FUNCTIONAL ANALYSIS OF MAMMALIAN HOMOLOGUES OF YEAST VRP1P USING S. cerevisiae

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Functional Analysis of Mammalian Homologues of
Yeast Vrp1p Using *S. cerevisiae*

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Abstract

Verprolin is an actin associated protein from yeast and it has three mammalian homologues, WIP, WIRE/WICH and CR16. *S. cerevisiae vrp1Δ* strain exhibits defects of growth at high temperature, endocytosis and actin patches polarization. Expression of WIP, WIRE or CR16 in *vrp1Δ* strain suppresses both the growth and endocytic defect. For WIP and WIRE, the interaction with actin is crucial for their activities while the actin binding motif of CR16 plays a redundant role with N-terminal proline rich region in suppressing the growth defect. A second actin binding motif of N-Vrp1p1-364 was identified and it is sufficient for actin patch polarization in the presence of Las17p binding domain. The actin binding motif plays a redundant role with the proline rich sequence of WH2/V domain to restore cell growth and actin patches polarization. The Vrp1p-Las17p interaction is crucial for Vrp1p364-817's localization and for complementation of cellular defects. Therefore, though all the verprolin family proteins have a homologous V domain and a WASP/Las17p binding domain, they may have different activities and mechanism in regulating the actin cytoskeleton in vivo.
It was in the context of changing population demographic landscape and decline of Sultanate in Moro territories that a power and identity vacuum existed. The space was filled through a reconnecting with the dormant Islamic consciousness that made possible through the return of Islamic scholars from the Middle East Islamic Universities. Chief among them was Ustadz Hashim Salamae an Al-Azhar alumnus. These scholars not only brought new political thinking but also the Middle East culture. Among the early signs of their influence were the replacement of traditional local Muslim dresses by a Middle East fashioned dresses. Customary rituals perceived deviant to Islam were prohibited. The Ustadz (religious scholar) slowly replaced the Pandita (Traditional Muslim preacher). Political and religious thinking began to echo contemporary Islamic thinkers in the Middle East, Hashim Salamat admitted drawing...
Summary

Verprolin is an actin associated protein from yeast and it has three mammalian homologues, WIP, WIRE/WICH and CR16. S. cerevisiae vrplΔ strain exhibits following defects: defect of growth at high temperature, endocytosis defect and actin patches polarization defect due to abnormal organization of actin cytoskeleton. WIP, WIRE and CR16 localize to actin patches in vrplΔ strain. Expression of WIP, WIRE or CR16 in vrplΔ strain suppresses both the growth defect and endocytic defect of this strain without correcting the actin patch polarization defect. The actin binding domain of WIRE and CR16, but not WIP, is crucial for the interaction with actin. The actin interaction of both WIP and WIRE, but not CR16, is critical for the suppression of growth and endocytosis defects of vrplΔ strain. The KLKK motif and N-terminal proline rich region of WIP play a redundant role in the interaction with actin and suppression of growth and endocytosis defects while the KLKK motif of WIRE is critical for both its activity and interaction with actin. Therefore, to WIP and WIRE, the actin interaction and activity in suppressing cellular defects of vrplΔ strain is co-related. The RLRK motif of CR16 is critical for the interaction with actin, but not critical for suppressing the growth defect. Actin binding motif RLRK of CR16 plays a redundant role with N-terminal proline rich region in suppressing the growth defect of vrplΔ strain.

Vrp1p1-364 can support growth of vrplΔ cells at high temperature while Vrp1p364-817 can support growth of vrplΔ cells at high temperature as well as actin patch
polarization. A second actin binding motif located in Vrp1p\textsubscript{1-364} was identified and either one of Vrp1p\textsubscript{1-364}'s two actin binding domains is sufficient for actin patch polarization in the presence of Las17p binding domain. Although the actin binding domain is critical for actin patch polarization, the actin binding motif plays a redundant role with the proline rich sequence located inside the WH2/V domain of Vrp1p to restore cell growth at high temperature and actin patches polarization. Las17p binding domain located at Vrp1p\textsubscript{760-817} is critical for Vrp1p's role in actin patch polarization, and fusing the Las17p binding domain to C-terminal of Vrp1p\textsubscript{1-364} enable Vrp1p\textsubscript{1-364} to restore actin patch polarization. The Las17p binding domain is essential for proper localization of Vrp1p\textsubscript{364-817} and for complement of growth and actin patches polarization defects. Fusing CAAX tag to C-terminal of Vrp1p\textsubscript{5A-364-817} enables it to localize to cortex. Vrp1p\textsubscript{5A-364-817-CAAX} fully restores cell viability at high temperature, but not actin patches polarization. Therefore, the Vrp1p-Las17p interaction mediated by MPKPR motif is crucial for Vrp1p\textsubscript{364-817}'s localization and for complement of cell growth and actin patches polarization defects.

Therefore, though all the verprolin family proteins present a homologous V domain and a WASP/Las17p binding domain, they may have different activities and mechanism in regulating the actin polymerization in vivo.
Abbreviations

3-AT .............................................................. 3-amino-1,2,4-triazole
A ................................................................. adenosine
a.a ............................................................. amino acid
AD .............................................................. DNA activation domain
Ade ............................................................. Adenine
Amp ........................................................... Ampicillin
Arp ............................................................ Actin related protein
ATP .......................................................... adenosine 5'-triphosphate
BD ............................................................. DNA binding domain
bp .............................................................. base pair
BSA ........................................................... Bovine serum albumin
°C .............................................................. degree Celsius
CR16 .......................................................... Glucocorticoid regulated protein
C-terminus .................................................. carboxy-terminus
DIC/Normarski .......................................... differential interference contrast
DMSO ......................................................... Dimethyl sulfoxide
dNTP ........................................................ deoxyribonucleotide triphosphate
DTT ............................................................. Dithiothreitol
ECL ............................................................. enhanced chemiluminescence
E.coli ........................................................... 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
His ............................................................ histidine
HRP .......................................................... horseradish peroxidase
IgG ............................................................ immunoglobulin G
IPTG ........................................................ Isopropyl-thio-β-D-galactopyranoside
Kan .......................................................... Kanamycin
Kb ........................................................... kilo base pair
kD/KDa ................................................... kilo dalton
LB ............................................................ Luria-Bertani medium
Political and religious thinking began to echo to Islam were prohibited. The Ustadz (religious scholar) slowly replaced the Pandita among the Muslim dresses by a Middle East fashioned dresses. Customary rituals perceived deviant these scholars not only brought new political thinking but also the Middle East culture. Possible through the return of Islamic scholars from the Middle East Islamic was filled through a reconnecting with the dormant Islamic consciousness that made second wave of Islam.
1 Introduction

1.1 Cell and cytoskeleton

Cells are the basic units of life. The eukaryotic cells are composed of cell membrane, organelles, nucleus and cytoplasm. The majority of cellular metabolism takes place in the cytosol which consists of enzymes to carry out biochemical reactions as well as proteins with structural functions. Nearly half of the total proteins in a cell are located in the cytosol. Protein complexes are assembled and disassembled throughout the cell cycle. If an eukaryotic cell is permeabilized by nonionic detergent the soluble protein from the cytoplasm will diffuse out, leaving behind the cytoskeletal filaments. The cytoskeleton is highly organized and some of the cellular proteins rely on the cytoskeletal filaments for localization to specific regions within the cell.

The eukaryotic cell contains at least three kinds of cytoskeletal filaments, actin filaments (also name as microfilaments), microtubules and intermediate filaments. Each kind of cytoskeletal filament is a polymer of different monomer proteins. Monomeric actin units assemble into microfilaments while heterodimeric tubulin subunits are building blocks of microtubules. Intermediate filaments are assembled by various families of intermediate filament subunit proteins. Both actin microfilaments and intermediate filaments are usually attached to plasma membrane proteins and form a skeleton to support the fragile plasma membrane. However only actin
filaments, but not intermediate filaments, are involved in cell movements. Nearly all the eukaryotic cells have all the three types of cytoskeleton filaments which are regulated both temporally and spatially. For example, in epithelial cells, the actin microfilaments are abundant in the apical region and they are associated with cell-cell junctions and microvilli. The cytoskeleton has been highly conserved during the evolution. Sequence alignment shows that there is a high degree of sequence homology between actin from yeast and human which are separated by millions of years of evolution. This suggests that actin is involved in critical cellular functions which are dependent on the conserved cytoskeleton. Mutations of cytoskeleton monomer proteins might disrupt the assembly or disassembly of filaments and their binding partners. Functional analysis using systemic deletion and site directed mutagenesis has identified some homologues of actin cytoskeleton associated proteins in various species.

Though most of the cells are spherical when they are cultured as a suspension culture, more commonly they adopt various shapes and sizes due to their internal cytoskeletons and attachments to extracellular matrix. Cytoskeleton is linked with extracellular matrix or the cytoskeleton of other cells through the plasma membrane. Cell cytoskeleton is dynamically regulated through assembly and disassembly of the cytoskeleton filaments to regulate the cell shape. Disassembly of the cytoskeleton in some region of the cell and assembly in other region will produce coordinated changes in cell shape which results in cell protrusion and migration. The distinctive
shape of cells depends on the organization and remodeling of actin filaments, and some proteins connecting microfilaments to the plasma membrane. The remodeling of actin filaments provides physical force through polymerization and depolymerization which promotes the movement of organelles along actin filaments. These microfilament-membrane binding proteins functions to hold the actin cytoskeleton frameworks to the plasma membrane. For example, the plasma membrane can be deformed to form microvillus or similar protrusion if it is attached to actin filament bundles.

1.2 Actin and actin cytoskeleton

The actin filaments are concentrated in the cell cortex which is a narrow zone located just beneath the cell membrane. The actin filaments are organized into a network so that most organelles are excluded from cortical cytoplasm. In some cells with dense cortical cytoskeleton, actin filaments are a part of the network to compartmentalize the cytosol and assist the cells to anchor to extracellular matrix. Since the actin cytoskeleton lies just beneath the cell plasma membrane, it provides the flexibility and strength of cell membrane which is crucial for cell movement (Lodish, et al., 2000). There are two mechanisms which have been proposed to explain the initiation of cell movement and generation of mechanical force. One mechanism postulates that the assembly and disassembly of microfilaments provide mechanical force which results in the change of cell shape. The other mechanism postulates that generation of force requires motor proteins which are enzymes utilizing ATP to walk along
microfilaments or microtubule and generating the force required for changing the cell shape. Some types of cell movement rely on both of these mechanisms (Lodish, et al., 2000). The length of filaments and the ratio between different cytoskeleton associated proteins can vary dramatically when the actin filaments are cross-linked to form actin structures and networks. This variety and flexibility of actin cytoskeleton allow the cells to keep the plasticity of cell shape and vary the cell shape instantly according to extracellular condition. In the cells which are migrating, the actin filaments are assembled rapidly in one region while disassembled in another region as the total cellular actin is limited.

Actin is the most abundant cellular protein as the actin structures cover almost all parts of the cell. Actin comprises 10% of the total proteins in muscle cells and 1-5% in non-muscle cells (Lodish, et al., 2000). Large amount of actins are located in special cellular structures such as microvilli and the concentration of actin there can be up to 5 mM while the average concentration in the whole cell is 0.1-0.5 mM. Actin is a globular protein with the molecular weight 42 kDa and is encoded by a highly conserved gene (Lodish, et al., 2000). Single cell organisms have one or two copy of actin genes while multi-cellular organisms have multiple genes encoding actin. For example, human has 6 actin genes encoding different isoforms of this protein such as $\alpha$-actin in muscle cells associated with contractile structures, $\beta$-actin in non-muscle cells located in the cell leading edge and related with actin polymerization, and $\gamma$-actin in non-muscle cells related with stress fibers (Lodish, et al., 2000).
There are two forms of actin inside the cell, they are globular monomer actin (G-actin) and filamentous polymer actin (F-actin). G-actin monomers can be assembled or polymerized into long helical F-actin polymers, and F-actin polymer filaments also can be depolymerized back into globular monomer actin. This is the basic and important process for the reorganization of dynamic actin cytoskeleton. Profilin promotes actin polymerization by accelerating the exchange of ADP to ATP in G-actin (Kovar and Pollard, 2004). New barbed ends of filaments are produced by severing existing filaments, uncapping existing filaments or de novo nucleation of actin subunits (Condeelis, 1993, Pollard and Borisy, 2003, Zigmond, 1996). Arp2/3 and formin are the two characterized actin nucleators present in yeast to mammalian cells (Moseley and Goode, 2006). The assembly of actin filament is promoted by actin nucleation mediated by Arp2/3 (Actin Related Protein) complex (Zigmond, 1996), which is composed of seven peptides.

1.3 Arp2/3 complex and actin polymerization

Arp2/3 complex consists of two actin related proteins, Arp2 and Arp3, together with another 5 subunits, Arc40, Acr35, Arc19, Arc18 and Arc15 (Korenbaum, et al., 1998). Both Arp2 and Arp3 share sequence similarity with actin and has been identified in many eukaryotes. Arp2 temperature sensitive mutants of S.cerevisiae exhibit cellular defects such as lack of endocytosis and abnormal actin organization (Moreau, et al., 1996, Moreau, et al., 1997). Arp2/3 complex is present at high concentration up to 10 uM in cytoplasm (Higgs, et al., 1999) and simulates actin polymerization by binding
to the side of existing actin filaments, capping the pointed end of actin filaments and forming the nucleation of filamental barbed ends. The barbed ends of actin filament is the starting base of a new branched actin filament (Mullins, et al., 1998). The new branched actin filament is elongated and thereby generating physical force to push the plasma membrane or cellular vacoules forward (Figure 1.1a).

