Δ* cells.

-: indicates no growth at 37°C or no localization to the cortical patches.

N: Not done.

3.2 Functional analysis of Wiskott Aldrich Syndrome (WAS) mutations using *S. cerevisiae las17Δ* cells

3.2.1 Introduction

S. cerevisiae las17Δ cells co-expressing WASP and WIP were able to grow at the restrictive temperature though the other defects in the strain namely endocytosis and actin patch polarization, were not rescued. The ability of WASP and WIP to suppress the growth defects of *las17Δ* cells requires the formation of WASP-WIP protein complex. If the interaction between WASP and WIP is abolished by deleting the WH1 domain of WASP or the WASP binding domain (WBD) of WIP, then WASP is unable to suppress the growth defects of *las17Δ* cells, in the presence of WIP. In this section, the effect of the Wiskott Aldrich Syndrome (WAS) missense mutations on the activity of WASP in suppressing the growth defects of *las17Δ* cells was examined.

3.2.2 Generation of WASP mutants with the missense mutations causing WAS

Mutations that lead to Wiskott Aldrich Syndrome (WAS) disease are missense mutations, null mutations or splice variants of WASP (Derry et al., 1994). There are around 53 known missense point mutations which cause the disease with varying degrees of severity (Imai et al., 2003). The effect of 52 WAS missense mutations on the activity of WASP in *las17Δ* cells was studied. The point mutations were individually generated in the DNA encoding WASP by site directed mutagenesis to generate individual WASP constructs each carrying a different missense mutation (Figs. 3.33, 3.34). A summary of the findings is listed in Table 3.7.

Figure 3.33 Locations of the Wiskott Aldrich Syndrome (WAS) mutations in WASP protein.

Figure 3.34 Locations of the Wiskott Aldrich Syndrome (WAS) WH1 domain mutations and the C-terminal mutations in WASP.

3.2.3 WASP mutants bearing missense mutations in the WH1 domain are unable to suppress growth defects of *las17Δ* cells

The activity of the 52 WASP mutants in *S. cerevisiae las17Δ* cells was studied by co-expressing individual WASP mutants with WIP in the *las17Δ* strain (IDY166). The relevant transformants were streaked on YPUAD plates to examine the growth of the strains at 24°C and 37°C. Of the 52 WASP mutants examined, 13 mutants were unable to restore growth of *las17Δ* cells at 37°C in the plate assay (Figs. 3.35-41; Table 3.7). The 13 mutations (E31K, L35H, G40V, L46P, W64R, G70W, C73Y, I85T, T111P, G125R, F128L, E133K and A134T) which abolished the activity of WASP are all located in the WH1 domain of WASP.

After analyzing the effect of the 52 WAS mutations on the activity of WASP in suppressing the growth defects of the *las17Δ* cells, it was found that 39/52 mutations did not affect the ability of WASP in suppressing the growth defects of *las17Δ* cells in the plate assay (Figs. 3.35-43; Table 3.7). In order to further characterize the effect of these 39 mutations on the activity of WASP at 37°C, a growth curve was plotted by measuring the OD₆₀₀ at 1 hour intervals for the strains expressing these WASP constructs. Visual

examination of the growth curve suggests that the WASP mutants were not as efficient as wild type WASP in suppressing the growth defects of the *las17Δ* cells (Figs. 3.35-43).

Figure 3.39 Effect of the Wiskott Aldrich Syndrome (WAS) missense mutations F84L, I85T, R86H, G89D, W97C and Q99R on the activity of WASP in *las17Δ* cells.

Figure 3.40 Effect of the Wiskott Aldrich Syndrome (WAS) missense mutations L105P, Y107C, T111P, H115Y, G119E and A124E on the activity of WASP in *las17Δ* cells.

Figure 3.43 Effect of the Wasp Aldrich Syndrome (WAS) miss mutations P373S, P459S, P476E, R477K, I481N and D485N on activity of WASP in *las17Δ* cells.

3.2.4 Localization of WASP mutants in *las17Δ* cells, in the presence of WIP

Wild type WASP localizes to the cortical actin patches of *las17Δ* cells in the presence of WIP. After examining the ability of the 52 WASP mutants to suppress the growth defects of *las17Δ* cells, the sub-cellular localization of the WASP mutants in the presence of WIP was examined. The DNA encoding GFP was fused to the C-terminus of the DNA encoding the WASP constructs to generate WASP-GFP fusion constructs. Wild type WASP and the WASP mutants fused with GFP were then expressed with WIP in *las17Δ* cells and the transformants were analyzed with a fluorescence microscope. Wild type WASP-GFP and the mutant WASP-GFP bearing the C-terminal region mutations were able to localize to the cortical patches at both 24°C and 37°C (Figs 3.51-52; Table 3.7).

In the case of the WASP WH1 domain mutants, of the 13 mutants which were unable to rescue the growth defects of *las17Δ* cells in the plate assay, WASP-GFP bearing each of the 5 mutations (W64R, G70W, T111P, G125R or F128L) were unable to localize to cortical patches at both 24°C and 37°C (Figs. 3.46-47, 3.49-50; Table 3.7). Mutant WASP-GFP bearing each of the remaining 8 mutations (E31K, L35H, G40V, L46P, C73Y, I85T, E133K or A134T) localized to cortical patches at 24°C but not at 37°C (Figs. 3.44-45, 3.47-48, 3.50; Table 3.7). Interestingly, WASP having the following 9 WH1 domain mutations (C43W, P58L, S82P, R86H, W97C, L105P, Y107C, H115Y or A124E) localized to the cortical patches at 24°C but did not localize to the cortical patches at 37°C (Figs 3.45-49; Table 3.7) even though these WASP mutants were functional in suppressing the growth defects of *las17Δ* at 37°C. Therefore, among the 40 mutations located in the WH1 domain of WASP, 22 mutations affect the localization of WASP to cortical patches in *las17Δ* cells at either 37°C or at both 24°C and 37°C (Table 3.7).

Figure 3.44 Sub cellular localization of mutant WASP-GFP bearing the Wiskott Aldrich Syndrome (WAS) mutations M6I, L27F, E31K and L35H in *las17Δ* cells.

Figure 3.45 Sub cellular localization of mutant WASP-GFP bearing the Wiskott Aldrich Syndrome (WAS) mutations L39P, G40V, R41G, C43W, T45M and L46P in *las17Δ* cells.

Figure 3.46 Sub cellular localization of mutant WASP-GFP bearing the Wiskott Aldrich Syndrome (WAS) mutations A47D, T48I, Q52H, A56V, P58L and W64R in *las17Δ* cells.

Figure 3.47 Sub cellular localization of mutant WASP-GFP bearing the Wiskott Aldrich Syndrome (WAS) mutations G70W, C73Y, V75M, Q77G, S82P and Y83C in *las17Δ* cells.

Figure 3.48 Sub cellular localization of mutant WASP-GFP bearing the Wiskott Aldrich Syndrome (WAS) mutations F84L, I85T, R86H, G89D, W97C and Q99R in *las17Δ* cells.

Figure 3.49 Sub cellular localization of mutant WASP-GFP bearing the Wiskott Aldrich Syndrome (WAS) mutations L105P, Y107C, T111P, H115Y, G119E and A124E in *las17Δ* cells.

Figure 3.50 Sub cellular localization of mutant WASP-GFP bearing the Wiskott Aldrich Syndrome (WAS) mutations G125R, F128L, E131K, E133K, A134T and R138P in *las17Δ* cells.

Figure 3.51 Sub cellular localization of mutant WASP-GFP bearing the Wiskott Aldrich Syndrome (WAS) mutations G187C, A236E, L270P, I294T, M307V and P359T in *las17Δ* cells.

Figure 3.52 Sub cellular localization of mutant WASP-GFP bearing the Wiskott Aldrich Syndrome (WAS) mutations P373S, P459S, P476E, R477K, I481N and D485N in *las17Δ* cells.

3.2.5 Changes in the expression of WASP in *las17Δ* cells caused by the mutations

It was shown earlier that the co-expression of WIP with WASP enhanced the stability of WASP in *las17Δ* cells which is dependent on interaction with WIP. Thus it is possible that some of the mutations in WASP might compromise the stability of WASP in yeast cells, leading to insufficient protein levels and inability to suppress the growth defect of *las17Δ* strain. Total protein was isolated from *las17Δ* cells co-expressing WIP and WASP-GFP bearing the WH1 domain mutations and analyzed by Western blot. Western blot analysis of the expression of recombinant WASP with the WH1 domain mutations showed that among the 13 mutants which were unable to suppress growth defect of *las17Δ* cells, protein expression could not be detected for WASP-GFP having each of the 7 mutations (G40V, W64R, G70W, T111P, G125R, F128L or E133K) but WASP-GFP having each of the remaining 6 mutations (E31K, L35H, L46P, C73Y, I85T or A134T) could be detected on the western blot (Fig. 3.53A). Additionally, protein expression could not be detected for recombinant WASP-GFP bearing the mutations C43W or A124E, though these mutations did not affect the activity of WASP in suppressing the growth defects of *las17Δ* cells (Figs. 3.36, 3.40, 3.53A).

Among the 9 mutants (G40V, C43W, W64R, G70W, T111P, A124E, G125R, F128L and E133K) which could not be detected on the western blot, WASP-GFP bearing the mutations G40V, C43W, A124E or E133K were able to localize to cortical patches at 24°C (Figs. 3.45, 3.49-50; Table 3.7). This suggests that even though WASP-GFP bearing these mutations could not be detected on the western blot, they were still expressed in sufficient quantities to mediate localization to the cortical patches which

could be detected under a fluorescence microscope. In the case of the mutant WASP-GFP bearing the remaining 5/9 mutations W64R, G70W, T111P, G125R or F128L which could not be detected earlier either in the western blot or by fluorescence microscopy, extended exposure of the X-ray film to the immunoblots revealed the bands pertaining to these proteins (Fig. 3.53B).

Figure 3.53 Expression of wild type WASP-GFP and mutant WASP-GFP bearing WH1 domain mutations.

- A. Total protein extract was prepared from IDY166 transformants growing at exponential phase at 24°C, co-expressing WIP and wild type WASP-GFP or mutant WASP-GFP having WH1 domain mutations. The extracted proteins were separated by SDS-PAGE and western blotted. The GFP fusion proteins were detected using anti-GFP serum and Hexokinase (loading control) was detected using anti-Hexokinase.
- B. The indicated WASP mutant derivatives were immunoblotted and detected by extended exposure.

WASP Constructs	Phenotype	Growth 37°C	Localization	
			24°C	37°C
WT		+	+	+
M 6 I	X	+	+	+
L 27 F	X	+	+	+
E 31 K	W/X		+	
L 35 H	W		+	
L 39 P	W/X	+	+	+
G 40 V	W		+	
R 41 G	X	+	+	+
C 43 W	W	+	+	
T 45 M	X	+	+	+
L 46 P	X		+	
A 47 D	X	+	+	+
T 48 I	X	+	+	+
Q 52 H	X	+	+	+
A 56 V	X	+	+	+
P 58 L	W/X	+	+	
W 64 R	W			
G 70 W	W			
C 73 Y	W		+	
V 75 M	W/X	+	+	+
Q 77 G	X	+	+	+
S 82 P	W	+	+	
Y 83 C	X	+	+	+
F 84 L	W/X	+	+	+
I 85 T	W		+	
R 86 H	W/X	+	+	
G 89 D	X	+	+	+
W 97 C	W/X	+	+	
Q 99 R	X	+	+	+
L 105 P	W	+	+	
Y 107 C	W/X	+	+	
T 111 P	W			
H 115 Y	W	+	+	
G 119 E	X	+	+	+
A 124 E	W	+	+	
G 125 R	W			
F 128 L	W			
E 131 K	W/X	+	+	+
E 133 K	W/X		+	
A 134 T	W		+	
R 138 P	W/X	+	+	+
G 187 C	X	+	+	+
A 236 E	X	+	+	+
L 270 P	N	+	+	+
I 294 T	N	+	+	+
M 307 V	X	+	+	+
P 359 T	W	+	+	+
P 373 S	W	+	+	+
P 459 S	W	+	+	+
K 476 E	W	+	+	+
R 477 K	X	+	+	+
I 481 N	X	+	+	+
D 485 N	W/X	+	+	+

Table 3.7 Functional studies on 52 WAS mutations and their associated phenotypes.

“+” indicates the ability to suppress growth defect or the ability to localize to the cortical patches in *las17Δ* cells in the presence of WIP, “-” indicates no growth of the strain at 37°C or no localization to the cortical patches.

W: Wiskott Aldrich syndrome (WAS),

X: X-linked thrombocytopenia (XLT),

N: X-linked neutropenia (XLN).

3.2.6 Mutant WASP bearing the mutations E31K, L35H, L46P, C73Y, I85T or A134T are expressed at both 24°C and 37°C

The following WH1 domain mutations, E31K, L35H, L46P, C73Y, I85T or A134T abolished the ability of WASP to suppress the growth defects of *las17Δ* cells. However, mutant WASP variants were expressed well enough that their localization to cortical patches at 24°C could be detected by fluorescence microscopy or by immunoblotting. It is possible that WASP bearing the 5 mutations (E31K, L35H, L46P, C73Y, I85T or A134T) were stable only at 24°C but at 37°C the proteins could be unstable thus explaining the inability of the mutant WASP to suppress the growth defects of the *las17Δ* cells. Therefore, the stability of WASP bearing the 5 mutations (E31K, L35H, L46P, C73Y, I85T or A134T) at 37°C was examined. The *las17Δ* cells co-expressing WIP and the mutant WASP were grown to exponential phase at 24°C and immediately processed for extraction or the cell culture was shifted to 37°C for 2 hours prior to the extraction of total protein. Total lysates from the strains grown at 24°C or 37°C were western blotted with anti-GFP antibody. Immunoblotting of the protein extracts showed that mutant WASP-GFP bearing the mutations E31K, L35H, L46P, C73Y, I85T or A134T were stably expressed at both 24°C and 37°C (Fig. 3.54).

Figure 3.54 Expression of mutant WASP-GFP bearing the mutations E31K, L35H, L46P, C73Y, I85T or A134T in *las17Δ* cells, in the presence of WIP at 24°C and 37°C.

Total protein extract was prepared from IDY166 transformants co-expressing WIP and wild type WASP-GFP or mutant WASP-GFP. The cells were grown to exponential phase at 24°C and processed immediately for protein extraction or shifted to 37°C for 2 hrs prior to the extraction of proteins. The extracted proteins were Western blotted and probed with anti-GFP serum and anti-Hexokinase serum.

3.2.7 A134T mutation in WASP does not affect WASP-WIP interaction in a Yeast Two Hybrid assay

Among the 52 Wiskott Aldrich Syndrome (WAS) mutations characterized in this study, it was determined that 5 mutations (E31K, L35H, L46P, C73Y, I85T or A134T) abolished the ability of WASP to suppress the growth defects of *las17Δ* even though the mutant proteins were stable at 37°C. This raises the possibility that the mutations might cause conformational changes which could affect the interaction of the mutant WASP with other cytoskeletal proteins.

Using Yeast Two Hybrid assay, it has been shown that truncated WIP (residues 377-503) representing the WASP binding region failed to interact with the WH1 domain (residues 1-170) of WASP having the A134T mutation (Stewart et al., 1999; Luthi et al., 2003). The effect of the A134T mutation on the interaction between full length WASP and WIP was not determined. Fluorescence microscopy revealed that WASP^{A134T}-GFP was able to localize to cortical patches of *las17Δ* cells in the presence of WIP at 24°C, possibly through interaction with WIP. Therefore, the interaction between the full length proteins WASP^{A134T} and WIP was examined in a Yeast Two Hybrid assay. Yeast PJ69-4A strain was transformed with plasmids expressing AD-WIP and BD-WASP or BD-WASP^{A134T} and the transformants were streaked on SD minimal media plates lacking histidine. The transformants co-expressing AD-WIP and BD-WASP/ BD-WASP^{A134T} were able to grow on SD minimal media plates lacking histidine (Fig. 3.55A), suggesting that full length WIP interacts with full length WASP^{A134T}. The interaction of WIP with WASP^{A134T} was then examined in a GST pull down assay. Plasmid expressing WIP-GST fusion was

introduced into *las17Δ* cells along with plasmid expressing WASP-GFP or WASP^{A134T}-GFP. Total lysate from transformants expressing the GST and GFP fusion proteins was obtained and clarified. GST beads were then added to the clarified cell lysate to pull down WIP-GST. After performing the pull down, the proteins bound to the beads were eluted, resolved by SDS PAGE and western blotted. The western blot membrane was probed with anti-GFP and anti-GST. Analysis of the interaction between WIP and the WASP constructs by GST pull down assay showed that WIP-GST was able to pull down wild type WASP-GFP but WASP^{A134T}-GFP did not bind to WIP-GST (Fig. 3.55B). Thus, based on the data from the Yeast Two Hybrid assay and the pull down assay, it can be concluded that the WASP^{A134T} mutant interacts weakly with WIP and that this interaction is sufficient to mediate localization at 24°C but not sufficient for suppressing the growth defect of the *las17Δ* strain at 37°C.

Figure 3.55 WASP^{A134T} does not interact with WIP

- A. *S. cerevisiae* skain PJ69-4A was co-transformed with plasmids expressing the BD fusion proteins and the AD fusion proteins in the combinations as indicated in the table. The transformants were streaked on SD minimal media plates lacking histidine and incubated at 30°C for 5 days. The growth of the strains on the plates (+) indicated an interaction between the AD and BD fusion proteins expressed by the strain. “-” indicates lack of interaction.
- B. *las17Δ* cells (IDY166) were co-transformed with plasmids expressing WIP-GST and WASP-GFP or WASP^{A134T}-GFP. The transformants were grown in YPUAD liquid at 24°C to exponential phase and a GST pull down assay was done. The GFP fusion proteins were detected using anti-GFP serum and WIP-GST was detected using anti-GST serum.
- C. Total cell lysate from *las17Δ* cells (IDY166) co-expressing WIP-GST and WASP-GFP or WASP^{A134T}-GFP was western blotted and probed with anti-GFP serum and anti-Hexokinase serum.

3.2.8 WASP^{A134T} interacts with *S. cerevisiae* actin and the SH3 domains of Myo5p, Hoflp and Bzzlp

Wild type WASP interacts with *S. cerevisiae* actin and the SH3 domains of Myo5p, Hoflp and Bzzlp in a Yeast Two Hybrid assay. Expression of WASP^{A134T} was unable to rescue the growth defects of *las17Δ* cells in the presence of WIP. The mutations in WASP^{A134T} abolished interaction with WIP in a pull down assay. In order to determine whether the mutation in WASP^{A134T} also affects the interaction of the mutant WASP with the yeast cytoskeletal proteins, a Yeast Two Hybrid assay was performed. The DNA encoding WASP^{A134T} was cloned into the vector pACT2 (pAD). pAD-WASP^{A134T} was introduced into the *S. cerevisiae* strain PJ69-4A along with each of the pBD vectors expressing actin or the SH3 domains of Myo5p, Hoflp and Bzzlp. The transformants were then screened for growth on SD minimal media plates lacking histidine. The transformants expressing BD-WASP and the AD domain fused with Actlp, Myo5p₁₀₈₅₋₁₂₁₉, Hoflp₅₅₁₋₆₆₉ or Bzzlp₄₉₄₋₆₆₃ were able to grow on SD minimal media plates lacking histidine (Table 3.8), suggesting that WASP^{A134T} is able to interact with actin and the SH3 domains of MyoSp, Hoflp and Bzzlp. Therefore, the mutation in WASP^{A134T} only affects the interaction of the protein with WIP but does not affect the interaction of the mutant WASP with the yeast cytoskeletal proteins.

Table 3.8 Interaction of WASP^{A134T} with *S.cerevisiae* actin and SH3 domains of Bzzlp, Hoflp and Myo5p.

	AD-WASP	AD-WASP ^{A134T}	AD-Vector
BD-Actlp	+	+	-
BD-Bzzlp ₄₉₄₋₆₆₃	+	+	-
BD-Hoflp ₅₅₁₋₆₆₉	+	+	-
BD-Myo5p ₁₀₈₅₋₁₂₁₉	+	+	-
BD-Vector	-	-	-

PJ69-4A transformants expressing the indicated fusion proteins were streaked on SD minimal media plates lacking histidine and incubated at 30°C for 5 days. The growth of the strains on the plates (+) indicated an interaction between the AD and BD fusion proteins expressed by the strain. “-” indicates lack of interaction.

3.3 Functional analysis of Las17p-Vrp1p interaction

3.3.1 Introduction

Las17p, the yeast homologue of WASP, is essential for the viability of *S. cerevisiae* cells at 37°C but not at 24°C (Li, 1997). Deletion of *LAS17/BEE1* results in defects in endocytosis and actin patch polarization at both permissive (24°C) and restrictive temperature (37°C) (Li, 1997; Madania et al., 1999), even though the *las17Δ* strain has 2 additional proteins (Myo3p and Myo5p) with an acidic domain (A) which are functionally redundant with the acidic domain of Las17p (Evangelista et al., 2000; Lechler et al., 2000). In contrast to the phenotype resulting from the deletion of the entire *LAS17* gene, deleting only the WH2/V domain and the acidic domain (A) of Las17p (Las17pΔVCA) does not cause major defects to the strain at either 24°C or 30°C (Winter et al., 1999). Thus we sought to determine the molecular mechanism by which Las17p lacking the acidic domain rescues the defects of *las17Δ* strain.

3.3.2 N-Las17p₁₋₃₆₈ complements the growth defects of *las17Δ* strain

Vrp1p is the *S. cerevisiae* homologue of mammalian WIP (Ramesh et al., 1997). Expression of either the N-terminal or the C-terminal region of Vrp1p in *vrp1Δ* strain is capable of suppressing the growth defect of the strain (Thanabalu and Munn, 2001), suggesting that the domains in the N-terminal and the C-terminal regions of Vrp1p can function independently to restore growth. Deletion or mutation of the *LAS17* gene results in a phenotype similar to the deletion of *VRP1* gene (Naqvi et al. 1998; Madania et al., 1999). The N-terminal region of Las17p contains the WH1 domain which mediates binding with Vrp1p (Naqvi et al., 1998) and the C-terminal VCA domain activates the

Arp2/3 complex (Rodal et al., 2003). A possibility that the N-terminal and the C-terminal fragments of Las17p can restore the growth defects of *las17Δ* strain was therefore investigated.

In order to express full length Las17p, N-Las17p₁₋₃₆₈ (N-terminal region of Las17p) and C-Las17p₃₇₀₋₆₃₃ (C-terminal region of *las17p*) in *las17Δ* strain, the DNA encoding Las17p, N-Las17p₁₋₃₆₈ and C-Las17p₃₇₀₋₆₃₃ under the transcriptional regulation of the LAS17 promoter were cloned in a yeast shuttle vector YCplac33 (Fig. 3.56). The plasmids expressing Las17p, N-Las17p₁₋₃₆₈ and C-Las17p₃₇₀₋₆₃₃ were introduced into a *las17Δ* strain (IDY223) by Lithium Acetate transformation. The transformants expressing Las17p, N-Las17p₁₋₃₆₈ and C-Las17p₃₇₀₋₆₃₃ were streaked on YPUAD plates to examine for growth at 37°C and a growth curve at 37°C was plotted by measuring the OD₆₀₀ of the liquid culture at 1 hour intervals. Transformants expressing N-Las17p₁₋₃₆₈ were able to grow at 37°C in both the plate assay and the liquid culture assay just like the transformants expressing full length Las17p, while the transformants expressing C-Las17p₃₇₀₋₆₃₃ were unable to grow at 37°C in either the plate assay or the liquid culture assay (Fig. 3.57).

Figure 3.56 Schematic map of Las17p, N-Las17p₁₋₃₆₈ and C-las17p₃₇₀₋₆₃₃ constructs.

Figure 3.57 Expression of N-Las17p₁₋₃₆₈ complements the growth defects of *las17Δ* cells.

- A. Empty vector or vector encoding Las17p, N-Las17p₁₋₃₆₈ or C-Las17p₃₇₀₋₆₃₃ was introduced into IDY223. The transformants were streaked on YPUAD plates to examine growth at 24°C and 37°C.
- B. A growth curve was plotted by measuring the OD₆₀₀ at 1 hour intervals for liquid culture of the above mentioned strains grown at 24°C and 37°C.

3.3.3 N-Las17p₁₋₃₆₈ restores endocytosis and **actin** patch polarization in *las17Δ* strain

Expression of N-Las17p₁₋₃₆₈ complements the growth defects of the *las17Δ* cells, but can it replace full length Las17p in complementing all the defects of the *las17Δ* strain? In order to answer this question, the ability of N-Las17p₁₋₃₆₈ to complement the defects in endocytosis and **actin** patch polarization of *las17Δ* cells was examined. *las17Δ* transformants expressing Las17p, N-Las17p₁₋₃₆₈ and C-Las17p₃₇₀₋₆₃₃ were analyzed for uptake of Lucifer Yellow (LY) in a fluid phase endocytosis assay. Expression of either Las17p or N-Las17p₁₋₃₆₈ in *las17Δ* cells enabled the uptake of Lucifer Yellow (LY) at

both 24°C and 37°C as indicated by the fluorescence in the vacuoles whereas the transformants expressing C-Las17p₃₇₀₋₆₃₃ showed no fluorescence in the vacuoles at either 24°C or 37°C (Fig. 3.58) just like the control cells harboring an empty vector. The fluorescence in the vacuoles of the *las17Δ* cells expressing N-Las17p₁₋₃₆₈ was comparable to that of the cells expressing Las17p (Fig. 3.58).

Figure 3.58 Expression of N-Las17p₁₋₃₆₈ rescues the endocytosis defects of *las17Δ* cells.

IDY223 transformants growing exponentially at 24°C and harboring empty vector, vector expressing Las17p, N-Las17p₁₋₃₆₈ or C-Las17p₃₇₀₋₆₃₃ were incubated with LY at 24°C or 37°C and washed thoroughly. For performing the assay at 37°C, the cells were incubated at 37°C for 2 hours prior to the addition of LY. LY signal was visualized by fluorescence microscopy. Upper panels: fluorescence optics. Lower panels: DIC optics. Bar, 5µm.

The distribution of **actin** patches in *las17Δ* cells expressing Las17p, N-Las17p₁₋₃₆₈ and C-Las17p₃₇₀₋₆₃₃ was then examined by staining the cells with Alexa-488-conjugated

phalloidin. The *S. cerevisiae las17Δ* cells expressing N-Las17p₁₋₃₆₈ showed polarization of actin patches at both 24°C and 37°C in contrast to the transformants expressing C-Las17p₃₇₀₋₆₃₃ where the actin patches were depolarized (Fig. 3.59A). The ability of N-Las17p₁₋₃₆₈ to rescue the cytoskeletal defect in *las17Δ* cells was further supported by the observation that the sensitivity of *las17Δ* cells to 1M salt was rescued by expression of N-Las17p₁₋₃₆₈ (Fig. 3.59B).

Figure 3.59 Expression of N-Las17p₁₋₃₆₈ restores actin patch polarization in *las17Δ* cells.

- A. *las17Δ* cells harboring empty vector or vector expressing Las17p, N-Las17p₁₋₃₆₈ or C-Las17p₃₇₀₋₆₃₃ were fixed immediately or were shifted to 37°C for 2 hours prior to fixing. The fixed cells were permeabilized and F-actin stained with Alexa-488-conjugated phalloidin. The actin patches were visualized by fluorescence microscopy. Bar, 5μm.
- B. The transformants harboring empty vector or vector expressing Las17p or N-Las17p₁₋₃₆₈ were streaked on YPUAD plate and YPUAD plate containing 1M NaCl and incubated at 24°C.

3.3.4 N-Las17p₁₋₃₆₈ localizes to the cortical actin patches of *las17Δ* cells

Las17p localizes to cortical actin patches through its own localization activity as well as through interaction with Vrp1p (Li, 1997; Lechler et al., 2001). In order to determine their localization, GFP fusion constructs of Las17p, N-Las17p₁₋₃₆₈ and C-Las17p₃₇₀₋₆₃₃ were generated. Centromeric plasmids expressing GFP, Las17p-GFP, N-Las17p₁₋₃₆₈-GFP or C-Las17p₃₇₀₋₆₃₃-GFP were introduced into the *las17Δ* strain and the transformants were analyzed by fluorescence microscopy. Live cell imaging showed that Las17p-GFP and N-Las17p₁₋₃₆₈-GFP localized to cortical patches in *las17Δ* cells at both 24°C and 37°C while GFP and C-Las17p₃₇₀₋₆₃₃-GFP showed diffused cytoplasmic staining (Fig. 3.60).

Figure 3.60 N-Las17p₁₋₃₆₈-GFP localizes to the cortical patches of *las17Δ* cells.

Vector encoding GFP, Las17p-GFP, N-Las17p₁₋₃₆₈-GFP or C-las17p₃₇₀₋₆₃₃-GFP was introduced into *las17Δ* cells. The transformants were grown in YPUAD liquid to exponential phase at 24°C. For microscopic visualization at 37°C, the cells were shifted to 37°C for 2 hours prior to the processing. GFP signal was visualized by fluorescence microscopy. Upper panels: FITC-fluorescence optics. Lower panels: DIC optics. Bar, 5μm.