Figure 1.1a, Dendritic Nucleation Treadmilling Model for Protrusion of the Leading Edge
(1) Extracellular signals activate receptors. (2) The associated signal transduction pathways produce active Rho-family GTPases and PIP2. (3) Activate WASp/Scar proteins. (4) WASp/Scar proteins bring together Arp2/3 complex and an actin monomer on the side of a preexisting filament to form a branch. (5) Rapid growth at the barbed end of the new branch (6) pushes the membrane forward. (7) Capping protein terminates growth within a second or two. (8) Filaments age by hydrolysis of ATP bound to each actin subunit (white subunits turn yellow) followed by dissociation of the γ phosphate (subunits turn red). (9) ADF/cofilin promotes phosphate dissociation, severs ADP-actin filaments and promotes dissociation of ADP-actin from filament ends. (10) Profilin catalyzes the exchange of ADP for ATP (turning the subunits white), returning subunits to (11) the pool of ATP-actin bound to profilin, ready to elongate barbed ends as they become available. (12) Rho-family GTPases also activate PAK and LIM kinase, which phosphorylates ADF/cofilin. This tends to slow down the turnover of the filaments.
The return of Islamic scholars from the Middle East Islamic Universities contributed to a reconnection with the dormant Islamic consciousness. Chief among them was Ustadz Hashim Salamae, an Al-Azhar alumnus. These scholars brought new political thinking and Middle East culture, as evidenced by the replacement of traditional local Muslim dresses by Middle East fashioned attire. Customary rituals perceived as deviant to Islam were prohibited. The Ustadz (religious scholar) began to replace the Pandita (Traditional Muslim preacher). Political and religious thinking began to echo contemporary Islamic thinkers in the Middle East, as Hashim Salamat admitted drawing from their religious thinking.

Cell survival depends on a well-organized and regulated response to extracellular conditions. Cell ECM and cell-cell interaction are crucial for cellular processes such as polarization, endocytosis, cytokinesis, motility, and metastasis. Cell motility plays an important role in human health as it is involved in immune response and cancer invasion (Lodish, et al., 2000). Remodeling and reorganization of actin cytoskeleton in cells are involved in cellular processes such as formation of filopodia and lamellipodia. Further exploration has lead to a better understanding of the network from upstream transmembrane receptors to downstream regulators of actin cytoskeleton. The Rho family GTPases such as Cdc42, Rac, and Rho are main regulators that trigger the transformation between various forms of actin cytoskeletal structures. It has been reported that Cdc42 and Rac are involved in filopodia (Hall, 1998) and lamellipodia (Higgs and Pollard, 1999) formation respectively. Some downstream regulators of Rho family GTPase include Wiskott Aldrich Syndrome protein family (Figure 1.1b) (WASP family, which includes WASP, N-WASP, WAVE1,
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The Arp2/3 complex is intrinsically inactive and dependent on its activators such as ActA and WASP family of proteins. ActA is a cell surface protein of Listeria bacteria and is required for utilizing host cell’s actin system to form a comet tail (Smith, et al., 1995, Welch, et al., 1998). WASP family of proteins (Wiskott-Aldrich Syndrome protein) include WASP (Derry, et al., 1994), N-WASP (Miki, et al., 1996), WAVE1 (Miki, et al., 1998), WAVE2 (Suetsugu, et al., 1999), WAVE3 (Suetsugu, et al., 1999) and the yeast homologue Las17p (Li, 1997). Both WASP and N-WASP have a conserved WH1 domain at the N-terminal and a VCA region (this region including a V/WH2 domain, a connecting region and an acidic domain) at the C-terminal (Paavilainen, et al., 2004). Arp2/3 complex alone exhibits a low activity of actin nucleation since there is a large separation between the Arp2 and Arp3 subunits (Robinson, et al., 2001). WASP family of proteins induces a large structural re-arrangement of Arp2/3 complex to push Arp2 and Arp3 subunits closer to catalyze actin nucleation (Goley, et al., 2004, Rodal, et al., 2005). Therefore the WASP family of proteins are important activators of Arp2/3 complex in the regulation of actin
polymerization.

1.4 WASP family of proteins

WASP is Wiskott-Aldrich Syndrome Protein and mutation of the gene encoding WASP leads to Wiskott-Aldrich syndrome (WAS) (Derry, et al., 1994). Wiskott-Aldrich syndrome is an X-linked inherited immune deficiency syndrome, characterized by eczema, thrombocytopenia, bleeding, and recurrent infections (Aldrich, et al., 1954, Ochs and Notarangelo, 2005). Most of the WAS patients die before the age of 10 if not treated by bone marrow transplantation (Rimm and Rappeport, 1990). WASP is expressed predominantly in haematopoietic cells. Lymphocytes from WAS patients show cytoskeletal abnormalities such as a relatively smooth surface with decreased microvilli in number and size (Thrasher, et al., 2000). Over-expression of WASP induces formation of actin clusters (Symons, et al., 1996). WASP binds to small GTPase, such as Cdc42 and Rac, via its GTPase binding domain (GBD). This suggests that WASP plays a role in actin polymerization and may provide a connection between Cdc42, Rac and the actin cytoskeleton (Machesky and Hall, 1997). N-WASP (neural WASP) was identified through its interaction with growth-factor receptor-bound protein (GRB2) (Miki, et al., 1996). N-WASP is abundant in neural tissue, but is also expressed in several other types of tissue. WASP and N-WASP share common domains, such as verprolin homology domain (V domain, also named as WASP Homologue 2 domain, WH2 domain) at the C-terminus, the cofillin homology domain (also named as central domain or connecting domain, C
domain) and the acidic domain (A domain) (Figure 1.1b). Three domains constitute VCA region in WASP while four domains constitute VVCA region in N-WASP due to an extra V domain. Screening for proteins with homology sequence of VCA region leads to the identification of WAVE1 (Miki, et al., 1998), WAVE2 (Suetsugu, et al., 1999) and WAVE3 (Suetsugu, et al., 1999). WAVE1 and WAVE3 are enriched in brain while WAVE2 is expressed ubiquitously. Las17p is the yeast homologue of WASP and N-WASP (Naqvi, et al., 1998). WAVE proteins are present in Dictyostelium, but not in yeast, suggesting that WAVE might be necessary for cell migration (Takenawa and Suetsugu, 2007). Thus WASP, N-WASP, WAVE1,2,3 and yeast homologues Las17, are members of the WASP family (Paavilainen, et al., 2004) which link extracellular signals to actin cytoskeleton reorganization.

It has been shown recently that the V domain of WASP/N-WASP interacts with G-actin, and the CA region interacts with Arp2/3 complex (Takenawa and Miki, 2001). The actin polymerization requires the formation of a nucleus made of 3 G-actin monomers. There are 2 actin related molecules in Arp2/3 complex which share sequence similarity with actin and play the role of actin monomers, thus the recruitment of the third actin monomer would lead to the formation of an actin nucleus required by actin polymerization. Therefore VCA region of WASP/N-WASP play an important role in the initiation of actin polymerization by functioning as a platform where actin monomers bind to Arp2/3 complex (Miki and Takenawa, 1998, Rohatgi, et al., 1999).
Both WASP and N-WASP have a GTPase binding domain (GBD) mediating the interaction with Cdc42, and a VCA region mediating the interaction with actin and Arp2/3 complex. In addition, N-WASP has an extra V domain at the N-terminal of VCA region (Figure 1.1b). GBD domain has been proposed to interact with the C-terminal VCA region of WASP and VVCA region of N-WASP, resulting in the autoinhibition of WASP/N-WASP activity (Miki, et al., 1998, Prehoda, et al., 2000, Rohatgi, et al., 2000). This inhibition can be relieved by the binding of various activator proteins such as Cdc42 (Cell Division cycle 42) and PIP2 (PtIns(4.5)P2, Phosphatidyl-inositol 4,5-diphosphate), which relieve the auto-inhibited conformation of WASP/N-WASP (Rohatgi, et al., 2000).

N-WASP/WASP has been shown to interact with a number of proteins such as WIP, WIRE/WICH and CR16, while Las17p (yeast WASP) has been shown to interact with Vrp1p (yeast WIP). All the four proteins which interact with WASP family of proteins are members of Verprolin family of proteins with a conserved V (Vrp1p domain, or WH2 WASP Homologue 2 domain) at the N-terminal and a WASP/N-WASP binding domain (for Vrp1p is Las17 binding domain) at the C-terminal. The activity of WASP/N-WASP is regulated by the binding of WIP (Martinez-Quiles, et al., 2001), WIRE/WICH (Aspenstrom, 2002, Kato, et al., 2002) or CR16 (Ho, et al., 2004), all of which are members of Verprolin protein family. Majority of WASP in the cell is present as a complex with WIP (Sasahara, et al., 2002) and N-WASP is present as a complex with WIRE (Kato, et al., 2002). Nearly half of the missense mutations
causing Wiskott–Aldrich Syndrome are point mutations in the WH1 domain of WASP (Imai, et al., 2003), and some of these mutations have been shown to affect WASP-WIP interaction (Stewart, et al., 1999), suggesting that WASP-WIP interaction is crucial for WASP’s activity. Therefore WIP together with other members of Verprolin protein family play a major role in the function of WASP family of proteins.

1.5 Verprolin family of proteins

Verprolin (Very rich proline protein) was initially identified as a very poline-rich protein in yeast (named as Vrp1p) as the result of a screening for vinculin gene in Saccharomyces cerevisiae (Donnelly, et al., 1993). Functional analysis of Vrp1p has lead to a better understanding of its important role in actin cytoskeleton organization and endocytosis (Donnelly, et al., 1993, Munn, et al., 1995). Verprolin proteins are widely expressed in eukaryotic organisms except plants (Aspenstrom, 2005). Fungi, yeast and insect have one isoform of verprolin while vertebrates have three isoforms encoding verprolin-like proteins. The yeast verprolin is Vrp1p (Munn, et al., 1995), and the mammalian verprolin family consists of three proteins: WASP-Interacting Protein (WIP) (Ramesh, et al., 1997), product of glucocorticoid-regulated gene (CR16) (Weiler, et al., 1996), and WIP-related protein (WIRE) (Aspenstrom, 2002). WIRE is also referred to as WICH (WIP and CR16 homologous protein) (Kato, et al., 2002). All members of Verprolin family are characterized by the presence of a V domain (also named as WASP Homologue 2 domain, WH2) at the N-terminus and a WBD (WASP Binding domain) at the C-terminus (Aspenstrom, 2005, Kato, et al., 2002).
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The gene encoding WIP is located on chromosome 2 in both mice and human. WIP was first identified as WASP interacting protein (Ramesh, *et al.*, 1997) and subsequently as a mammalian functional homologue of Vrp1p as expression of WIP suppressed the growth and endocytosis defect of yeast *vrp1-1* mutant strain (Vaduva, *et al.*, 1999). CR16 was initially identified as a neural protein (Masters, *et al.*, 1996) before being shown to share an sequence homology of up to 25% with WIP (Ho, *et al.*, 2001). WIRE/WICH share 30-40% identity to WIP or CR16 (Aspenstrom, 2002, Kato, *et al.*, 2002). All members also contain proline rich regions at the N-terminal as well as at other part of this molecule suggesting that they are proline rich which might explain the aberrant migration on SDS PAGE. For example, WIP has a calculated molecular weight of 52 kDa but migrates as a 65 kDa protein (Ramesh, *et al.*, 1997). CR16 has a calculated molecular weight of 49 kDa but migrates as a 85 kDa protein (Ho, *et al.*, 2001).

Verprolin plays an important role in actin dynamics by two different mechanisms: signaling to actin dynamics mediated by the WASP (Wiskott-Aldrich syndrome protein) family proteins, and, influencing actin polymerization independent on WASP family proteins (Anton and Jones, 2006).

1.6 Functions of Verprolin proteins dependent on WASP

WIP is a multifunctional protein and was initially identified as a WASP interacting protein using the yeast two hybrid system (Ramesh, *et al.*, 1997). WIP is the first
identified mammalian member of Verprolin family including WIP, WIRE, CR16 and yeast Vrp1p. As the first mammalian protein of Verprolin family, WIP has been studied and analyzed better than either CR16 or WIRE. Consistent with its name, WASP interacting protein, WIP carries out its function to regulate the actin cytoskeleton through interaction with WASP family of proteins by the WASP binding domain (WBD) at the C-terminus. It has been reported that 95% of WASP in lymphocytes are complexed with WIP (Sasahara, et al., 2002) while WIRE and CR16 have been reported to form a tight complex with N-WASP (Ho, et al., 2001, Kato, et al., 2002). It has been shown that WIP is a chaperone for WASP, as WASP is rapidly degraded in the absence of WIP (de la Fuente, et al., 2007). WIP inhibits Cdc42-mediated activation of N-WASP suggesting that one of the functions of WIP is to stabilize WASP/N-WASP in an inactive closed conformation (Martinez-Quiles, et al., 2001). Expression of WASP Binding domain of WIRE suppresses the microspike formation induced by N-WASP (Kato, et al., 2002), and ectopic expression of WIRE reduces the receptor mediated endocytosis in a N-WASP dependent manner (Aspenstrom, 2004). WIP is crucial for localizing WASP activity for actin polymerization mediated motility of vaccinia (Scaplehorn, et al., 2002), and for the formation of immunological synapse after TCR ligation (Sasahara, et al., 2002). In addition, WIP synergizes with N-WASP to induce filopodia when overexpressed in a fibroblast (Moreau, et al., 2000). WIP and WASP play complementary roles in chemotaxis (Gallego, et al., 2006). In yeast, cortical localization and activity of C-terminus of Vrp1p to suppress the growth and actin patches polarization defects,
were mediated by the interaction with Las17p, the yeast homologue of WASP (Thanabalu and Munn, 2001).

1.7 Function of Verprolin proteins independent of WASP

Though WASP/N-WASP are important binding partners of Verprolin family of proteins, not all functions of Verprolin family proteins are related directly to WASP/N-WASP interactions (Anton and Jones, 2006). Verprolin family proteins exhibit functions independent of WASP/N-WASP. WIP, WIRE and CR16 contain three to six potential profilin binding sites (Actin Based Motility homology-2, ABM-2) which are implicated in binding to the actin monomer binding proteins (Holt and Koffier, 2001, Purich and Southwick, 1997). This motifs has the consensus sequence of XPPPPP where X can be A, S, L, or G (Anton and Jones, 2006). The verprolin proteins bind to both G- and F-actin as well as profilin (Aspenstrom, 2004). All the three mammalian Verprolins contain a V domain at the N-terminus which mediates the interaction with actin, and contain a proline rich region inside the V domain which has been shown to mediate the interaction with Profilin or SH3 (Src Homology 3) domain containing proteins. Cortactin is an actin-binding SH3 domain-containing protein, and its interaction with WIP is mediated by the SH3 domain. WIP increases the efficiency of cortactin-mediated actin polymerization in a concentration dependent manner (Kinley, et al., 2003). Nck, a ubiquitous adaptor molecule forms a complex with WIP and profilin indicating that Nck couple extracellular signals to the cytoskeleton via its interaction with Verprolin proteins (Anton, et al., 1998).
adaptor protein CrkL binds directly to WIP to form a complex which is recruited to lipid rafts and the immunological synapse (Sasahara, et al., 2002). Therefore, due to the presence of proline rich regions, Verprolin proteins are likely to interact with SH3 (Src Homology 3) domain-containing proteins and involved in the signaling pathway as well as remodeling of actin cytoskeleton.

Both WIP and WIRE/WICH inhibit actin depolymerization rates in a concentration dependent manner (Kato, et al., 2002, Martinez-Quiles, et al., 2001) and stabilize the cellular F-actin content (Martinez-Quiles, et al., 2001, Ramesh, et al., 1997). WIRE/WICH has been reported to cross link actin filaments though the central proline rich region instead of WASP binding domain (Kato and Takenawa, 2005). Consistent with this finding, WIP was observed to co-localize with stress fibers and filopodia (Vetterkind, et al., 2002), WIRE was observed to localize along bundles of actin filaments as well as in F-actin containing protrusions at the cell edges independent of WASP (Aspenstrom, 2004). It has been shown that mutant WIRE which has lost the interaction with WASP, induces reorganization of actin filament system (Aspenstrom, 2004). WIRE induces the cross-linking of actin filaments resulting in straight bundled actin filaments, and induces the formation of thick actin fibers in cultured fibroblast. This actin cross linking activity is WASP independent, but modified by the presence of WASP (Kato, et al., 2002). N-terminal of Vrp1p, the yeast member of Verprolin family of proteins, is sufficient to restore cell viability at high temperature in vrplA strain, as N-Vrp1p does not interact with Las17p
suggested that N-Vrp1p has activities independent of Las17p (Thanabalu and Munn, 2001). Therefore, though WASP/N-WASP are the main binding partner of the Verprolin proteins, the verprolin proteins have roles in regulation of actin cytoskeleton independent of WASP family of proteins. The WASP independent function of Verprolin proteins may be similar to that of other actin filament binding proteins, such as tropomyosin, which stabilize actin filaments and prevent depolymerization of F-actin (Broschat, et al., 1989, Ono and Ono, 2002).

1.8 WIP

WIP is the first mammalian Verprolin family member identified by a genetic screen using WASP as bait and initially characterized as WASP interacting protein (WIP) which induces actin polymerization and redistribution in lymphoid cells (Ramesh, et al., 1997). WIP is a proline-rich protein with a verprolin homology domains (V domain) at the N-terminus, which is also called WASP homology (WH2) domains (residues 1-50) (Ramesh, et al., 1997). V domain is found in many proteins that regulate the actin cytoskeleton, including Verprolin and WASP family of proteins. V domain of WIP contains a KLKK motif which has been reported to be important for actin binding (Vaduva, et al., 1997). WIP also contains a number of proline rich putative SH3-binding motifs that have the sequence PPPfXP where “f” represents a hydrophobic residue. One of the SH3-binding motifs GRSGPXPPXP of WIP has been implicated in binding to SH3 domain-containing proteins and some of these proteins participate in the signaling cascade regulating cytoskeleton (Mirey, et al., 2005). WIP
also contains three XPPPPP sequences (Actin-Based Motility 2 sequence, ABM2 motif, X can be represented by A, L, S, or G), which binds to profilin, a protein that promotes actin polymerization (Ramesh, et al., 1997). The presence of SH3-binding motifs and ABM2 motif in WIP suggests that it might link the signal-transduction machinery to the remodeling of actin cytoskeleton (Figure 1.2).

![Diagram of WIP, CR16, and WIRE proteins]

Figure 1.2 Schematic representation of the mammalian verprolin family WIP, CR16 and WIRE. The verprolin family proteins share the presence of a V domain including a proline rich region at the N-terminus, a WASP/N-WASP binding domain at the C-terminus, and a proline rich region with repeat PxxP motifs in the middle.

Most of WASP and WIP molecules exist as a complex inside the cells (Sasahara, et al., 2002). WASP and WIP purified from bacteria are able to bind to each other, suggesting that posttranslational modification is not necessary for the interaction (Ho, et al., 2006). WASP binding domain (WBD) at the C-terminal of WIP binds to the WH1 domain at the N-terminal of WASP. Majority of point mutations in WASP patients are located in its WH1 domain, even though this domain represents only 18%
of the WASP sequence (de Saint Basile, et al., 1996). Some of the mutations that cause WAS have been shown to disrupt the WASP-WIP interaction, suggesting that the interaction is crucial for their function (Stewart, et al., 1999). The interaction between Verprolin and WASP/N-WASP is crucial for some, but not all, functions of Verprolin as well as WASP/N-WASP. Binding to Las17p (yeast homologue of WASP) is essential for C-Vrp1p (yeast Verprolin) to suppress growth and actin patches polarization defects in vrp1Δ strain (Thanabalu and Munn, 2001).

WIP interacts with both globular and filamentous actin. The presence of 3 ABM-2 motifs of WIP indicates possible interaction between WIP and profilin, which is involved in regulation of actin polymerization. WIP localizes to cortical actin patches in yeast (Vaduva, et al., 1999) and actin rich structures such as the base of filopodia, lamellipodia and stress fibers (Kinley, et al., 2003, Martinez-Quiles, et al., 2001, Vetterkind, et al., 2002) in mammalian cells. The cortical actin filament network is disrupted in WIP-deficient lymphocytes (Anton, et al., 2002). Over expression of WIP in human B cells stabilizes actin filaments (Ramesh, et al., 1997), leads to increased F-actin content, and induces the formation of subcortical patches of actin (Ramesh, et al., 1997) indicating that the mechanism for WIP to stimulate actin polymerization through binding partner such as profilin, which is different from its inhibitory effect on WIP-WASP/N-WASP (Anton and Jones, 2006). Endocytosis is an important cellular process through which the plasma membrane is invaginated into the cell resulting in the formation of a vesicle. Expression of WIP rescues the endocytosis and
the ability of growth at elevated temperature of verprolin deficient yeast cells (Vaduva, et al., 1999). Further more, WIP was reported to associate with N-WASP and cortactin which contribute to endocytosis (Cao, et al., 2003, Otsuki, et al., 2003). WIP stabilizes not only actin filaments, but also WASP (Chou, et al., 2006, de la Fuente, et al., 2007). WIP binds to tyrosine kinase Syk and protects it from degradation by proteasome in B cells (Kettner, et al., 2004).

1.9 WIRE

As a member of Verprolin family of proteins, WIRE has a closer evolutionary relationship with WIP than CR16 (Aspenstrom, 2005) (Figure 6.1). WIRE was identified as a verprolin protein simultaneously by Aspenstrom (Aspenstrom, 2002) and as WICH by Kato (Kato, et al., 2002) since it exhibits a conserved V domain at N-terminus and a WASP binding domain at C-terminus. WIRE/WICH was shown to bind strongly to N-WASP but weakly to WASP (Kato, et al., 2002) and localized to actin filaments in transiently transfected porcine aortic endothelial cells with PDGF-β (Platelet derived growth factor) receptor (Anton, et al., 2003). Ectopically expressed WIRE lead to formation of peripheral protrusions composed of filopodia and lamellipodia-like structures if the cells were treated with PDGF (Aspenstrom, 2002). Ectopic expression of WIRE induced actin microspike formation through cooperation with N-WASP and in addition, expression of the WASP binding region (or WASP associating region) suppressed microspike formation induced by N-WASP (Kato, et al., 2002). This result is consistent with the data of Aspenstrom, reporting that in cells
ectopically expressing WIRE, the endocytosis of PDGF-β receptor was drastically reduced and this reduction was WASP dependent and a direct interaction of WASP and WIRE is needed (Aspenstrom, 2002). WIRE re-localizes WASP to actin filaments when both of WIRE and WASP were co-expressed and this re-localization need the direct interaction between WIRE and WASP (Aspenstrom, 2002). The evidence indicates that WIRE has a role in the WASP mediated organization of the actin cytoskeleton.

1.10 CR16

Among the three members of mammalian Verprolin family, CR16 is the least characterized. CR16 was identified as a glucocorticoid regulated gene expressed in subpopulations of neurons in the brain, including the hippocampus (Masters, et al., 1996). The CR16 open reading frame encodes a 45 kDa protein containing 32% proline. The antibody identifies a protein doublet of 67 and 70 kDa by sodium-dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) from hippocampal extracts, However, when hippocampal extracts are electrophoresed on non-denaturing polyacrylamide gels, the CR16 protein migrates as a 48 kDa protein which correlates well with the calculated size (Weiler, et al., 1996). Sequence alignment reveals 12 sequence homologies to the SH3 binding domain consensus sequence XPXXPPP. In addition, CR16 has at least 36 copies of the PXXP motif, which is contained in all known SH3 binding domains (Weiler, et al., 1996).
CR16 is present in a tight complex with native N-WASP (Ho, *et al.*, 2001). Although N-WASP is expressed ubiquitously, CR16 is found predominately in the brain. CR16 and N-WASP co-localize in hippocampal neurons and filopodia of growth cone. CR16 binds both G- and F-actin but does not affect the kinetics of actin polymerization mediated by N-WASP and the Arp2/3 complex. CR16 shares 25% sequence identity with WIP. Like WIP and WIRE, CR16 is characterized by a V domain at the N-terminus and a WASP binding domain at the C-terminus (Ho, *et al.*, 2001). Therefore CR16 is a member of Verprolin family of proteins.

### 1.11 Vrp1p


Vrp1p can be divided into two functional modules: an N-terminal peptide, Vrp1p_{1-364} and a C-terminal peptide Vrp1p_{364-817} (Thanabalu and Munn, 2001). Both Vrp1p_{1-364} and Vrp1p_{364-817} were found to restore growth at restrictive temperature to *vrplΔ*
Introduction


1.12 \textit{S.cerevisiae}: model for the study of actin cytoskeleton

Actin cytoskeleton is essential in a wide variety of cellular functions, including cell morphogenesis, cell polarity, cytokinesis, endocytosis, cell motility, cell adhesion and migration (Ono, 2007). Reorganization of the actin cytoskeleton is regulated both spatially and temporally but the mechanism is still not very clear. \textit{S.cerevisiae} is an
excellent model system for the study of actin cytoskeleton dynamics because it has a
relatively simple actin cytoskeleton and offers powerful experimental tools for genetic
manipulation. The unicellular eukaryote Saccharomyces cerevisiae, budding yeast, is
very useful in elucidating the role of actin and actin cytoskeleton associated proteins
because many of the actin associated proteins identified in S. cerevisiae have often
been found to have counterparts with similar functions in mammalian system
(Moseley and Goode, 2006). This indicates the usefulness of S. cerevisiae to carry out
functional analysis of mammalian actin cytoskeleton related proteins in this
unicellular eukaryote.

The actin cytoskeleton in S. cerevisiae comprises of cortical actin patches and
Actin cables are thick filaments that align along the mother-bud axis with their tips
focused at sites of actin patch polarization (Adams and Pringle, 1984, Amberg, 1998,
Engqvist-Goldstein and Drubin, 2003, Lew and Reed, 1993). The cortical actin
patches undergo characteristic changes through out the cell cycle, and the patches are
polarized towards nascent bud sites and the tips of small buds. The re-organization of
cortical actin patches is regulated by cortical patch proteins, such as Las17p/Bee1p
(Li, 1997), Vrp1p (Munn, et al., 1995), Myo3p (Evangelista, et al., 2000), Myo5p
(Goodson, et al., 1996), Rvs167p (Balguerie, et al., 1999) and the Arp2/3 complex
(Pruyne and Bretscher, 2000). These actin patch proteins are involved in the uptake
step of endocytosis at the plasma membrane (Munn, et al., 1995, Wendland, et al.,
1998). Actin cables that extend along the mother-bud axis are required for polarized growth (Pruyne, et al., 1998) as well as cell viability at high temperature and cytokinesis. Thus, assays of endocytosis, actin patches polarization and viability at high temperature can be used to evaluate the integrity of actin cytoskeleton.