In order to determine whether N-Las17p₁₋₃₆₈-GFP localizes to cortical actin patches, Arc40p-RFP was expressed in the cells expressing Las17p-GFP and N-Las17p₁₋₃₆₈-GFP. The transformants were examined under a fluorescence microscope using the GFP and RFP filter sets. Some of the Las17p-GFP and N-Las17p₁₋₃₆₈-GFP patches were found to co-localize with Arc40p-RFP patches (Fig. 3.61A). The expression of N-Las17p₁₋₃₆₈-GFP and C-Las17p₃₇₀₋₆₃₃-GFP in the *las17Δ* cells was then examined. Total protein extract prepared from the transformants expressing N-Las17p₁₋₃₆₈-GFP and C-Las17p₃₇₀₋₆₃₃-GFP was resolved by SDS PAGE and analyzed by western blot. Immunoblotting of protein extracts showed that both N-Las17p₁₋₃₆₈-GFP and C-Las17p₃₇₀₋₆₃₃-GFP are expressed at comparable levels (Fig. 3.61B). Thus, N-Las17p₁₋₃₆₈ can functionally replace Las17p in complementing the growth, endocytosis and actin patch polarization defects in *S. cerevisiae las17Δ* cells.

Figure 3.61 N-Las17p₁₋₃₆₈ localizes to the cortical actin patches of *las17Δ* cells.

- A** *las17Δ* cells co-expressing Arc40p-RFP and Las17p-GFP or N-las17p₁₋₃₆₈-GFP were grown in YPUAD liquid at 24°C. GFP and RFP were visualized by fluorescence microscopy. Arrows point to co-localizing patches. Bar, 5μm.
- B.** Total protein extract was prepared from *las17Δ* cells expressing N-Las17p₁₋₃₆₈-GFP or C-Las17p₃₇₀₋₆₃₃-GFP. The extracted proteins were separated by SDS-PAGE and Western blotted. The GFP fusion proteins were detected using anti-GFP serum and Hexokinase (loading control) was detected using anti-Hexokinase serum.

3.3.5 N-Las17p₁₋₃₆₈ interacts with SH3 domain containing proteins and Vrplp in a Yeast Two Hybrid assay

Las17p has been shown to interact with many cytoskeletal proteins including Vrplp, Actlp, Bzzlp, Hoflp, Myo3p, Myo5p and Rvs167p (Naqvi et al., 1998; Soulard et al., 2002; Naqvi et al., 2001; Evangelista et al., 2000; Lechler et al., 2000; Colwill et al., 1999). Most of these proteins are essential for growth and also for endocytosis and actin patch polarization in *S. cerevisiae*. SH3 domain containing proteins such as Bzzlp, Hoflp, Myo3p, Myo5p and Rvs167p bind to Las17p proline rich motifs. The LBD domain of Vrplp mediates interaction with the WH1 domain of Las17p. In full-length Las17p, the region of the protein located between the residues 369-633 encompasses the VCA domain and proline rich motifs. N-Las17p₁₋₃₆₈ encompasses the WH1 domain and some proline rich motifs. The interaction of N-Las17p₁₋₃₆₈ with Vrplp, *S. cerevisiae* actin and the SH3 domain containing proteins was examined in a Yeast Two Hybrid assay. pAD-N-Las17p₁₋₃₆₈ was introduced into the *S. cerevisiae* strain PJ69-4A along with each of the pBD vectors expressing the C-terminal region of Vrplp, Actlp or the SH3 domains of Myo3p, Myo5p, Hoflp, Bzzlp and Rvs167p. The transformants were then tested for growth on SD minimal media plates lacking histidine. The transformants co-expressing AD-N-Las17p₁₋₃₆₈ and the BD fusion of Vrplp₃₆₄₋₈₁₇ and the SH3 domains of Myo5p, Hoflp, Bzzlp and Rvs167p were able to grow on SD minimal media plates lacking histidine (Table 3.9). The Yeast Two Hybrid assay suggests that N-Las17p₁₋₃₆₈ interacts with the C-terminal region of Vrplp and the SH3 domains of Myo5p, Hoflp, Bzzlp and Rvs167p. Although N-Las17p₁₋₃₆₈ lacks the VCA domain, the protein is

functional and complements the defects of *las17Δ* cells possibly through its interaction with other cytoskeletal proteins like Vrp1p, Myo5p, Hof1p, Bzz1p and Rvs167p.

Table 3.9 Interaction of N-Las17p₁₋₃₆₈ with actin, Vrp1p and SH3 domain containing proteins.

	AD-N-Las17p ₁₋₃₆₈	AD-Vector
BD-Act1p	-	-
BD-Vrp1p ₃₆₄₋₈₁₇	+	-
BD-Bzz1p ₄₉₄₋₆₆₃	+	-
BD-Hof1p ₅₅₁₋₆₆₉	+	-
BD-Myo3p ₁₁₁₈₋₁₁₈₈	-	-
BD-Myo5p ₁₀₈₅₋₁₂₁₉	+	-
BD-Rvs167p ₄₂₃₋₄₈₂	+	-
Vector	-	-

Plasmids expressing the indicated BD and AD fusion proteins were introduced into the strain PJ69-4A, the transformants were streaked on SD minimal media plates lacking histidine and incubated at 30°C for 5 days. The growth of the strains on the plates (+) indicated an interaction between the AD and BD fusion proteins. “-” indicates lack of interaction.

3.3.6 Isolation of temperature sensitive mutant of N-Las17p₁₋₃₆₈

Although N-Las17p₁₋₃₆₈ lacks the VCA domain, expression of N-Las17p₁₋₃₆₈ rescues the growth defects of *las17Δ* cells. The function of the domains of N-Las17p₁₋₃₆₈ can be studied by introducing mutations into the DNA encoding N-Las17p₁₋₃₆₈ which compromise the activity of the protein. A random PCR mutagenesis was performed as described (Leung et al., 1989). First, the plasmid coding for N-Las17p₁₋₃₆₈ (pN-Las17p₁₋₃₆₈) was used as a template for the PCR amplification using primers which would amplify the region encompassing the restriction endonuclease sites for KpnI and *EcoRV* on the DNA encoding N-Las17p₁₋₃₆₈. The PCR was carried out in the presence of Mn²⁺ ions in order to induce random mutagenesis. The PCR product was isolated and purified. Next, pN-Las17p₁₋₃₆₈ was digested with the restriction endonucleases KpnI and *EcoRV*, the vector backbone was isolated and purified. Then, the vector backbone and the PCR product were mixed and introduced into the *las17Δ* strain (IDY223) by Lithium acetate

transformation (Schematic in Fig. 3.62). After a period of incubation at 24°C, the transformants were then replica plated onto YPUAD plates and growth of the colonies at 37°C was examined. The transformants which did not grow at 37°C were selected from the replica plated cells grown at 24°C and further tested for their ability to carry out fluid phase endocytosis (Lucifer Yellow uptake) at 24°C and the distribution of actin patches at 24°C was also examined. The additional assays were carried out to ensure that the observed temperature sensitivity is not due to lack of expression/ poor expression of N-Las17p₁₋₃₆₈.

Figure 3.62 PCR mutagenesis of the DNA encoding N-Las17p₁₋₃₆₈.

Plasmid pN-Las17p₁₋₃₆₈ was digested with the restriction endonucleases *KpnI* and *EcoRV* and the vector backbone was purified. The DNA coding for N-Las17p₁₋₃₆₈ at the region flanking the restriction enzyme sites *KpnI* and *EcoRV* was PCR amplified in the presence of Mn²⁺ and the PCR product was purified. The purified PCR product and vector backbone were mixed and introduced into *S. cerevisiae las17Δ* cells.

Among the temperature-sensitive transformants which were screened for fluid phase endocytosis and actin patch polarization at 24°C, one transformant met the criteria for selection and the plasmid from the transformant was isolated and sequenced. Sequence analysis revealed two mutations in the DNA encoding N-Las17p₁₋₃₆₈ predicted to result in two amino acid substitutions (L80T and H94L), N-Las17p₁₋₃₆₈^{TL}. In order to ensure that the observed temperature sensitivity is due to the two mutations and not due to other mutations in other regions of the plasmid, the two mutations were engineered by site directed mutagenesis to generate the plasmid clone pN-Las17p^{TL} which encodes for N-Las17p₁₋₃₆₈^{TL} (Fig. 3.63). Plasmids encoding N-Las17p₁₋₃₆₈ or N-Las17p₁₋₃₆₈^{TL} were introduced into *las17Δ* cells and the transformants were examined for growth at 37°C. The mutant cells expressing N-Las17p₁₋₃₆₈^{TL} were unable to grow at 37°C in neither the plate assay, nor the liquid culture assay (Fig. 3.64) suggesting that the inability of N-Las17p₁₋₃₆₈^{TL} to suppress the growth defects of *las17Δ* cells is indeed due to the mutations present in the protein.

Figure 3.63 Schematicmap of N-Las17p₁₋₃₆₈^{TL}, N-Las17p₁₋₃₆₈^T and N-las17p₁₋₃₆₈^L.

Figure 3.64 The mutant, N-Las17p₁₋₃₆₈^{TL} does not rescue growth defects of *las17Δ* cells.

- A. Empty vector or vector encoding N-Las17p₁₋₃₆₈ or N-Las17p₁₋₃₆₈^{TL} was introduced into *las17Δ* cells. The transformants were streaked on YPUAD plates to examine growth at 24°C and 37°C.
- B. A growth curve was plotted by measuring the OD₆₀₀ at 1 hour intervals for liquid culture of the above mentioned strains grown at 24°C and 37°C.

3.3.7 N-Las17p₁₋₃₆₈^{TL} restores fluid phase endocytosis and polarization of actin patches of *las17Δ* cells at 24°C but not at 37°C

las17Δ cells expressing N-Las17p₁₋₃₆₈^{TL} were unable to grow at 37°C. The ability of N-Las17p₁₋₃₆₈^{TL} to rescue the defects in endocytosis and actin patch polarization in *las17Δ* cells at both 24°C and 37°C was examined. Expression of N-Las17p₁₋₃₆₈^{TL} in *las17Δ* cells enabled vacuolar uptake of Lucifer Yellow (LY) at 24°C but not at 37°C (Fig. 3.65) as indicated by the fluorescence in the vacuoles at 24°C but not at 37°C. The distribution of actin patches in *las17Δ* cells expressing N-Las17p₁₋₃₆₈ and N-Las17p₁₋₃₆₈^{TL} was then

examined by staining the actin patches with Alexa-488-conjugated phalloidin. Expression of N-Las17p₁₋₃₆₈^{TL} in the *las17Δ* cells restored polarization of actin patches distribution at 24°C but the actin patches were depolarized at 37°C (Fig. 3.66).

Figure 3.65 N-Las17p₁₋₃₆₈^{TL} does not rescue the defects in endocytosis of *las17Δ* cells at 37°C.

IDY223 transformants harboring empty vector, vector expressing **Las17p**, N-Las17p₁₋₃₆₈ or N-Las17p₁₋₃₆₈^{TL} were tested for LY uptake at 24°C or 37°C. For performing the assay at 37°C, the cells were incubated at 37°C for 2 hours prior to the addition of LY. LY signal was visualized by fluorescence microscopy. Upper panels: fluorescence optics. Lower panels: DIC optics. Bar, 5μm.

Figure 3.66 Expression of N-Las17p₁₋₃₆₈^{TL} restores actin patch polarization in *las17Δ* cells only at 24°C and not at 37°C.

las17Δ cells expressing Las17p, N-Las17p₁₋₃₆₈ or N-Las17p₁₋₃₆₈^{TL} were fixed immediately or were shifted to 37°C for 2 hours prior to fixing. The fixed cells were permeabilized and F-actin stained with Alexa-488-conjugated phalloidin. The actin patches were visualized by fluorescence microscopy. Bar, 5 μm.

3.3.8 N-Las17p₁₋₃₆₈^{TL} localizes to cortical patches of *las17Δ* cells

N-Las17p₁₋₃₆₈^{TL} was unable to rescue the growth, endocytosis or actin patch polarization defects of *las17Δ* cells at 37°C. N-Las17p₁₋₃₆₈ localizes to cortical patches at both 24°C and 37°C. Thus the localization of N-Las17p₁₋₃₆₈^{TL} in *las17Δ* cells was examined to determine whether N-Las17p₁₋₃₆₈^{TL} was non functional at 37°C due to any defects in its localization. A translation fusion construct N-Las17p₁₋₃₆₈^{TL}-GFP was generated and expressed in *las17Δ* cells and the localization was studied by fluorescence microscopy. N-Las17p₁₋₃₆₈^{TL}-GFP localized to the cortical patches of *las17Δ* cells at both 24°C and 37°C (Fig. 3.67). However, there were fewer N-Las17p₁₋₃₆₈^{TL}-GFP patches at 37°C as compared to 24°C. In order to determine whether N-Las17p₁₋₃₆₈^{TL}-GFP localizes to cortical actin patches, Arc40p-RFP was expressed in cells expressing N-Las17p₁₋₃₆₈^{TL}-GFP and subjected to epifluorescence microscopy. Some of the N-Las17p₁₋₃₆₈^{TL}-GFP patches were found to co-localize with Arc40p-RFP patches (Fig. 3.68A)

Figure 3.67 N-Las17p₁₋₃₆₈^{TL}-GFP localizes to the cortical patches of *las17Δ* cells.

Transformants expressing GFP, Las17p-GFP, N-Las17p₁₋₃₆₈-GFP or N-Las17p₁₋₃₆₈^{TL}-GFP were grown in YPUAD liquid. GFP signal was visualized in living cells by fluorescence microscopy. Upper panels: FITC-fluorescence optics. Lower panels: DIC optics. Bar, 5μm.

Figure 3.68 N-Las17p₁₋₃₆₈^{TL}-GFP expression is comparable to that of N-Las17p₁₋₃₆₈-GFP and localizes to the cortical actin patches of *las17Δ* cells.

- A. Strain IDY223 was co-transformed with vectors expressing Arc40p-RFP and N-Las17p₁₋₃₆₈-GFP or N-Las17p₁₋₃₆₈^{TL}-GFP. The transformants were grown in YPUAD liquid to exponential phase at 24°C. GFP and RFP were visualized by fluorescence microscopy. Arrows point to co-localizing patches. Bar, 5μm.
- B. Total protein extract was prepared from IDY223 transformants growing exponentially at 24°C and harboring vector expressing N-Las17p₁₋₃₆₈-GFP or N-Las17p₁₋₃₆₈^{TL}-GFP. The extracted proteins were separated by SDS-PAGE and Western blotted. The GFP fusion proteins were detected using anti-GFP serum and Hexokinase (loading control) was detected using anti-Hexokinase serum.

The expression of N-Las17p₁₋₃₆₈^{TL}-GFP was then examined to determine whether the inability of N-Las17p₁₋₃₆₈^{TL} to suppress the defects in *las17Δ* was due to reduced expression of the protein. Total protein was extracted from *las17Δ* cells expressing N-Las17p₁₋₃₆₈-GFP or N-Las17p₁₋₃₆₈^{TL}-GFP. The extracted proteins were resolved by SDS PAGE and analyzed by western blot. Western blot analysis revealed that N-Las17p₁₋₃₆₈^{TL}-GFP was expressed at levels comparable to that of N-Las17p₁₋₃₆₈-GFP (Fig. 3.68B).

3.3.9 L80T and H94L mutations abolish the activity of N-Las17p₁₋₃₆₈ in *las17Δ* cells

The mutant, N-Las17p₁₋₃₆₈^{TL} which was isolated by PCR mutagenesis was unable to rescue the defects in the *las17Δ* strain at 37°C. The locations of the two mutations L80T and H94L in N-Las17p₁₋₃₆₈^{TL} are not contiguous but they are located close to each other in the protein. The ability of N-Las17p₁₋₃₆₈ having either of the mutations L80T and H94L, to suppress the defects in *las17Δ* cells was examined. Mutations L80T and H94L were generated individually in N-Las17p₁₋₃₆₈ through site directed mutagenesis (Fig. 3.63). Plasmids coding for N-Las17p₁₋₃₆₈^T and N-Las17p₁₋₃₆₈^L were introduced into the *las17Δ* strain and the transformants were assessed for growth at 37°C. Mutant cells expressing N-Las17p₁₋₃₆₈^T and N-Las17p₁₋₃₆₈^L were able to grow at 37°C in both the plate assay and the liquid culture assay (Fig. 3.69). Then the ability of N-Las17p₁₋₃₆₈^T and N-Las17p₁₋₃₆₈^L to restore endocytosis and actin patch polarization in *las17Δ* cells was examined at 24°C and 37°C. Expression of N-Las17p₁₋₃₆₈^T or N-Las17p₁₋₃₆₈^L in *las17Δ* cells enabled the cells to take up Lucifer Yellow (LY) at both 24°C and 37°C (Fig. 3.70). Similarly, the actin patches were polarized at both 24°C and 37°C in the mutant cells expressing N-Las17p₁₋₃₆₈^T or N-Las17p₁₋₃₆₈^L (Fig. 3.71).

Figure 3.69 N-Las17p₁₋₃₆₈ with single mutations L80T or H94L complements the growth defects of *las17Δ* cells.

- A. Empty vector or vector encoding N-Las17p₁₋₃₆₈, N-Las17p₁₋₃₆₈^T or N-Las17p₁₋₃₆₈^L was introduced into *las17Δ* cells. The transformants were streaked on YPUAD plates to examine growth at 24°C and 37°C.
- B. A growth curve was plotted by measuring the OD₆₀₀ at 1 hour intervals for liquid culture of the indicated strains grown at 37°C.

Next, the sub cellular localization of N-Las17p₁₋₃₆₈^T-GFP and N-Las17p₁₋₃₆₈^L-GFP was examined in *las17Δ* cells. Both N-Las17p₁₋₃₆₈^T-GFP and N-Las17p₁₋₃₆₈^L-GFP localized to the cortical patches of *las17Δ* cells at both 24°C and 37°C (Fig. 3.72). Thus N-Las17p₁₋₃₆₈^T and N-Las17p₁₋₃₆₈^L behave like wild type N-Las17p₁₋₃₆₈ in rescuing the defects in *las17Δ* strain. Thus both mutations, L80T and H94L are needed to compromise the activity of N-Las17p₁₋₃₆₈ in *las17Δ* cells.

Figure 3.70 Expression N-Las17p₁₋₃₆₈^T or N-Las17p₁₋₃₆₈^L restores endocytosis in *las17Δ* cells. *las17Δ* cells harboring empty vector, vector expressing Las17p, N-Las17p₁₋₃₆₈, N-Las17p₁₋₃₆₈^T or N-Las17p₁₋₃₆₈^L were incubated with LY at 24°C or 37°C and washed thoroughly. For performing the assay at 37°C, the cells were incubated at 37°C for 2 hours prior to the addition of LY. LY signal was visualized by fluorescence microscopy. Upper panels: fluorescence optics. Lower panels: DIC optics. Bar, 5μm.

Figure 3.71 Expression of N-Las17p₁₋₃₆₈^T or N-Las17p₁₋₃₆₈^L restores actin patch polarization in *las17Δ* cells.

Figure 3.72 N-Las17p₁₋₃₆₈^T-GFP and N-Las17p₁₋₃₆₈^L-GFP localize to the cortical patches of *las17Δ* cells. *las17Δ* cells expressing GFP, Las17p-GFP, N-Las17p₁₋₃₆₈-GFP, N-Las17p₁₋₃₆₈^T-GFP or N-Las17p₁₋₃₆₈^L-GFP were grown in YPUAD liquid to exponential phase. GFP signal was visualized in living cells by fluorescence microscopy. Upper panels: FITC-fluorescence optics. Lower panels: DIC optics. Bar, 5μm.

3.3.10 Expression of N-Las17p₁₋₃₆₈^{TL} from a high copy (2μ) plasmid suppresses the growth defects of *las17Δ* cells

In the experiments done earlier, the activity of all the Las17p constructs including N-Las17p₁₋₃₆₈^{TL} had been studied in *las17Δ* cells by expressing the proteins from CEN plasmid (YCplac33). Expression of N-Las17p₁₋₃₆₈^{TL} from YCplac33 was unable to suppress the defects in growth, endocytosis or actin patch polarization at 37°C though endocytosis and actin patch polarization were restored at 24°C (Figs. 3.64-66). Immunoblotting of protein extracts from *las17Δ* cells expressing N-Las17p₁₋₃₆₈-GFP or

N-Las17p₁₋₃₆₈^{TL}-GFP suggests that the expression of the mutant N-Las17p₁₋₃₆₈^{TL}-GFP was similar to that of the wild type protein (Fig. 3.68B). In order to determine whether expression of N-Las17p₁₋₃₆₈^{TL} from a high copy plasmid would improve the activity of the protein and thus enable it to suppress the defects in *las17Δ* cells at 37°C, the DNA encoding N-Las17p₁₋₃₆₈^{TL} was cloned into a 2μ vector (YEplac195) to generate the plasmid pHC-N-Las17p₁₋₃₆₈^{TL}. The 2μ vector encoding N-Las17p₁₋₃₆₈^{TL} or the CEN vector encoding N-Las17p₁₋₃₆₈ were introduced into *las17Δ* cells and the transformants were streaked on YPUAD plates to examine for growth at 37°C and a growth curve at 37°C was also plotted. Expression of N-Las17p₁₋₃₆₈^{TL} from a 2μ vector suppressed the growth defects of the *las17Δ* cells in both the plate assay and the liquid culture assay (Fig. 3.73). However, visual examination of the growth curve for the liquid culture assay suggests that N-Las17p₁₋₃₆₈^{TL} expressed from a 2μ vector was not as efficient as wild type N-Las17p₁₋₃₆₈ expressed from a CEN vector in suppressing the growth defects of *las17Δ* cells (Fig. 3.73B).

Then, the expression of N-Las17p₁₋₃₆₈^{TL} expressed from 2μ plasmid was studied by tagging the protein with GFP. N-Las17p₁₋₃₆₈-GFP (CEN), N-Las17p₁₋₃₆₈^{TL}-GFP (CEN) and N-Las17p₁₋₃₆₈^{TL}-GFP (2μ) were expressed in *las17Δ* cells. Total protein was extracted from the transformants and the expression of the GFP fusion proteins was studied by Western blot. Western blot analysis revealed that N-Las17p₁₋₃₆₈^{TL}-GFP (2μ) was strongly expressed as compared to the expression of N-Las17p₁₋₃₆₈-GFP (CEN) and N-Las17p₁₋₃₆₈^{TL}-GFP (CEN) (Fig. 3.74).

Figure 3.73 Expression of N-Las17p₁₋₃₆₈^{TL} from 2μ plasmid complements the growth defect of *las17Δ* cells.

- A. Empty vector (YCplac33), YCplac33 encoding N-Las17p₁₋₃₆₈ and N-Las17p₁₋₃₆₈^{TL} or YEplac195 encoding N-Las17p₁₋₃₆₈^{TL} were introduced into *las17Δ* cells. The transformants were streaked on YPUAD plates to examine growth at 24°C and 37°C.
- B. A growth curve was plotted by measuring the OD₆₀₀ at 1 hour intervals for liquid culture of the indicated strains grown at 37°C.

Figure 3.74 Expression of N-Las17p₁₋₃₆₈^{TL} from 2μ plasmid leads to strong expression in *las17Δ* cells.

Total protein extract was prepared from IDY223 transformants growing exponentially at 24°C and harboring vector YCplac33 expressing N-Las17p₁₋₃₆₈-GFP, N-Las17p₁₋₃₆₈^{TL}-GFP or vector YEplac195 expressing N-Las17p₁₋₃₆₈^{TL}-GFP. The extracted proteins were resolved by SDS-PAGE and Western blotted. The GFP fusion proteins were detected using anti-GFP serum and Hexokinase (loading control) was detected using anti-Hexokinase serum.

3.3.11 Expression of N-Las17p₁₋₃₆₈^{TL} from 2μ plasmid restores endocytosis but not actin patch polarization in *las17Δ* cells at 37°C

Expression of N-Las17p₁₋₃₆₈^{TL} from 2μ plasmid was able to suppress the growth defects of the *las17Δ* cells. The ability of the transformants expressing N-Las17p₁₋₃₆₈^{TL} (2μ) to carry out fluid phase endocytosis and the distribution of actin patches in the transformants was examined. Expression of N-Las17p₁₋₃₆₈^{TL} from 2μ plasmid in *las17Δ* cells enabled the transformants to take up LY at both 24°C and 37°C (Fig. 3.75). Furthermore, N-Las17p₁₋₃₆₈^{TL} (2μ) was able to restore polarization of the actin patches in *las17Δ* cells at 24°C but the actin patches remained depolarized at 37°C (Fig. 3.76). Therefore, over expression of the mutant, N-Las17p₁₋₃₆₈^{TL} is unable to suppress all the defects of *las17Δ* cells.

Figure 3.75 Expression of N-Las17p₁₋₃₆₈^{TL} from 2μ plasmid restores endocytosis in *las17Δ* cells at 37°C. IDY223 transformants harboring empty vector (YCplac33), YCplac33 expressing *Las17p*, N-Las17p₁₋₃₆₈, N-Las17p₁₋₃₆₈^{TL} or YEplac195 expressing N-las17₁₋₃₆₈^{TL} were incubated with LY at 24°C or 37°C and washed thoroughly. LY signal was visualized by fluorescence microscopy. Upper panels: fluorescence optics. Lower panels: DIC optics. Bar, 5μm.

Figure 3.76 Expression of N-Las17p₁₋₃₆₈^{TL} from 2μ plasmid restores actin patch polarization in *las17Δ* cells only at 24°C and not at 37°C.

The actin patches in *las17Δ* transformants harboring empty vector (YCplac33), YCplac33 encoding Las17p, N-Las17p₁₋₃₆₈, N-Las17p₁₋₃₆₈^{TL} or YEplac195 expressing N-las17p₁₋₃₆₈^{TL} were stained and visualized by fluorescence microscopy. Bar, 5μm.

3.3.12 Mutations in N-Las17p₁₋₃₆₈^{TL} abolish interaction with Vrplp in a pull down assay

Las17p has been shown to interact with many cytoskeletal proteins like Vrplp, Bzzlp, Hoflp, Myo3p, Myo5p and Rvs167p (Naqvi et al., 1998; Soulard et al., 2002; Naqvi et al., 2001; Evangelista et al., 2000; Lechler et al., 2000; Colwill et al., 1999). N-Las17p₁₋₃₆₈ was able to interact with Vrplp, Hoflp, Bzzlp and Rvs167p in the Yeast Two Hybrid assay. Thus, the inability of N-Las17p₁₋₃₆₈^{TL} to suppress the defects of *las17Δ* strain could be due to the loss of interaction with other cytoskeletal regulators. It has been shown that the amino acid residues in the region 20-122 of Las17p mediate interaction with Vrplp (Lechler et al., 2001) (Fig. 3.77). Since the mutations in N-Las17p₁₋₃₆₈^{TL} fall in the region of the protein involved in binding with Vrplp, the interaction of N-Las17p₁₋₃₆₈^{TL} with Vrplp was examined through a pull down assay. The DNA encoding the polyhistidine-tag (His-tag) was fused to the C-terminus of the DNA encoding the C-terminal region of Vrplp (364-817) to generate the fusion construct C-Vrplp-HisT. Plasmid expressing C-

Vrp1p-HisT was co-transformed with plasmids expressing GFP, N-Las17p₁₋₃₆₈-GFP or N-Las17p₁₋₃₆₈^{TL}-GFP into *vrp1Δ* cells. Lysates from the transformants expressing the His-tag and the GFP fusion proteins were processed for pull down analysis. The membrane was probed with anti-HisT and anti-GFP to detect C-Vrp1p-HisT and GFP respectively. The pull down assay showed that C-Vrp1p-HisT pulled down N-Las17p₁₋₃₆₈-GFP but did not pull down N-Las17p₁₋₃₆₈^{TL}-GFP (Fig. 3.78A), suggesting that the mutation in N-Las17p₁₋₃₆₈^{TL}-GFP affects the interaction with C-Vrp1p-HisT. Lysates from the transformants co-expressing the His-tag and GFP fusion proteins were western blotted and probed with anti-GFP serum to verify the expression of the GFP fusion proteins (Fig. 3.78C).

* - The location of the L80T and H94L mutations in N-Las17p₁₋₃₆₈

Figure 3.77 Schematic representation of the domains of Las17p and the location of the L80T and H94L mutations. The arrow heads indicate the position of the residues 206 and 368. The Vrp1p binding region in Las17p is highlighted in green, the four PxxP motifs (P- proline residue; x- any amino acid) involved in binding with Myo5p are highlighted in yellow and the VCA domain is highlighted in grey.

3.3.13 N-Las17p₁₋₃₆₈^{TL} interacts with the SH3 domain of Myo5p in a pull down assay

Type I myosins are involved in Arp2/3 complex dependent actin polymerization at the sites of endocytosis (Geli et al., 2000). Type I myosins can interact with Las17p by direct binding between the SH3 domain of the Type I myosins with the proline rich regions in Las17p. Yeast Two Hybrid screening has indicated that the consensus sequence PxxxxPxxP (P- proline; x-any amino acid) represents a binding site for interaction with Myo3p and Myo5p (Evangelista et. al., 2000). Las17p has 4 potential PxxxxPxxP motifs (Fig. 3.77) while Vrp1p has 22 such motifs. Apart from direct interaction between Type I myosins and Las17p, it has also been suggested that the Type I myosins possibly form a multi protein complex with Las17p by binding with proteins that also interact with Las17p (Evangelista et al., 2000; Lecher et al., 2000). Abolishing the interaction between Las17p and Myo3p or Myo5p causes defects in growth and actin patch polarization in yeast cells (Evangelista et al., 2000). Therefore, the interaction of N-Las17p₁₋₃₆₈ and N-Las17p₁₋₃₆₈^{TL} with the SH3 domain of Myo5p was examined. A Myo5p-SH3-HisT construct was generated and co-expressed with GFP, N-Las17p₁₋₃₆₈-GFP or N-Las17p₁₋₃₆₈^{TL}-GFP in *las17Δ* cells. Ni-NTA agarose beads were used to perform pull down experiments for Myo5p-SH3-HisT. The proteins bound to the beads were eluted and analyzed by western blotting with anti-HisT and anti-GFP to detect Myo5p-SH3-HisT and GFP respectively. The pull down assay showed that Myo5p-SH3-HisT was able to pull down both N-Las17p₁₋₃₆₈-GFP and N-Las17p₁₋₃₆₈^{TL}-GFP (Fig. 3.78B). However, N-Las17p₁₋₃₆₈-GFP band was more intense than the N-Las17p₁₋₃₆₈^{TL}-GFP band suggesting that more molecules of N-Las17p₁₋₃₆₈-GFP are in the complex with Myo5p-SH3 domain

Lysates from the *las17Δ* cells co-expressing the GFP fusion and His-tag fusion proteins were western blotted and probed with anti-GFP serum (Fig. 3.78D).