1.13 Objectives of this study

WASP family of proteins is regulator of actin polymerization mediated by Arp2/3 complex. WIP, WIRE/WICH, CR16 and Vrp1p are members of Verprolin protein family which play a role in the activity of WASP family of proteins. Further more, Verprolin proteins have functions independent of WASP family of proteins. AMY88 is a vrp1Δ strain of S.cerevisia which exhibits actin cytoskeleton related cellular defects such as endocytosis, growth defect at elevated temperature and actin patches polarization defect (Thanabalu and Munn, 2001). Expression of human WIP using a strong inducible promoter in vrp1-l mutant strain corrects the growth defects of this strain (Vaduva et al., 1999). This suggests that human WIP has activities similar to that of Vrp1p. I will express verprolin family proteins in yeast vrp1Δ strain to characterize functional domains of these proteins which are essential for growth at high temperature, endocytosis and actin patches polarization. I propose to carry out deletion analysis of Verprolin proteins to identify the localization signal and the smallest fragments which can rescue the growth defect of vrp1Δ strain. I will also carry out site directed mutagenesis of any motifs or proteins binding sites present in the functional domain to analyze the significance of these motifs or binding sites in...
rescuing the cellular defects of \textit{vrp1A} strain and further more to identify the cooperation effect of these motifs and domains. The domain identified will be sub-cloned into the bait vector of yeast two hybrid system and used to carry out a yeast two hybrid assay. This will allow characterization of the binding partners including WASP which is involved in cytoskeletal remodeling. Mapping the functional domains of Verprolin proteins will lead to a better understanding of the role of Verprolin proteins in polarization and proliferation in both yeast and mammalian cells, and the roles of WASP and WIP in the Wiskott Aldrich Syndrome.
2 Methods and Materials

2.1 Materials

2.1.1 Chemicals, reagents, enzymes, antibodies and kits

All the general chemicals, reagents and solvents were of analytical grade, purchased from Sigma Aldrich Chemical Company Ltd. (St. Louis, Mo, USA), unless otherwise stated. Solutions were sterilized by autoclaving or by passing through a 0.22µm filter if required.

Restriction endonucleases, T4 DNA ligase, and some of other enzymes were obtained from New England Biolabs (Ipswich, MA, USA), Fermentas (Hanover, MD, USA) or Roche (Indianapolis, IN, USA). The DNA polymerase *pfu* was from Stratagene (La Jolla, CA, USA). The DNA polymerase was from Biotools (Nave, Madrid, Spain). Lysozyme was purchased from Sigma-Aldrich (St. Louis, Mo, USA). Plasmid Miniprep kit, PCR purification kit and Gel Extraction Kits were purchased from Qiagen (Valencia, CA, USA). Site directed mutagenesis kit was purchased from Stratagene (La Jolla, CA, USA). *Escherichia coli* BL21 (DE3) and DH5α were purchased from Invitrogen (Carlsbad, CA, USA).

Protein molecular weight marker was from Bio-Rad Laboratories (Hercules, CA, USA). DNA loading ladder was from New England Biolabs or Fermentas. The dNTP
Methods and Materials

(dTTP, dATP, dGTP and dCTP) mixtures were purchased from Invitrogen. 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) or Fermentas. Ampicillin and kanamycin Sulfate were purchased from Sigma-Aldrich or Invitrogen. Rabbit Anti-GFP, anti-GST, Alexa Fluor® 488 goat anti-rabbit IgG were from Molecular Probes (Eugene, OR, USA) or Santa Cruz Biotechnology (Santa Cruz, CA, USA). AbI PRISM DNA sequencing kit (Big Dye) was purchased from Applied Biosystems (Foster City, CA, USA). Ni²⁺-NTA resin and GST sepharose were purchased from Amersham Biosciences (Buckinghamshire, UK).

2.1.2 Solution and buffer

TAE (Tris-acetate-EDTA, 50×)

50× stock solution, 1 liter: 242 g of Tris base, 57.1 ml of glacial acetic acid, 100 ml of 0.5 M EDTA, pH 8.0.

Agarose gel

1% (w/v) agarose in 1× TAE buffer was heated in microwave oven till cleared.

6×DNA loading buffer

0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol FF, 40% (w/v) sucrose in H₂O, Stored at 4°C.
Elution buffer for Ni-NTA beads

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

Elution buffer for GST beads

5-10 mM reduced glutathione in PBS.

Washing buffer for Ni-NTA beads

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

Washing buffer for GST beads

1% Triton X-100 in PBS

5x Tris-glycine electrophoresis buffer

25 mM Tris (15.1 g/L), 250 mM glycine (94 g/L) pH 8.3, 0.1% SDS (50 ml 10% SDS stock/L)

SDS-PAGE gel-loading buffer (2x)

100 mM Tris-HCl (pH 6.8), 200 mM dithiothreitol, 4% SDS, 0.2% Bromophenol blue, 20% (v/v) glycerol, Stored at -20°C.

30% Acrylamide mix

Dissolve 29 g acrylamide and 1 g N,N'-methylene-bioacrylamide in about 70 ml water, then adjust to 100 ml. The pH should be or less than 7.0. Store at 4°C.

10% Ammonium persulfate

Dissolve 1 g ammonium persulfate in 10 ml water. Store at 4°C.
Methods and Materials

Destaining solution

Methanol: H₂O: glacial acetic acid = 450:450:100

Staining solution

0.25% Brilliant Blue R-250 in destaining solution.

10x TE buffer

1.0 M Tris-HCl, 10 mM EDTA, pH 7.5.

10x LiAC

1 M lithium acetate, adjust pH 7.5 with dilute acetic acid and autoclave.

2.2 Medium and Strains

2.2.1 Bacterial strains and medium

Several bacterial strains were used in the study. The *E.coli* (Escherichia coli) strain, DH5α was used throughout the study for all cloning procedures, plasmid amplification, purification and for protein expression using the Tac promoter (pGEX plasmid). *E.coli* BL21(DE3) was used for the protein expression using the T7 promoter (pET plasmid). *E.coli* was either cultured in LB broth (1% bacto tryptone, 0.5% bacto yeast extract, 1% NaCl, pH7.0) or maintained on LB agar plates (LB broth containing 2% bacto agar) at 37°C. When recombinant plasmid was introduced into the host bacterial, the bacterial cells were cultured in the media supplemented with 75 ug/ml of ampicillin or 30 ug/ml of kanamycin.
2.2.2 Yeast strains

Yeast strains used in this study are listed in Table 2-1. Yeast strain PJ69-4A was a gift from Philip James, University of Wisconsin and used as the host strain for yeast two-hybrid assay.

Table 2.1, Yeast strains used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>RH1657</td>
<td>MATa his4 leu2 ura3 lys2</td>
<td>Riezman laboratory strain</td>
</tr>
<tr>
<td>AMY88</td>
<td>MATa his4 leu2 ura3 lys2 vrplA::KanMx bar1</td>
<td>Thanabalu. T. et al., 2001</td>
</tr>
<tr>
<td>IDY166</td>
<td>MATa his3 leu2 ura3 trp1 las17A::URA3</td>
<td>Naqvi et al., 1998</td>
</tr>
<tr>
<td>PJ69-4A</td>
<td>MATa his3 leu2 ura3 trp1 gal4A gal80A met2::GAL7-lacZ GAL2-ADE2 LYS2::GAL1-HIS3</td>
<td>James et al., 1996</td>
</tr>
</tbody>
</table>

2.2.3 Growth condition and maintenance of yeast strains

Yeast cells used in this study were cultured in YPD (Yeast Extract/Peptone/Dextrose) Medium, Synthetic Defined (SD) Media or SD lacking the appropriate amino acids for plasmid maintenance. Yeast media were prepared using standard methods (Sherman, F. 1991). YPUAD is YPD supplemented with 40 μg/ml adenine and 20 μg/ml uracil. SD minimal medium and YPD were prepared as described (Adams, et al., 1997). SD-Trp-Leu-His+3AT (3-aminotriazole) medium was used for testing the interaction of yeast two hybrid assay where 3AT was used to suppress the leaky expression of HIS3, limit histidine biosynthesis and cell growth. For preparation of solid media, 2% of bacto-agar was added to liquid broth before autoclaving.
Yeast cells were streaked onto YPUAD plates (2% Casein Peptone; 1% Yeast Extract; 2% Glucose; 0.004% Uracil; 0.002% Adenine; 2% Agar) and incubated at 30°C (for Wild type strain) or 24°C (for mutant strain) for 2-4 days. Temperature sensitive mutants were grown at the permissive temperature such as 24°C and analyzed at the restrictive temperature of 37°C. Colonies are collected with sterile loops, re-suspended in YPUAD with 20% glycerol (2% Casein Peptone, 1% Yeast Extract, 2% Glucose, 0.004% Uracil; 0.002% Adenine, 20% Glycerol) and frozen at -80°C for long term storage. Stock plates were sealed with Parafilm and stored at 4°C.

2.3 DNA manipulation

General recombinant DNA methods were performed as described by Sambrook (Sambrook et al., 1989). PCR (polymerase chain reaction) was performed with thermostable DNA polymerase. Purification of plasmids and amplified DNA fragments was carried out using Qiagen purification kit. Restriction endonucleases enzyme digestion was carried out using the appropriate buffers supplied by New England Biolabs. Roche DNA ligase was used for ligation of DNA fragments.

2.3.1 Vectors

Plasmids were used for the expression of protein in E.coli and pET series of vectors which contain T7 promoter were used for expression of proteins in BL21 (DE3) cells. PGEX and other vectors which contain Tac promoter were used for the expression of
proteins in DH5α. pACT2 vector which contains Gal4p Activation domain, and pAS2-1 vector which contains Gal4p DNA binding domain were used for yeast two hybrid. YCplac series low copy number and YEplac series high copy number plasmids with different selective marker were used for the expression of proteins in yeast mutant cells. Table 2.2 gives the name of plasmids and their applications.

Table 2.2 Commercial plasmids used in this study

<table>
<thead>
<tr>
<th>Vector Name</th>
<th>Resistance</th>
<th>Selective Marker</th>
<th>Expression Host</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Puc19</td>
<td>Amp</td>
<td>-</td>
<td>-</td>
<td>For sub-cloning</td>
</tr>
<tr>
<td>pHt24a</td>
<td>Amp</td>
<td>-</td>
<td>DH5α</td>
<td>Expressing GST fusion proteins</td>
</tr>
<tr>
<td>pET24a</td>
<td>Kan</td>
<td>-</td>
<td>BL21(DE3)</td>
<td>Expressing His tag fusion proteins</td>
</tr>
<tr>
<td>pACT2</td>
<td>Amp</td>
<td>Leu</td>
<td>PJ69-4A</td>
<td>Generating AD fusion proteins</td>
</tr>
<tr>
<td>pAS2-1</td>
<td>Amp</td>
<td>Trp</td>
<td>PJ69-4A</td>
<td>Generating BD fusion proteins</td>
</tr>
<tr>
<td>pCDNA3.1</td>
<td>Amp</td>
<td>-</td>
<td>Mammalian</td>
<td>Protein expression in Mammalian cell</td>
</tr>
<tr>
<td>YCplac22</td>
<td>Amp</td>
<td>Trp</td>
<td>Yeast</td>
<td>Protein expression in yeast cell</td>
</tr>
<tr>
<td>YCplac33</td>
<td>Amp</td>
<td>Ura</td>
<td>Yeast</td>
<td>Protein expression in yeast cell</td>
</tr>
<tr>
<td>YCplac111</td>
<td>Amp</td>
<td>Leu</td>
<td>Yeast</td>
<td>Protein expression in yeast cell</td>
</tr>
<tr>
<td>YEplac112</td>
<td>Amp</td>
<td>Trp</td>
<td>Yeast</td>
<td>Protein expression in yeast cell</td>
</tr>
<tr>
<td>YEplac195</td>
<td>Amp</td>
<td>Ura</td>
<td>Yeast</td>
<td>Protein expression in yeast cell</td>
</tr>
<tr>
<td>YEplac181</td>
<td>Amp</td>
<td>Leu</td>
<td>Yeast</td>
<td>Protein expression in yeast cell</td>
</tr>
</tbody>
</table>

2.3.2 Plasmid construction

Standard DNA techniques (Sambrook, et al, 1989) and DH5α bacterial strains were used for the construction of recombinant plasmids and amplification of new plasmid constructs. Plasmid constructs are listed in Appendix-1.

2.3.3 Primers for PCR reaction

The oligodeoxyribonucleotides primers were used for PCR amplification, introducing
new restriction sites, sequencing, deletion mutagenesis, and site directed mutagenesis (SDM).

2.3.4 Polymerase chain reaction (PCR)

The DNA polymerase, Taq and Pfu Turbo polymerase were used for the PCR amplification to get DNA fragment for cloning. The Taq polymerase which has comparably low fidelity, was mainly used for the colony screening PCR, checking for the presence of recombinant plasmids in bacteria. DNA polymerase obtained from Biotools was also used to amplify gene coding CR16 because of the high GC content of CR16 gene, even though the enzyme has low fidelity. Pfu was mainly used for amplification of other DNA fragments because of its high fidelity.

PCR reaction cock tail (50 μl reaction volume) was made before running the programme.
5 μl 10× reaction buffer,
2 μl 2 mM dNTP mixture,
0.2 ul template plasmid,
2 μl 10 uM forward primer,
2 μl 10μM reverse primer,
1 U of polymerase.
Sterile water was added to make cock tail up to 50 ul.

The mixture was added into thin-wall 0.2 ml PCR tube and put into PTC-100 Peltier Thermal Cycler. Reaction was performed using the following programme:
95 °C 4 min, 95 °C 45 sec, 50-65 °C, 45 sec, 72 °C for Taq polymerase for certain time (1kb/min) depending on the size of the prospective PCR product. Generally 25 cycles were applied for amplification PCR reaction or 30 cycles for screening PCR.
2.3.5 Optimized condition for PCR amplification of DNA encoding CR16

The presence of proline rich regions reduces the output of PCR product for CR16, but not for WIP, WIRE or Vrplp. PCR programme was specially optimized for amplification of CR16 gene and DNA polymerase was screened for the most efficient enzyme for PCR of CR16. DNA polymerase from Biotools and the following programme were finally found to have the highest efficiency for PCR of CR16, but still quite inefficient compared with WIP or WIRE.

95 °C 4 min,
95 °C 1 min,
75°C 3 min,
75°C 10 min,
\{ repeat 25 cycles

2.3.6 Isolation and purification of DNA fragments

2.3.6.1 DNA electrophoresis

2.5 ul Ethidium Bromid (10 mg/ml) was added to 50 ml of 1% molten agarose after the agarose cooled down to 70-80°C. Agarose was poured into gel casting tray. PCR products or other DNA samples mixed with loading dye were loaded into the gel soaked in 1× TAE buffer and gel electrophoresis was carried out at 100 V for 1 hour. The DNA bands were visualized using UV trans-illuminator and excised from the gel using scalpel.
2.3.6.2 Isolation of DNA fragments

DNA bands of correct size were cut off from the agarose gel and the DNA molecules were recovered and purified using Qiagen Gel Extraction Kit according to the manufacture instruction.

2.3.7 Digestion and ligation of insert DNA into vector

Purified DNA fragment was digested with restriction endonucleases. All digestions were performed at 37°C for 1-2 hours (for BamHI and EcoRI, incubation time was kept below 1.5 hours due to star activity of these enzymes). Normally 5 units of enzymes were used to digest 1 µg of plasmid DNA. Most of the restriction enzymes used in the study were purchased from New England Biolabs.

Purified insert DNA and linearized vector DNA were ligated using 1 unit of DNA ligase in ligation buffer (0.3 M Tris-HCl, pH 7.8; 0.1 M MgCl2; 0.1 M DTT and 5 mM ATP) for 2-16 hours at appropriate temperature (25°C for 2-4 hours, and 4°C or 16°C for 16 hours). The molar ratio of insert:vector was usually 3:1 or 5:1.

2.3.8 Amplification and isolation of plasmid DNA

2.3.8.1 Preparation of competent E.coli. cells

Small aliquot of DH5α stored in -80°C was thawed on ice and inoculated in 2 ml LB broth and incubated overnight at 37°C for. The overnight culture was transferred into 200 ml of fresh LB medium and incubated in a shaking incubator at 37°C till OD600
reaches 0.4 - 0.5. The culture was transferred to pre-cooled sterile centrifuge bottles and immediately put on ice. The cells were spun down at 2,600 g (5,500 rpm) at 4 °C for 10 minute before being re-suspended in 20 ml of ice cold sterile 100 mM MgCl₂. Cells were incubated in MgCl₂ solution for 2-4 hours to make the cells transformation competent. 20 ml sterile ice cold 50% glycerol was added to the cell suspension and mixed. Maintaining cells at 4°C at all time was crucial to prepare cells with high competence. 200 µl aliquot of the cell suspension was put into ice cold tube and dipped into liquid nitrogen before being stored at -80°C.

2.3.8.2 Preparation of electroporation competent E.coli cells

For the preparation of competent bacteria cells for electroporation, a single fresh colony of E.coli strain DH5α was incubated within 5 ml of LB broth at 37°C with 250 rpm shaking overnight. 5 ml of the overnight culture was inoculated into a 2 liter flask with 500 ml LB broth and shaken at 200 rpm in 37°C until the OD₆₀₀ of 0.5. The culture was put on ice for 30 minutes after being transferred into sterile centrifuge bottles. Cells were pelleted by centrifuging at 5000 rpm in 4°C for 15 minutes using BECKMAN Coulter™ Avanti™ J-25 Centrifuge (BECKMAN, U.S.A). Cell pellet was re-suspended in 500 ml ice-cold sterile water and spun down at 5000 rpm in 4°C for 15 minutes. Cell pellets were re-suspended in 250 ml of ice-cold sterile water and centrifuged at 5000 rpm in 4°C for 15 minutes. Cell pellets were re-suspended in 10 ml 10% glycerol and transferred to another new sterile centrifuge bottle. The cells were spun down at 10,000 rpm in 4°C for 15 minutes. The cell pellets were
re-suspended in a final volume of 1 ml 10% glycerol. The suspensions were frozen in 200 µl aliquots in liquid nitrogen and stored in -80°C. During the preparation of electroporation competent cells, incubation on ice is crucial for preparing cells with high competence.

2.3.8.3 *E.coli* transformation

Competent cells which had been stored in -80°C were thawed on ice for 5 min. 20 µl ligation reaction mixture was added to 50-100 µl competent cells and mixed gently. Cells were incubated on ice for 30 min before applying a 90 sec heat shock in a 42°C water bath. Cells were spread directly onto LB-Amp plates. If the antibiotics marker kanamycin is used for selection, cells were incubated in LB without antibiotics for 1 hour before spreading on kanamycin plate.

2.3.8.4 Electroporation of *E. coli* cells

The electroporation cuvettes were chilled on ice for 5 minutes before use. The electro-competent *E.coli* DH5α cells were thawed and incubated on ice. 1 µl of DNA in TE buffer was added to 50 µl electro-competent cell and gently mixed. The mixture was transferred into the electroporation cuvette and left on ice for 2 min. Configuration of Gene Pulser XcellTM Electroporation System (BIO-RAD, U.S.A) was set up as 25 uF capacitor, 2.5 KV and the pulse controller unit to 200 Ω for 2 mm cuvette. The cuvette was put in the sliding cuvette holder and pulsed at 2.5 KV. 1ml of ice chilled LB was added into the cuvette after a single pulse. The mixture was
transferred to an eppendorf tube and incubated at 37°C for 30 min. The cell suspension was spun down and the pellet was re-suspended in 100 μl water and plated on selective plates with antibiotics. DNA containing too much salt will make the sample too conductive and cause arcing at high voltage.

2.3.8.5 Amplification of plasmid

Single colony was picked and inoculated in LB together with antibiotics in 37°C for overnight (usually for 16 hours). The plasmid DNA was extracted using Qiagen Miniprep Kit according to manufacturer’s instruction.

2.3.9 Verification of recombinant DNA constructs

2.3.9.1 Restriction digest

Restriction enzyme digestion was employed to screen for recombinant clones. The enzymes which recognized unique restriction digest sites at either end of insert DNA, were selected to digest the plasmids. Insert DNA and vector DNA were separated by DNA electrophoresis and visualized by UV trans-illuminator. Restriction enzymes used in the study were purchased from New England Biolabs. All digestions were performed at 37°C for 2 hours except for a few enzymes which needs special optimized digestion temperature such as 25°C. Normally 5 units of enzymes were used to digest 1 μg of plasmid DNA.
2.3.9.2 Colony screening PCR

Colony screening PCR is an economical method to check for the presence of recombinant plasmids. In order to check for the presence of insert DNA cloned into the vector, the colonies were screened by PCR using the cells as template. The screening PCR was performed as follows: Single colonies were inoculated in LB liquid with antibiotic and cultured at 37°C, 250 rpm until the OD_{600} of the culture reach about 0.8. 2 μl of the culture was used as template for the PCR reaction. DNA polymerase from Biotools was used to amplify DNA fragments. Only the bacteria containing the recombinant plasmid will have a PCR product. The rest of liquid culture from positive colonies would be used for the plasmid isolation and purification. Usually restriction digestion of the isolated plasmid will be performed for further confirmation of the presence of recombinant plasmids.

2.3.10 Site directed mutagenesis (SDM)

Mutagenesis is a fundamentally important DNA technology which seeks to change the base sequence of DNA and test its effect on protein function. The mutagenesis can be conducted in vivo (in studies of model organisms, or cultured cells) or in vitro. The mutagenesis can be directed to a specific site in a pre-determined way (site-directed mutagenesis), or can be random. If a gene has been cloned and a functional assay of the product is available, it is very useful to employ a form of mutagenesis in vitro which results in alteration of specific amino acids of the gene product in a predetermined way. This is very useful when attempting to evaluate the contribution
of a specific amino acid to the biological function of a protein.

Oligonucleotide mismatch mutagenesis is a popular method of introducing a predetermined single nucleotide change into a cloned gene. Two methods based on mismatch of oligonucleotide and PCR amplification were used for site directed mutagenesis.

2.3.10.1 Site directed mutagenesis based on one-step PCR

The first method was using commercial site directed mutagenesis kit from Stratagene.

Cocktail for PCR:
DNA from Miniprep: 0.25 ul
Primer 1 (10 uM): 2 ul
Primer 2 (10 uM): 2 ul
dNTP (2 uM): 5 ul
10 x buffer: 5 ul
Pfu (2.5 unit/ul): 1 ul
Quick solution (optional): 3 ul
Water: 31.75 ul
Total: 50 ul

PCR programme:
95°C 1 min,
95°C 50 sec, 60°C 50 sec, 68°C for 1min/kb, run 18 cycles,
68°C 7min.

The PCR product was digested by 10 unit of DpnI (from NEB) at 37°C for 1 hour. 20 ul of DpnI digested PCR product was mixed with competent cells and incubated on ice for 30 min. The cells were heat shocked at 42°C for 90 sec and incubated for another 30 min on ice. All the competent cells were inoculated in LB broth without any antibiotic for 30 min before spreading onto LB plate with antibiotics.
Methods and Materials

Step 1
Plasmid Preparation

Gene in plasmid with target site (●) for mutation

Step 2
Temperature Cycling

Denature the plasmid and anneal the oligonucleotide primers (●) containing the desired mutation (×)

Using the nonstrand-displacing action of PfuTurbo DNA polymerase, extend and incorporate the mutagenic primers resulting in nicked circular strands

Step 3
Digestion

Digest the methylated, nonmutated parental DNA template with Dpn I

Step 4
Transformation

Transform the circular, nicked dsDNA into XL1-Blue supercompetent cells

After transformation, the XL1-Blue supercompetent cells repair the nicks in the mutated plasmid

LEGEND
- Parental DNA plasmid
- Mutagenic primer
- Mutated DNA plasmid

Fig.2.1 QuikChange® Site-Directed Mutagenesis (Stratagene)
A modified protocol was used for SDM instead of protocol provided by Stratagene.
2.3.10.2 Site directed mutagenesis based on two-step PCR

The second method used to generate site directed mutation was the two step overlay extension PCR. Two sets of complementary primers were designed and synthesized where the intended mutational sequences were located. Two individual PCR were set up to output two fragments of the gene with mutation (Figure 2.2). These two DNA fragment were mixed and used as template for each other by adding another two primers (Figure 2.2).

![Diagram of Site Directed Mutagenesis (SDM) by two step PCR.](image)

Figure 2.2 Site Directed Mutagenesis (SDM) by two step PCR. (Human Molecular Genetics, third edition)

Site directed mutagenesis shown above can result in an amplified product with a specific pre-determined mutation located in a central segment. PCR reactions are amplifying overlapping segments of DNA containing an introduced mutation (by deliberate base mismatching using a mutant primer - 1M or 2M). After the two products are combined, denatured and allowed to re-anneal, the DNA polymerase can extend the 3' end of heteroduplexes with recessed 3' ends. Thereafter, a full length product with the introduced mutation in a central segment can be amplified by using the outer primers 1 and 2 only.
2.3.11 Deletion mutagenesis

Systematic deletion analysis was carried out using PCR. Primers were designed for amplifying gene fragments and series of truncated DNA fragments were cloned into plasmid vector. Heterologous expression and analysis of these truncated proteins lead to the construction of a map of functional domains of these proteins.

2.3.12 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:

\[
\text{DNA concentration (ug/ul)} = \text{OD}_{260} \times \text{Dilution factor} \times \text{A}/1000;
\]

A=50 ug/ml if double stranded DNA or plasmid,

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 or/and other UV absorbers, and it is advisable to re-precipitate the DNA.

OD_{260}/OD_{280} higher than 2.0 indicates possible contamination caused by RNA.

2.3.13 DNA sequencing

DNA sequencing reaction was performed before sending samples for analysis. 8 ul BigDye terminator, 0.7 ul of 10 mM sequencing primer, 2.5 purified plasmid, 0.5 ul DMSO and 8.3 ul water was gently mixed in a 200 ul PCR thin wall tube. Sequencing
PCR programme was run as following:

95°C for 5 min,
96°C for 10 sec, 50°C for 5 sec, 60°C for 4 min, 30 cycles,

The adding of 0.5 ul DMSO to the 20 ul reaction volume helps to sequencing of plasmids which are bigger than 10 Kb or templates with secondary structure, high annealing temperature, GC rich sequence, repeats and etc. Product of sequencing PCR was sent to Tan Tock Seng Hospital for analysis on ABI Genetic Analyzer 3100.

2.4 Yeast manipulation

2.4.1 Yeast transformation

Yeast colonies were inoculated in 25ml YPUAD or SD and incubated overnight at 24°C (for mutant strains) or 30°C (for wild type strains) with shaking at 250 rpm. The following day, the overnight culture was diluted into another fresh 25 ml YPUAD or SD (to produce an OD_{600}=0.2) and incubated at 24°C or 30°C for another 3 or more hours with shaking. Cells were collected by centrifugation at 1000 × g for 5 min at RT and re-suspended in 25 ml sterile water. The cells were centrifuged again and re-suspended in 1.0 ml 100 mM LiAc and transferred into a Eppendorf tube. Cells were pelleted at top speed for 15 sec and re-suspended in a final volume of 250 μl 100 mM LiAc. 50 μl aliquot of yeast cells were transferred into each eppendorf tubes. Cells were pelleted and resuspended with transformation mix including 240 μl 50% PEG, 5 μl Salmon Sperm DNA (10mg/ml), 36 μl 1.0 M LiAc, 1–2 μg plasmid DNA in 75 μl Water. The tubes were vortexed continuously for 1 min and incubated at RT for
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30 min; then heated shock in a water bath at 42°C for 20 min. Tubes were centrifuged at 6,000 rpm for no more than 15 seconds and cell pellets were resuspended in 300 µl of water. 100 µl cell suspension were plated onto selective SD plates.