Figure 3.78 Interaction of N-Las17p₁₋₃₆₈ and N-Las17p₁₋₃₆₈^{TL} with C-Vrp1p and Myo5p-SH3 in a pull down assay.

- A. *vrp1Δ* cells were co-transformed with plasmids expressing C-Vrp1p-HisT and GFP, N-Las17p₁₋₃₆₈-GFP or N-Las17p₁₋₃₆₈^{TL}-GFP. The transformants were grown in YPUAD liquid at 24°C to exponential phase and a His-tag pull down assay was done. The GFP fusion proteins were detected using anti-GFP serum and C-Vrp1p-HisT was detected using anti-HisT serum.
- B. A His-tag pull down assay was performed for *las17Δ* cells co-expressing Myo5p-SH3-HisT and GFP, N-Las17p₁₋₃₆₈-GFP or N-Las17p₁₋₃₆₈^{TL}-GFP. The GFP fusion proteins were detected using anti-GFP serum and Myo5p-SH3-HisT was detected using anti-HisT serum.
- C. Lysates from *vrp1Δ* cells co-expressing C-Vrp1p-HisT and GFP, N-Las17p₁₋₃₆₈-GFP or N-Las17p₁₋₃₆₈^{TL}-GFP were western blotted and probed with anti-GFP serum and anti-Hexokinase serum.
- D. Lysates from *las17Δ* cells co-expressing Myo5p-SH3-HisT and GFP, N-Las17p₁₋₃₆₈-GFP or N-Las17p₁₋₃₆₈^{TL}-GFP were western blotted and probed with anti-GFP serum and anti-Hexokinase serum.

3.3.14 N-Las17p₁₋₃₆₈^{TL} interacts with the SH3 domains of Hoflp, Bzzlp and Rvs167p in a Yeast Two Hybrid assay

N-Las17p₁₋₃₆₈ is able to interact with SH3 domain containing proteins like Myo5p, Hoflp, Bzzlp and Rvs167p. The mutations in N-Las17p₁₋₃₆₈^{TL} abolish interaction of the protein with Vrp1p and also significantly affect the interaction with the SH3 domain of Myo5p. In order to determine whether the mutations in N-Las17p₁₋₃₆₈^{TL} affect interaction with the SH3 domains of Hoflp, Bzzlp and Rvs167p, a Yeast Two Hybrid assay was performed. Plasmid expressing AD-N-Las17p₁₋₃₆₈^{TL} was introduced into the *S. cerevisiae* strain PJ69-4A along with each of the pBD vectors expressing the SH3 domains of Hoflp, Bzzlp and Rvs167p. The transformants co-expressing AD-N-Las17p₁₋₃₆₈^{TL} and each of the BD fusion with the SH3 domains of Hoflp, Bzzlp and Rvs167p were able to grow on SD minimal media plates lacking histidine (Table 3.10), suggesting that N-Las17p₁₋₃₆₈^{TL} was able to interact with the SH3 domains of Hoflp, Bzzlp and Rvs167p.

Table 3.10 Interaction of N-Las17p₁₋₃₆₈^{TL} with SH3 domain containing proteins.

	AD-N-Las17p ₁₋₃₆₈	AD-N-Las17p ₁₋₃₆₈ ^{TL}	AD-Vector
BD-Bzzlp₄₉₄₋₆₆₃	+	+	-
BD-Hoflp₅₅₁₋₆₆₉	+	+	-
BD-Rvs167p₄₂₃₋₄₈₂	+	+	-
BD-Vector	-	-	-

PJ69-4A transformants co-expressing the indicated AD and BD fusion proteins were streaked on SD minimal media plates lacking histidine. The growth of the strains on the plates (+) indicates an interaction between the AD and BD fusion proteins. “-“ indicates lack of interaction.

3.3.15 WH1 (1-206) domain of Las17p forms a complex with the SH3 domain of Myo5p

Vrp1p has been shown to bind directly to type I myosins like Myo5p (Anderson et al., 1998) and it has been suggested that Vrp1p has 22 PxxxxPxxP motifs involved in binding with Myo5p (Evangelista et al., 2000). Therefore, it is possible that in the pull down assay done earlier with Myo5p-SH3-HisT, native Vrp1p molecules present in the cell lysate would have also bound to Myo5p-SH3-HisT on the Ni NTA beads. This in turn suggests that wild type N-Las17p₁₋₃₆₈-GFP was pulled down because of its direct interaction with Myo5p-SH3 as well as due to its interaction with Vrp1p. On the other hand, the mutant N-Las17p₁₋₃₆₈^{TL}-GFP which does not interact with Vrp1p was pulled down only due to its direct interaction with Myo5p-SH3 accounting for the reduced band intensity in the western blot. In order to test this hypothesis, a GFP-tagged construct, N-Las17p₁₋₂₀₆-GFP (Fig. 3.79) which encodes the Vrp1p binding region but lacks the Myo5p-SH3 binding motifs was made.

Figure 3.79 Schematic map of N-Las17p₁₋₂₀₆ and N-Las17p₂₀₇₋₃₆₈

The interaction of N-Las17p₁₋₂₀₆-GFP with the His-tagged SH3 domain of Myo5p was examined by pull down assays. The western blot membrane was probed with anti-GFP and anti-HisT to detect GFP and Myo5p-SH3-HisT respectively. The results showed that Myo5p-SH3-HisT was able to pull down N-Las17p₁₋₂₀₆-GFP (Fig. 3.80A), suggesting

that N-Las17p₁₋₂₀₆-GFP and Myo5p-SH3-HisT from a complex. Therefore, N-Las17p₁₋₂₀₆ is able to interact with Myo5p-SH3 domain even though it lacks the proline rich motifs.

Figure 3.80 N-Las17p₁₋₂₀₆ interacts with Myo5p-SH3 in a pull down assay.

A. *las17Δ* cells were co-transformed with plasmids expressing Myo5p-SH3-HisT and N-Las17p₁₋₂₀₆-GFP or GFP. The transformants were grown in YPUAD liquid at 24°C to exponential phase and a His-tag pull down assay was done. The GFP fusion proteins were detected using anti-GFP serum and Myo5p-SH3-HisT was detected using anti-HisT serum.

B. Cell lysates from *las17Δ* cells co-expressing Myo5p-SH3-HisT and GFP or N-Las17p₁₋₂₀₆-GFP were western blotted and probed with anti-GFP serum and anti-Hexokinase serum.

3.3.16 WH1 domain of Las17p does not bind to the SH3 domain of Myo5p

N-Las17p₁₋₂₀₆ was able to interact with the SH3 domain of Myo5p in a pull down assay, even though the WH1 domain lacks the proline rich motifs (encompassed by residues 207-368) involved in interaction with SH3 domains. This raises the possibility that the interaction between N-Las17p₁₋₂₀₆ and the SH3 domain is mediated through additional proteins. In order to determine whether N-Las17p₁₋₂₀₆ is able to bind directly to the Myo5p-SH3 domain, a Yeast Two Hybrid assay was performed. PJ69-4A cells transformed with plasmids expressing BD-Myo5p-SH3 and AD-N-Las17p₂₀₇₋₃₆₈ were able to grow on SD minimal media plates lacking histidine while there was no growth of the transformants expressing BD-Myo5p-SH3 and AD-N-Las17p₁₋₂₀₆ (Table 3.11),

suggesting that N-Las17p₁₋₂₀₆ does not interact directly with Myo5p-SH3. However, it is also possible that BD-Myo5p-SH3 and AD-N-Las17p₁₋₂₀₆ do form a complex but the complex is unable to travel to the nucleus for reporter activation, thus giving a negative result in the Yeast Two Hybrid assay. The findings of the Yeast Two Hybrid assay need to be verified by performing pull down assays with purified proteins in order to be certain that N-Las17p₁₋₂₀₆ and Myo5p-SH3 do not interact directly with each other.

Table 3.11 Only N-Las17p₂₀₇₋₃₆₈ interacts with Myo5p-SH3 in a Yeast Two Hybrid assay.

	BD-Myo5p ₁₀₈₅₋₁₂₁₉	BD-Vector
AD-N-Las17p ₁₋₂₀₆	-	-
AD-N-Las17p ₂₀₇₋₃₆₈	+	-
AD-Vector	-	-

S. cerevisiae strain PJ69-4A was co-transformed with plasmids expressing the BD fusion proteins and the AD fusion proteins in combinations as indicated in the table. The transformants were streaked on SD minimal media plates lacking histidine and incubated at 30°C for 5 days. The growth of the strains on the plates (+) indicated an interaction between the AD and BD fusion proteins expressed by the strain. "-" indicates lack of interaction.

3.3.17 Expression of Las17p-WH1 domain rescues the growth defects of *las17Δ* cells but does not restore endocytosis or actin patch polarization at 37°C

One of the possible reasons as to why the mutant, N-Las17p₁₋₃₆₈^{TL} is unable to rescue the defects of *las17Δ* cells is the inability of the mutant to interact with Vrp1p. The WH1 domain of Las17p interacts with Vrp1p and also with the SH3 domain of Myo5p (Fig. 3.80) in a pull down assay, even though it lacks the proline rich motifs which have been shown to be involved in the direct interaction between Las17p and Myo5p (Evangelista et al., 2000). In order to determine whether expression of the Las17p-WH1 domain is capable of rescuing the defects in *las17Δ* cells, plasmid encoding N-Las17p₁₋₂₀₆ was

introduced into *las17Δ* cells and the transformants were streaked on YPUAD plates to examine for growth at 37°C and a growth curve was also plotted by measuring the OD₆₀₀ at 37°C for liquid culture of the strains. Mutant cells expressing N-Las17p₁₋₂₀₆ were able to grow at 37°C in the plate assay (Fig. 3.81A) but in the liquid culture assay, the growth rate of the cells expressing N-Las17p₁₋₂₀₆ was significantly reduced compared to cells expressing N-Las17p₁₋₃₆₈ (Fig 3.81B).

Figure 3.81 Expression of N-Las17p₁₋₂₀₆ rescues the growth defects of *las17Δ* cells.

- A. *las17Δ* cells harboring vector or vector encoding N-Las17p₁₋₃₆₈ or N-Las17p₁₋₂₀₆ were streaked on YPUAD plates to examine growth at 24°C and 37°C.
- B. A growth curve was plotted by measuring the OD₆₀₀ at 1 hour intervals for liquid culture of the indicated strains grown at 37°C.

The distribution of actin patches in the cells expressing N-Las17p₁₋₂₀₆ and the ability of the cells to carry out fluid phase endocytosis was examined. The transformants expressing N-Las17p₁₋₃₆₈ and N-Las17p₁₋₂₀₆ were incubated with Lucifer Yellow (LY) at

24°C and 37°C and the uptake of LY into the vacuoles was examined under a fluorescence microscope. Expression of N-Las17p₁₋₂₀₆ in *las17Δ* cells enabled the cells to take up the dye at 24°C as indicated by the fluorescence in the vacuoles but the cells were unable to take up the dye at 37°C (Fig. 3.82). The distribution of **actin** patches in the transformants expressing N-Las17p₁₋₂₀₆ was studied by staining the cells with Alexa-488-conjugated phalloidin. The **actin** patches were polarized at 24°C in the transformants expressing N-Las17p₁₋₂₀₆ but the patches were depolarized at 37°C (Fig. 3.83). Therefore, the proline rich motifs located between the amino acid residues 207-368 of N-Las17p₁₋₃₆₈ are also essential for the activity of the protein in rescuing the defects of *las17Δ* cells at 37°C.

Figure 3.82 N-Las17p₁₋₂₀₆ does not rescue the defects in endocytosis of *las17Δ* cells at 37°C. *las17Δ* cells harboring empty vector, vector expressing Las17p, N-Las17p₁₋₃₆₈ or N-Las17p₁₋₂₀₆ were tested for LY uptake at 24°C and 37°. LY signal was visualized by fluorescence microscopy. Upper panels: fluorescence optics. Lower panels: DIC optics. Bar, 5μm.

Figure 3.83 Expression of N-Las17p₁₋₂₀₆ restores actin patch polarization in *las17Δ* cells only at 24°C and not at 37°C.

IDY223 transformants harboring empty vector, vector expressing Las17p, N-Las17p₁₋₃₆₈ or N-Las17p₁₋₂₀₆ were fixed immediately or were shifted to 37°C for 2 hours prior to fixing. The fixed cells were permeabilized and F-actin stained with Alexa-488-conjugated phalloidin. The actin patches were visualized by fluorescence microscopy. Bar, 5μm.

3.3.18 The WH1 domain of Las17p is able to localize to cortical patches of *las17Δ* cells

Expression of the WH1 domain of Las17p was able to rescue the growth defects of *las17Δ* cells, even though the other defects in the strain were not rescued. Next, the sub cellular localization of Las17p-WH1 domain was examined by fluorescence microscopy. N-Las17p₁₋₂₀₆-GFP localized to the cortical patches of *las17Δ* cells at both 24°C and 37°C (Fig. 3.84). Then, in order to determine whether N-Las17p₁₋₂₀₆-GFP localizes to cortical actin patches, Arc40p-RFP was expressed in cells expressing N-Las17p₁₋₂₀₆-GFP. Some of the N-Las17p₁₋₂₀₆-GFP patches were found to co-localize with Arc40p-RFP patches (Fig. 3.85A).

Figure 3.84 N-Las17p₁₋₂₀₆-GFP localizes to the cortical patches of *las17Δ* cells.

las17Δ cells expressing GFP, Las17p-GFP, N-Las17p₁₋₃₆₈-GFP or N-Las17p₁₋₂₀₆-GFP were grown in YPUAD liquid at 24°C. For visualizing localization at 37°C, the cells were shifted to 37°C for 2 hours. GFP signal was visualized in living cells by fluorescence microscopy. Upper panels: FITC-fluorescence optics. Lower panels: DIC optics. Bar, 5μm.

The expression of N-Las17p₁₋₂₀₆-GFP was then examined to determine whether the inability of N-Las17p₁₋₂₀₆ to rescue all the defects of *las17Δ* was due to reduced expression of the protein. Total protein was extracted from *las17Δ* cells expressing N-Las17p₁₋₂₀₆-GFP and N-Las17p₁₋₃₆₈-GFP and the expression of the GFP fusion proteins was studied by Western blot. Western blot analysis showed that the expression of N-Las17p₁₋₂₀₆-GFP was comparable to that of N-Las17p₁₋₃₆₈-GFP (Fig. 3.85B).

Figure 3.85 N-Las17p₁₋₂₀₆ is expressed as well as N-Las17p₁₋₃₆₈ and localizes to the cortical actin patches of *las17Δ* cells.

- A. Arc40p-RFP and N-Las17p₁₋₃₆₈-GFP or N-Las17p₁₋₂₀₆-GFP were co-expressed in *las17Δ* cells. The transformants were grown in YPUAD liquid at 24°C. GFP and RFP were visualized by fluorescence microscopy. Arrows point to co-localizing patches. Bar, 5μm.
- B. *las17Δ* cells expressing N-Las17p₁₋₃₆₈-GFP or N-Las17p₁₋₂₀₆-GFP were grown exponentially at 24°C and total protein was prepared. The extracted proteins were separated by SDS-PAGE and Western blotted. The GFP fusion proteins were detected using anti-GFP serum and Hexokinase (loading control) was detected using anti-Hexokinase serum.

3.3.19 Expression of N-Las17p₁₋₂₉₄ is able to rescue the growth defects of *las17Δ* cells without rescuing the endocytosis and actin patch polarization defects at 37°C

In the previous experiments, it was shown that expression of even the Las17p-WH1 domain was able to rescue the growth defects of *las17Δ* cells though the defects in endocytosis and actin patch polarization were not rescued at 37°C. This shows that the region of the protein located between the amino acid residues 207-368 of N-Las17p₁₋₃₆₈ is also essential for the activity of the protein in rescuing the defects of *las17Δ* cells. The restriction enzyme *KpnI* cuts the DNA coding for N-Las17p₁₋₃₆₈, at the nucleic acid position 880 which corresponds to the amino acid position 294. Therefore, a DNA

fragment coding for N-Las17p₁₋₂₉₄ would include the WH1 domain and also half of the region bounded by the amino acid residues 207-368 of N-Las17p₁₋₃₆₈. The conveniently located *Kpn*I restriction enzyme site was utilized to clone a DNA fragment which encodes for N-Las17p₁₋₂₉₄ into the expression vector YCplac33 (Fig. 3.86). Plasmid expressing N-Las17p₁₋₂₉₄ was introduced into *las17Δ* cells and the transformants were assessed for growth at 37°C. Expression of N-Las17p₁₋₂₉₄ restored growth of the transformants at 37°C in the plate assay (Fig. 3.87A) but in the liquid culture assay, the growth of the cells expressing N-Las17p₁₋₂₉₄ was not as efficient as the cells expressing N-Las17p₁₋₃₆₈ (Fig. 3.87B). The localization of N-Las17p₁₋₂₉₄ was studied by expressing a translation fusion construct of GFP and N-Las17p₁₋₂₉₄ in *las17Δ* cells. N-Las17p₁₋₂₉₄-GFP localized to the cortical patches of *las17Δ* cells at both 24°C and 37°C (Fig. 3.88).

Figure 3.86 Schematicmap of N-Las17p₁₋₂₉₄, N-Las17p₁₋₃₂₂, N-las17p₁₋₃₃₉ and N-Las17p₁₋₃₅₀.

Figure 3.87 Expression of N-Las17p₁₋₂₉₄ rescues the growth defects of *las17Δ* cells.

- A.** Empty vector or vector encoding N-Las17p₁₋₃₆₈, N-Las17p₁₋₂₀₆ or N-Las17p₁₋₂₉₄ was introduced into IDY223. The transformants were streaked on YPUAD plates to examine growth at 24°C and 37°C.
- B.** A growth curve was plotted by measuring the OD₆₀₀ at 1 hour intervals for the liquid culture.

Figure 3.88 N-Las17p₁₋₂₉₄-GFP localizes to the cortical patches of *las17Δ* cells. *las17Δ* cells expressing GFP, Las17p-GFP, N-Las17p₁₋₃₆₈-GFP or N-Las17p₁₋₂₉₄-GFP were grown in YPUAD liquid to exponential phase at 24°C. The cells were immediately processed for microscopic visualization or they were shifted to 37°C for 2 hours prior to the processing. GFP signal was visualized in living cells by fluorescence microscopy. Upper panels: FITC-fluorescence optics. Lower panels: DIC optics. Bar, 5μm.

Then the distribution of **actin** patches and the ability of the transformants expressing N-Las17p₁₋₂₉₄ to carry out fluid phase endocytosis were examined. The cells expressing N-Las17p₁₋₂₉₄ were incubated with Lucifer yellow (LY) at 24°C and 37°C and the uptake of LY into the vacuoles was examined under a fluorescence microscope. The mutant cells expressing N-Las17p₁₋₂₉₄ were able to take up the dye at 24°C as indicated by the fluorescence in the vacuoles but the cells were unable to take up the dye at 37°C (Fig. 3.89).

Figure 3.89 N-Las17p₁₋₂₉₄ does not rescue the defects in endocytosis of *las17Δ* cells at 37°C.

IDY223 transformants growing exponentially at 24°C and harboring empty vector, vector expressing Las17p, N-Las17p₁₋₃₆₈ or N-Las17p₁₋₂₉₄ were incubated with LY at 24°C or 37°C and washed thoroughly. LY signal was visualized by fluorescence microscopy. Upper panels: fluorescence optics. Lower panels: DIC optics. Bar, 5μm.

The distribution of **actin** patches in the transformants expressing N-Las17p₁₋₂₉₄ was studied by staining the cells with Alexa-488-conjugated phalloidin. The **actin** patches were polarized at 24°C but depolarized at 37°C in the transformants expressing N-Las17p₁₋₂₉₄ (Fig. 3.90). Therefore, the region of the protein between amino acid residues 295-368 of N-Las17p₁₋₃₆₈ is essential for the activity of the protein in rescuing the defects in endocytosis and **actin** patch polarization at 37°C.

Figure 3.90 Expression of N-Las17p₁₋₂₉₄ restores **actin** patch polarization in *las17Δ* cells only at 24°C and not at 37°C.

Actin patches in *las17Δ* cells expressing Las17p, N-Las17p₁₋₃₆₈ or N-Las17p₁₋₂₉₄ were stained and visualized by fluorescence microscopy. Bar, 5μm.

3.3.20 Systematic deletion of the proline rich regions between amino acid residues 295-368 of N-Las17p₁₋₃₆₈

Expression of N-Las17p₁₋₂₉₄ was able to rescue the growth defect of *las17Δ* cells but was unable to restore endocytosis and **actin** patch polarization at 37°C, suggesting that the region enclosed by the amino acid residues 295-368 of N-Las17p₁₋₃₆₈ which is rich in **proline** rich regions is essential for the activity of the protein in rescuing the defects in endocytosis and **actin** patch polarization at 37°C. The **proline** rich regions located between amino acid residues 295-368 were systematically deleted to generate plasmids

encoding N-Las17p₁₋₃₂₂, N-Las17p₁₋₃₃₉ and N-Las17p₁₋₃₅₀ (Fig. 3.86). *las17Δ* cells expressing each of the three proteins N-Las17p₁₋₃₂₂, N-Las17p₁₋₃₃₉ and N-Las17p₁₋₃₅₀ were able to grow on YPUAD agar plates at 37°C (Fig. 3.91A).

Figure 3.91 Systematic deletion of the **proline** rich regions between amino acid residues 295-368 of N-Las17p₁₋₃₆₈.

- A. Empty vector or vector encoding N-Las17p₁₋₃₆₈, N-Las17p₁₋₃₅₀, N-Las17p₁₋₃₃₉, N-Las17p₁₋₃₂₂ or N-Las17p₁₋₂₉₄ was introduced into *las17Δ* cells. The transformants were streaked on YPUAD plates to examine growth at 24°C and 37°C.
- B. A growth curve was plotted by measuring the OD₆₀₀ at 1 hour intervals for liquid culture of the indicated strains grown at 37°C.

After examining the growth of cells in the plate assay, a growth curve was plotted for liquid culture of the cells expressing N-Las17p₁₋₃₆₈, N-Las17p₁₋₃₅₀, N-Las17p₁₋₃₃₉ and N-Las17p₁₋₃₂₂. The results of the liquid culture assay showed that there was a progressive

decrease in the growth rate of the cells in proportion to the size of the deletion of the proline rich regions. Visual examination of the growth curve suggests that the growth rate of the *las17Δ* cells expressing N-Las17p₁₋₃₆₈ and N-Las17p₁₋₃₅₀ is similar (Fig. 3.91B). Similarly, *las17Δ* cells expressing N-Las17p₁₋₃₃₉ and N-Las17p₁₋₃₂₂ also grew at the same rate (Fig. 3.91B). However, the rate of growth of *las17Δ* cells expressing N-Las17p₁₋₃₅₀ was higher than that of the cells expressing N-Las17p₁₋₃₃₉ (Fig. 3.91B), suggesting that the region of the protein encompassed by the amino acid residues from 340-349 of N-Las17p₁₋₃₆₈ plays an important role in complementing growth defects of *las17Δ* cells.

Figure 3.92 N-Las17p₁₋₃₂₂ does not rescue the defects in endocytosis of *las17Δ* cells at 37°C.

IDY223 transformants harboring empty vector, vector expressing Las17p, N-Las17p₁₋₃₆₈, N-Las17p₁₋₃₅₀, N-Las17p₁₋₃₃₉ or N-Las17p₁₋₃₂₂ were tested for LY uptake at 24°C and 37°C. LY signal was visualized by fluorescence microscopy. Upper panels: fluorescence optics. Lower panels: DIC optics. Bar, 5μm.

After examining the effect of the deletion of the **proline** rich regions on the activity of the N-terminal region of Las17p in suppressing the growth defects of *las17Δ* cells, the ability of the *las17Δ* cells to carry out fluid phase endocytosis was examined by incubating the cells in Lucifer Yellow (LY) at 24°C and 37°C. The transformants expressing N-Las17p₁₋₃₅₀ and N-Las17p₁₋₃₃₉ were able to take up the dye at both 24°C and 37°C but the mutant cells expressing N-Las17p₁₋₃₂₂ were able to take up the dye only at 24°C but not at 37°C (Fig. 3.92).

Figure 3.93 N-Las17p₁₋₃₃₉ and N-Las17p₁₋₃₂₂ are unable to restore **actin** patch polarization in *las17Δ* cells at 37°C.

- A. IDY223 transformants growing exponentially at 24°C and harboring empty vector, vector expressing Las17p, N-Las17p₁₋₃₆₈, N-Las17p₁₋₃₅₀, N-Las17p₁₋₃₃₉ or N-Las17p₁₋₃₂₂ were fixed, permeabilized and F-actin stained with Alexa-488-conjugated phalloidin. The actin patches were visualized by fluorescence microscopy. Bar, 5μm.
- B. The indicated strains were streaked on YPUAD plate and YPUAD plate containing 1M NaCl and incubated at 24°C.

Then the distribution of **actin** patches in *las17Δ* cells expressing N-Las17p₁₋₃₅₀, N-Las17p₁₋₃₃₉ and N-Las17p₁₋₃₂₂ was examined by phalloidin staining. **Actin** patches were polarized at both 24°C and 37°C only in the mutant cells expressing N-Las17p₁₋₃₅₀ but in the *las17Δ* cells expressing N-Las17p₁₋₃₃₉ and N-Las17p₁₋₃₂₂, polarization of **actin** patches was restored at 24°C but not at 37°C (Fig. 3.93A). The ability of N-Las17p₁₋₃₅₀, N-Las17p₁₋₃₃₉ and N-Las17p₁₋₃₂₂ to rescue the **cytoskeletal** defects of *las17Δ* cells at 24°C was further supported by the observation that *las17Δ* cells expressing the three proteins were able to grow on YPUAD plates containing 1M NaCl (Fig. 3.93B). N-Las17p₁₋₃₅₀-GFP, N-Las17p₁₋₃₃₉-GFP and N-Las17p₁₋₃₂₂-GFP localized to the cortical patches of *las17Δ* cells at both 24°C and 37°C (Fig. 3.94).

Figure 3.94 N-Las17p₁₋₃₅₀-GFP, N-Las17p₁₋₃₃₉-GFP and N-Las17p₁₋₃₂₂-GFP localize to the cortical patches of *las17Δ* cells.

las17Δ cells expressing GFP, Las17p-GFP, N-Las17p₁₋₃₆₈-GFP, N-Las17p₁₋₃₅₀-GFP, N-Las17p₁₋₃₃₉-GFP or N-Las17p₁₋₃₂₂-GFP were grown in YPUAD liquid. GFP signal was visualized in living cells by fluorescence microscopy. Upper panels: FITC-fluorescence optics. Lower panels: DIC optics. Bar, 5μm.

3.3.21 Mutation of the **proline** rich regions (323-329, **342-348** or **352-358**) in **N-Las17p₁₋₃₆₈** has no effect on the activity of **N-Las17p₁₋₃₆₈**

Expression of N-Las17p₁₋₃₂₂ suppressed the growth defects of *las17Δ* but did not restore endocytosis or actin patch polarization at 37°C. This suggests that the region of N-Las17p₁₋₃₆₈ between the residues 323-368 is essential for the activity of N-Las17p₁₋₃₆₈ in restoring endocytosis and actin patch polarization in *las17Δ* cell at 37°C. The region enclosed by the residues 323-368 in N-Las17p₁₋₃₆₈ is rich in proline residues. It has been suggested that Myo5p binds to the PxxxxPxxP motif (P- proline; x- any amino acid) present in both Las17p and Vrp1p (Evangelista et al., 2000). Las17p has 4 potential PxxxxPxxP motifs (Evangelista et al., 2000) one of which is located in the region flanked by the residues 323-368 (Fig. 3.77). The proline residues between the regions 323-329, 342-348 or 352-358 of N-Las17p₁₋₃₆₈ were mutated to alanine through site directed mutagenesis to generate plasmids coding for N-Las17p₁₋₃₆₈³²³, N-Las17p₁₋₃₆₈³⁴² or N-Las17p₁₋₃₆₈³⁵² respectively (Fig. 3.95).

Figure 3.95 Schematic map of N-Las17p₁₋₃₆₈³²³, N-Las17p₁₋₃₆₈³⁴² and N-Las17p₁₋₃₆₈³⁵².