Accurate concentration and volume of PEG3550 added to the mixture helps to increase the efficiency of yeast transformation. For co-transforming two plasmids together into yeast cell, efficiency is 10 times lower than that of single plasmid transformation of yeast. Thus more cells after co-transformation should be spread onto SD selective plate.

2.4.2 DNA extraction from yeast

2.4.2.1 Plasmid DNA extraction from yeast

Yeast cells were scraped from plates and suspended in 50 µl S-Buffer (10 mM KPO4, 10 mM EDTA; pH 7.2; 50 mM Mercaptoethanol; 1.5 mg/mL Zymolase). The suspension was incubated at 37°C for 30 min. 50 µl lysis buffer (0.25 M Tris-Hcl; 25 mM EDTA; pH 7.5; 2.5% SDS) was added into the suspension with gentle vortex. The mixture was incubated in 65°C for 30 min. 88 µl 3 M KOAc was added and the tube was chilled on ice for 10 min. The mixture was centrifuged for 10 min at 14,500 rpm and the supernatant was transferred into a new tube containing 1 ml of absolute ethanol. After incubated on ice for 10 min, the tube was centrifuged for 10 min at 14,500 rpm, then the supernatant was discarded. The pellet was washed with 70% ethanol and then air-dried. The final DNA was resuspended in 20 µl TE with RNase.
2.4.2.2 Genomic DNA extraction from yeast

Single colony was inoculated into 10 ml of appropriate media such as YPUAD medium and grown in 30°C for overnight. For some mutant strains longer incubation will be necessary for accumulating enough cells. Cells were diluted in the same medium the next morning to about OD<sub>600</sub> 0.2 and incubated for 3-6 hours till OD<sub>600</sub> reach 0.5-0.6. Cells was spun down at 5000 rpm for 10 min. Supernatant was discarded, and the pellet resuspended in 0.5 ml of 1 M sorbitol, and 0.1 ml of 0.5 M EDTA. The suspension was transferred to 1.5 ml eppendorf tube. 50 ul of 5-10 mg/ml yeast lytic enzyme was added into this eppendorf tube and the mixture was incubated at 37°C for 1 hour. Tubes were spun down at top speed for 30 sec. Cells were resuspended in 0.5 ml Tris-EDTA buffer (50 mM Tris, 20 mM EDTA, pH 7.5). 50 ul of 10% SDS was added with thorough mix. The mixture was incubated at 65°C for 30 min. 0.3 ml of 5 M potassium acetate was added into the same tube and incubated on ice for 1 hour. Tube was spun at top speed for 5 min. Supernatant was transferred into a new eppendorf tube and 1 ml of isopropanol of room temperature was added and mixed. The tube was left on the bench for 5 min, and spun briefly. Pellet was washed with 1 ml 70% ethanol. Pellet was collected by another spin for 5 min and air dried for half an hour (Do not over dry). Pellet was resuspended in 100 ul of TE buffer of pH 7.5. 5 ul of 10 mg/ml RNase was added and incubated together at 37°C for 1 hour and extracted with phenol twice. 30 ul of DNA precipitation buffer (3 M Potassium(K), 5M Acetate, pH 5.8) was added and mixed, followed by addition of 200 ul of 100% ethanol. The tube was incubated on ice for 5 min and spun for 10 min.
at top speed. The supernatant was discarded and the pellet was washed with 1 ml of 70% ethanol, vortexed and spun for 5 min. Supernatant was discarded, and pellet was briefly air dried and resuspended in 0.1-0.3 ml TE buffer.

2.4.3 Protein extraction from yeast

2.4.3.1 Extraction of protein from yeast for western blot

Cells picked from a single colony were inoculated in 25 ml of YPUAD medium overnight. Overnight culture was diluted using YPUAD to OD_{600} ~ 0.2 on the next morning. Cells growing at exponential phase were harvested when OD_{600} reached to 0.5. Cells of total 7 OD_{600} Unit were pelleted by centrifugation at 1000xg. The cell pellet was resuspended in 25ml sterile water and spun again using the same centrifugation setup. Pellet was resuspended in 1 ml cold 1×TE buffer and transferred to an eppendorf tube. Cells were collected by centrifugation at 14,500 rpm for 15 seconds at RT and resuspended in 240 μl mixture containing 1.85 M NaOH and 1.06 M β-mercaptoethanol. Tube was incubated on ice for 10 min, and equal volume (240 ul) of 20% TCA was added and gently mixed by pipetting up and down. After being incubated on ice for 10 min, cells were centrifuged at 14,500 rpm for 10 min and the pellet was washed with 1 ml ice-cold acetone. The protein pellet was collected by centrifugation at 14,500 rpm for 10 min and resuspended in a final volume of 200 μl Urea buffer (containing 8 M Urea, 50 mM Tris, pH6.8 and 2% SDS). The resuspension of pellet in room temperature is a slow process and need around 1-2 hours. Finally 15 μl of protein sample was loaded onto SDS-PAGE gel for
electrophoresis.

2.4.3.2 Extraction of native protein from yeast for biochemical assay

Yeast colonies were inoculated in 25ml YPUAD and incubated at 30°C (wild type) or 24°C (mutant strain) for overnight with shaking at 250 rpm to stationary phase. The overnight culture was diluted into 200 ml YPUAD (to produce an OD$_{600}$=0.2) and incubated at same temperature for 3 hours with shaking till OD$_{600}$ reached 0.5-0.6. Cells were transferred to several 50 ml falcon tubes and centrifuged at 2000 × g for 5 min at 4°C. Cell pellets were resuspended in 50 ml sterile water and centrifuged again. Cells were resuspended in 4 ml water and distributed into 4 eppendorf tubes and spun down for 15 sec at top speed. The supernatant was discarded. Pellet in each tube was resuspended in 0.2 ml of ice cold lysis buffer (PBS with protease inhibitors 5 ul/g dried cells pellet). Glass beads were added to each tube till the surface of suspension reached to the 0.5 ml scale bar. The tubes were placed in the Mini-Beadbeater$^{TM}$ (Biospec Product, Bartlesville, OK, USA) located in cold room, apply the high speed homogenize programme for 4 x 30 sec with 1 min interval. A small hole was made at the bottom of each tube. The rear part of each tube was put into another new eppendorf tube individually. Spin for 1 min at 6000 rpm in 4°C and collect the supernatant. Add 200 ul lysis buffer to the beads and spin again to collect more of the washings. Discard the inner tube with glass beads. Spin the outer tube with washings at top speed for 5 min in 4°C to remove cells debris. Supernatant was collected and transferred into a new eppendorf tube. The native proteins with lysis buffer in the new
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tube are ready for biochemical assays.

2.4.4 Assay for viability at high temperature

Yeast colonies from selective plate were picked using sterile tooth picks and streaked onto YPUAD plate to obtain single colonies. The YPUAD plate was incubated at 37°C for 3 days.

2.4.5 Viability of yeast on high salt plate

Yeast colonies from selective plate was picked using sterile tooth picks and streaked onto YPUAD-NaCl plate (Usually NaCl concentration was 1M-0.5M). The YPUAD plate was incubated at 24°C for 5 days.

2.4.6 Endocytosis assay

Yeast colonies were inoculated in 25ml YPUAD and incubated at 30°C (wild type) or 24°C (mutant strain) with shaking (200 rpm) overnight. The overnight culture was diluted into 25 ml YPUAD (to produce an OD<sub>600</sub>=0.1-0.2) and incubated at same temperature for around 3 hours with shaking till OD<sub>600</sub> reached 0.4-0.5. 1 ml of cells were pipetted out and transferred to a 1.5 ml eppendorf tube. The lid of the tube was punctured with a large Gauge needle (such as BD PrecisionGlide™ Needle 18G11/2 TW) several times to make holes on the lid. Gently spin down cells in micro-centrifuge at 3000 rpm for 1 minutes at room temperature. The supernatant was removed and the cell pellet was re-suspend in 90 ul of fresh YPUAD. 10 ul of Lucifer Yellow stock solution (50 mg/ml Lucifer yellow carbohydrazide, dilithium salt in
H$_2$O) was added to the tube. Aerate the sample with several flicking of tube. Cells were incubated at 24°C for 1 hour with occasionally flicking to aerate the sample or with constant shaking. Endocytosis was stopped by adding 1 ml ice cold wash buffer (50 mM Na$_2$H$_2$PO$_4$, 10 mM NaF, 10 mM Na azide, pH 7) and immediately put on ice. Cells were gently spun down at 3000 rpm for 2 min at room temperature. Supernatant was removed gently by pipetting and cells were resuspended in 1 ml 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 ul of ice cold washing buffer and left on ice. 1 ul of cell suspension was pipetted onto a clean microscope slide. A cover slip was placed on the cell suspension immediately and pressed gently and evenly. Cells on slides were observed using microscope under fluorescein isothiocyanate (FITC) filter to view Lucifer yellow, and under Nomarski (differential interference contrast) filter to view the vacuole morphology.

Cell density is critical for this assay. Thus the cell density was maintained around 1 x $10^7$ cells/ml. Lucifer yellow accumulates in the vacuole in wild type cells. It also binds non-specifically to cell wall in all cells. Mutant cells such as vrplA strain show surface staining only and no vacuolar staining. Cells should not be spun down at high speed in order to maintain normal vacuolar morphology.

For endocytosis assay at high temperature such as 37°C, cells were shifted from 24°C to 37°C for 1 hour before addition of Lucifer yellow dye, subsequently they were
inoculated at 37°C for another 1 hour before washing as described above.

2.4.7 Actin patches polarization assay by quick F-actin staining

Yeast colonies were inoculated in 25ml YPUAD and incubated at 30°C (wild type) or 24°C (mutant strain) overnight with shaking at 200 rpm to stationary phase. Next morning the overnight culture was diluted into 25 ml YPUAD (to produce an OD$_{600}$=0.1-0.2) and incubated at same temperature for around 3 hours with shaking till OD$_{600}$ reached 0.4-0.5. 900 ul cells of exponential growth phase were pipetted into a new eppendorf tube and mixed with 100 ul of 37% formaldehyde by pipetting. Cells were incubated in room temperature for 1-10 min. Cells were spun down at 3000 rpm for 3 min. Cell pellet was washed 3 times with PBS and permeabilized by resuspension of cells in PBS with 1% Triton X-100 for 30 sec. Cells were continuously pipetted up and down during this 30 sec. Cells were spun down and washed 3 times with PBS, resuspended in 50 ul of PBS with 0.1 mg/ml rhodamine or Alexa fluorescence 488 conjugated phalloidin and incubated for 5 min at room temperature. Cells were washed 3 or more times with PBS to reduce the fluorescence background. The cell pellet was resuspended in 10 ul of PBS. 1 ul of cell suspension was put onto a glass slide and a cover slip was placed, gently and evenly, on this drop of cells immediately. Cells were viewed under appropriate filter set.
2.4.8 Yeast two hybrid assay

2.4.8.1 Yeast two hybrid assay

As a useful technique, yeast two-hybrid screening is used to identify novel protein interactions and define interacting domains. The bait gene was expressed as a fusion to the GAL4 DNA-binding domain (BD), while yeast genomic libraries were fused with GAL4 activation domain (AD) in different frames (AD-library). When bait BD fused protein and library AD fused proteins interact, the GAL4-BD and GAL4-AD were brought into proximity, thus activating transcription of two reporter genes (ADE2 and HIS3).

Plasmids pBD-bait and mammalian genomic libraries fused with activation domain in different frames were sequentially transformed into yeast strain PJ69-4A. The transformant was selected on SD-Trp-Leu-His (with 2 mM 3-AT) plates. After incubated at 30°C for 5-10 days, colonies were streaked out onto SD-Trp-Leu-Ade plates for another one week to eliminate false positives. Plasmids were extracted from those colonies cells, and then transformed into *E.coli* by electroporation. Plasmids extracted from *E.coli* are sent for DNA sequencing. Novel proteins or domains were found with prospective interaction after alignment of sequencing result with protein data base.
2.4.8.2 Yeast two hybrid assay for protein-protein interaction

Yeast two-hybrid assay is also used to identify the interaction among known proteins or domains. Verprolin family of genes were cloned into pAS2-1 plasmid downstream of GAL4 DNA-binding domain (BD), while other known gene or deletions were cloned into pACT2 plasmid downstream of GAL4 activation domain (AD). When these two proteins interact, the GAL4-BD and GAL4-AD were brought into proximity and transcription of two reporter genes (ADE2 and HIS3) was activated. Transformant cells with activated transcription were selected on two different selective plate, SD-Trp-Leu-His with 2mM 3-AT. 3-AT is used to function as inhibitor of unspecific activation of report gene transcription.

2.5 Western blot analysis

Specific proteins were detected by antibody using Western Blot. After SDS-PAGE electrophoresis, proteins were transferred to a Protran® Nitrocellulose Transfer Membrane (from Schleicher & Schuell, Germany) in transfer buffer (20 mM Tris-HCl, 144 mM glycine, 20% methanol) using a mini Trans-Blot Electrophoretic Transfer Cell (BIO-RAD, USA) at 85 volts for 2 hours in 4°C. It is essential for a good transfer result to make sure that there is not air bubble between any of two layers when assembling sponge-paper-gel-membrane sandwich in transfer cassette because the protein would be trapped in the filter papers by the air bubble.