Plasmids coding for N-Las17p₁₋₃₆₈³²³, N-Las17p₁₋₃₆₈³⁴² or N-Las17p₁₋₃₆₈³⁵² were introduced into *las17Δ* cells and the transformants were assessed for growth at 37°C. Expression of N-Las17p₁₋₃₆₈³²³, N-Las17p₁₋₃₆₈³⁴² or N-Las17p₁₋₃₆₈³⁵² suppressed the

growth defects of *las17Δ* cells at 37°C in both the plate assay and liquid culture assay (Fig. 3.96).

Figure 3.96 Mutation of the proline rich regions at 323-329, 342-348 or 352-358 does not affect the ability of N-Las17p₁₋₃₆₈ to rescue the growth defects of *las17Δ* cells.

- A. Empty vector or vector encoding N-Las17p₁₋₃₆₈, N-Las17p₁₋₃₆₈³²³, N-Las17p₁₋₃₆₈³⁴² or N-Las17p₁₋₃₆₈³⁵² was introduced into IDY223. The transformants were streaked on YPUAD plates to examine growth at 24°C and 37°C.
- B. A growth curve was plotted by measuring the OD₆₀₀ at 1 hour intervals for liquid culture of the indicated strains grown at 37°C.

The transformants expressing N-Las17p₁₋₃₆₈³²³, N-Las17p₁₋₃₆₈³⁴² or N-Las17p₁₋₃₆₈³⁵² were examined for their ability to carry out fluid phase endocytosis by incubating the cells with Lucifer Yellow (LY) at 24°C and 37°C. Expression of N-Las17p₁₋₃₆₈³²³, N-Las17p₁₋₃₆₈³⁴² or N-Las17p₁₋₃₆₈³⁵² enabled the uptake of LY into the vacuoles, as indicated by the fluorescence in the vacuoles (Fig. 3.97). The distribution of actin patches in the

transformants expressing N-Las17p₁₋₃₆₈³²³, N-Las17p₁₋₃₆₈³⁴² or N-Las17p₁₋₃₆₈³⁵² was examined by staining the cells with Alexa-488-conjugated phalloidin. The transformants expressing N-Las17p₁₋₃₆₈³²³, N-Las17p₁₋₃₆₈³⁴² or N-Las17p₁₋₃₆₈³⁵² showed polarized distribution of **actin** patches (Fig. 3.98). Therefore, mutating the **proline** residues in the **proline** rich regions 323-329, 342-348 or 352-358 does not affect the ability of N-Las17p₁₋₃₆₈ to rescue the defects of the *las17Δ* strain. This also raises the possibility that instead of mutating just one of the three **proline** rich regions, two of the **proline** rich regions or all the three regions need to be mutated to affect the activity of N-Las17p₁₋₃₆₈.

Figure 3.97 Expression of N-Las17p₁₋₃₆₈³²³, N-Las17p₁₋₃₆₈³⁴² or N-Las17p₁₋₃₆₈³⁵² rescues the defects in endocytosis of *las17Δ* cells at 24°C and 37°C.

las17Δ cells expressing Las17p, N-Las17p₁₋₃₆₈, N-Las17p₁₋₃₆₈³²³, N-Las17p₁₋₃₆₈³⁴² or N-Las17p₁₋₃₆₈³⁵² were tested for LY uptake at 24°C and 37°C. Upper panels: fluorescence optics. Lower panels: DIC optics. Bar, 5 μm.

Figure 3.98 Expression of N-Las17p₁₋₃₆₈³²³, N-Las17p₁₋₃₆₈³⁴² or N-Las17p₁₋₃₆₈³⁵² restores actin patch polarization in *las17Δ* cells at both 24°C and 37°C.

Exponentially growing *las17Δ* cells expressing Las17p, N-Las17p₁₋₃₆₈, N-Las17p₁₋₃₆₈³²³, N-Las17p₁₋₃₆₈³⁴² or N-Las17p₁₋₃₆₈³⁵² were fixed, permeabilized and F-actin stained with Alexa-488-conjugated phalloidin. The actin patches were visualized by fluorescence microscopy. Bar, 5μm.

3.3.22 Full length Las17p with mutation is able to suppress growth defects of *las17Δ* cells

The mutations in N-Las17p₁₋₃₆₈^{TL} abolished the ability of the N-terminal region of Las17p to suppress the defects in *las17Δ* strain. In order to examine if the presence of the L80T and H94L mutations affect the activity of full length Las17p, site directed mutagenesis was used to construct a plasmid coding for full length Las17p bearing the L80T and H94L amino acid substitutions (Fig. 3.99). Plasmid expressing Las17p^{TL} was introduced into *las17Δ* cells and the transformants were examined for growth on YPUAD plates at 37°C and a growth curve at 37°C was plotted by measuring the OD₆₀₀ of the liquid culture at 1 hour intervals. Transformants expressing Las17p^{TL} grew at 37°C in both the plate assay and the liquid culture assay, similar to *las17Δ* cells expressing full length Las17p (Fig. 3.100).

Figure 3.99 Schematic map of Las17p^{TL}, Las17p₁₋₅₂₇, Las17p₁₋₅₂₇^{TL}, N-Las17p₁₋₃₆₈VCA and N-Las17p₁₋₃₆₈^{TL}VCA.

Figure 3.100 Expression of the full length mutant, Las17p^{TL} rescues the growth defects of *las17Δ* cells.

- A. Empty vector or vector encoding Las17p or Las17p^{TL} was introduced into *las17Δ* cells. The transformants were streaked on YPUAD plates to examine growth at 24°C and 37°C.
- B. A growth curve was plotted by measuring the OD₆₀₀ at 1 hour intervals for liquid culture of the indicated strains grown at 37°C.

Las17p^{TL}-GFP localized to the cortical patches of *las17Δ* cells at both 24°C and 37°C (Fig. 3.101) and the expression of Las17p^{TL}-GFP was detected by western blotting of total cell lysates (Fig. 3.102).

Figure 3.101 Las17p^{TL}-GFP localizes to the cortical patches of *las17Δ* cells. *las17Δ* cells expressing GFP, Las17p-GFP or Las17p^{TL}-GFP were grown in YPUAD liquid to exponential phase at 24°C. The cells were immediately processed for microscopic visualization or they were shifted to 37°C for 2 hours prior to the processing. GFP signal was visualized in living cells by fluorescence microscopy. Upper panels: FITC-fluorescence optics. Lower panels: DIC optics. Bar, 5μm.

Figure 3.102 Expression of Las17p^{TL} in *las17Δ* cells.

Total protein extract was prepared from IDY223 transformants growing exponentially at 24°C and harboring vector expressing Las17p-GFP or Las17p^{TL}-GFP. The extracted proteins were separated by SDS-PAGE and Western blotted. The GFP fusion proteins were detected using anti-GFP serum and Hexokinase (loading control) was detected using anti-Hexokinase serum.

3.3.23 Expression of Las17p^{TL} rescues the endocytosis defect but rescues the actin patch polarization defect of *las17Δ* strain at only 24°C

Expression of the full length mutant, Las17p^{TL} was able to rescue the growth defects of the *las17Δ* cells. The ability of Las17p^{TL} to rescue the defects in endocytosis and actin patch polarization in *las17Δ* cells was then examined. Expression of Las17p^{TL} enabled vacuolar uptake of Lucifer Yellow (LY) at both 24°C and 37°C as indicated by the vacuolar fluorescence (Fig. 3.103), which was similar to that seen in mutant cells expressing wild type Las17p. The cortical actin patches were polarized at 24°C in the transformants expressing Las17p^{TL} but at 37°C, the actin patches were depolarized (Fig. 3.104).

Figure 3.103 Expression of Las17p^{TL} rescues the defects in endocytosis of *las17Δ* cells.

las17Δ cells harboring empty vector, vector expressing Las17p or Las17p^{TL} were incubated with LY at 24°C or 37°C and washed thoroughly. For performing the assay at 37°C, the cells were incubated at 37°C for 2 hours prior to the addition of LY. LY signal was visualized by fluorescence microscopy. Upper panels: fluorescence optics. Lower panels: DIC optics. Bar, 5μm.

Figure 3.104 Expression of Las17p^{TL} does not restore actin patch polarization in *las17Δ* cells at 37°C.

Exponentially growing *las17Δ* cells harboring empty vector or vector expressing Las17p or Las17p^{TL} were fixed, permeabilized and F-actin stained with Alexa-488-conjugated phalloidin. The actin patches were visualized by fluorescence microscopy. Bar, 5μm.

3.3.24 Las17p^{TL} does not interact with Vrp1p in a pull down assay

The ability of Las17p^{TL} to interact with Vrp1p was determined by performing a His-tag pull down assay. Plasmid expressing C-Vrp1p-HisT was co-transformed with plasmid expressing Las17p-GFP or Las17p^{TL}-GFP into *vrp1Δ* cells. The pull down assay showed that C-Vrp1p-HisT was able to pull down only Las17p-GFP but not the mutant, Las17p^{TL}-GFP (Fig. 3.105), suggesting that Las17p^{TL}-GFP was unable to interact with C-Vrp1p-HisT.

C-Vrp1p-HisT pull down

Figure 3.105 Las17p^{TL} does not interact with C-Vrp1p in a pull down assay.

IDY223 was co-transformed with plasmids expressing C-Vrp1p-HisT and Las17p-GFP or Las17p^{TL}-GFP. The transformants were grown in YPUAD liquid at 24°C to exponential phase and a His-tag pull down assay was done. The GFP fusion proteins were detected using anti-GFP serum and C-Vrp1p-HisT was detected using anti-HisT serum.

3.3.25 The VCA domain of Las17p^{TL} is critical for rescuing endocytosis defect of *las17Δ* cells at 37°C

Expression of the N-terminal mutant, N-Las17p₁₋₃₆₈^{TL} did not rescue the growth defect of *las17Δ* cells while endocytosis and actin patch polarization are restored at only 24°C and not 37°C. In contrast to the behavior of the N-terminus mutant, expression of the full length mutant, Las17p^{TL} in *las17Δ* cells rescues the defects in growth and endocytosis but does not restore actin patch polarization at 37°C. Next, the function of the VCA domain in the activity of Las17p^{TL} was examined. Plasmids encoding Las17p₁₋₅₂₇ and Las17p₁₋₅₂₇^{TL} which had a deletion of the VCA domain were constructed (Fig. 3.99). *las17Δ* cells expressing Las17p₁₋₅₂₇ or Las17p₁₋₅₂₇^{TL} were able to grow on YPUAD plates at 37°C (3.106A) but in the liquid culture assay, the cells expressing Las17p₁₋₅₂₇^{TL} were not as efficient as the cells expressing Las17p₁₋₅₂₇ (Fig 3.106B). The sub-cellular localization of Las17p₁₋₅₂₇ and Las17p₁₋₅₂₇^{TL} was studied by fluorescence microscopy. Las17p₁₋₅₂₇-GFP and Las17p₁₋₅₂₇^{TL}-GFP localized to the cortical patches of *las17Δ* cells at both 24°C and 37°C (Fig. 3.107). Immunoblotting of protein extracts from *las17Δ* cells expressing Las17p₁₋₅₂₇-GFP and Las17p₁₋₅₂₇^{TL}-GFP suggests that the expression of the mutant, Las17p₁₋₅₂₇^{TL}-GFP was similar to that of Las17p₁₋₅₂₇-GFP (Fig. 3.108).

Figure 3.106 Expression of Las17p₁₋₅₂₇^{TL} rescues the growth defects of *las17Δ* cells.

- A. Empty vector or vector encoding Las17p, Las17p₁₋₅₂₇ or Las17p₁₋₅₂₇^{TL} was introduced into *las17Δ* cells. The transformants were streaked on YPAD plates to examine growth at 24°C and 37°C.
- B. A growth curve was plotted by measuring the OD₆₀₀ at 1 hour intervals for liquid culture of the indicated strains grown at 37°C.

Figure 3.107 Las17p₁₋₅₂₇^{TL}-GFP localizes to the cortical patches of *las17Δ* cells.

Vector encoding GFP, Las17p-GFP, Las17p₁₋₅₂₇-GFP or Las17p₁₋₅₂₇^{TL}-GFP was introduced into IDY223. GFP signal was visualized in living cells by fluorescence microscopy. Upper panels: FITC-fluorescence optics. Lower panels: DIC optics. Bar, 5 μm.

Figure 3.108 Expression of Las17p₁₋₅₂₇^{TL} in *las17Δ* cells. Total protein extract was prepared from exponentially growing *las17Δ* cells expressing Las17p₁₋₅₂₇-GFP or Las17p₁₋₅₂₇^{TL}-GFP. The extracted proteins were separated by SDS-PAGE and Western blotted with anti-GFP and anti-Hexokinase antisera.

Then the distribution of **actin** patches and the ability of the *las17Δ* cells expressing Las17p₁₋₅₂₇ and Las17p₁₋₅₂₇^{TL} to carry out fluid phase endocytosis were examined. Expression of the wild type protein, Las17p₁₋₅₂₇ in *las17Δ* cells restored fluid phase endocytosis at both 24°C and 37°C as indicated by the fluorescence in the vacuoles (Fig. 3.109). The *las17Δ* cells expressing Las17p₁₋₅₂₇^{TL} were able to take up the dye at 24°C but not at 37°C (Fig. 3.109).

Figure 3.109 Expression of Las17p₁₋₅₂₇^{TL} does not restore endocytosis in *las17Δ* cells at 37°C. *las17Δ* cells expressing Las17p, Las17p₁₋₅₂₇ or Las17p₁₋₅₂₇^{TL} were incubated with LY at 24°C or 37°C and washed thoroughly. For performing the assay at 37°C, cells were incubated at 37°C for 2 hours prior to the addition of LY. LY signal was visualized by fluorescence microscopy. Upper panels: fluorescence optics. Lower panels: DIC optics. Bar, 5 μm.

Similarly, the **actin** patches were polarized at 24°C in the transformants expressing Las17p₁₋₅₂₇ or Las17p₁₋₅₂₇^{TL} (Fig. 3.110) but at 37°C, the patches were polarized only in the transformants expressing the wild type protein, Las17p₁₋₅₂₇ (Fig. 3.110).

Figure 3.110 Expression of Las17p₁₋₅₂₇^{TL} does not restore **actin** patch polarization in *las17Δ* cells at 37°C. *las17Δ* cells growing exponentially at 24°C and harboring empty vector, vector expressing Las17p, Las17p₁₋₅₂₇ or Las17p₁₋₅₂₇^{TL} were fixed immediately or were shifted to 37°C for 2 hours prior to fixing. The fixed cells were permeabilized and F-actin stained with Alexa-488-conjugated phalloidin. The **actin** patches were visualized by fluorescence microscopy. Bar, 5μm.

3.3.26 Expression of N-Las17p₁₋₃₆₈^{TL} fused with the VCA domain suppresses the growth defects of *las17Δ* cells

Expression of N-Las17p₁₋₃₆₈^{TL} in *las17Δ* cell does not suppress the defects of the strain at 37°C though endocytosis and **actin** patch polarization are restored at 24°C (Figs. 3.64-66). N-Las17p₁₋₃₆₈^{TL} does not interact with Vrp1p as indicated by the pull down assay (Fig. 3.78A). The pull down assay with the Myo5p-SH3 domain also suggests that N-Las17p₁₋₃₆₈^{TL} interacts with a lesser fraction of Myo5p-SH3 domain as compared to the wild type protein (Fig. 3.788). These findings indicate that N-Las17p₁₋₃₆₈^{TL} is defective possibly due to its lack of or sub-optimal binding to cytoskeletal proteins like Vrp1p and Myo5p respectively. In *vitro* studies have shown that the VCA domain of Las17p is capable of

activating the Arp2/3 complex (Rodal et al. 2003). We reasoned that fusing the Las17p-VCA domain to N-Las17p₁₋₃₆₈^{TL} could compensate for the lack of binding/ less binding of N-Las17p₁₋₃₆₈^{TL} with Vrp1p/ Myo5p. The DNA encoding the Las17p-VCA domain was amplified by PCR and fused to the C-terminal end of the DNA encoding N-Las17p₁₋₃₆₈ and N-Las17p₁₋₃₆₈^{TL} (Fig. 3.99). The plasmids expressing N-Las17p₁₋₃₆₈VCA and N-Las17p₁₋₃₆₈^{TL}VCA were introduced into *las17Δ* cells and the transformants were assessed for growth at 37°C. Expression of N-Las17p₁₋₃₆₈^{TL}VCA enabled the transformants to grow at 37°C in the plate assay (Fig. 3.111) and also restored fluid phase endocytosis at 24°C and 37°C (Fig. 3.112). However, expression of N-Las17p₁₋₃₆₈^{TL}VCA restored actin patch polarization in *las17Δ* cells only at 24°C (Fig. 3.113). The localization of N-Las17p₁₋₃₆₈^{TL}VCA was examined by generation the GFP fusion construct N-Las17p₁₋₃₆₈^{TL}VCA-GFP. N-Las17p₁₋₃₆₈^{TL}VCA-GFP localized to the cortical patches of *las17Δ* cells at 24°C and 37°C (Fig. 3.114).

Figure 3.111 Expression of N-Las17p₁₋₃₆₈^{TL}VCA rescues growth defects of *las17Δ* cells in the plate assay. Empty vector or vector encoding N-Las17p₁₋₃₆₈, N-Las17p₁₋₃₆₈VCA or N-Las17p₁₋₃₆₈^{TL}VCA was introduced into IDY223. The transformants were streaked on YPUAD plates to examine growth at 24°C and 37°C.

Figure 3.112 Expression of N-Las17p₁₋₃₆₈^{TL}VCA rescues the defects in endocytosis of *las17Δ* cells at 37°C. *las17Δ* cells expressing N-Las17p₁₋₃₆₈, N-Las17p₁₋₃₆₈^{TL}, N-Las17p₁₋₃₆₈VCA or N-Las17p₁₋₃₆₈^{TL}VCA were tested for LY uptake at 24°C and 37°C. LY signal was visualized by fluorescence microscopy. Upper panels: fluorescence optics. Lower panels: DIC optics. **Bar**, 5μm.

Figure 3.113 Expression of N-Las17p₁₋₃₆₈^{TL}VCA does not restore **actin** patch polarization in *las17Δ* cells at 37°C. *las17Δ* cells growing exponentially at 24°C and harboring empty vector, vector expressing Las17p, N-Las17p₁₋₃₆₈, N-Las17p₁₋₃₆₈VCA or N-Las17p₁₋₃₆₈^{TL}VCA were fixed immediately or shifted to 37°C for 2 hours prior to fixing. The fixed cells were permeabilized and F-actin stained with Alexa-488-conjugated phalloidin. The **actin** patches were visualized by fluorescence microscopy. **Bar**, 5μm.

Figure 3.114 N-Las17p₁₋₃₆₈VCA-GFP and N-Las17p₁₋₃₆₈^{TL}VCA-GFP localize to the cortical patches of *las17Δ* cells.

Vector encoding GFP, Las17p-GFP, N-Las17p₁₋₃₆₈-GFP, N-Las17p₁₋₃₆₈VCA-GFP or N-Las17p₁₋₃₆₈^{TL}VCA-GFP was introduced into *las17Δ* cells and the transformants were grown in YPUAD liquid. GFP signal was visualized in living cells by fluorescence microscopy. Upper panels: FITC-fluorescence optics. Lower panels: DIC optics. Bar, 5μm.

Las17p Constructs	Growth 37°C	Localization		Endocytosis		Actin patch polarization	
		24°C	37°C	24°C	37°C	24°C	37°C
Las17p	+	+	+	+	+	+	+
N-Las17p₁₋₃₆₈	+	+	+	+	+	+	+
N-Las17p₃₇₀₋₆₃₃							
N-Las17p₁₋₃₆₈^{TL}		+	+	+		+	
N-Las17p₁₋₃₆₈^T	+	+	+	+	+	+	+
N-Las17p₁₋₃₆₈^L	+	+	+	+	+	+	+
N-Las17p₁₋₃₆₈^{TL} (2μ)	+	N	N	+	+	+	
N-Las17p₁₋₂₀₆	+	+	+	+		+	
N-Las17p₁₋₂₉₄	+	+	+	+		+	
N-Las17p₁₋₃₂₂	+	+	+	+		+	
N-Las17p₁₋₃₃₉	+	+	+	+	+	+	
N-Las17p₁₋₃₅₀	+	+	+	+	+	+	+
N-Las17p₁₋₃₆₈³²³	+	N	N	+	+	+	+
N-Las17p₁₋₃₆₈³⁴²	+	N	N	+	+	+	+
N-Las17p₁₋₃₆₈³⁵²	+	N	N	+	+	+	+
Las17p^{TL}	+	+	+	+	+	+	
Las17p₁₋₅₂₇	+	+	+	+	+	+	+
Las17p₁₋₅₂₇^{TL}	+	+	+	+		+	
N-Las17p₁₋₃₆₈VCA	+	+	+	+	+	+	+
N-Las17p₁₋₃₆₈^{TL}VCA	+	+	+	+	+	+	

Table 3.12 Summary of functional studies on the expression of Las17p constructs in *las17Δ* strain. The suppression of the growth defect, defect in endocytosis or actin patch polarization is indicated by “+” while “-” indicates otherwise. Similarly for the localization studies, “+” indicates localization to the cortical actin patches and “-” indicates inability to localize to the cortical patches. N indicates studies not done.

4 Discussion

4.1 Functional analysis of WASP family of proteins

In *S. cerevisiae*, **actin** filaments are organized into three prominent structures: cortical **actin** patches, **actin** cables and the actomyosin ring; of which the **actin** patches make up most of the filamentous **actin** in the yeast cell. **Actin** patches are in a constant state of flux having slow lateral motion followed by rapid internalization into the cytoplasm. The motility of the patches at the cortex and in the cytoplasm is facilitated by **actin** polymerization mediated by a host of proteins (reviewed in Moseley & Goode, 2006). The assembly of G-**actin** to form the F-**actin** polymer is a tightly regulated process which involves the nucleation of the **actin** filaments by proteins called **actin** nucleators such as Arp2/3 complex. The Arp2/3 complex nucleates **actin** assembly by mimicking the barbed end of an **actin** filament (Pollard et al., 2002). The Arp2/3 complex has minimal nucleation activity on its own and it has to be activated by nucleation promotion factors (NPF) (Mullins et al., 1998; Welch et al., 1998; reviewed in Pollard, 2007). The Wiskott Aldrich Syndrome protein (WASP) is one of the best characterized member of the Class I family of NPFs. WASP is a 502 residue **proline** rich protein that is expressed predominantly in hematopoietic cells (Derry et al., 1994). Mutations in the gene coding for WASP cause the genetic disease Wiskott Aldrich Syndrome (WAS), an X-linked disease (Derry et al., 1994). The primary symptoms of Wiskott Aldrich Syndrome (WAS) are thrombocytopenia, eczema and bleeding disorders. WASP is the founding member of the WASP family which in mammals include WASP, N-WASP, WAVE1, WAVE2 and WAVE3. WASP is expressed only in hematopoietic cells while the **paralogs** are ubiquitous (Miki et al., 1996; Miki et al., 1998). WASP consists of the WH1 domain at

the N-terminal region followed by the GTPase binding domain (GBD), proline rich region and lastly the verprolin homology-cofilin homology-acidic domain (VCA domain) (Fig. 1.6; reviewed in Pollard, 2007). The WH1 domain of WASP interacts with mammalian Verprolins (WIP, WIRE and CR16) (Ramesh et al., 1997; Aspenstrom, 2004; Ho et al., 2001). Mutations causing Wiskott Aldrich Syndrome (WAS) have been shown to abolish WASP-WIP interaction (Stewart et al., 1999) and majority of missense mutations are located within the WH1 domain.

Las17p is the *S. cerevisiae* homologue of mammalian WASP with similar domain organization as that of WASP but without the GTPase binding domain (GBD) (Moseley and Goode, 2006). Las17p, unlike WASP has been shown to be constitutively active and able to promote actin polymerization even in the absence of Cdc42p (Rodal et al., 2003). The WH1 domain of Las17 interacts with the Las17p binding domain (LBD) of Vrp1p (Naqvi et al., 1998). Vrp1p is the yeast homologue of mammalian WIP (Ramesh et al., 1997). The proline rich regions of Las17p interact with SH3 domain containing proteins like Bzz1p, Hof1p, Myo3p, Myo5p and Rvs167p (Soulard et al., 2002; Naqvi et al., 2001; Evangelista et al., 2000; Lechler et al., 2000; Colwill et al., 1999). The VCA domain at the C-terminal region of Las17p binds and activates the Arp2/3 complex (Rodal et al., 2003).

In this study, the functional significance of WASP-WIP interaction and Las17p-Vrp1p interaction has been examined in *las17Δ* strains.

4.2 Functional analysis of WASP-WIP interaction using *S. cerevisiae las17Δ*

4.2.1 WASP suppresses the growth defects of *las17Δ* cells only in the presence of WIP

Expression of human WASP in *S. cerevisiae las17Δ* cells did not suppress the growth defects of the strain (Fig. 3.3) while co-expression of WASP with WIP was able to rescue the growth defects at 37°C (Fig 3.5). WASP localizes to the cortical actin patches of *las17Δ* cells only in the presence of WIP and co-expression with WIP also improves the stability of WASP (Fig. 3.8-10). Abolishing WASP binding to WIP impairs WASP localization to the cortical patches though the localization of WIP remains unaffected (Fig. 3.16, 18). Expression of WIP alone in the *las17Δ* strain does not rescue the growth defects of the strain (Fig. 3.7) suggesting that the growth of the *las17Δ* cells at 37°C requires the combined activity of WASP and WIP.

Las17p has been shown to localize to the cortical actin patches in *S. cerevisiae* cells (Li, 1997) and this in turn implies that WASP is unable to rescue the defects of *las17Δ* strain due to its inability to localize to the cortical actin patches of *las17Δ* cells in the absence of WIP. In *S. cerevisiae* cells, Las17p and Vrp1p function as a complex (Thanabalu et al., 2007). WIP is the human homologue of yeast Vrp1p and in mammalian cells, WASP and WIP form a complex similar to the complex formed by Las17p and Vrp1p in yeast cells (Naqvi et al., 1998). It has also been shown that expression of WIP in *vrp1Δ* cells rescues the growth defects of the mutant strain (Vaduva et al., 1999) suggesting that WIP is capable of participating in the cascade of events regulating cytoskeleton in *S. cerevisiae*.

In mammalian cells, WIP is crucial for localization of WASP both in a vaccinia-based actin motility system and to the immune synapse after T cell receptor (TCR) ligation (Moreau et al., 2000; Sasahara et al., 2002). Additionally, abolishing WASP-WIP interaction in macrophages not only affects chemotaxis but also podosome formation in the cells (Tsuboi, 2007). Considering that podosomes are also actin rich structures on the plasma membrane, similar to the cortical actin patches in yeast cells, it is very likely that WIP plays an important role in the localization and activity of WASP.

4.2.2 WASP and WIP function as a complex in suppressing the growth defects of *las17Δ* cells

WASP is able to suppress the growth defects of IDY166 in the presence of WIP and the apparent role of WIP in the activity of the WASP-WIP complex is to target the complex to the cortical actin patches. This in turn raises a question as to whether WASP can suppress growth defect of IDY166 if in the absence of WIP, WASP is targeted to the plasma membranes through other means. Fusion of a CAAX box motif (GCCIIIC) at the carboxyl terminal of proteins confers them with strong cortical localization (Golsteyn et al., 1997). It has been shown that fusing Ras1p's CAAX box (Powers et al., 1984) to GFP localizes GFP to the plasma membrane of *S. cerevisiae* (Thanabalu and Munn, 2001) and it was determined that fusing the CAAX box to human and *S. cerevisiae* cytoskeletal proteins improved their activity in *S. cerevisiae* mutant strains. Fusing CAAX to N-terminal region of Vrp1p (N-Vrp1p) enhanced the activity of N-Vrp1p-CAAX in *vrp1Δ* cells (Thanabalu et al., 2001) and similarly, the activity of mammalian Verprolins like WIP and CR16 was enhanced in *vrp1Δ* cells by the fusion of CAAX box (unpublished

data from our lab). However, in the case of WASP, localization of WASP to the plasma membrane by fusing CAAX did not enable WASP-CAAX to suppress the growth defect of IDY166, in the absence of WIP (Fig. 3.11A). Therefore, localization of WASP to plasma membrane alone is not sufficient for the activity of WASP in *S. cerevisiae* cells, suggesting that WIP has additional roles besides mediating WASP localization. Additionally, it is also possible that instead of a general localization to the plasma membrane through fusion of CAAX to WASP, WASP needs to be targeted specifically to the cortical actin patches in order for the protein to participate in the cytoskeletal machinery.

Fusion of CAAX box to WASP did not obviate the necessity for WIP in order for WASP to suppress the growth defects of the *las17Δ* strain. So apart from mediating localization of WASP to cortical patches, what are the other ways in which WIP influences WASP activity in the *las17Δ* strain? A possible role for WIP in the stabilization of WASP became apparent on comparing the expression of WASP in *las17Δ* cells both in the presence and absence of WIP. The expression of WASP was significantly higher in cells co-expressing WASP and WIP than in the cells expressing only WASP (Fig. 3.10A). This observation that the stability of WASP is improved in the presence of WIP is not restricted to the yeast system as similar findings have been reported in other model systems. In *Caenorhabditis elegans*, the expression of WSP-1 (*C. elegans* WASP) is lower in the organisms treated with RNAi for WIP-1 (*C. elegans* WIP) (Sawa and Takenawa, 2006). Moreover, during the last one year, 5 reports have demonstrated that the stabilization of WASP is mediated by WIP in mammalian cells and that in the

absence of WIP, WASP is rapidly degraded (Chou et al., 2006; Konno et al., 2007; Tsuboi, 2007; de la Fuente et al., 2007; Dong et al., 2007). However, in contrast to the stabilization of WASP by WIP, the stability of WIP is not enhanced significantly by the co-expression of WASP and WIP in *S. cerevisiae las17Δ* cells (Fig. 3.10B). Therefore, based on the results presented in this study and in the published literature, it is possible that human WASP is degraded by the proteases expressed in *S. cerevisiae* and binding with WIP protects WASP from degradation.