Running condition is constant voltage such as 85 V for 2 hours or 65 V for overnight.
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The current should be around 200-250 mA. If current is high than 250 mA, transfer buffer should be replaced by another batch and better cooling system would be applied. For the protein which molecular weight is less than 50 kDa, the transfer time should not exceed 2 hours as some small molecules could go through the Nitrocellulose Transfer Membrane. If the molecular weight of protein is more than 120 kD, the transfer time should be prolonged and not less than 2 hours.

After transfer, the membrane was placed in a shaking container and blocked with 5% milk in PBS on a rocker platform for 0.5-1 hour at room temperature. Membrane was then incubated with appropriate dilution of primary antibody in PBS with 2-5% milk on a rocker plate for 1 hour at room temperature or overnight at 2-8 °C. Membrane was washed 3 x 10 min with washing buffer (PBS with 0.05% Triton X-100) to remove unbound antibodies and non-specific bound proteins. The membrane was incubated with 1:10000 diluted, horse radish peroxidase conjugated secondary antibody in PBS containing 5% milk at room temperature for 1-2 hours in a shaking container. Membrane was washed 3 x 10 min with washing buffer again to remove unbound antibodies and non-specific bound proteins. The membrane was finally incubated with the mixture of SuperSignal® West Pico Chemiluminescent Substrate (1 ml SuperSignal® West Pico Stable Peroxide Solution mixed with 1 ml SuperSignal® West Pico Luminol Enhancer Solution, PIERCE, U.S.A) for 5 minutes. Signal was detected using Hyper (Amersham Pharmacia Biotech, UK) film to expose for proper time until bands were visualized properly.
For western blot, optimal antibody concentration varied greatly from 1:200 to 1:10⁴ and should be determined experimentally. Concentration of milk in PBS varied from 2-5% and helped to reduce the unspecific bands.
Results

3 Functional analysis of WIP and WIRE in \textit{vrp1A} strain

WIP and WIRE are mammalian members of Verprolin family of proteins. Deficiency of WIP causes profound defects in the actin filament network in lymphocytes (Anton, \textit{et al.}, 2002). Vrp1p is an actin associated protein and is involved in cell polarity and endocytosis (Donnelly, \textit{et al.}, 1993, Munn, \textit{et al.}, 1995). Deletion of VRP1 gene leads to cytoskeletal defects such as loss of actin patches polarization, loss of fluid phase endocytosis, loss of viability at elevated temperature or on plates with high salt concentration. WIP and Vrp1p are functional homologues as expression of WIP suppresses the growth defect as well as cytoskeletal defects of \textit{vrp1} mutant yeast strain (Vaduva, \textit{et al.}, 1999).

\textit{S.cerevisiae} has a well characterized genetic background and only one copy of VRP1 gene, functional analysis of mammalian Verprolin proteins using yeast system will give clearer result than using mammalian system. Thus, WIP or WIRE was expressed either from a low or a high copy plasmid in \textit{vrp1A} strain to check its activity in suppressing cellular defects such as endocytosis, viability at high temperature or actin patches polarization. Deletion analysis and site directed mutagenesis will help to delineate the functional domains of WIP and WIRE.
3.1 Expression of WIP in vrplA strain

3.1.1 Mammalian WIP suppress the growth and endocytosis defects of yeast vrplA strain

3.1.1.1 WIP expressed from low copy plasmid does not suppress the growth defect of vrplA strain

Vrp1p is an actin associated protein and S. cerevisia with vrpl deletion are unable to grow at high temperature and have defects in endocytosis and actin patch polarization. Expression of WIP using Gal inducible promoter is able to suppress the growth defects of both vrpl-l and vrplA strain while WIP is able to suppress endocytic defect of vrpl-l but unable to suppress that of vrplA strain (Vaduva, et al., 1999). In vrpl-l strain, it is not possible to distinguish the contribution of WIP in suppressing the growth defect of vrpl-l because of the presence of mutant Vrp1p with uncharacterized activities. It will be helpful to establish a system where the mammalian WIP is able to suppress the growth defects of vrplA strain and use the system to characterize the functional domains of WIP.

In order to express WIP in vrplA strain (AMY88), the cDNA encoding WIP gene was amplified using PCR and placed under the transcriptional regulation of VRPl promoter. This construct was placed on a low copy plasmid Ycplac111 (Figure 3.1). This plasmid was introduced into vrplA strain by lithium acetate transformation and the transformants were analyzed for growth at high temperature. WIP expressed from
a low copy plasmid was unable to suppress the growth, endocytosis or actin patches polarization defect of this strain (data not shown). This lack of complementation could be due to several reasons, including poor localization and poor expression of WIP in *S.cerevisiae*.

![Diagram](image)

Figure 3.1 Construction map of WIP DNA encoding WIP was fused to the 5' end of DNA encoding GST, and cloned into Ycplac111, the low copy plasmid under the control of VRP1 promoter.

### 3.1.1.2 WIP localizes to cortical patches in *vrp1A* strain

In order to check for the localization of WIP in yeast cells, DNA encoding GFP was fused to the 3' end of DNA encoding WIP. This WIP-GFP construct was introduced into *vrp1A* strain on low copy plasmid and the transformants were viewed under a fluorescent microscope. WIP-GFP was found to localize to cortical patches (Figure 3.2) similar to Vrp1p (Thanabal and Munn, 2001). Addition of CAAX box motif to N-Vrp1p, the yeast homologues of WIP, has been shown to enhance the localization to
cell membrane and activity of N-Vrp1p in vrplA (Thanabalu and Munn, 2001). Fusion of GST has been known to stabilize ectopic proteins which are unstable in host cells (Yang, et al., 2005). In order to check whether we can enhance the activity of WIP we fused the CAAX motif (C is cysteine residue, A is aliphatic residue, X can any type of residues. The sequence of CAAX box used for this research is CIIC) (Powers, et al., 1984) or GST to the C-terminus of WIP and introduced the low copy plasmid expressing WIP-CAAX or WIP-GST construct into vrplA cells. Addition of GST did not improve the activity of WIP in vrplA cells (Figure 3.3A) and the CAAX motif did not improve the activity of WIP (data not shown). The lack of activity of WIP expressed from low copy plasmid is probably due to insufficient expression.

3.1.1.3 WIP-GFP localized to actin patches

As mentioned above, WIP localized to cortical patches in vrplA strain. In order to check whether these patches were cortical actin patches, plasmid expressing WIP-GFP and Arc40-RFP were co-transformed into vrplA strain. Arc40p is a component of Arp2/3 complex which localizes to cortical actin patches (Winter, et al., 1999). Cells expressing both WIP-GFP and Arc40-RFP were viewed under fluorescence microscopy using GFP and RFP filter respectively (Figure 3.2). Yeast two hybrid assay showed that there is no interaction between WIP and Arc40p (data not shown), therefore the observed co-localization of WIP and Arc40p is not due to physical interaction between these two proteins, indicating that WIP localizes to
Functional analysis of WIP and WIRE

cortical actin patches independent of Arc40p.
Figure 3.2 WIP localizes to actin patches
WIP-GFP was co-expressed with Acr40p-RFP in *vrplA* strain. Transformant cells expressing GFP or WIP-GFP with Acr40p-RFP were viewed under GFP filter or RFP filter. Bar, 5 μm.

3.1.1.4 WIP expressed from high copy plasmid suppressed growth and endocytosis defects of *vrplA* strain

As mention above, WIP localizes to cortical patches, but is not able to suppress the growth defect of *vrplA* strain. This lack of function of WIP is not due to the lack of localization to cortical patches, but might due to low expression or poor stability in yeast cells. It has been shown that deletion of the V domain of WIP leads to loss of protein stability (Vaduva, *et al.*, 1999), and that translation fusion with GST has been shown to stabilize proteins. Therefore we placed the WIP-GST on YEplac181, a high copy plasmid to enhance the expression and stability of this protein. This plasmid was
then introduced into \textit{vrp1A} strain and the transformants were analyzed for growth at high temperature. The \textit{vrp1A} transformants were able to grow at 37°C (Figure 3.3A) suggesting that WIP-GST expressed from a high copy plasmid was able to suppress the growth defect of \textit{vrp1A} strain. The \textit{vrp1A} transformants were analysed for fluid phase endocytosis and actin patch polarization. Expression of WIP-GST from a high copy plasmid suppressed the endocytosis defect but not the actin patch polarization defect of \textit{vrp1A} strain (Figure 3.3B and Figure 3.4A). WIP-GST expressed from high copy plasmid suppressed the growth defect while WIP or WIP-GST expressed from low copy plasmid did not, indicating that copy number of the gene encoding WIP and consequently the expression level are crucial for the suppression of growth defect in \textit{vrp1A} strain. It was reported that the ability to suppress the actin patches polarization defect was correlated with the ability to grow on high salt (1M NaCl) YPD plate (Thanabalu and Munn, 2001). The cells expressing WIP-GST from high copy plasmid were streaked on YPUAD plate with 1 M NaCl and incubated in 24°C for 5 days. These cells were not able to grow on 1 M NaCl YPUAD plate (data not shown) confirming that the WIP-GST expressed from high copy plasmid was not able to suppress the actin patches polarization defect.
Figure 3.3 WIP-GST expressed from high copy plasmid suppresses the growth defect of \( vrplA \) strain

A) DNA encoding WIP-GST was introduced into \( vrplA \) cells, transformants were streaked on YPUAD and incubated at 24°C and 37°C for 3 days respectively. “LC” indicates the expression from low copy plasmid, “HC” indicates the expression from high copy plasmid.

B) Actin staining of \( vrplA \) cells expressing WIP-GST from low copy plasmid or high copy plasmid respectively. Cells growing to exponential phase were fixed using formaldehyde and permeabilized with Triton X-100. Cells were then stained using Alexa fluorescence 488 conjugated phalloidin. Bar, 5 \( \mu m \).
Figure 3.4 WIP-GST expressed from high copy plasmid suppresses the endocytosis defect of \textit{vrrp1A} strain

A) Endocytosis assay of \textit{vrrp1A} cells expressing WIP-GST fusion proteins from low copy plasmid or high copy plasmid respectively. Cells growing to exponential phase were re-inoculated with YPUAD including Lucifer Yellow dye for one hour before viewed under the fluorescence microscope.

B) WIP-GST was introduced into \textit{vrrp1A} strain and expressed from low copy plasmid or high copy plasmid respectively. Cells expressing fusion proteins were incubated in YPUAD, and lysed when the OD$_{600}$ reached 0.5. the cell lysate was analysed by western blot. Fusion proteins were detected using anti-GST. Anti-Hexokinase was used to indicate the level of total proteins.
3.1.1.5 WIP interacts with actin but not Las17p

Yeast two hybrid assay was carried out to verify the interaction between WIP and actin or Las17p. The yeast two hybrid system that we are using is able to identify the interaction between proteins by the ability of transformants to grow on SD(-Trp-Leu-His) plates.

Human WIP interacts with WASP and Las17p is the yeast homologues of WASP. In order to check whether the human WIP interacts with Las17p, DNA encoding WIP was cloned into pACT2 (activation domain), DNA encoding Las17p was cloned into pAS2-1 (binding domain) and both of them were co-transformed into PJ69-4A. The yeast transformants with both of the two plasmids (pAD-WIP and pBD-Las17) were selected on SD (-Trp-Leu) plates and streaked on SD(-Trp-Leu-His) plates to check the growth. There was no visible growth on SD(-Trp-Leu-His) plates (Figure 3.5) indicating that Las17p does not interact with WIP. The yeast transformants expressing AD-WIP and BD-Actin were able to grow on SD(-Trp-Leu-His) showing that WIP interacts with actin (Figure 3.5).
3.1.2 The V domain but not the actin binding motif, is important for the function of WIP in vrp1A strain

3.1.2.1 V domain of WIP is crucial for suppressing the growth defect

Vrp1p interacts with actin through residues 1-70 (WH2/V domain) and the actin binding motif has been identified as the KLKK motif (Vaduva, et al., 1997). To address the role of WH2/V domain in WIP function, this domain (N-terminal 1-50 residues) was deleted and WIP51-503-GST (WIPΔV-GST) expressed in vrp1A strain from a high copy plasmid. Expression of WIP51-503-GST in vrp1A failed to correct the growth or endocytosis defect of vrp1A strain (Figure 3.6), suggesting that the V domain is essential for WIP’s ability to suppress growth and endocytosis defect.
Functional analysis of WIP and WIRE

Figure 3.6 Growth and endocytosis assay of \textit{vrp1A} cells expressing WIP\textsubscript{51-503}-GST from high copy plasmid.

A) Transformants of \textit{vrp1A} cells expressing WIP-GST, WIP\textsuperscript{4A}-GST or WIP\textsubscript{51-503}-GST from high copy plasmid were streaked on YPUAD plate and incubated at 37°C or 24°C for 3 days.

B) Cells of \textit{vrp1A} strain expressing WIP-GST, WIP\textsubscript{51-503}-GST from high copy plasmid were inoculated in YPUAD to exponential phase, and incubated in YPUAD with Lucifer Yellow dye for one hour before viewed under fluorescence microscope.