Abolishing the interaction between WASP and WIP by deleting the WASP binding domain (WBD) of WIP (Table 3.3), also abolishes the ability of WASP to suppress the growth defects of *las17Δ* cells (Fig. 3.15). Moreover WASP is not stabilized by WIP₁₋₄₀₃ (Fig. 3.17). Deleting the WH1 domain of WASP also abolished the interaction between WASP and WIP (Table 3.3) and the mutant WASP₁₃₉₋₅₀₂ is unable to suppress the growth defects of *las17Δ* cells in the presence of WIP (Fig. 3.15). However, in contrast to the other case where WASP is not stabilized by WIP₁₋₄₀₃, the lack of interaction between WASP₁₃₉₋₅₀₂ and wild type WIP does not destabilize WASP₁₃₉₋₅₀₂. On the contrary, WASP₁₃₉₋₅₀₂ was stably expressed both in the presence and absence of WIP (Fig. 3.17). In human platelets, WASP is susceptible to proteolytic degradation by Calpain (Shcherbina et al., 2001). It has also been suggested that when WIP binds with the WH1 domain of WASP, the WASP binding domain of WIP wraps around the WH1 domain of WASP and thus protects WASP from Calpain mediated degradation (de la Fuente et al. 2007). Therefore based on the data for WASP₁₃₉₋₅₀₂ expression in *S. cerevisiae* cells, it can be inferred that the WH1 domain of WASP is targeted by the proteases expressed in

S. cerevisiae. Deleting the WH1 domain of WASP would thus hinder proteolytic degradation by the *S. cerevisiae* proteases. However, a *S. cerevisiae* homologue of human Calpain has not yet been identified, so the mechanism of degradation of WASP in *S. cerevisiae* is not clear. Targeted localization of WASP₁₃₉₋₅₀₂ to the plasma membrane through fusion of the CAAX box does not enable WASP₁₃₉₋₅₀₂-CAAX to suppress the growth defects of the *las17Δ* strain both in the presence and absence of WIP (Fig. 3.19). This suggests that the physical binding of WASP with WIP is essential than just stabilization or localization of WASP to the plasma membrane.

4.2.3 Functional interaction between WASP and WIP

From the data presented so far, it is clear that WASP and WIP work as a complex in suppressing the growth defects of the *las17Δ* strain. However, function of WASP-WIP complex in the *las17Δ* strain is unclear. Does the WASP-WIP complex perform a similar role as that of the Las17p-Vrp1p complex in regulating the actin cytoskeleton in *S. cerevisiae*? In *vitro* studies have shown that the VCA domain of WASP promotes actin polymerization through the activation of the Arp2/3 complex (Yamaguchi et al., 2000). The VCA domain of WASP contains a KLKK motif which mediates interaction with actin and it also contains a DDW motif which mediates interaction with the Arp2/3 complex (Pollard, 2007). If the VCA domain of WASP is deleted, the resulting construct, WASP₁₋₄₂₄ is unable to suppress the growth defects of *las17Δ* cells, even in the presence of WIP (Fig. 3.20). Similarly, mutating the DDW motif in the VCA domain also abolishes the ability of WASP to suppress the growth defects of the *las17Δ* cells (Fig. 3.24). Deletion or mutation in the VCA domain has no effect on the stability of the

mutant WASP (Fig. 3.23, 27) or the localization to the cortical actin patches (Fig. 3.21-22, 3.25-26). Although WASP does not require the VCA domain either for stability or localization, a functional VCA domain is essential for the activity of the WASP-WIP complex in suppressing the growth defects of the *las17Δ* strain. Since the function of the VCA domain is to activate the Arp2/3 complex, it can be inferred that the WASP-WIP complex promotes actin polymerization in *S. cerevisiae*, through the activation of the Arp2/3 complex.

The ability of the WASP-WIP complex to suppress the growth defects of *las17Δ* cells, not only involves the VCA domain of WASP but also the V domain present in WIP. Deleting the V domain of WIP abolishes the interaction of WIP₅₁₋₅₀₃ with actin but does not affect interaction with WASP (Table 3.5). WIP₅₁₋₅₀₃ still localizes to the cortical patches (Fig. 3.29), stabilizes WASP (Fig. 3.31) and also mediates the localization of WASP to the cortical actin patches (Figs. 3.30, 32). However, co-expression of WASP and WIP₅₁₋₅₀₃ does not suppress the growth defects of the *las17Δ* cells (Fig. 3.28). This suggests that the V domain of WIP is essential for the activity of the WASP-WIP complex, possibly by providing additional actin molecules to the complex for optimal actin polymerization. NMR studies have been used to map the binding region between the WASP-WH1 domain and the WIP-WBD domain (Volkman et al., 2002), however, the orientation of WIP relative to WASP in the WASP-WIP complex is not clear. It is possible that in the WASP-WIP complex, WIP is oriented in a manner such that the C-terminal region of WIP containing the WBD domain is bound to the N-terminal region of WASP and the N-terminal region of WIP containing the V domain is facing the VCA

domain of WASP (Fig. 4.1). In *vitro* actin polymerization studies done to compare the efficiency of the VCA domains of WASP, N-WASP, WAVE1, WAVE2 and WAVE3 to promote actin polymerization have revealed that the VCA domain of N-WASP was most effective in activating the Arp2/3 complex (Yamaguchi et al., 2000). It was suggested that the presence of 2 V domains in N-WASP as compared to 1 V domain in WASP and WAVE1-3 could account for the higher activity of the VCA domain of N-WASP. Moreover, fusing an extra V domain to the WAVE1-VCA domain improved the activity of the WAVE1-VCA domain to the same level as that of the N-WASP-VCA domain (Yamaguchi et al., 2000). Therefore, it is possible that the V domain of WIP needs to be oriented such that it is in close proximity to the WASP-VCA domain in order for optimal activity of the WASP-WIP complex in suppressing the growth defects of the *las17Δ* cells. Additionally, the experiments done using the WIPAV (WIP₅₁₋₅₀₃) constructs also indicate the possibility that mutations in WIP might also contribute to the clinical manifestations of WAS. There is at least one reported case of a patient with clinical presentation of WAS in the absence of detectable mutation on WASP (Konno et al., 2007) suggesting that in this patient, mutations in other proteins cause the disease.

4.2.4 Is the activity of the WASP-WIP complex dependent on interaction with *S. cerevisiae* cytoskeletal proteins?

Las17p interacts with SH3 domain containing proteins like Bzz1p, Hof1p, Myo3p, Myo5p and Rvs167p (Soulard et al., 2002; Naqvi et al., 2001; Evangelista et al., 2000; Lechler et al., 2000; Colwill et al., 1999). Most of these proteins are required for the viability of *S. cerevisiae* at restrictive temperature and in some cases, deletion of more

than one SH3 domain containing protein (Myo3p and Myo5p) is lethal to the cells (Geli et al., 1996; Goodson et al., 1996; Moseley and Goode, 2006). The interaction of WASP with the SH3 domain of Bzz1p, Hof1p, Myo3p, Myo5p and Rvs167p was examined by Yeast Two Hybrid and found that WASP interacts with the SH3 domains of Hof1p, Bzz1p and MyoSp (Table 3.2). Similar to wild type WASP, WASP with deletion or mutation in the VCA domain also interacted with the SH3 domains of Hof1p, Bzz1p and MyoSp (Table 3.4). Hof1p is related to *S. pombe* Cdc15 and human PSTPIP1 (Ren et al., 2005). Hof1p and its homologues are associated with cytokinesis in eukaryotic cells (Lippincott and Li, 2000). In *S. cerevisiae* cells Hof1p expression is cell cycle regulated, expression commences during anaphase and persists until the completion of cytokinesis (Vallen et al., 2000). Deletion of *HOF1* results in temperature sensitive growth defect at 37°C and *HOF1* deletion in combination with the deletion of additional genes associated with cytokinesis like Myo1 is lethal to the cells (Vallen et al., 2000). Hof1p localizes to the bud neck and the localization of Hof1p is dependent on additional cytoskeletal proteins like Las17p, Vrp1p and type I myosins (Thanabalu and Munn, 2001; Naqvi et al., 2001). It is possible that WASP might play a similar role as that of Las17p in regulating the activity of Hof1p.

The interaction of WASP with Bzz1p is interesting because, Bzz1p has been postulated to be the *S. cerevisiae* homologue of human Toca-1 (Moseley and Goode, 2006). In *vitro* actin polymerization studies carried out using purified recombinant cytoskeletal proteins have shown that WIP inhibits Cdc42 mediated activation of N-WASP (Martinez-Quiles et al., 2001). It was also reported recently that this inhibitory activity of WIP can be

neutralized by the addition of Toca-1 to the reaction mixture containing N-WASP, WIP and Cdc42 (Ho et al., 2004). In *S. cerevisiae*, deletion of *BZZ1* has no obvious phenotype (Soulard et al., 2002) and both Las17p and Bzz1p lack Cdc42p binding domains. Therefore, it is unclear whether Bzz1p regulation of the WASP-WIP complex is similar to that of Toca-1 in mammalian cells. Since Bzz1p has been shown to interact with other cytoskeletal proteins through its SH3 domain (Soulard et al., 2002), it is possible that Bzz1p plays the role of an adaptor to mediate the interaction of the WASP-WIP complex with other *S. cerevisiae* cytoskeletal proteins.

Both Vrp1p and Las17p have been shown to interact with type I myosins like Myo5p (Evangelista et al., 2000). It is suggested that PxxxxPxxP (P- proline residue, x- any amino acid) present in both Vrp1p (22 motifs) and Las17p (4 motifs) mediates binding with Myo5p (Evangelista et al., 2000). Pattern match analysis of the WASP protein sequence suggests that there are around 10 PxxxxPxxP motifs in WASP all of which are located between the residues 346-404. It is possible that in a manner similar to that of Las17p, the PxxxxPxxP motifs in WASP also mediate the interaction between WASP and the SH3 domain of Myo5p. Human WIP is also capable of binding with Myo5p (unpublished data from our lab) just like the binding of Vrp1p with Myo5p. WIP has the actin binding V domain and Myo5p has the Arp2/3 complex activating CA domain (Ramesh et al., 1997; Moseley and Goode, 2006). Assuming that WIP and Myo5p are able to form a complex in *S. cerevisiae*, then why does expression of WIP alone in the *las17Δ* strain not suppress the growth defects in the strain? After all expression of WIP in *vrp1Δ* strain is able to suppress the growth defects of the strain (Vaduva et al., 1999)

though it is unclear whether the activity of WIP in *vrp1Δ* strain is mediated by any endogenous yeast cytoskeletal protein like Las17p or Myo5p. One possibility is that even though Myo5p possesses the Arp2/3 complex activating CA domain, it needs to be "activated" by WASP in order to utilize the CA domain to promote actin polymerization. Another possibility is that WASP, WIP and Myo5p form a complex and this complex would require the combined activity of the V domain of WIP, VCA domain of WASP and the CA domain of Myo5p in order to suppress the growth defects of the *las17Δ* strain.

From the data presented in this study, the following model can be postulated for the activity of the WASP-WIP complex in *las17Δ* strain (Fig. 4.1). WIP localizes to the cortical actin patches of *las17Δ* cells. WASP is recruited to the cortical actin patches through its interaction with WIP. Binding of WIP with the WH1 domain of WASP stabilizes WASP and protects it from proteolytic degradation. In the WASP-WIP complex, WIP could be oriented in a manner such that the V domain of WIP is in close proximity to the VCA domain of WASP. The activity of the WASP-WIP complex possibly involves the recruitment of the endogenous *S. cerevisiae* SH3 domain containing proteins, especially Myo5p which can bind with both WASP and WIP. The CA domain of Myo5p could also promote actin polymerization by working in tandem with the V domain of WIP and the VCA domain of WASP. Regardless of the exact mechanism of action of the WASP-WIP complex in *S. cerevisiae las17Δ* cells, the results presented in this study show that WASP and WIP function as a complex. Recent work from various laboratories also suggest that even in mammalian cells, WASP and WIP function as a complex and that one of the functions of WIP is to stabilize WASP (Chou et al., 2006;

Konno et al., 2007; Tsuboi, 2007; de la Fuente et al., 2007; Dong et al., 2007). It is unclear whether WASP needs to be activated by *S. cerevisiae* Cdc42p as postulated in actin polymerization, experiments with WASP and human Cdc42 (Higgs and Pollard, 2000). Recent work in our lab employing fluorescence microscopy to analyze conformational changes in WASP in the presence of human Cdc42 has suggested that Cdc42 does not bring about any significant change in WASP conformation (Lim et al., 2007).

Figure 4.1 A working model for WASP-WIP activity.

4.3 Functional analysis of Wiskott Aldrich Syndrome (WAS) mutations using *S. cerevisiae las17Δ* cells

In the previous section, it was shown that WASP is able to suppress the growth defects of *S. cerevisiae las17Δ* cells in the presence of WIP. WASP and WIP function as a complex and the activity of the complex requires the V domain of WIP and the VCA domain of WASP. In this study, the effect of 52 WAS missense mutations on the activity of WASP

in suppressing the growth defects of *las17Δ* cells was examined. The missense mutations were individually generated in the plasmid pWASP by site directed mutagenesis to generate 52 plasmids encoding mutant WASP. The 52 plasmids were co-transformed with plasmid expressing WIP into the *las17Δ* strain IDY166. The localization of the mutant WASP-GFP in the *las17Δ* cells was examined by fluorescence microscopy and the ability of the mutants to suppress the growth defects of the strain was examined in both a plate assay and liquid culture assay.

4.3.1 Mutations in the WH1 domain cause severe defects in the activity of WASP-WIP complex in *las17Δ* cells

Missense mutations in the WH1 domain cause the disease with varying degrees of severity. Among the 40 WH1 domain mutations, 14 mutations cause XLT, 15 mutations cause Classic WAS and 11 mutations cause either XLT or WAS depending upon the particular case (Table 3.7). The following 13 WH1 domain mutations abolished the ability of WASP to suppress the growth defects of the *las17Δ* cells: E31K, L35H, G40V, L46P, W64R, G70W, C73Y, I85T, T111P, G125R, F128L, E133K and A134T (Figs. 3.35-41; Table 3.7). The remaining 27 out of 40 mutations also affected the activity of WASP to a certain degree as evident from the liquid culture assay but the mutants were able to restore growth of the *las17Δ* cells in the plate assay (Figs. 3.35-41), so these mutations are not as severe as the 13 mutations described earlier.

Among these 13 WASP mutants which were unable to restore growth of the strain at 37°C in the plate assay, WASP bearing the mutations W64R, G70W, T111P, G125R or

F128L were extremely unstable and they did not localize to the cortical patches even at 24°C (Fig. 3.53; Table 3.7). Incidentally, all the 5 mutations (W64R, G70W, T111P, G125R or F128L) cause the severe form of the disease, Classic WAS. It is possible that these mutations could inherently make WASP unstable or they could affect the interaction of WASP with WIP and thus leading to loss of stability. The following 6/13 mutations E31K, L35H, L46P, C73Y, I85T and A134T did not affect the localization of mutant WASP to the cortical patches at 24°C but abolished localization at 37°C even though the proteins were expressed at 37°C (Table 3.7; Fig. 3.54). A possible explanation for why the 6 mutations affect the activity of WASP was provided on examining how the A134T mutation affected WASP-WIP interaction. It has been reported earlier that introducing the A134T mutation in WASP (WASP^{A134T}) abolishes WASP-WIP interaction (Stewart et al., 1999). The interaction between WASP^{A134T} and WIP was examined using Yeast Two Hybrid and GST pull down assays. The results of the Yeast two Hybrid and pull down experiments were contradictory, WASP^{A134T} interacted with WIP in the Yeast Two Hybrid but no interaction between WASP^{A134T} and WIP could be detected in the pull down assay (Fig. 3.55). It is possible that there is weak interaction between WASP^{A134T} and WIP, which can be detected in the Yeast Two Hybrid assay but the interaction is not strong enough to be visualized using the pull down assay. The weak interaction between WASP^{A134T} and WIP would also account for the localization of WASP^{A134T} to the cortical patches at 24°C but under stringent conditions (37°C), the mutant is unable to suppress the growth defects of the strain even though it is expressed at 37°C.

4.3.2 Mutations outside the WH1 domain do not significantly affect WASP activity in *las17Δ* cells

Among the 12 C-terminal region mutations examined, the mutations causing Classic WAS are all located in the proline rich region (P359T and P373S) and the VCA domain (P459S, K476E and D485N). The rest of the mutations in the VCA domain (R477K and I481N) and the mutations in the Basic region (G187C and A236E) cause XLT. It is unclear why the mutations in the Basic region, proline rich region or VCA domain cause the disease. It is possible that these mutations cause conformational changes in the protein which might affect the activity of the protein or the interaction with other cytoskeletal proteins. However, almost all of the 12 C-terminal region mutations had no effect on the activity of WASP in suppressing the growth defects of the *las17Δ* strain (Figs. 3.42-43; Table 3.7) and the mutant WASP were also able to localize to the cortical patches (Figs. 3.51-52; Table 3.7). The only exception was for the mutation D485N, as *las17Δ* strain expressing WASP^{D485N} grew at a slower rate than the other mutants in the liquid culture assay though the strain was able to grow as well as the others in the plate assay. The D485N mutation lies in the VCA domain of WASP and among the C-terminal region mutations, this particular mutation affects patients with the most frequency, 2 cases with Classic WAS and 1 case with XLT have been reported (Imai et al., 2003). It is possible that the missense mutations in the Basic region, proline rich region and the VCA domain affect the activity of WASP only in human cells but these mutations are not severe enough to compromise the activity of the mutant protein in *S. cerevisiae las17Δ* cells.

Among the 12 C-terminal region mutations, 3 mutations are located in the GBD domain of WASP: L270P and I294T which cause XLN and M307V which causes XLT. Mutant WASP bearing these mutations suppressed the growth defects of the *las17Δ* strain in the presence of WIP (Fig. 3.42; Table 3.7) and the mutants also localized to the cortical patches in the presence of WIP (Fig. 3.51; Table 3.7). It is suggested that the L270P and I294T mutations in the GBD domain destabilize the inactive conformation in WASP and thus making the mutant WASP constitutively active. In vitro actin polymerization studies have shown that mutant WASP bearing the L270P (WASP^{L270P}) mutation is able to promote actin polymerization even in the absence of Cdc42 (Devriendt et al., 2001). In the same assay, it was also shown that wild type WASP was able to promote actin polymerization to the same extent as the mutant, only in the presence of Cdc42 (Devriendt et al., 2001). This suggests that all the manifestations associated with XLN are caused due to unrestricted actin polymerization carried out by WASP having the L270P and I294T mutations. Expression of the constitutively active mutant WASP^{L270P} in *las17Δ* strain suppresses the growth defects of the strain only in the presence of WIP (Fig. 3.12). WASP^{L270P} is also unable to localize to the cortical actin patches on its own and it requires WIP to mediate localization to the cortical patches (Fig. 3.13). Targeted localization of WASP^{L270P} to the plasma membrane by fusion of the CAAX box also does not obviate the necessity for WIP in order for WASP^{L270P} to suppress the growth defects of the *las17Δ* strain (Fig. 3.14). These findings suggest that even though mutations causing XLN might possibly make WASP constitutively active in vitro, the mutant WASP still requires the presence of WIP to suppress the growth defect of *las17Δ* cells. As suggested in the WASP-WIP model (Fig. 4.1), the V domain of WIP regulates the

activity of the VCA domain of WASP possibly by providing additional actin molecules. This could hold true even for constitutively active WASP. It is not clear why the M307V mutation causes XLT rather than XLN even though it is located within the GBD domain. One possibility is that the M307V mutation which is located at the very fringe of the GBD domain (encompassed by the residues 242-309), does not make WASP constitutively active and therefore causes XLT instead of XLN.

4.4 Functional analysis of Las17p-Vrp1p interaction

4.4.1 The VCA domain of Las17p is redundant for rescuing the defects of *las17Δ* strain

Las17p shares a similar domain structure as that of WASP, a WH1 domain at the N-terminal region and the VCA domain at the C-terminal region, but lacks a GTPase binding domain (GBD) (Moseley and Goode, 2006). In mammalian cells like monocytes, deletion of the VCA domain affects the ability of the cells to form phagocytic cups (Tsuboi and Meerloo, 2007). However in yeast cells, the VCA domain of Las17p is possibly redundant with the rest of the domains in the protein. Expression of N-Las17p₁₋₃₆₈ (N-terminal region of Las17p) rescued the growth defects of the strain and also restored endocytosis and actin patch polarization in the *las17Δ* cells to the same extent as that of full length Las17p (Fig. 3.57-59). Interestingly, expression of C-Las17p₃₇₀₋₆₃₃ which comprises of the Arp2/3 complex activating VCA domain, failed to rescue any of the defects in the mutant strain (Figs. 3.57-59). N-Las17p₁₋₃₆₈ localized to the cortical patches of the cells at both 24°C and 37°C but C-Las17p₃₇₀₋₆₃₃ failed to localize to the cortical patches even at 24°C though its expression was comparable to that of N-Las17p₁₋

368 (Fig. 3.60-61) suggesting that the localization motif which mediates Las17p localization to the cortical patches is located in the N-terminal region of the protein. These findings indicate that Las17p promotes actin polymerization through its VCA domain as well as through its binding partners. Testing for N-Las17p₁₋₃₆₈ interaction with *S. cerevisiae* cytoskeletal proteins suggests that it interacts with Vrplp, Bzzlp, Hoflp, Myo5p and Rvs167p (Table 3.10). It is possible that the activity of N-Las17p₁₋₃₆₈ could be mediated by these cytoskeletal proteins.

4.4.2 The N-terminal region of Las17p functions through interaction with Vrplp

A mutant, N-Las17p₁₋₃₆₈^{TL} was isolated which had the missense mutations L80T and H94L. Expression of N-Las17p₁₋₃₆₈^{TL} failed to rescue the growth defects of the *las17Δ* strain at 37°C and restored endocytosis and actin patch polarization only at 24°C (Figs. 3.64-66). The expression of N-Las17p₁₋₃₆₈^{TL} was comparable to that of the wild type protein and the mutant localized to the cortical patches at both 24°C and 37°C though there were fewer patches at 37°C as compared to 24°C (3.67-68B). Over-expression of N-Las17p₁₋₃₆₈^{TL} from a high copy plasmid (2μ) suppressed the growth and endocytosis defects but restored actin patch polarization only at 24°C (Figs. 3.73, 75-76). These findings suggest that the loss in activity of N-Las17p₁₋₃₆₈^{TL} is not due to lack of adequate expression but due to other factors.

Las17p has been shown to interact with many cytoskeletal proteins like Vrplp, Bzzlp, Hoflp, Myo3p, Myo5p and Rvs167p (Naqvi et al., 1998; Soulard et al., 2002; Naqvi et al., 2001; Evangelista et al., 2000; Lechler et al., 2000; Colwill et al., 1999). N-Las17p₁₋

368 was able to interact with Vrp1p, Hof1p, Bzz1p and Rvs167p in the Yeast Two Hybrid assay. The two mutations in N-Las17p₁₋₃₆₈^{TL}, L80T and H94L fall within the region which has been shown to mediate the interaction of Las17p with Vrp1p (Lechler et al., 2001) (Fig. 3.77). Therefore, interaction of N-Las17p₁₋₃₆₈^{TL} with the Vrp1p was examined in a pull down assay and found that N-Las17p₁₋₃₆₈^{TL} did not interact with Vrp1p whereas the wild type protein could (Fig. 3.78A). These findings suggest that the activity of N-Las17p₁₋₃₆₈^{TL} was compromised due to lack of binding with Vrp1p.

4.4.3 Vrp1p promotes the formation of **Vrp1p-Las17p-Myo5p** complex

Type I myosins (Myo3p and Myo5p) are unconventional myosins which possess the Arp2/3 complex activating CA domain at their C-terminal region (Brown 1997; Geli et al., 2000). Las17p and Vrp1p interact with the type I myosin and it is suggested that this interaction is mediated through the PxxxxPxxP motifs (4 in Las17p and 22 in Vrp1p) (Evangelista et al., 2000). The presence of a larger number of PxxxxPxxP motifs in Vrp1p as compared to Las17p suggests that Vrp1p could bind with a larger fraction of type I myosin than Las17p but there is no experimental evidence to prove this.

Abolishing the interaction between Las17p and the type I myosins causes defects in growth and actin patch polarization in yeast cells (Evangelista et al., 2000). It is possible that the type I myosins work as a complex with Las17p in regulating cytoskeleton especially since they possess a CA domain. Interestingly, the 4 PxxxxPxxP motifs in Las17p which are postulated to mediate binding between Las17p and type I myosins are all located within the N-terminal region of the protein. The interaction of N-Las17p₁₋₃₆₈

and N-Las17p₁₋₃₆₈^{TL} with the SH3 domain of Myo5p was examined in a pull down assay, which showed that both the wild type protein and the mutant interacted with Myo5p-SH3 (Fig. 3.78B). However, N-Las17p₁₋₃₆₈ interacted with a larger fraction of Myo5p-SH3 as compared with the mutant, N-Las17p₁₋₃₆₈^{TL}. N-Las17p₁₋₃₆₈^{TL} interacts with a lesser fraction of Myo5p-SH3 possibly due to two reasons. One reason could be that the mutations may cause a drastic conformational change in N-Las17p₁₋₃₆₈^{TL} which in turn affects direct binding with Myo5p-SH3. The second reason could be that the lack of interaction between N-Las17p₁₋₃₆₈^{TL} and Vrp1p in turn affects interaction between N-Las17p₁₋₃₆₈^{TL} and Myo5p-SH3. The second reason seems more plausible since, the WH1 domain of Las17p (N-Las17p₁₋₂₀₆) interacts with Myo5p-SH3 in a pull down assay even though this fragment lacks any PxxxxPxxP motifs (Fig. 3.80). Yeast Two Hybrid testing showed that there is no direct binding between N-Las17p₁₋₂₀₆ and Myo5p-SH3 (Table 3.11, the results need to be verified by performing a pull down assay with purified components). These results suggest that Vrp1p plays a significant role in the formation of Vrp1p-Las17p-Myo5p complex. Thus, a complex formed by N-Las17p₁₋₃₆₈ and Vrp1p would be able to bind with 26 molecules of Myo3p/ Myo5p (4 molecules for Las17p and 22 molecules for Vrp1p). This would in theory provide the N-Las17p₁₋₃₆₈-Vrp1p complex with 26 Arp2/3 complex activating CA domains (Fig. 4.2) while the mutant, N-Las17p₁₋₃₆₈^{TL} has access to only 4 molecules of type I myosins through direct binding. This limited binding of type I myosins with N-Las17p₁₋₃₆₈^{TL} could be enough to restore endocytosis and actin patch polarization in *las17Δ* cells at 24°C but not sufficient to rescue the defects of the strain at 37°C. Interaction with Vrp1p is also essential for the activity of full length Las17p because, Las17p^{TL} which does not interact with Vrp1p (Fig.

3.105) is unable to restore **actin patch polarization** at 37°C (Fig. 3.104) even though it possesses the VCA domain.

Figure 4.2 Vrp1p mediates Las17p interaction with a larger fraction of Myo5p molecules.

Las17p has 4 Myo5p binding motifs for direct interaction with Myo5p while Vrp1p has 22 Myo5p binding motifs. The Las17p-Vrp1p complex could possibly bind with 26 Myo5p molecules.

4.4.4 Are the Myo5p binding motifs essential for the activity of N-Las17p₁₋₃₆₈?

The WH1 domain of Las17p (N-Las17p₁₋₂₀₆) is capable of interacting with Myo5p and this interaction is mediated by Vrp1p. Based on the **pull down** assay of N-Las17p₁₋₃₆₈ and N-Las17p₁₋₃₆₈^{TL} with Myo5p-SH3, we can conclude that N-Las17p₁₋₃₆₈ interacts with a larger **fraction** of Myo5p through its binding with Vrp1p rather than by direct binding with Myo5p. Does this mean that the Myo5p binding sites located on N-Las17p₁₋₃₆₈ are redundant? The WH1 domain of Las17p (N-Las17p₁₋₂₀₆) lacks the Myo5p binding motifs but is still capable of binding with Myo5p though its interaction with Vrp1p. Expression of N-Las17p₁₋₂₀₆ suppressed the growth defects of *las17Δ* cells and also restored endocytosis and actin patch polarization at 24°C but not at 37°C (Figs. 3.81-83). These findings suggest that the activity of the N-terminal region of Las17p in rescuing the defects of *las17Δ* cells requires both the WH1 domain and the PxxxxPxxP motifs which

are all located outside the WHI domain. Why are the Myo5p binding motifs essential for the activity of N-Las17p₁₋₂₀₆ when it has access to a larger fraction of Myo5p through interaction with Vrplp? An obvious possibility is that N-Las17p₁₋₃₆₈ needs to bind with the maximum number of Myo5p molecules via both direct and indirect interaction in order to suppress the cytoskeletal defects in *las17Δ* cells. This was substantiated by the observation that deletion of the proline rich regions in N-Las17p₁₋₃₆₈ affects restoration of actin patch polarization in *las17Δ* cells at 37°C (Fig. 3.93A).

4.4.5 Activity of the VCA domain is necessary at the initial stages of actin patch assembly

The N-terminal region of Las17p (N-Las17p₁₋₃₆₈) is capable of complementing the deficiency of full length Las17p in *las17Δ* cells (Figs. 3.57-59). The activity of N-Las17p₁₋₃₆₈ possibly involves interaction with type I myosins both by direct and indirect binding. The VCA domain of Las17p (residues 528-633) consists of the CA region which binds and activates the Arp2/3 complex and the V domain which has the actin binding KLKK motif (Moseley and Goode, 2006). Is the VCA domain necessary for the activity of full length Las17p? The N-terminal mutant, N-Las17p₁₋₃₆₈^{TL} does not suppress any of the defects of the *las17Δ* strain at 37°C (Figs. 3.64-66). In contrast to N-Las17p₁₋₃₆₈^{TL}, the full length mutant, Las17p^{TL} restores growth and endocytosis at 37°C though actin patches are still depolarized at 37°C (Figs. 3.100, 103-104). If the VCA domain is directly fused to N-Las17p₁₋₃₆₈^{TL}, the recombinant protein N-Las17p₁₋₃₆₈^{TL}VCA behaves like Las17p^{TL} as it is able to suppress the defects of *las17Δ* cells except for restoring actin patch polarization at 37°C (Figs. 3.111-113). Additionally, if the VCA domain is deleted

from full length Las17p^{TL} (N-Las17p₁₋₅₂₇^{TL}), it is able to partially suppress the growth defects but not the endocytosis and actin patch polarization defects at 37°C (Figs. 3.106, 109-110). These findings suggest that improved activity of Las17p^{TL} as compared to N-Las17p₁₋₃₆₈^{TL} is mainly due to the VCA domain and that there is limited contribution by the segment between the N-terminal region and the VCA domain (residues 369-527) at least for suppressing the defects in endocytosis and actin patch polarization. Therefore, the VCA domain is essential for the activity of full length Las17p and it possibly has a redundant role with that of interaction of Las17p with Vrp1p and Myo5p. The VCA domain of Las17p may also be associated with assembly and maturation of actin patches. The assembly of actin patches takes place in 3 stages: early recruitment or non motile stage followed by the intermediate stage of slow patch motility and finally the fast patch motility stage concomitant with the disassembly of the endocytic coat (Kaksonen et al., 2003). The motility of the actin patch components was studied by live cell imaging techniques and found that the recruitment of patch components takes place in an orderly fashion (Kaksonen et al., 2003 and 2005; Jonsdottir and Lee, 2004; Newpher et al., 2006; Sun et al., 2006 and 2007; Toshima et al., 2006; Okreglak and Drubin, 2007). Las17p is among the first proteins to be recruited to the putative actin patch along with another NPF, Pan1p (Kaksonen et al., 2003). The other NPFs like Myo3p, Myo5p and Abp1p are possibly recruited to the actin patches at the intermediate stage of assembly (Kaksonen et al., 2003; Jonsdottir and Lee, 2004). Vrp1p appears at the patches only after the recruitment of Las17p but before the appearance of Myo5p (Sun et al., 2006). This suggests that Vrp1p possibly mediates the recruitment of Myo5p to the actin patches even though Myo5p is capable of binding directly with Las17p. During the intermediate stage

of patch assembly, actin patches display random movement along the plasma membrane cortex which is dependent on actin polymerization (Kaksonen et al., 2003) initiated by the type I Myosins. In addition to the type I Myosins, Las17p and Panlp also possibly play a major role in the motility of the patch during the intermediate stage.

Based on the results discussed so far, it is apparent that in order for Las17p to rescue the defects of the *las17Δ* strain, maximum activity is necessary for restoration of actin patch polarization at 37°C, followed by that for restoration of endocytosis at 37°C and viability at 37°C. Lastly, restoration of actin patch polarization and endocytosis at 24°C requires minimum activity from Las17p. A model can thus be proposed for the activity of Las17p in restoring endocytosis and actin patch polarization in *las17Δ* cells (Fig. 4.3). Las17p is able to restore actin patch polarization at 37°C only if it forms a complex with Vrplp irrespective of the activity of its own VCA domain. Restoration of endocytosis at 37°C is not as stringent as that of restoration of actin patch polarization at the same temperature. Thus Las17p need not function as a complex with Vrplp and is capable of employing its VCA domain for rescuing the endocytosis defect at 37°C. Restoration of endocytosis and actin patch polarization at 24°C requires minimum activity, Las17p neither needs to form a complex with Vrplp nor does it have to utilize its VCA domain. Instead, Las17p can possibly rescue the defects at 24°C through direct binding with type I myosins like Myo5p.

Figure 4.3 Domains of Las17p required for rescuing the cellular defects of *las17Δ* cells.

- Restoring endocytosis and actin patch polarization at 24°C:** Expression of the N-terminal mutant of Las17p which does not form a complex with Vrp1p, is sufficient to restore endocytosis and actin patch polarization at 24°C. This suggests that Las17p does not require the activity of the VCA domain or complex formation with Vrp1p in order to restore endocytosis and actin patch polarization at 24°C.
- Restoring growth at 37°C:** Restoring growth at 37°C requires additional activities than that necessary for restoring endocytosis and actin patch polarization at 24°C. Restoration of growth at 37°C requires either a functional VCA domain of Las17p or N-Las17p₁₋₃₆₈ (lacking the VCA domain) needs to form a complex with Vrp1p.
- Restoring endocytosis at 37°C:** Restoring endocytosis at 37°C requires the presence of the VCA domain of Las17p. In the absence of the VCA domain of Las17p, N-Las17p₁₋₃₆₈ needs to form a complex with Vrp1p in order to restore endocytosis at 37°C. As compared to the activity required for restoring growth at 37°C, restoring endocytosis at 37°C requires additional activity because, it was observed that expression of only the WH1 domain of Las17p which is capable of complex formation with Vrp1p, restored growth at 37°C without restoring endocytosis at 37°C.
- Restoring actin patch polarization at 37°C:** Restoring actin patch polarization at 37°C requires optimal activity whereby Las17p/ Vrp1p complex formation is essential irrespective of the presence of the VCA domain of Las17p.

4.5 Conclusion

I have used *S. cerevisiae las17Δ* strain for functional analysis of mammalian WASP as well as the yeast WASP (Las17p). Using this strain, I was able to show that mammalian

WASP and WIP work as a complex in suppressing the growth defect of the strain. This is in contrast to published data which suggests that WIP keeps WASP in an inactive state. I was also able to show that the V domain of WIP and the VCA domain of WASP are both essential for the WASP-WIP complex to rescue the growth defects of *las17Δ* cells. The Wiskott Aldrich Syndrome (WAS) missense mutations in the VCA domain of WASP do not have any significant effect on WASP activity in *las17Δ* cells. However, a number of mutations in the WH1 domain abolished the ability of WASP to rescue the growth defects of *las17Δ* cells, emphasizing the importance of WASP-WIP interaction. The yeast WASP, Las17p functions similar to WASP by forming a complex with yeast WIP, Vrp1p for rescuing the defects of *las17Δ* cells. However, in contrast to WASP, the VCA domain of Las17p is essential only under conditions when Las17p is unable to form a complex with Vrp1p. The activity of the WASP-WIP complex and the Las17p-Vrp1p complex probably involves contribution from other *S. cerevisiae* cytoskeletal proteins, especially the type I myosins. There are many similarities and also differences in the activity of WASP and Las17p in *las17Δ* cells and our data has given valuable insight regarding how some of the constructs suppress the cytoskeletal defects of the mutant strain. On comparison of the biological activity of the human and yeast proteins, we find that the WASP-WIP complex is only able to suppress the growth defects of the *las17Δ* strain but the defects in endocytosis and actin patch polarization are not rescued. This further substantiates the observation that it is possible to rescue the growth defect without rescuing other cytoskeletal defects of the strain (Thanabalu and Munn, 2001). Alternatively, it is also possible that there is partial complementation of the endocytosis and actin polarization defects which is below the detection limit of the assays but is

sufficient to allow growth at restrictive temperature. The difference in the activity between WASP and Las17p could also be due to differences in the interaction of these proteins with other *S. cerevisiae* cytoskeletal proteins. Interestingly, the number of putative Myo5p binding motifs in the WASP-WIP complex and the Las17p-Vrp1p complex is similar with 26 Myo5p binding motifs in the Las17p-Vrp1p complex and 25 Myo5p binding sites in the WASP-WIP complex. It is likely that not all the predicted sites mediate interaction with Myo5p and it is also possible that the physical interaction may not translate into biological activity. Further experiments need to be performed to examine the biochemical activity of human WASP in promoting actin polymerization in the presence of *S. cerevisiae* cytoskeletal proteins. This would also give us a better understanding of the functions of their human homologues.

Notwithstanding the difference in the level of activity between WASP and Las17p in *S. cerevisiae las17Δ* cells, the fundamental conclusion in this study that WASP and WIP work as a complex has been substantiated by publications from other laboratories working on mammalian cells (Chou et al., 2006; Konno et al., 2007; Tsuboi, 2007; de la Fuente et al., 2007; Dong et al., 2007). Further studies along this line would lead to a better understanding of the molecular mechanism of actin polymerization and would enable better therapeutic strategies for alleviating the symptoms of Wiskott Aldrich Syndrome.

References

- Adams, A. E., and Pringle, J. R. (1984).** Relationship of actin and tubulin distribution to bud growth in wild-type and morphogenetic-mutant *Saccharomyces cerevisiae*. *J Cell Biol* 98, 934-945.
- Adams, R. J., and Pollard, T. D. (1986).** Propulsion of organelles isolated from *Acanthamoeba* along actin filaments by myosin-I. *Nature* 322, 754-756.
- Amberg, D. C. (1998).** Three-dimensional imaging of the yeast actin cytoskeleton through the budding cell cycle. *Mol Biol Cell* 9, 3259-3262.
- Anderson, B. L., Boldogh, I., Evangelista, M., Boone, C., Greene, L. A., and Pon, L. A. (1998).** The Src homology domain 3 (SH3) of a yeast type I myosin, Myo5p, binds to verprolin and is required for targeting to sites of actin polarization. *J Cell Biol* 141, 1357-1370.
- Ariga, T., Yamada, M., Wada, T., Saitoh, S., and Sakiyama, Y. (1999).** Detection of lymphocytes and granulocytes expressing the mutant WASP message in carriers of Wiskott-Aldrich syndrome. *Br J Haematol* 104, 893-900.
- Aspenstrom, P. (2004).** The mammalian verprolin homologue WIRE participates in receptor-mediated endocytosis and regulation of the actin filament system by distinct mechanisms. *Exp Cell Res* 298, 485-498.
- Ayscough, K. R., Stryker, J., Pokala, N., Sanders, M., Crews, P., and Drubin, D. G. (1997).** High rates of actin filament turnover in budding yeast and roles for actin in establishment and maintenance of cell polarity revealed using the actin inhibitor latrunculin-A. *J Cell Biol* 137, 399-416.
- Ayscough, K. R. (2000).** Endocytosis and the development of cell polarity in yeast require a dynamic F-actin cytoskeleton. *Curr Biol* 10, 1587-1590.
- Banin, S., Gout, I., and Brickell, P. (1999).** Interaction between Wiskott-Aldrich Syndrome protein (WASP) and the Fyn protein-tyrosine kinase. *Mol Biol Rep* 26, 173-177.
- Barylko, B., Binns, D. D., and Albanesi, J. P. (2000).** Regulation of the enzymatic and motor activities of myosin I. *Biochim Biophys Acta* 1496, 23-35.

Bear, J. E., Rawls, J. F., and Saxe, C. L., 3rd (1998). SCAR, a WASP-related protein, isolated as a suppressor of receptor defects in late Dictyostelium development. *J Cell Biol* 142, 1325-1335.

Belmont, L. D., Orlova, A., **Drubin**, D. G., and Egelman, E. H. (1999). A change in actin conformation associated with filament instability after Pi release. *Proc Natl Acad Sci U S A* 96, 29-34.

Bi, E., Maddox, P., Lew, D. J., Salmon, E. D., **McMillan**, J. N., Yeh, E., and Pringle, J. R. (1998). Involvement of an actomyosin contractile ring in *Saccharomyces cerevisiae* cytokinesis. *J Cell Biol* 142, 1301-1312.

Brown, S. S. (1997). Myosins in yeast. *Curr Opin Cell Biol* 9, 44-48.

Carrier, M. F. (1991). Nucleotide hydrolysis in cytoskeletal assembly. *Curr Opin Cell Biol* 3, 12-17.

Carlsson, A. E., Shah, A. D., **Elking**, D., Karpova, T. S., and Cooper, J. A. (2002). Quantitative analysis of actin patch movement in yeast. *Biophys J* 82, 2333-2343.

Caron, E. (2002). Regulation of Wiskott-Aldrich syndrome protein and related molecules. *Curr Opin Cell Biol* 14, 82-87.

Chang, F. S., Stefan, C. J., and Blumer, K. J. (2003). A WASp homolog powers actin polymerization-dependent motility of endosomes in vivo. *Curr Biol* 13, 455-463.

Chou, H. C., Anton, I. M., Holt, M. R., Curcio, C., Lanzardo, S., Worth, A., Burns, S., Thrasher, A. J., Jones, G. E., and Calle, Y. (2006). WIP regulates the stability and localization of WASP to podosomes in migrating dendritic cells. *Curr Biol* 16, 2337-2344.

Collins, K., Sellers, J. R., and Matsudaira, P. (1990). Calmodulin dissociation regulates brush border myosin I (110-kD-calmodulin) mechanochemical activity in *vitro*. *J Cell Biol* 110, 1137-1147.

Colwill, K., Field, D., Moore, L., Friesen, J., and Andrews, B. (1999). In *vivo* analysis of the domains of yeast Rvs167p suggests Rvs167p function is mediated through multiple protein interactions. *Genetics* 152, 881-893.

Cooper, J. A., Buhle, E. L., Jr., Walker, S. B., Tsong, T. Y., and **Pollard**, T. D. (1983). Kinetic evidence for a monomer activation step in actin polymerization. *Biochemistry* 22, 2193-2202.

de la Fuente, M. A., Sasahara, Y., Calamito, M., Anton, I. M., Elkhail, **A.**, **Gallego**, M. D., Suresh, K., Siminovitch, K., Ochs, H. D., Anderson, K. C., *et al.* (2007). WIP is a chaperone for Wiskott-Aldrich syndrome protein (WASP). *Proc Natl Acad Sci U S A* 104, 926-931.

Derry, J. M., Ochs, H. D., and Francke, U. (1994). Isolation of a novel gene mutated in Wiskott-Aldrich syndrome. *Cell* 78,635-644.

Dong, X., Patino-Lopez, G., Candotti, F., and Shaw, S. (2007). Structure-function analysis of WIP role in TCR-stimulated NFAT activation: Evidence that WIP/WASP dissociation is not required and that the WIP N-terminus is inhibitory. *J Biol Chem.* 282, 30303-30310.

Donnelly, S. F., Pocklington, M. J., Pallotta, D., and Orr, E. (1993). A proline-rich protein, verprolin, involved in cytoskeletal organization and cellular growth in the yeast *Saccharomyces cerevisiae*. *Mol Microbiol* 10, 585-596.

Duncan, M. C., Cope, M. J., **Goode**, B. L., Wendland, B., and **Drubin**, D. G. (2001). Yeast Eps15-like endocytic protein, Panlp, activates the Arp2/3 complex. *Nat Cell Biol* 3, 687-690.

Eitzen, G., Wang, L., Thorngren, N., and Wickner, W. (2002). Remodeling of organelle-bound actin is required for yeast vacuole fusion. *J Cell Biol* 158,669-679.

Evangelista, M., Klebl, B. M., Tong, A. H., Webb, B. A., Leeuw, T., Leberer, E., Whiteway, M., Thomas, D. Y., and Boone, C. (2000). A role for myosin-I in actin assembly through interactions with Vrplp, Beelp, and the Arp2/3 complex. *J Cell Biol* 148,353-362.

Evangelista, M., Pruyne, D., **Amberg**, D. C., Boone, C., and Bretscher, A. (2002). Formins direct Arp2/3-independent actin filament assembly to polarize cell growth in yeast. *Nat Cell Biol* 4, 260-269.

Gallwik, D., and Seidel, R. (1980). Molecular cloning of the actin gene from yeast *Saccharomyces cerevisiae*. *Nucleic Acids Res* 8, 1043-1059.

Geli, M. I., and Riezman, H. (1996). Role of type I myosins in receptor-mediated endocytosis in yeast. *Science* 272,533-535.

- Geli, M. I., Wesp, A., and Riezman, H. (1998). Distinct functions of calmodulin are required for the uptake step of receptor-mediated endocytosis in yeast: the type I myosin Myo5p is one of the calmodulin targets. *Embo J* 17, 635-647.
- Geli, M. I., Lombardi, R., Schmelzl, B., and Riezman, H. (2000). An intact SH3 domain is required for myosin I-induced actin polymerization. *Embo J* 19, 4281-4291.
- Gertler, F. B., Niebuhr, K., Reinhard, M., Wehland, J., and Soriano, P. (1996). Mena, a relative of VASP and Drosophila Enabled, is implicated in the control of microfilament dynamics. *Cell* 87, 227-239.
- Goley, E. D., Rodenbusch, S. E., Martin, A. C., and Welch, M. D. (2004). Critical conformational changes in the Arp2/3 complex are induced by nucleotide and nucleation promoting factor. *Mol Cell* 16, 269-279.
- Goley, E. D., and Welch, M. D. (2006). The ARP2/3 complex: an actin nucleator comes of age. *Nat Rev Mol Cell Biol* 7, 713-726.
- Goode, B. L., Rodal, A. A., Barnes, G., and Drubin, D. G. (2001). Activation of the Arp2/3 complex by the actin filament binding protein Abp1p. *J Cell Biol* 153, 627-634.
- Goodson, H. V., and Spudich, J. A. (1995). Identification and molecular characterization of a yeast myosin I. *Cell Motil Cytoskeleton* 30, 73-84.
- Goodson, H. V., Anderson, B. L., Warrick, H. M., Pon, L. A., and Spudich, J. A. (1996). Synthetic lethality screen identifies a novel yeast myosin I gene (MYO5): myosin I proteins are required for polarization of the actin cytoskeleton, *J Cell Biol* 133, 1277-1291.
- Haarer, B. K., Petzold, A., Lillie, S. H., and Brown, S. S. (1994). Identification of MYO4, a second class V myosin gene in yeast. *J Cell Sci* 107 (Pt 4), 1055-1064.
- Higgs, H. N., and Pollard, T. D. (1999). Regulation of actin polymerization by Arp2/3 complex and WASp/Scar proteins. *J Biol Chem* 274, 32531-32534.
- Higgs, H. N., and Pollard, T. D. (2000). Activation by Cdc42 and PIP(2) of Wiskott-Aldrich syndrome protein (WASP) stimulates actin nucleation by Arp2/3 complex. *J Cell Biol* 150, 1311-1320.
- Higuchi, R., Krummel, B., and Saiki, R. K. (1988). A general method of in vitro preparation and specific mutagenesis of DNA fragments: study of protein and DNA interactions. *Nucleic Acids Res* 16, 7351-7367.

- Hill, K. L., Catlett, N. L., and **Weisman, L. S. (1996)**. Actin and myosin function in directed vacuole movement during cell division in *Saccharomyces cerevisiae*. *J Cell Biol* 135, 1535-1549.
- Ho, H. Y., Rohatgi, R., Ma, L., and Kirschner, M. W. (2001). CR16 forms a complex with N-WASP in brain and is a novel member of a conserved proline-rich actin-binding protein family. *Proc Natl Acad Sci U S A* 98, 11306-11311.
- Ho, H. Y., Rohatgi, R., Lebensohn, A. M., Le, M., Li, J., Gygi, S. P., and Kirschner, M. W. (2004). Toca-1 mediates Cdc42-dependent actin nucleation by activating the N-WASP-WIP complex. *Cell* 118,203-216.
- Huang, F. Y., Chiu, N. C., and Liu, S. C. (1997). Penicillin-resistant pneumococcal infections in children. *J Formos Med Assoc* 96, 414-418.
- Huckaba, T. M., Gay, A. C., Pantalena, L. F., Yang, H. C., and Pon, L. A. (2004). Live cell imaging of the assembly, disassembly, and actin cable-dependent movement of endosomes and actin patches in the budding yeast, *Saccharomyces cerevisiae*. *J Cell Biol* 167, 519-530.
- Hwang, E., Kusch, J., Barral, Y., and Huffaker, T. C. (2003). Spindle orientation in *Saccharomyces cerevisiae* depends on the transport of microtubule ends along polarized actin cables. *J Cell Biol* 161,483-488.
- Imai, K., Nonoyama, S., and Ochs, H. D. (2003). WASP (Wiskott-Aldrich syndrome protein) gene mutations and phenotype. *Curr Opin Allergy Clin Immunol* 3,427-436.
- Johnston, G. C., Prendergast, J. A., and Singer, R. A. (1991). The *Saccharomyces cerevisiae* MYO2 gene encodes an essential myosin for vectorial transport of vesicles. *J Cell Biol* 113, 539-551.
- Jonsdottir, G. A., and Li, R. (2004). Dynamics of yeast Myosin I: evidence for a possible role in scission of endocytic vesicles. *Curr Biol* 14, 1604-1609.
- Jung, G., Remmert, K., Wu, X., Volosky, J. M., and Hammer, J. A., 3rd (2001). The *Dictyostelium* CARMIL protein links capping protein and the Arp2/3 complex to type I myosins through their SH3 domains. *J Cell Biol* 153, 1479-1497.
- Kaksonen, M., Sun, Y., and **Drubin, D. G. (2003)**. A pathway for association of receptors, adaptors, and actin during endocytic internalization. *Cell* 115,475-487.

- Kaksonen, M., Toret, C. P., and **Drubin, D. G. (2005)**. A modular design for the clathrin- and actin-mediated endocytosis machinery. *Cell* 123, 305-320.
- Kato, M., **Miki, H.**, Kurita, S., **Endo, T.**, Nakagawa, H., Miyamoto, S., and Takenawa, T. **(2002)**. WICH, a novel verprolin homology domain-containing protein that functions cooperatively with N-WASP in actin-microspike formation. *Biochem Biophys Res Commun* 291, 41-47.
- Kilmartin, J. V., and Adams, A. E. **(1984)**. Structural rearrangements of tubulin and actin during the cell cycle of the yeast *Saccharomyces*. *J Cell Biol* 98, 922-933.
- Kim, A. S., Kakalis, L. T., **Abdul-Manan, N.**, Liu, G. A., and Rosen, M. K. **(2000)**. Autoinhibition and activation mechanisms of the Wiskott-Aldrich syndrome protein. *Nature* 404, 151-158.
- Kinosian, H. J., Selden, L. A., Estes, J. E., and Gershman, L. C. **(1993)**. Nucleotide binding to actin. Cation dependence of nucleotide dissociation and exchange rates. *J Biol Chem* 268, 8683-8691.
- Kleenebrecht, J., Selow, J., and **Winkler, W. (1982)**. The mouse mutant limb-deformity (ld). *Anat Anz* 152, 313-324.
- Konno, A., Kirby, M., Anderson, S. A., Schwartzberg, P. L., and Candotti, F. **(2007)**. The expression of Wiskott-Aldrich syndrome protein (WASP) is dependent on WASP-interacting protein (WIP). *Int Immunol* 19, 185-192.
- Korn, E. D., Carlier, M. F., and Pantaloni, D. **(1987)**. Actin polymerization and ATP hydrolysis. *Science* 238, 638-644.
- Krisiukeniene, A., **Sitkauskiene, B.**, and Sakalauskas, R. **(2003)**. [Wiskott-Aldrich syndrome: the possibilities of diagnosis and treatment]. *Medicina (Kaunas)* 39, 211-216.
- Kubler, E., and Riezman, H. **(1993)**. Actin and fimbrin are required for the internalization step of endocytosis in yeast. *Embo J* 12, 2855-2862.
- Lechler, T., Shevchenko, A., and Li, R. **(2000)**. Direct involvement of yeast type I myosins in Cdc42-dependent actin polymerization. *J Cell Biol* 148, 363-373.
- Lechler, T., Jonsdottir, G. A., Klee, S. K., Pellman, D., and Li, R. **(2001)**. A two-tiered mechanism by which Cdc42 controls the localization and activation of an Arp2/3-activating motor complex in yeast. *J Cell Biol* 155, 261-270.

- Lee, W. L., Bezanilla, M., and **Pollard**, T. D. (2000). Fission yeast myosin-I, Myolp, stimulates actin assembly by Arp2/3 complex and shares functions with WASp. *J Cell Biol* 151, 789-800.
- Leverrier, Y., Lorenzi, R., Blundell, M. P., Brickell, P., Kinnon, C., Ridley, A. J., and Thrasher, A. J. (2001). Cutting edge: the Wiskott-Aldrich syndrome protein is required for efficient phagocytosis of apoptotic cells. *J Immunol* 166, 4831-4834.
- Li, R. (1997). Bee1, a yeast protein with homology to Wiskott-Aldrich syndrome protein, is critical for the assembly of cortical actin cytoskeleton. *J Cell Biol* 136, 649-658.
- Lillie, S. H., and Brown, S. S. (1994). Immunofluorescence localization of the unconventional myosin, Myo2p, and the putative kinesin-related protein, Smylp, to the same regions of polarized growth in *Saccharomyces cerevisiae*. *J Cell Biol* 125, 825-842.
- Lim, R. P., Misra, A., Wu, Z., and Thanabalu, T. (2007). Analysis of conformational changes in WASP using a split YFP. *Biochem Biophys Res Commun* 362, 1085-1089.
- Lippincott, J., and Li, R. (1998). Sequential assembly of myosin II, an IQGAP-like protein, and filamentous actin to a ring structure involved in budding yeast cytokinesis. *J Cell Biol* 140, 355-366.
- Lippincott, J., and Li, R. (2000). Involvement of PCH family proteins in cytokinesis and actin distribution. *Microsc Res Tech* 49, 168-172.
- Lodish**, H., Berk, A., Zipursky, S.L., Matsudaira, P., Baltimore, D. and **Darnell**, J.E. (2000). *Molecular cell Biology*. W.H. Freeman and Company.
- Ma, L., Rohatgi, R., and Kirschner, M. W. (1998). The Arp2/3 complex mediates actin polymerization induced by the small GTP-binding protein Cdc42. *Proc Natl Acad Sci U S A* 95, 15362-15367.
- Machesky, L. M., Atkinson, S. J., Ampe, C., Vandekerckhove, J., and **Pollard**, T. D. (1994). Purification of a cortical complex containing two unconventional actins from *Acanthamoeba* by affinity chromatography on profilin-agarose. *J Cell Biol* 127, 107-115.
- Machesky, L. M., and **Insall**, R. H. (1998). Scar1 and the related Wiskott-Aldrich syndrome protein, WASP, regulate the actin cytoskeleton through the Arp2/3 complex. *Curr Biol* 8, 1347-1356.
- Machesky, L. M., **Mullins**, R. D., Higgs, H. N., Kaiser, D. A., Blanchoin, L., May, R. C., Hall, M. E., and **Pollard**, T. D. (1999). Scar, a WASp-related protein, activates

nucleation of actin filaments by the Arp2/3 complex. *Proc Natl Acad Sci U S A* 96, 3739-3744.

Madania, A., Dumoulin, P., Grava, S., Kitamoto, H., Scharer-Brodbeck, C., **Soulard, A.**, Moreau, V., and **Winsor, B. (1999)**. The *Saccharomyces cerevisiae* homologue of human Wiskott-Aldrich syndrome protein Las17p interacts with the Arp2/3 complex. *Mol Biol Cell* 10,3521-3538.

Martinez-Quiles, N., Rohatgi, R., Anton, I. M., Medina, M., Saville, S. P., **Miki, H.**, Yamaguchi, H., Takenawa, T., **Hartwig, J. H.**, Geha, R. S., and Ramesh, N. (2001). WIP regulates N-WASP-mediated actin polymerization and filopodium formation. *Nat Cell Biol* 3, 484-491.

Meng, L., **Rajmohan, R.**, Yu, S., and Thanabalu, T. (2007). Actin binding and proline rich motifs of CR16 play redundant role in growth of *vrp1Delta* cells. *Biochem Biophys Res Commun* 357,289-294.

Miki, H., Miura, K., and Takenawa, T. (1996). N-WASP, a novel actin-depolymerizing protein, regulates the cortical cytoskeletal rearrangement in a PIP2-dependent manner downstream of tyrosine kinases. *Embo J* 15,5326-5335.

Miki, H., Suetsugu, S., and Takenawa, T. (1998). WAVE, a novel WASP-family protein involved in actin reorganization induced by Rac. *Embo J* 17,6932-6941.

Millard, T. H., Sharp, S. J., and **Machesky, L. M. (2004)**. Signalling to actin assembly via the WASP (Wiskott-Aldrich syndrome protein)-family proteins and the Arp2/3 complex. *Biochem J* 380, 1-17.

Mooseker, M. (1993). Myosin superfamily: a multitude of myosins. *Curr Biol* 3, 245-248.

Mooseker, M. S., and Cheney, R. E. (1995). Unconventional myosins. *Annu Rev Cell Dev Biol* 11,633-675.

Moseley, J. B., and **Goode, B. L. (2006)**. The yeast actin cytoskeleton: from cellular function to biochemical mechanism. *Microbiol Mol Biol Rev* 70, 605-645.

Moulding, D. A., Blundell, M. P., Spiller, D. G., White, M. R., Cory, G. O., Calle, Y., **Kempski, H.**, Sinclair, J., **Ancliff, P. J.**, Kinnon, C., *et al.* (2007). Unregulated actin polymerization by WASp causes defects of mitosis and cytokinesis in X-linked neutropenia. *J Exp Med* 204, 2213-2224.

- Mulholland, J., Wesp, A., Riezman, H., and Botstein, D. (1997). Yeast **actin** cytoskeleton mutants accumulate a new class of Golgi-derived secretory vesicle. *Mol Biol Cell* 8, 1481-1499.
- Mullins, R. D., Heuser, J. A., and Pollard, T. D.** (1998). The interaction of Arp2/3 complex with **actin**: nucleation, high affinity pointed end capping, and formation of branching networks of filaments. *Proc Natl Acad Sci U S A* 95, 6181-6186.
- Munn, A. L., Stevenson, B. J., Geli, M. I., and Riezman, H. (1995). **end5**, **end6**, and **end7**: mutations that cause **actin** delocalization and block the internalization step of endocytosis in *Saccharomyces cerevisiae*. *Mol Biol Cell* 6, 1721-1742.
- Naqvi, N. I., Eng, K., Gould, K. L., and Balasubramanian, M. K. (1999). Evidence for F-actin-dependent and -independent mechanisms involved in assembly and stability of the medial actomyosin ring in fission yeast. *Embo J* 18, 854-862.
- Naqvi, S. N., Zahn, R., Mitchell, D. A., Stevenson, B. J., and Munn, A. L. (1998). The WASp homologue Las17p functions with the WIP homologue End5p/verprolin and is essential for endocytosis in yeast. *Curr Biol* 8, 959-962.
- Naqvi, S. N., Feng, Q., Boulton, V. J., Zahn, R., and Munn, A. L. (2001). Vrp1p functions in both actomyosin ring-dependent and Hof1p-dependent pathways of cytokinesis. *Traffic* 2, 189-201.
- Newpher, T. M., and Lemmon, S. K. (2006). Clathrin is important for normal **actin** dynamics and progression of Sla2p-containing patches during endocytosis in yeast. *Traffic* 7, 574-588.
- Ochs, H. D., Slichter, S. J., Harker, L. A., Von Behrens, W. E., Clark, R. A., and Wedgwood, R. J. (1980). The Wiskott-Aldrich syndrome: studies of lymphocytes, granulocytes, and platelets. *Blood* 55, 243-252.
- Ochs, H. D., and Thrasher, A. J. (2006). The Wiskott-Aldrich syndrome. *J Allergy Clin Immunol* 117, 725-738; quiz 739.
- Okreglak, V., and **Drubin, D. G.** (2007). Cofilin recruitment and function during **actin**-mediated endocytosis dictated by **actin** nucleotide state. *J Cell Biol* 178, 1251-1264.
- Pantaloni, D., Le Clainche, C., and Carlier, M. F. (2001). Mechanism of **actin**-based motility. *Science* 292, 1502-1506.

- Paustian, T. and Roberts, G. (2007). The Microbial World: They rule. University of Wisconsin-Madison.
- Pollard, T. D. (1986). Assembly and dynamics of the actin filament system in nonmuscle cells. J Cell Biochem 31, 87-95.
- Pollard, T. D. (2002). Formins initiate new actin filaments. Nat Cell Biol 4, E191.
- Pollard, T. D. (2007). Regulation of actin filament assembly by Arp2/3 complex and formins. Annu Rev Biophys Biomol Struct 36, 451-477.
- Powers, S., Kataoka, T., Fasano, O., Goldfarb, M., Strathern, J., Broach, J., and Wigler, M. (1984). Genes in *S. cerevisiae* encoding proteins with domains homologous to the mammalian ras proteins. Cell 36, 607-612.
- Pruyne, D. W., Schott, D. H., and Bretscher, A. (1998). Tropomyosin-containing actin cables direct the Myo2p-dependent polarized delivery of secretory vesicles in budding yeast. J Cell Biol 143, 1931-1945.
- Pruyne, D., and Bretscher, A. (2000). Polarization of cell growth in yeast. J Cell Sci 113 (Pt 4), 571-585.
- Pruyne, D., Evangelista, M., Yang, C., Bi, E., Zigmond, S., Bretscher, A., and Boone, C. (2002). Role of formins in actin assembly: nucleation and barbed-end association. Science 297, 612-615.
- Purich, D. L., and Southwick, F. S. (1997). ABM-1 and ABM-2 homology sequences: consensus docking sites for actin-based motility defined by oligoproline regions in *Listeria* ActA surface protein and human VASP. Biochem Biophys Res Commun 231, 686-691.
- Qualmann, B., Kessels, M. M., and Kelly, R. B. (2000). Molecular links between endocytosis and the actin cytoskeleton. J Cell Biol 150, F111-116.
- Rajmohan, R., Meng, L., Yu, S., and Thanabalu, T. (2006). WASP suppresses the growth defect of *Saccharomyces cerevisiae* *las17Delta* strain in the presence of WIP. Biochem Biophys Res Commun 342, 529-536.
- Ramesh, N., Anton, I. M., Hartwig, J. H., and Geha, R. S. (1997). WIP, a protein associated with wiskott-aldrich syndrome protein, induces actin polymerization and redistribution in lymphoid cells. Proc Natl Acad Sci U S A 94, 14671-14676.

- Reinhard, M., Giehl, K., Abel, K., Haffner, C., Jarchau, T., Hoppe, V., Jockusch, B. M., and Walter, U. **(1995)**. The proline-rich focal adhesion and microfilament protein VASP is a ligand for profilins. *Embo J* 14, 1583-1589.
- Ren, G., Wang, J., Brinkworth, R., Winsor, B., Kobe, B., and Munn, A. L. **(2005)**. Verprolin cytokinesis function mediated by the Hof one trap domain. *Traffic* 6, 575-593.
- Riezman, H. **(1985)**. Endocytosis in yeast: several of the yeast secretory mutants are defective in endocytosis. *Cell* 40, 1001-1009.
- Riezman, H., Munn, A., Geli, M. I., and Hicke, L. **(1996)**. Actin-, myosin- and ubiquitin-dependent endocytosis. *Experientia* 52, 1033-1041.
- Robinson, R. C., Turbedsky, K., Kaiser, D. A., Marchand, J. B., Higgs, H. N., Choe, S., and Pollard, T. D. **(2001)**. Crystal structure of Arp2/3 complex. *Science* 294, 1679-1684.
- Rodal, A. A., Manning, A. L., Goode, B. L., and Drubin, D. G. **(2003)**. Negative regulation of yeast WASp by two SH3 domain-containing proteins. *Curr Biol* 13, 1000-1008.
- Rodal, A. A., Sokolova, O., Robins, D. B., Daugherty, K. M., Hippenmeyer, S., Riezman, H., Grigorieff, N., and Goode, B. L. **(2005)**. Conformational changes in the Arp2/3 complex leading to actin nucleation. *Nat Struct Mol Biol* 12, 26-31.
- Rohatgi, R., Ma, L., Miki, H., Lopez, M., Kirchhausen, T., Takenawa, T., and Kirschner, M. W. **(1999)**. The interaction between N-WASP and the Arp2/3 complex links Cdc42-dependent signals to actin assembly. *Cell* 97, 221-231.
- Rohatgi, R., Nollau, P., Ho, H. Y., Kirschner, M. W., and Mayer, B. J. **(2001)**. Nck and phosphatidylinositol 4,5-bisphosphate synergistically activate actin polymerization through the N-WASP-Arp2/3 pathway. *J Biol Chem* 276, 26448-26452.
- Rosenfeld, S. S., and Renner, B. **(1994)**. The GPQ-rich segment of Dictyostelium myosin IB contains an actin binding site. *Biochemistry* 33, 2322-2328.
- Sagot, I., Klee, S. K., and Pellman, D. **(2002)**. Yeast formins regulate cell polarity by controlling the assembly of actin cables. *Nat Cell Biol* 4, 42-50.
- Sasahara, Y., Rachid, R., Byrne, M. J., de la Fuente, M. A., Abraham, R. T., Ramesh, N., and Geha, R. S. **(2002)**. Mechanism of recruitment of WASP to the

immunological synapse and of its activation following TCR ligation. *Mol Cell* 10, 1269-1281.

Sawa, M., and Takenawa, T. (2006). *Caenorhabditis elegans* WASP-interacting protein homologue WIP-1 is involved in morphogenesis through maintenance of WSP-1 protein levels. *Biochem Biophys Res Commun* 340,709-717.

Schmidt, A., Hannah, M. J., and Huttner, W. B. (1997). Synaptic-like microvesicles of neuroendocrine cells originate from a novel compartment that is continuous with the plasma membrane and devoid of transferrin receptor. *J Cell Biol* 137,445-458.

Schott, D. H., Collins, R. N., and Bretscher, A. (2002). Secretory vesicle transport velocity in living cells depends on the myosin-V lever arm length. *J Cell Biol* 156, 35-39.

Shcherbina, A., Miki, H., Kenney, D. M., Rosen, F. S., Takenawa, T., and **Remold-O'Donnell**, E. (2001). WASP and N-WASP in human platelets differ in sensitivity to protease calpain. *Blood* 98,2988-2991.

Simon, H. U., Mills, G. B., Hashimoto, S., and Siminovitch, K. A. (1992). Evidence for defective transmembrane signaling in B cells from patients with Wiskott-Aldrich syndrome. *J Clin Invest* 90, 1396-1405.

Soulard, A., Lechler, T., Spiridonov, V., Shevchenko, A., Shevchenko, A., Li, R., and **Winsor**, B. (2002). *Saccharomyces cerevisiae* Bzzlp is implicated with type I myosins in actin patch polarization and is able to recruit actin-polymerizing machinery in *vitro*. *Mol Cell Biol* 22, 7889-7906.

Stewart, D. M., **Tian**, L., and Nelson, D. L. (1999). Mutations that cause the Wiskott-Aldrich syndrome impair the interaction of Wiskott-Aldrich syndrome protein (WASP) with WASP interacting protein. *J Immunol* 162,5019-5024.

Suetsugu, S., Miki, H., and Takenawa, T. (1999). Identification of two human WAVE/SCAR homologues as general actin regulatory molecules which associate with the Arp2/3 complex. *Biochem Biophys Res Commun* 260,296-302.

Sun, Y., Martin, A. C., and **Drubin**, D. G. (2006). Endocytic internalization in budding yeast requires coordinated actin nucleation and myosin motor activity. *Dev Cell* 11, 33-46.

- Sun, Y., Carroll, S., Kaksonen, M., Toshima, J. Y., and **Drubin, D. G. (2007)**. PtdIns(4,5)P₂ turnover is required for multiple stages during clathrin- and actin-dependent endocytic internalization. *J Cell Biol* 177,355-367.
- Symons, M., **Derry, J. M.**, Karlak, B., Jiang, S., Lemahieu, V., **McCormick, F.**, Francke, U., and Abo, A. **(1996)**. Wiskott-Aldrich syndrome protein, a novel effector for the GTPase CDC42Hs, is implicated in actin polymerization. *Cell* 84, 723-734.
- Takenawa, T., and Miki, H. **(2001)**. WASP and WAVE family proteins: key molecules for rapid rearrangement of cortical actin filaments and cell movement. *J Cell Sci* 114, 1801-1809.
- Thanabalu, T., and Munn, A. L. **(2001)**. Functions of Vrp1p in cytokinesis and actin patches are distinct and neither requires a WH2/V domain. *Embo J* 20, 6979-6989.
- Thanabalu, T., Rajmohan, R., Meng, L., Ren, G., Vajjhala, P. R., and Munn, A. L. **(2007)**. Verprolin function in endocytosis and actin organization. Roles of the Las17p (yeast WASP)-binding domain and a novel C-terminal actin-binding domain. *Febs J* 274, 4103-4125.
- Toshima, J. Y., Toshima, J., Kaksonen, M., Martin, A. C., King, D. S., and **Drubin, D. G. (2006)**. Spatial dynamics of receptor-mediated endocytic trafficking in budding yeast revealed by using fluorescent alpha-factor derivatives. *Proc Natl Acad Sci U S A* 103, 5793-5798.
- Trifaro, J. M., and Vitale, M. L. **(1993)**. Cytoskeleton dynamics during neurotransmitter release. *Trends Neurosci* 16,466-472.
- Tsuboi, S. **(2007)**. Requirement for a complex of Wiskott-Aldrich syndrome protein (WASP) with WASP interacting protein in podosome formation in macrophages. *J Immunol* 178, 2987-2995.
- Uruno, T., Liu, J., Zhang, P., Fan, Y., Egile, C., Li, R., Mueller, S. C., and Zhan, X. **(2001)**. Activation of Arp2/3 complex-mediated actin polymerization by cortactin. *Nat Cell Biol* 3, 259-266.
- Vaduva, G., Martin, N. C., and Hopper, A. K. **(1997)**. Actin-binding verprolin is a polarity development protein required for the morphogenesis and function of the yeast actin cytoskeleton. *J Cell Biol* 139, 1821-1833.

- Vaduva, G., Martinez-Quiles, N., Anton, I. M., Martin, N. C., Geha, R. S., Hopper, A. K., and Ramesh, N. (1999). The human WASP-interacting protein, WIP, activates the cell polarity pathway in yeast. *J Biol Chem* 274, 17103-17108.
- Valdez-Taubas, J., and Pelham, H. R. (2003). Slow diffusion of proteins in the yeast plasma membrane allows polarity to be maintained by endocytic cycling. *Curr Biol* 13, 1636-1640.
- Vallen, E. A., Caviston, J., and Bi, E. (2000). Roles of Hoflp, Bnlp, Bnrlp, and myolp in cytokinesis in *Saccharomyces cerevisiae*. *Mol Biol Cell* 11, 593-611.
- Volkman, B. F., Prehoda, K. E., Scott, J. A., Peterson, F. C., and Lim, W. A. (2002). Structure of the N-WASP EVH1 domain-WIP complex: insight into the molecular basis of Wiskott-Aldrich Syndrome. *Cell* 111, 565-576.
- Waddle, J. A., Karpova, T. S., Waterston, R. H., and Cooper, J. A. (1996). Movement of cortical actin patches in yeast. *J Cell Biol* 132, 861-870.
- Watts, F. Z., Shiels, G., and Orr, E. (1987). The yeast MYO1 gene encoding a myosin-like protein required for cell division. *Embo J* 6, 3499-3505.
- Weaver, A. M., Karginov, A. V., Kinley, A. W., Weed, S. A., Li, Y., Parsons, J. T., and Cooper, J. A. (2001). Cortactin promotes and stabilizes Arp2/3-induced actin filament network formation. *Curr Biol* 11, 370-374.
- Weed, S. A., Karginov, A. V., Schafer, D. A., Weaver, A. M., Kinley, A. W., Cooper, J. A., and Parsons, J. T. (2000). Cortactin localization to sites of actin assembly in lamellipodia requires interactions with F-actin and the Arp2/3 complex. *J Cell Biol* 151, 29-40.
- Weiler, M. C., Smith, J. L., and Masters, J. N. (1996). CR16, a novel proline-rich protein expressed in rat brain neurons, binds to SH3 domains and is a MAP kinase substrate. *J Mol Neurosci* 7, 203-215.
- Welch, M. D., Iwamatsu, A., and Mitchison, T. J. (1997). Actin polymerization is induced by Arp2/3 protein complex at the surface of *Listeria monocytogenes*. *Nature* 385, 265-269.
- Welch, M. D., Rosenblatt, J., Skoble, J., Portnoy, D. A., and Mitchison, T. J. (1998). Interaction of human Arp2/3 complex and the *Listeria monocytogenes* ActA protein in actin filament nucleation. *Science* 281, 105-108.

- Welch, M. D., and **Mullins**, R. D. (2002). Cellular control of actin nucleation. *Annu Rev Cell Dev Biol* 18, 247-288.
- Williams, B. D., and Waterston, R. H. (1994). Genes critical for muscle development and function in *Caenorhabditis elegans* identified through lethal mutations. *J Cell Biol* 124,475-490.
- Winter, D., **Podtelejnikov**, A. V., Mann, M., and Li, R. (1997). The complex containing actin-related proteins Arp2 and Arp3 is required for the motility and integrity of yeast actin patches. *Curr Biol* 7, 519-529.
- Winter, D. C., Choe, E. Y., and Li, R. (1999). Genetic dissection of the budding yeast Arp2/3 complex: a comparison of the *in vivo* and structural roles of individual subunits. *Proc Natl Acad Sci U S A* 96,7288-7293.
- Wu, C., **Lytvyn**, V., Thomas, D. Y., and Leberer, E. (1997). The phosphorylation site for Ste20p-like protein kinases is essential for the function of myosin-I in yeast. *J Biol Chem* 272,30623-30626.
- Yamaguchi, H., **Miki**, H., Suetsugu, S., Ma, L., Kirschner, M. W., and Takenawa, T. (2000). Two tandem verprolin homology domains are necessary for a strong activation of Arp2/3 complex-induced actin polymerization and induction of microspike formation by N-WASP. *Proc Natl Acad Sci U S A* 97, 12631-12636.
- Yarar, D., To, W., Abo, A., and Welch, M. D. (1999). The Wiskott-Aldrich syndrome protein directs actin-based motility by stimulating actin nucleation with the Arp2/3 complex. *Curr Biol* 9, 555-558.
- Yin, H., Pruyne, D., Huffaker, T. C., and Bretscher, A. (2000). Myosin V orientates the mitotic spindle in yeast. *Nature* 406, 1013-1015.
- Zhang, J., Shehabeldin, A., da Cruz, L. A., Butler, J., Somani, A. K., **McGavin**, M., Kozieradzki, I., dos Santos, A. O., **Nagy**, A., Grinstein, S., *et al.* (1999). Antigen receptor-induced activation and cytoskeletal rearrangement are impaired in Wiskott-Aldrich syndrome protein-deficient lymphocytes. *J Exp Med* 190, 1329-1342.

Appendix 1: Plasmids used in this study

Plasmid	Description	Reference
YCplac22	TRP1 CEN4 ARS plasmid	Gietz et. al., 1988
YCplac33	URA3 CEN4 ARS plasmid	Gietz et. al., 1988
YCplac111	LEU2 CEN4 ARS plasmid	Gietz et. al., 1988
YEplac112	TRP1 2 μ m ORI plasmid	Gietz et. al., 1988
YEplac195	URA3 2 μ m ORI plasmid	Gietz et. al., 1988
YEplac181	LEU2 2 μ m ORI plasmid	Gietz et. al., 1988
pACT2	LEU2 vector with Gal4p DNA activation domain	Clontech
pAS2-1	TRP1 vector with Gal4p DNA activation domain	Clontech
pWASP	YEplac112 with WASP	This study
pWASP-CAAX	YEplac112 with WASP-CAAX	This study
pWASP ^{L270P} -CAAX	YEplac112 with WASP ^{L270P} -CAAX	This study
pWASP ^{AAA}	YEplac112 with WASP (targeted mutation from DEW to AAA)	This study
pWASP Δ WH1	YEplac112 with WASP (residues 139-502)	This study
pWASP Δ WH1-CAAX	YEplac112 with WASP (residues 139-502) fused with CAAX	This study
pWASP Δ VCA	YEplac112 with WASP (residues 1-424)	This study
pWASP ^{M6I}	YEplac112 with WASP (targeted mutation M6I)	This study
pWASP ^{L27F}	YEplac112 with WASP (targeted mutation L27F)	This study
pWASP ^{E31K}	YEplac112 with WASP (targeted mutation E31K)	This study
pWASP ^{L35H}	YEplac112 with WASP (targeted mutation L35H)	This study
pWASP ^{L39P}	YEplac112 with WASP (targeted mutation L39P)	This study
pWASP ^{G40V}	YEplac112 with WASP (targeted mutation G40V)	This study
pWASP ^{R41G}	YEplac112 with WASP (targeted mutation R41G)	This study
pWASP ^{C43W}	YEplac112 with WASP (targeted mutation C43W)	This study
pWASP ^{T45M}	YEplac112 with WASP (targeted mutation T45M)	This study
pWASP ^{L46P}	YEplac112 with WASP (targeted mutation L46P)	This study
pWASP ^{A47D}	YEplac112 with WASP (targeted mutation A47D)	This study
pWASP ^{T48I}	YEplac112 with WASP (targeted mutation T48I)	This study
pWASP ^{Q52H}	YEplac112 with WASP (targeted mutation Q52H)	This study
pWASP ^{A56V}	YEplac112 with WASP (targeted mutation A56V)	This study
pWASP ^{P58L}	YEplac112 with WASP (targeted mutation P58L)	This study
pWASP ^{W64R}	YEplac112 with WASP (targeted mutation W64R)	This study
pWASP ^{G70W}	YEplac112 with WASP (targeted mutation G70W)	This study
pWASP ^{C73Y}	YEplac112 with WASP (targeted mutation C73Y)	This study
pWASP ^{V75M}	YEplac112 with WASP (targeted mutation V75M)	This study
pWASP ^{Q77G}	YEplac112 with WASP (targeted mutation Q77G)	This study
pWASP ^{S82P}	YEplac112 with WASP (targeted mutation S82P)	This study
pWASP ^{Y83C}	YEplac112 with WASP (targeted mutation Y83C)	This study

pWASP ^{F84L}	YEplac112 with WASP (targeted mutation F84L)	This study
pWASP ^{I85T}	YEplac112 with WASP (targeted mutation I85T)	This study
pWASP ^{R86H}	YEplac112 with WASP (targeted mutation R86H)	This study
pWASP ^{G89D}	YEplac112 with WASP (targeted mutation G89D)	This study
pWASP ^{W97C}	YEplac112 with WASP (targeted mutation W97C)	This study
pWASP ^{Q99R}	YEplac112 with WASP (targeted mutation Q99R)	This study
pWASP ^{L105P}	YEplac112 with WASP (targeted mutation L105P)	This study
pWASP ^{Y107C}	YEplac112 with WASP (targeted mutation Y107C)	This study
pWASP ^{T111P}	YEplac112 with WASP (targeted mutation T111P)	This study
pWASP ^{H115Y}	YEplac112 with WASP (targeted mutation H115Y)	This study
pWASP ^{G119E}	YEplac112 with WASP (targeted mutation G119E)	This study
pWASP ^{A124E}	YEplac112 with WASP (targeted mutation A124E)	This study
pWASP ^{G125R}	YEplac112 with WASP (targeted mutation G125R)	This study
pWASP ^{F128L}	YEplac112 with WASP (targeted mutation F128L)	This study
pWASP ^{E131K}	YEplac112 with WASP (targeted mutation E131K)	This study
pWASP ^{E133K}	YEplac112 with WASP (targeted mutation E133K)	This study
pWASP ^{A134T}	YEplac112 with WASP (targeted mutation A134T)	This study
pWASP ^{R138P}	YEplac112 with WASP (targeted mutation R138P)	This study
pWASP ^{G187C}	YEplac112 with WASP (targeted mutation G187C)	This study
pWASP ^{A236E}	YEplac112 with WASP (targeted mutation A236E)	This study
pWASP ^{L270P}	YEplac112 with WASP (targeted mutation L270P)	This study
pWASP ^{I294T}	YEplac112 with WASP (targeted mutation I294T)	This study
pWASP ^{M307V}	YEplac112 with WASP (targeted mutation M307V)	This study
pWASP ^{P359T}	YEplac112 with WASP (targeted mutation P359T)	This study
pWASP ^{P373S}	YEplac112 with WASP (targeted mutation P373S)	This study
pWASP ^{P459S}	YEplac112 with WASP (targeted mutation P459S)	This study
pWASP ^{P476E}	YEplac112 with WASP (targeted mutation P476E)	This study
pWASP ^{R477K}	YEplac112 with WASP (targeted mutation R477K)	This study
pWASP ^{I481N}	YEplac112 with WASP (targeted mutation I481N)	This study
pWASP ^{D485N}	YEplac112 with WASP (targeted mutation D485N)	This study
pWIP	YCplac111 with full length WIP	This study
pWIPAV	YCplac111 with WIP (residues 51-503)	This study
pWIPΔWBD	YCplac111 with WIP (residues 1-403)	This study
pWIP-GST	YCplac111 with full length WIP-GST	This study
pLG1	YCplac111 with GFP	This study
pTG1	YCplac22 with GFP	This study
pUG1	YCplac33 with GFP	This study
pLG1-CAAX	YCplac111 with GFP-CAAX	This study
pLG2	YCplac111 with GST	This study
pArc40p-RFP	YCplac33 encoding HIS3 and Arc40p-RFP	This study

pVrplp-GFP	YCplac22 with Vrplp-GFP	This study
p-Las17p	YCplac33 with full length Las17p	This study
p-Las17p ^{TL}	YCplac33 with full length Las17p ^{TL}	This study
pLas17p ₁₋₅₂₇	YCplac33 with Las17p (residues 1-527)	This study
pLas17p ₁₋₅₂₇ ^{TL}	YCplac33 with Las17p ^{TL} (residues 1-527)	This study
pC-Las17p ₃₇₀₋₆₃₃	YCplac33 with Las17p (residues 370-633)	This study
pN-Las17p ₁₋₃₆₈	YCplac33 with Las17p (residues 1-368)	This study
pN-Las17p ₁₋₃₆₈ ^T	YCplac33 with Las17p ^T (residues 1-368)	This study
pN-Las17p ₁₋₃₆₈ ^L	YCplac33 with Las17p ^L (residues 1-368)	This study
pN-Las17p ₁₋₃₆₈ ^{TL}	YCplac33 with Las17p ^{TL} (residues 1-368)	This study
pHC-N-Las17p ₁₋₃₆₈ ^{TL}	YEplac195 with N-Las17p ₁₋₃₆₈ ^{TL}	This study
pN-Las17p ₁₋₃₅₀	YCplac33 with Las17p (residues 1-350)	This study
pN-Las17p ₁₋₃₃₉	YCplac33 with Las17p (residues 1-339)	This study
pN-Las17p ₁₋₃₂₂	YCplac33 with Las17p (residues 1-322)	This study
pN-Las17p ₁₋₂₉₄	YCplac33 with Las17p (residues 1-294)	This study
pN-Las17p ₁₋₂₀₆	YCplac33 with Las17p (residues 1-206)	This study
pN-Las17p ₁₋₂₀₆ ^{TL}	YCplac33 with Las17p ^{TL} (residues 1-206)	This study
pN-Las17p ₂₀₇₋₃₆₈	YCplac33 with Las17p (residues 207-368)	This study
pN-Las17p-VCA	YCplac33 with Las17p (residues 1-368 fused with residues 528-633)	This study
pN-Las17p ^{TL} -VCA	YCplac33 with Las17p ^{TL} (residues 1-368 fused with residues 528-633)	This study
pC-Vrplp-HisT	YCplac111 with Vrplp (residues 364-817) fused with His tag	This study
pTMyo5p(SH3)-HisT	YCplac22 with Myo5p (residues 1085-1219) fused with His tag	This study
pWASP-GFP	YEplac112 with WASP-GFP	This study
pWASP-GFP-CAAX	YEplac112 with WASP-GFP-CAAX	This study
pWASP ^{AAA} -GFP	YEplac112 with WASP ^{AAA} -GFP	This study
pWASP Δ WH1-GFP	YEplac112 with WASP Δ WH1-GFP	This study
	Y	This study
pWASP ^{M6I} -GFP	YEplac112 with WASP ^{M6I} -GFP	This study
		This study
pWASP ^{E31K} -GFP	YEplac112 with WASP ^{E31K} -GFP	This study
pWASP ^{L35H} -GFP	YEplac112 with WASP ^{L35H} -GFP	This study
pWASP ^{L39P} -GFP	YEplac112 with WASP ^{L39P} -GFP	This study
pWASP ^{G40V} -GFP	YEplac112 with WASP ^{G40V} -GFP	This study
pWASP ^{R41G} -GFP	YEplac112 with WASP ^{R41G} -GFP	This study
pWASP ^{C43W} -GFP	YEplac112 with WASP ^{C43W} -GFP	This study
pWASP ^{T45M} -GFP	YEplac112 with WASP ^{T45M} -GFP	This study
pWASP ^{L46P} -GFP	YEplac112 with WASP ^{L46P} -GFP	This study
pWASP ^{A47D} -GFP	YEplac112 with WASP ^{A47D} -GFP	This study

pWASP ^{T48I} -GFP	YEplac112 with WASP ^{T48I} -GFP	This study
pWASP ^{Q52H} -GFP	YEplac112 with WASP ^{Q52H} -GFP	This study
pWASP ^{A56V} -GFP	YEplac112 with WASP ^{A56V} -GFP	This study
pWASP ^{P58L} -GFP	YEplac112 with WASP ^{P58L} -GFP	This study
pWASP ^{W64R} -GFP	YEplac112 with WASP ^{W64R} -GFP	This study
pWASP ^{G70W} -GFP	YEplac112 with WASP ^{G70W} -GFP	This study
pWASP ^{C73Y} -GFP	YEplac112 with WASP ^{C73Y} -GFP	This study
pWASP ^{V75M} -GFP	YEplac112 with WASP ^{V75M} -GFP	This study
pWASP ^{Q77G} -GFP	YEplac112 with WASP ^{Q77G} -GFP	This study
pWASP ^{S82P} -GFP	YEplac112 with WASP ^{S82P} -GFP	This study
pWASP ^{Y83C} -GFP	YEplac112 with WASP ^{Y83C} -GFP	This study
pWASP ^{F84L} -GFP	YEplac112 with WASP ^{F84L} -GFP	This study
pWASP ^{I85T} -GFP	YEplac112 with WASP ^{I85T} -GFP	This study
pWASP ^{R86H} -GFP	YEplac112 with WASP ^{R86H} -GFP	This study
pWASP ^{G89D} -GFP	YEplac112 with WASP ^{G89D} -GFP	This study
pWASP ^{W97C} -GFP	YEplac112 with WASP ^{W97C} -GFP	This study
pWASP ^{Q99R} -GFP	YEplac112 with WASP ^{Q99R} -GFP	This study
pWASP ^{L105P} -GFP	YEplac112 with WASP ^{L105P} -GFP	This study
pWASP ^{Y107C} -GFP	YEplac112 with WASP ^{Y107C} -GFP	This study
pWASP ^{T111P} -GFP	YEplac112 with WASP ^{T111P} -GFP	This study
pWASP ^{H115Y} -GFP	YEplac112 with WASP ^{H115Y} -GFP	This study
pWASP ^{G119E} -GFP	YEplac112 with WASP ^{G119E} -GFP	This study
pWASP ^{A124E} -GFP	YEplac112 with WASP ^{A124E} -GFP	This study
pWASP ^{G125R} -GFP	YEplac112 with WASP ^{G125R} -GFP	This study
pWASP ^{F128L} -GFP	YEplac112 with WASP ^{F128L} -GFP	This study
pWASP ^{E131K} -GFP	YEplac112 with WASP ^{E131K} -GFP	This study
pWASP ^{E133K} -GFP	YEplac112 with WASP ^{E133K} -GFP	This study
pWASP ^{A134T} -GFP	YEplac112 with WASP ^{A134T} -GFP	This study
pWASP ^{R138P} -GFP	YEplac112 with WASP ^{R138P} -GFP	This study
pWASP ^{G187C} -GFP	YEplac112 with WASP ^{G187C} -GFP	This study
pWASP ^{A236E} -GFP	YEplac112 with WASP ^{A236E} -GFP	This study
pWASP ^{L270P} -GFP	YEplac112 with WASP ^{L270P} -GFP	This study
pWASP ^{I294T} -GFP	YEplac112 with WASP ^{I294T} -GFP	This study
pWASP ^{M307V} -GFP	YEplac112 with WASP ^{M307V} -GFP	This study
pWASP ^{P359T} -GFP	YEplac112 with WASP ^{P359T} -GFP	This study
pWASP ^{P373S} -GFP	YEplac112 with WASP ^{P373S} -GFP	This study
pWASP ^{P459S} -GFP	YEplac112 with WASP ^{P459S} -GFP	This study
pWASP ^{P476E} -GFP	YEplac112 with WASP ^{P476E} -GFP	This study
pWASP ^{R477K} -GFP	YEplac112 with WASP ^{R477K} -GFP	This study
pWASP ^{I481N} -GFP	YEplac112 with WASP ^{I481N} -GFP	This study

pWASP ^{D485N} -GFP	YEplac112 with WASP ^{D485N} -GFP	This study
pNLS-WASP ^{W64R} -YFP	YEplac112 with NLS-WASP ^{W64R} -YFP	This study
pNLS-WASP ^{G70W} -YFP	YEplac112 with NLS-WASP ^{G70W} -YFP	This study
pNLS-WASP ^{T111P} -YFP	YEplac112 with NLS-WASP ^{T111P} -YFP	This study
pNLS-WASP ^{G125R} -YFP	YEplac112 with NLS-WASP ^{G125R} -YFP	This study
pNLS-WASP ^{F128L} -YFP	YEplac112 with NLS-WASP ^{F128L} -YFP	This study
pWIP-GFP	YCplac111 with WIP-GFP	This study
pWIPΔV-GFP	YCplac111 with WIPΔV-GFP	This study
pWIPΔWBD-GFP	YCplac111 with WIPΔWBD-GFP	This study
pLas17p-GFP	YCplac33 with Las17p-GFP	This study
pLas17p ^{TL} -GFP	YCplac33 with Las17p ^{TL} -GFP	This study
pLas17p ₁₋₅₂₇ -GFP	YCplac33 with Las17p ₁₋₅₂₇ -GFP	This study
pLas17p ₁₋₅₂₇ ^{TL} -GFP	YCplac33 with Las17p ₁₋₅₂₇ ^{TL} -GFP	This study
pC-Las17p ₃₇₀₋₆₃₃ -GFP	YCplac33 with C-Las17p ₃₇₀₋₆₃₃ -GFP	This study
pN-Las17p ₁₋₃₆₈ -GFP	YCplac33 with N-Las17p ₁₋₃₆₈ -GFP	This study
pN-Las17p ₁₋₃₆₈ ^T -GFP	YCplac33 with N-Las17p ₁₋₃₆₈ ^T -GFP	This study
pN-Las17p ₁₋₃₆₈ ^L -GFP	YCplac33 with N-Las17p ₁₋₃₆₈ ^L -GFP	This study
pN-Las17p ₁₋₃₆₈ ^{TL} -GFP	YCplac33 with N-Las17p ₁₋₃₆₈ ^{TL} -GFP	This study
pHC-N-Las17p ₁₋₃₆₈ ^{TL} -GFP	YEplac195 with N-Las17p ₁₋₃₆₈ ^{TL} -GFP	This study
pN-Las17p ₁₋₃₅₀ -GFP	YCplac33 with N-Las17p ₁₋₃₅₀ -GFP	This study
pN-Las17p ₁₋₃₃₉ -GFP	YCplac33 with N-Las17p ₁₋₃₃₉ -GFP	This study
pN-Las1	YCplac33 with N-Las17p ₁₋₃₂₂ -GFP	This study
pN-Las1	YCplac33 with N-Las17p ₁₋₂₉₄ -GFP	This study
pN-Las17p ₁₋₂₀₆ -GFP	YCplac33 with N-Las17p ₁₋₂₀₆ -GFP	This study
pN-Las17p ₁₋₂₀₆ ^{TL} -GFP	YCplac33 with N-Las17p ₁₋₂₀₆ ^{TL} -GFP	This study
pN-Las17p ₂₀₇₋₃₆₈ -GFP	YCplac33 with N-Las17p ₂₀₇₋₃₆₈ -GFP	This study
pN-Las17p-VCA-GFP	YCplac33 with N-Las17p-VCA-GFP	This study
pN-Las17p ^{TL} -VCA-GFP	YCplac33 with N-Las17p ^{TL} -VCA-GFP	This study
pA-WASP	pACT2 with WASP	This study
pA-WASP ^{A134T}	pACT2 with WASP ^{A134T}	This study
pA-WASP ^{L270P}	pACT2 with WASP ^{L270P}	This study
pA-WASP ^{AAA}	pACT2 with WASP ^{AAA}	This study
pA-WASPΔWH1	pACT2 with WASPAWH1	This study
pA-WASPΔVCA	pACT2 with WASPAVCA	This study
pA-WIP	pACT2 with WIP	This study
pA-Myo3p(SH3)	pACT2 with Myo3p (residues 1118-1188)	This study
pA-Myo5p(SH3)	pACT2 with Myo5p (residues 1085-1219)	This study
pA-Hof1p(SH3)	pACT2 with Hof1p (residues 551-669)	This study
pA-Bzz1p(SH3)	pACT2 with Bzz1p (residues 494-663)	This study
pA-Rvs167p(SH3)	pACT2 with Rvs167p (residues 423-482)	This study

pA-Vrp1p ₃₆₄₋₈₁₇	pACT2 with Vrp1p (residues 364-817)	This study
pA-Las17p	pACT2 with Las17p	This study
pA-Las17p ^{TL}	pACT2 with Las17p ^{TL}	This study
pA-N-Las17p ₁₋₃₆₈	pACT2 with N-Las17p ₁₋₃₆₈	This study
pA-N-Las17p ₁₋₃₆₈ ^{TL}	pACT2 with N-Las17p ₁₋₃₆₈ ^{TL}	This study
pA-N-Las17p ₁₋₂₀₆	pACT2 with N-Las17p ₁₋₂₀₆	This study
pA-N-Las17p ₂₀₇₋₃₆₈	pACT2 with N-Las17p ₂₀₇₋₃₆₈	This study
pB-WASP	pAS2-1 with WASP	This study
pB-WASP ^{Δ134T}	pAS2-1 with	This study
pB-WASP ^{L270P}	pAS2-1 with WASP ^{L270P}	This study
pB-WASP ^{AAA}	pAS2-1 with WASP ^{AAA}	This study
pB-WASP ^{ΔWH1}	pAS2-1 with WASP ^{ΔWH1}	This study
pB-WASP ^{ΔVCA}	pAS2-1 with WASP ^{ΔVCA}	This study
pB-WIP	pAS2-1 with WIP	This study
pB-Myo3p(SH3)	pAS2-1 with (residues 1118-1188)	This study
pB-Myo5p(SH3)	pAS2-1 with (residues 1085-1219)	This study
pB-Hof1p(SH3)	pAS2-1 with (residues 551-669)	This study
pB-Bzz1p(SH3)	pAS2-1 with (residues 494-663)	This study
pB-Rvs167p(SH3)	pAS2-1 with (residues 423-482)	This study
pB-Act1p	pAS2-1 with Act1p	This study
pB-Vrp1p ₃₆₄₋₈₁₇	pAS2-1 with Vrp1p (residues 364-817)	This study
pB-Las17p	pAS2-1 with Las17p	This study
pB-Las17p ^{TL}	pAS2-1 with Las17p ^{TL}	This study
pB-N-Las17p ₁₋₃₆₈	pAS2-1 with N-Las17p ₁₋₃₆₈	This study
pB-N-Las17p ₁₋₃₆₈ ^{TL}	pAS2-1 with N-Las17p ₁₋₃₆₈ ^{TL}	This study
pB-N-Las17p ₁₋₂₃₄	pAS2-1 with N-Las17p ₁₋₂₃₄	This study
pB-N-Las17p ₁₋₂₀₆	pAS2-1 with N-Las17p ₁₋₂₀₆	This study
pB-N-Las17p ₂₀₇₋₃₆₈	pAS2-1 with N-Las17p ₂₀₇₋₃₆₈	This study

Appendix 2A: Primers used for WASP constructs

Primer	Sequence	Remarks
WAS-F	5' CAT GCC ATG GGA ATG AGT GGG GGC CCA ATG GGA G 3'	1-22 of WASP c-DNA
WAS-R	5' CGC GGA TCC GTC ATC CCA TTC ATC ATC TTC ATC TTC 3'	1480-1506 of WASP c-DNA
WASP_SEQ1	5' TGA AGA GAG AAG AGG AGG GC 3'	501-520 of WASP c-DNA
WAS dWH1-F	5' CAT GCC ATG GCC CTC GTG CAG GAG AAG ATA CA 3'	415-437 of WASP c-DNA
WAS-dVCA-R	5' CGC GGA TCC GCC CCC GGC AGG CAC CAG AG 3'	1253-1272 of WASP c-DNA
WASP-DDW-R	5' CGC GGA TCC GTC ATC CGC CGC CGC ATC TTC ATC TTC ATC 3'	1477-1506 of WASP c-DNA
M6I-F	5' CATG CC ATG GGA AGT GGG GGC CCA ATC GGA GGA AGG CCC GGG 3'	Forward primer -mutation Met 6 Phe
L27F-F	5' ATA CCC TCC ACC CTC TTC CAG GAC CAC GAG AAC 3'	Forward primer -mutation Leu 27 Phe
L27F-R	5' GTT CTC GTG GTC CTG GAA GAG GGT GGA GGG TAT 3'	Reverse primer -mutation Leu 27 Phe
E31K-F	5' CTC CTC CAG GAC CAC AAG AAC CAG CGA CTC TTT 3'	Forward primer -mutation Glu 31 Lys
E31K-R	5' AAA GAG TCG CTG GTT CTT GTG GTC CTG GAG GAG 3'	Reverse primer -mutation Glu 31 Lys
L35H-F	5' CAC GAG AAC CAG CGA CAC TTT GAG ATG CTT GGA 3'	Forward primer for • mutation Leu 35 His
L35H-R	5' TCC AAG CAT CTC AAA GTG TCG CTG GTT CTC GTG 3'	Reverse primer -mutation Leu 35 His
L39P-F	5' CGA CTC TTT GAG ATG CCT GGA CGA AAA TGC TTG 3'	Forward primer -mutation Leu 39 Pro
L39P-R	5' CAA GCA TTT TCG TCC AGG CAT CTC AAA GAG TCG 3'	Reverse primer -mutation Leu 39 Pro
G40V-F	5' CTC TTT GAG ATG CTT GTA CGA AAA TGC TTG ACG 3'	Forward primer -mutation Gly 40 Val
G40V-R	5' CGT CAA GCA TTT TCG TAC AAG CAT CTC AAA GAG 3'	Reverse primer -mutation Gly 40 Val
R41G-F	5' TTT GAG ATG CTT GGA GGA AAA TGC TTG ACG CTG 3'	Forward primer -mutation Arg 41 Gly
R41G-R	5' CAG CGT CAA GCA TTT TCC TCC AAG CAT CTC AAA 3'	Reverse primer -mutation Arg 41 Gly
C43W-F	5' ATG CTT GGA CGA AAA TGG TTG ACG CTG GCC ACT 3'	Forward primer -mutation Cys 43 Trp
C43W-R	5' AGT GGC CAG CGT CAA CCA TTT TCG TCC AAG CAT 3'	Reverse primer -mutation Cys 43 Trp
T45M-F	5' GGA CGA AAA TGC TTG ATG CTG GCC ACT GCA GTT 3'	Forward primer -mutation Thr 45 Met
T45M-R	5' AAC TGC AGT GGC CAG CAT CAA GCA TTT TCG TCC 3'	Reverse primer -mutation

		Thr 45 Met
L46P-F	5' CGA AAA TGC TTG ACG CCG GCC ACT GCA GTT GTT 3'	Forward primer -mutation Leu 46 Pro
L46P-R	5' AAC AAC TGC AGT GGC CGG CGT CAA GCA TTT TCG 3'	Reverse primer -mutation Leu 46 Pro
A47D-F	5' AAA TGC TTG ACG CTG GAC ACT GCA GTT GTT CAG 3'	Forward primer -mutation Ala 47 Asp
A47D-R	5' CTG AAC AAC TGC AGT GTC CAG CGT CAA GCA TTT 3'	Reverse primer -mutation Ala 47 Asp
T48I-F	5' TGC TTG ACG CTG GCC ATT GCA GTT GTT CAG CTG 3'	Forward primer -mutation Thr 48 Ile
T48I-R	5' CAG CTG AAC AAC TGC AAT GGC CAG CGT CAA GCA 3'	Reverse primer -mutation Thr 48 Ile
Q52H-F	5' GCC ACT GCA GTT GTT CAC CTG TAC CTG GCG CTG 3'	Forward primer -mutation Gln 52 His
Q52H-R	5' CAG CGC CAG GTA CAG GTG AAC AAC TGC AGT GGC 3'	Reverse primer -mutation Gln 52 His
A56V-F	5' GTT CAG CTG TAC CTG GTG CTG CCC CCT GGA GCT 3'	Forward primer -mutation Ala 56 Val
A56V-R	5' AGC TCC AGG GGG CAG CAC CAG GTA CAG CTG AAC 3'	Reverse primer -mutation Ala 56 Val
P58L-F	5' CTG TAC CTG GCG CTG CTC CCT GGA GCT GAG CAC 3'	Forward primer -mutation Pro 58 Leu
P58L-R	5' GTG CTC AGC TCC AGG GAG CAG CGC CAG GTA CAG 3'	Reverse primer -mutation Pro 58 Leu
W64R-F	5' CCT GGA GCT GAG CAC AGG ACC AAG GAG CAT TGT 3'	Forward primer -mutation Trp 64 Arg
W64R-R	5' ACA ATG CTC CTT GGT CCT GTG CTC AGC TCC AGG 3'	Reverse primer -mutation Trp 64 Arg
G70L-F	5' ACC AAG GAG CAT TGT TGG GCT GTG TGC TTC GTG 3'	Forward primer -mutation Gly 70 Leu
G70L-R	5' CAC GAA GCA CAC AGC CCA ACA ATG CTC CTT GGT 3'	Reverse primer -mutation Gly 70 Leu
C73Y-F	5' CAT TGT GGG GCT GTG TAC TTC GTG AAG GAT AAC 3'	Forward primer -mutation Cys 73 Tyr
C73Y-R	5' ATT ATC CTT CAC GAA GTA CAC AGC CCC ACA ATG 3'	Reverse primer -mutation Cys 73 Tyr
V75M-F	5' GGG GCT GTG TGC TTC ATG AAG GAT AAC CCC CAG 3'	Forward primer -mutation Val 75 Met
V75M-R	5' CTG GGG GTT ATC CTT CAT GAA GCA CAC AGC CCC 3'	Reverse primer -mutation Val 75 Met
D77G-F	5' GTG TGC TTC GTG AAG GGT AAC CCC CAG AAG TCC 3'	Forward primer -mutation Asp 77 Gly
D77G-R	5' GGA CTT CTG GGG GTT ACC CTT CAC GAA GCA CAC 3'	Reverse primer -mutation Asp 77 Gly
S82P-F	5' GAT AAC CCC CAG AAG CCC TAC TTC ATC CGC CTT 3'	Forward primer -mutation

		Ser 82 Pro
S82P-R	5' AAG GCG GAT GAA GTA GGG CTT CTG GGG GTT ATC 3'	Reverse primer -mutation Ser 82 Pro
Y83C-F	5' AAC CCC CAG AAG TCC TGC TTC ATC CGC CTT TAC 3'	Forward primer -mutation Tyr 83 Cys
Y83C-R	5' GTA AAG GCG GAT GAA GCA GGA CTT CTG GGG GTT 3'	Reverse primer -mutation Tyr 83 Cys
F84L-F	5' CCC CAG AAG TCC TAC CTC ATC CGC CTT TAC GGC 3'	Forward primer -mutation Phe 84 Leu
F84L-R	5' GCC GTA AAG GCG GAT GAG GTA GGA CTT CTG GGG 3'	Reverse primer -mutation Phe 84 Leu
I85T-F	5' CAG AAG TCC TAC TTC ACC CGC CTT TAC GGC CTT 3'	Forward primer -mutation Ile 85 Thr
I85T-R	5' AAG GCC GTA AAG GCG GGT GAA GTA GGA CTT CTG 3'	Reverse primer -mutation Ile 85 Thr
R86H-F	5' AAG TCC TAC TTC ATC CAC CTT TAC GGC CTT CAG 3'	Forward primer -mutation Arg 86 His
R86H-R	5' CTG AAG GCC GTA AAG GTG GAT GAA GTA GGA CTT 3'	Reverse primer -mutation Arg 86 His
G89D-F	5' TTC ATC CGC CTT TAC GAC CTT CAG GCT GGT CGG 3'	Forward primer -mutation Gly 89 Asp
G89D-R	5' CCG ACC AGC CTG AAG GTC GTA AAG GCG GAT GAA 3'	Reverse primer -mutation Gly 89 Asp
W97C-F	5' GCT GGT CGG CTG CTC TGC GAA CAG GAG CTG TAC 3'	Forward primer -mutation Trp 97 Cys
W97C-R	5' GTA CAG CTC CTG TTC GCA GAG CAG CCG ACC AGC 3'	Reverse primer -mutation Trp 97 Cys
Q99R-F	5' CGG CTG CTC TGG GAA CGG GAG CTG TAC TCA CAG 3'	Forward primer -mutation Gln 99 Arg
Q99R-R	5' CTG TGA GTA CAG CTC CCG TTC CCA GAG CAG CCG 3'	Reverse primer -mutation Gln 99 Arg
L105P-F	5' GAG CTG TAC TCA CAG CCT GTC TAC TCC ACC CCC 3'	Forward primer -mutation Leu 105 Pro
L105P-R	5' GGG GGT GGA GTA GAC AGG CTG TGA GTA CAG CTC 3'	Reverse primer -mutation Leu 105 Pro
Y107C-F	5' TAC TCA CAG CTT GTC TGC TCC ACC CCC ACC CCC 3'	Forward primer -mutation Leu 105 Pro
Y107C-R	5' GGG GGT GGG GGT GGA GCA GAC AAG CTG TGA GTA 3'	Reverse primer -mutation Leu 105 Pro
T111P-F	5' GTC TAC TCC ACC CCC CCC CCC TTC TTC CAC ACC 3'	Forward primer -mutation Thr 111 Pro
T111P-R	5' GGT GTG GAA GAA GGG GGG GGG GGT GGA GTA GAC 3'	Reverse primer -mutation Thr 111 Pro
H115Y-F	5' CCC ACC CCC TTC TTC TAC ACC TTC GCT GGA GAT 3'	Forward primer -mutation His 115 Tyr
H115Y-R	5' ATC TCC AGC GAA GGT GTA GAA GAA GGG GGT GGG 3'	Reverse primer -mutation

		His 115 Tyr
G119E-F	5' TTC CAC ACC TTC GCT GAA GAT GAC TGC CAA GCG 3'	Forward primer -mutation Gly 119 Glu
G119E-R	5' CGC TTG GCA GTC ATC TTC AGC GAA GGT GTG GAA 3'	Reverse primer -mutation Gly 119 Glu
A124E-F	5' GGA GAT GAC TGC CAA GAG GGG CTG AAC TTT GCA 3'	Forward primer -mutation Ala 124 Glu
A124E-R	5' TGC AAA GTT CAG CCC CTC TTG GCA GTC ATC TCC 3'	Reverse primer -mutation Ala 124 Glu
G125R-F	5' GAT GAC TGC CAA GCG CGG CTG AAC TTT GCA GAC 3'	Forward primer -mutation Gly 125 Arg
G125R-R	5' GTC TGC AAA GTT CAG CCG CGC TTG GCA GTC ATC 3'	Reverse primer -mutation Gly 125 Arg
F128L-F	5' CAA GCG GGG CTG AAC CTT GCA GAC GAG GAC GAG 3'	Forward primer -mutation Phe 128 Leu
F128L-R	5' CTC GTC CTC GTC TGC AAG GTT CAG CCC CGC TTG 3'	Reverse primer -mutation Phe 128 Leu
E131K-F	5' CTG AAC TTT GCA GAC AAG GAC GAG GCC CAG GCC 3'	Forward primer -mutation Glu 131 Lys
E131K-R	5' GGC CTG GGC CTC GTC CTT GTC TGC AAA GTT CAG 3'	Reverse primer -mutation Glu 131 Lys
E133K-F	5' TTT GCA GAC GAG GAC AAG GCC CAG GCC TTC CGG 3'	Forward primer -mutation Glu 133 Lys
E133K-R	5' CCG GAA GGC CTG GGC CTT GTC CTC GTC TGC AAA 3'	Reverse primer -mutation Glu 133 Lys
A134T-F	5' GCA GAC GAG GAC GAG ACC CAG GCC TTC CGG GCC 3'	Forward primer -mutation Ala 134 Thr
A134T-R	5' GGC CCG GAA GGC CTG GGT CTC GTC CTC GTC TGC 3'	Reverse primer -mutation Ala 134 Thr
R138P-F	5' GAG GCC CAG GCC TTC CCG GCC CTC GTG CAG GAG 3'	Forward primer -mutation Arg 138 Pro
R138P-R	5' CTC CTG CAC GAG GGC CGG GAA GGC CTG GGC CTC 3'	Reverse primer -mutation Arg 138 Pro
G187C-F	5' GGT GGA GAC CAA GGA TGC CCT CCA GTG GGT CCG 3'	Forward primer -mutation Gly 187 Cys
G187C-R	5' CGG ACC CAC TGG AGG GCA TCC TTG GTC TCC ACC 3'	Reverse primer -mutation Gly 187 Cys
A236E-F	5' AAG AAG ATC AGC AAA GAG GAT ATT GGT GCA CCC 3'	Forward primer -mutation Ala 236 Glu
A236E-R	5' GGG TGC ACC AAT ATC CTC TTT GCT GAT CTT CTT 3'	Reverse primer -mutation Ala 236 Glu
L270P-F	5' CCA GAT CTG CGG AGT CCG TTC TCC AGG GCA GGA	Forward primer -mutation Leu 270 Pro
L270P-R	5' TCC TGC CCT GGA GAA CGG ACT CCG CAG ATC TGG	Reverse primer -mutation Leu 270 Pro
I294T-F	5' CTT ATC TAC GAC TTC ACT GAG GAC CAG GGT GGG 3'	Forward primer -mutation

		Ile 294 Thr
I294T-R	5' CCC ACC CTG GTC CTC AGT GAA GTC GTA GAT AAG 3'	Reverse primer -mutation Ile 294 Thr
M307V-F	5' GCT GTG CGG CAG GAG GTG AGG CGC CAG GAG CCA 3'	Forward primer -mutation Met 307 Val
M307V-R	5' TGG CTC CTG GCG CCT CAC CTC CTG CCG CAC AGC 3'	Reverse primer -mutation Met 307 Val
P359T-F	5' CCA ACA CCC CGG GGA ACC CCA CCC CCA GGC CGA 3'	Forward primer -mutation Pro 359 Thr
P359T-R	5' TCG GCC TGG GGG TGG GGT TCC CCG GGG TGT TGG 3'	Reverse primer -mutation Pro 359 Thr
P373S-F	5' CCA CCA CCA CCC CCT TCA GCT ACT GGA CGT TCT 3'	Forward primer -mutation Pro 373 Ser
P373S-R	5' AGA ACG TCC AGT AGC TGA AGG GGG TGG TGG TGG 3'	Reverse primer -mutation Pro 373 Ser
P459S-F	5' TCA GCG CTG CAG CCA TCA CCT CAG AGC TCA GAG 3'	Forward primer -mutation Pro 459 Ser
P459S-R	5' CTC TGA GCT CTG AGG TGA TGG CTG CAG CGC TGA 3'	Reverse primer -mutation Pro 459 Ser
K476E-F	5' ATG CAC GTG ATG CAG GAG AGA AGC AGA GCC ATC 3'	Forward primer -mutation Lys 476 Glu
K476E-R	5' GAT GGC TCT GCT TCT CTC CTG CAT CAC GTG CAT 3'	Reverse primer -mutation Lys 476 Glu
R477K-F	5' CAC GTG ATG CAG AAG AAA AGC AGA GCC ATC CAC 3'	Forward primer -mutation Arg 477 Lys
R477K-R	5' GTG GAT GGC TCT GCT TTT CTT CTG CAT CAC GTG 3'	Reverse primer -mutation Arg 477 Lys
I481N-F	5' AAG AGA AGC AGA GCC AAC CAC TCC TCC GAC GAA 3'	Forward primer -mutation Ile 481 Asn
I481N-R	5' TTC GTC GGA GGA GTG GTT GGC TCT GCT TCT CTT 3'	Reverse primer -mutation Ile 481 Asn
D485N-F	5' GCC ATC CAC TCC TCC AAC GAA GGG GAG GAC CAG 3'	Forward primer -mutation Asp 485 Asn
D485N-R	5' CTG GTC CTC CCC TTC GTT GGA GGA GTG GAT GGC 3'	Reverse primer -mutation Asp 485 Asn

Appendix 2B: Primers used for Las17p constructs

LasPro-R	5' CTAG TCT AGA CAT ATT AAA TCA CGC CTA TCA ACA CTT AAA G 3'	Binds to 3' end of LAS17 promoter
LasNco-F	5' CAT GCC ATG GGA CTC CTA AAC TCT TCA GAT AAG 3'	1-27 of Las17p c-DNA
LasBam-R	5' CGC GGA TCC CCA ATC ATC ACC ATT GTC CAT ATC G 3'	1875-1899 of Las17p c-DNA
Las370-F	5' CTAG TCT AGA GCC ATG GCCGGTGGAAATAGCCTCCTT CC 3'	1108-1130 of Las17p c-DNA
Las80-F	5' ATC TGG GAC CAA GAA ACG TAT GTG AAT TTT GAA 3'	Forward primer -mutation Leu 80 Thr
Las80-R	5' TTC AAA ATT CAC ATA CGT TTC TTG GTC CCA GAT 3'	Reverse primer -mutation Leu 80 Thr
Las94-F	5' GAC CGT ACT TTT TTT CTT ACA TTT GAG ATG GAA 3'	Forward primer -mutation His 94 Leu
Las94-R	5' TTC CAT CTC AAA TGT AAG AAA AAA AGT ACG GTC 3'	Reverse primer -mutation His 94 Leu
Las207Nco-F	5' CATG CC ATG GGA AAT TCG ACA ACA GCA TCC GCA CCG 3'	619-642 of Las17p c-DNA
Las295Nco-F	5' CATG CC ATG GGA CAG CAG CAG TTT AAT CAA GCT CCT TC 3'	883-908 of Las17p c-DNA
Las206Bam-R	5' CGC GGA TCC GAT ATC GGA AAA TGA ACT TGT TTG GTC 3'	592-618 of Las17p c-DNA
Las294Bam-R	5' CGC GGA TCC AGG TAC CGG AAA TGG AAA TGG GTT TG 3'	856-882 of Las17p c-DNA
Las322-R	5' CGC GGA TCC CCG ATT ATT TCT GTT AGG CAA CTG TG 3'	940-966 of Las17p c-DNA
Las339-R	5' CGC GGA TCC AAC ACC TGA ACC TTC AGT AGT GGT AC 3'	991-1017 of Las17p c-DNA
Las350-R	5' CGC GGA TCC ACG CCT TGG CGG AGG TGG AGG 3'	1030-1050 of Las17p c-DNA
Las17VCA-F	5' CATG CC ATG GGA AGA TCT TTC CTA ACT CAA CAA CCT CAA TCT GG 3'	1525-1550 of Las17p c-DNA