C) Cells of \textit{vrp1A} strain expressing WIP-GST or WIP\textsuperscript{4A}-GST from high copy plasmid were lysed and the cell lysate was assessed by electrophoresis. GST fusion proteins were detected by anti-GST antibodies and total protein was evaluated by anti-Hexokinase.
3.1.2.2 V domain of WIP is crucial for interacting with actin

DNA encoding WIP_{51-503} (WIPΔV) was cloned into pACT2 (DNA activation domain, AD) and the DNA encoding Act1p was cloned into pAS2-1 (DNA binding domain, BD). Both of these two recombinant plasmids were introduced into pJ69-4A strain to check for interaction between WIP_{51-503} and actin. WIP_{51-503} does not interact with actin as assessed by yeast two hybrid assay (Figure 3.7, Table 3.1). This shows that WIP’s interaction with actin is mediated by the V domain and this domain is crucial for WIP’s activity in *S.cerevisiae*.

Figure 3.7 Yeast two hybrid assay for determining the interaction between WIP truncates and mutants with Actin
Each of WIP truncates was cloned into pACT2, Actin was cloned into pAS2-1. Both AD-WIP mutants and BD-Actin were introduced into PJ69-4A, and transformant cells were streaked on SD-Trp-Leu-His plate.

Table 3.1 Yeast two hybrid assay.

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<tr>
<td>AD-WIP_{1-503}</td>
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<tr>
<td>AD-WIP_{51-503}</td>
<td>-</td>
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<tr>
<td>AD-WIP^{4A}</td>
<td>+</td>
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<td>AD-WIP^{LNK}</td>
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<tr>
<td>AD-WIP^{4A-LNK}</td>
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</table>
3.1.2.3 Actin binding motif is not crucial for suppressing the growth or endocytosis defects of *vrp1A* cells

It was reported that the verprolin family of proteins have a conserved actin binding motif “KLKK” (Aspenstrom, 2005). Though the consensus sequence of this actin binding motif is “KLKK”, in the case of WIP, there is an extra lysine in the N-terminal of “KLKK” motif representing “KKLKK”. To address whether “KKLKK” motif is important for the function of WH2/V domain, we mutated the KKLKK motif to “AALAA” (named as 4A) using site directed mutagenesis. WIP^{4A}-GST mutant under the transcriptional regulation of VRP1 promoter on a high copy plasmid was introduced into *vrp1A* strain. The *vrp1A* transformants expressing WIP^{4A}-GST were able to grow at 37°C and take up lucifer yellow dye (Figure 3.8 A and B) indicating that the KKLKK motif of WIP is not crucial for suppressing the growth or endocytosis defect of *vrp1A* strain. This result was inconsistent with the report in which the “KLKK” mutation had been shown to be crucial for its activity to suppress growth defect (Vaduva, *et al.*, 1999). Since WIP^{4A}-GST is still able to suppress growth and endocytosis defect of *vrp1A* strain, there are probably some other regions which are responsible for suppressing the defects.
Figure 3.8 KKLKK motif of WIP is not critical for suppressing the growth or endocytosis defect
A) Growth assay of \( vrplA \) cells expressing \( WIP^{4A} \)-GST, \( WIP_{51-503} \)-GST or WIP-GST from high copy plasmid.
B) Endocytosis assay of \( vrplA \) cells expressing \( WIP^{4A} \)-GST.
C) Western blot for assessing the expression of GST fusion proteins using anti-GST antibody. Total protein was evaluated using Anti-Hexokinase antibody. Bar, 5 \( \mu m \).
3.1.2.4 Actin binding motif is not essential for the interaction between WIP with actin

In order to verify whether the K$_{44}$KLK$_{48}$ motif is the only actin binding site of WIP, we cloned the DNA fragment encoding WIP$^{4A}$ into pACT2 and introduced pAD-WIP$^{4A}$ into pJ69-4A together with pBD-Actin to check whether the K$_{44}$KLAA$_{48}$ or A$_{44}$ALAA$_{48}$ mutation would abolish interaction with actin. Transformants of PJ69-4A cells expressing both pAD-WIP$^{4A}$ and pBD-Actin grew on SD-Trp-Leu-His plate indicating that mutating KKLKK to AALAA does not abolish WIP’s interaction with actin (Figure 3.7, Table 3.1). This also indicates the existence of other unknown actin binding sites in V domain of WIP since deletion of the WH2/V domain abolished interaction with actin. This also suggests that the V domain is important for WIP’s ability to suppress the growth defect and important for interaction with actin while KKLKK is not essential for activity or actin binding.

3.1.2.5 LNK motif of WIP is not essential for actin binding

A conserved LNK motif was reported to be present among WASP and verprolin family of protein (Miki, et al., 1996) and to be a potential actin binding motif of low-affinity (Marchand, et al., 2001). Sequence analysis showed that there is a 25LNK$_{27}$ motif in the N-terminus of WIP. Thus 25LNK$_{27}$ motif was mutated to 25AAA$_{27}$ (WIP$^{LND}$). The experiments showed that both WIP$^{LND}$-GST (LNK mutation) and WIP$^{4A-LND}$-GST (combination of KKLKK and LNK mutations) expressed from
Functional analysis of WIP and WIRE

high copy plasmid suppressed the growth and endocytosis defect of vrplΔ strain (Figure 3.9 A and B). Yeast two hybrid assay showed that both WIP\textsuperscript{L NK} and WIP\textsuperscript{AA-L NK} interact with actin (Figure 3.7, Table 3.1). This indicates that the LNK motif of WIP is not essential for either actin interaction or for suppressing the growth defect.

3.1.3 The KKLKK motif and proline rich region play redundant role in suppressing the growth and endocytosis defect

3.1.3.1 The N-terminal proline rich region is not crucial for the suppression of growth or endocytosis defect

WIP\textsubscript{1-503}, but not WIP\textsubscript{51-503} (WIP\textsuperscript{AV}) expressed from high copy plasmid suppresses the defect of growth in vrplΔ strain, indicating that there are certain motifs crucial for this function of WIP located in the region 1-50. It has been reported that WIP and WIRE have ABM-2 motifs which are implicated in binding to profilin (Holt and Koffer, 2001, Purich and Southwick, 1997) at the N-terminus. Profilin is an actin binding protein that promotes actin polymerization (Korenbaum, et al., 1998). Thus it raises an interesting question whether the profilin binding motif at the N-terminal of WIP is crucial for the suppression of growth and endocytosis defect of vrplΔ strain. DNA encoding WIP\textsubscript{14-503} was fused to GST and expressed using VRP1 Promoter from a high copy plasmid in vrplΔ strain. WIP truncate without the first 13 residues of N-terminal (WIP\textsubscript{14-503}) expressed from high copy plasmid suppresses the growth and endocytosis defect (Figure 3.10), indicating that proline rich region WIP\textsubscript{1-13} is not essential for the suppression of growth or endocytosis defect. Thus there should be
Figure 3.9 LNK motif of WIP is not crucial for suppressing the growth or endocytosis defect of vrplA strain

A) Growth at 37°C of vrplA cells expressing WIP\textsuperscript{LNK}-GST or WIP\textsuperscript{4A-LNK}-GST from high copy plasmid. Transformant vrplA cells were streaked on YPUAD plates and incubated at 24°C and 37°C respectively for 3 days. 

B) Transformant of vrplA cells growing at exponential phase expressing WIP\textsuperscript{LNK}-GST or WIP\textsuperscript{4A-LNK}-GST from high copy plasmid were incubated in fresh YPUAD media including Lucifer Yellow for 1 hour before analysis using fluorescence microscope.

C) Cells of vrplA strain expressing WIP-GST, WIP\textsuperscript{4A}-GST, WIP\textsuperscript{LNK}-GST or WIP\textsuperscript{4A-LNK}-GST from high copy plasmid were lysed and the cell lysate was assessed by electrophoresis. GST fusion proteins were detected by anti-GST antibody and total protein was evaluated by anti-Hexokinase.
other motifs located in residues 1-50 which are crucial for the activity of WIP to restore endocytosis and cell viability at high temperature.

3.1.3.2 The KKLKK motif and proline rich region play redundant role in suppressing the growth and endocytosis defect of vrplA strain

Alignment of verprolin family of proteins shows that there are three conserved regions: a V domain at N-terminal including a proline rich region and a conserved actin binding motif KKLKK locating in the residues 1-50; and a C-terminal WASP/N-WASP/Las17p binding domain which mediates the interaction with WASP family of proteins. Single deletion of N-terminal proline rich region of WIP does not abolish the suppression of growth or endocytosis defect, and single site directed mutagenesis of KKLKK motif (WIP^{4A}) does not abolish the suppression of growth or endocytosis defect while deletion of V domain abolishes WIP’s ability to suppress either of these defects in vrplA strain. Therefore I hypothesized that KKLKK motif and proline rich region may play redundant role in suppressing the growth and endocytosis defect of vrplA strain.

In order to test the hypothesis, deletion mutation of the proline rich region in N-terminal of WIP, was combined with site directed mutagenesis of actin binding site through replacement of KKLKK by AALAA (WIP^{4A}). WIP^{4A}_{14-503}-GST was expressed from high copy plasmid in vrplA cells. vrplA transformants expressing WIP^{4A}_{14-503}-GST did not grow at 37°C on YPUAD plate and failed to take up Lucifer
Functional analysis of WIP and WIRE

yellow suggesting that WIP$^{4A,14-503}$-GST does not restore the endocytosis or cell growth at high temperature (Figure 3.10, Table 3.2). This indicates that the N-terminal proline rich region and KKLKK motif of WIP play redundant roles in suppressing the growth and endocytosis defect of $vrplA$ strain.

3.1.4 Actin binding, but not KKLKK motif, is essential for WIP to suppress the growth defect

As data showed above, with deletion of putative profilin binding site and mutation of KKLKK motif, WIP lost the ability to suppress of growth defect at high temperature in $vrplA$ strain. In order to determine whether the inability of WIP mutant to suppress the growth defect of $vrplA$ strain is due to lack of actin interaction, WIP$^{4A,14-503}$ or WIP$^{14-503}$ was cloned into pACT2 and fused to the C-terminus of activation domain of Gal4p transcription factor. Then either of the plasmid was co-transformed into PJ69-4A together with pBD-Actin. PJ69-4A transformants with pAD-WIP$^{4A,14-503}$ and pBD-Actin, did not grow on SD-Trp-Leu-His plate while cells of pAD-WIP$^{14-503}$ grew, indicating that WIP$^{4A,14-503}$, but not WIP$^{14-503}$, has lost the interaction with actin (Table 3.2). Therefore it suggests that interaction between WIP and actin is essential for suppressing the growth and endocytosis defect of $vrplA$ strain. This indicates that the 13 amino acids are involved in the interaction with actin though it is not clear whether this is a direct or indirect interaction, and indicates that the first 13 proline rich residues of WIP co-operate with the KKLKK motif and play a redundant role in the suppression of growth and endocytosis defect mediated by actin interaction.
Figure 3.10 The KKLKK motif and proline rich region play redundant role for the suppression of grow and endocytosis defect

A) Growth at 37°C of vrplA cells expressing WIP14-GST, WIP14-503-GST or WIP14-503-GST from high copy plasmid. Transformant vrplA cells were streaked on YPUAD plates and incubated at 24°C and 37°C respectively for 3 days before the growth was analysed.

B) Cells of vrplA strain expressing WIP-GST, WIP14-GST, WIP14-503-GST or WIP14-503-GST from high copy plasmid were lysed and the cell lysate was assessed by electrophoresis. GST fusion proteins were detected by GST antibody and total protein was evaluated by Anti-Hexokinase.

C) Transformant vrplA cells of exponential phase expressing WIP14-GST, WIP14-503-GST or WIP14-503-GST were incubated in fresh YPUAD media including Lucifer Yellow for 1 hour before viewed under fluorescence microscope. Bar, 5 μm.
Table 3.2 Suppression of growth defect in \textit{vrplA} strain and interaction with actin of WIP truncates. (In this thesis, if there is no other annotate, the term "Actin" refers to yeast actin, the expression product of gene Act1)

<table>
<thead>
<tr>
<th></th>
<th>Growth at 37°C</th>
<th>Endocytosis</th>
<th>Interaction with actin</th>
</tr>
</thead>
<tbody>
<tr>
<td>WIP</td>
<td>1</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>WIP$^{4A}$</td>
<td>1 4A</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>WIP$^{14-503}$</td>
<td>14</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>WIP$^{4A}_{14-503}$</td>
<td>14 4A</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>WIP$^{51-503}$</td>
<td>51</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

In order to detect for direct interaction between the proline rich sequence (1-13) and actin, attempts were made to express the V domain (1-50) as a GST fusion protein in \textit{E.coli}. However there was degradation and the fusion protein could not be purified.

### 3.1.5 Mammalian WIP interacts with yeast SH3 domain-containing proteins

Vrp1p has been shown to interact with many proteins with SH3 domain, such as Myo3p (Evangelista, \textit{et al.}, 2000), Myo5p (Goodson, \textit{et al.}, 1996), Hof1p (Ren, \textit{et al.}, 2005), Mti1p (Mochida, \textit{et al.}, 2002), Bzz1p (Soulard, \textit{et al.}, 2002) and Rvs167p (Munn, \textit{et al.}, 1995). Most of these proteins have also been shown to be essential for growth at high temperature as well as endocytosis and actin patch polarization. It is possible that WIP is unable to suppress the actin patch polarization defect because WIP does not interact with one or more of the proteins which are known to interact
with Vrp1p. To check for the interaction between the proteins with SH3 domain and WIP, we sub-cloned DNA encoding WIP into pAS2-1 and sub-cloned the DNA encoding SH3 domains of the above 6 proteins into pACT2 individually. pBD-WIP was introduced into pJ69-4A together with one of the pAD-SH3 domains constructs to check for interaction using the yeast two hybrid. Yeast two hybrid results showed that WIP interacts with SH3 domains of Hoflp and Bzzlp while interaction with Myo3p, Myo5p, Mti1p and Rvs167p was weak (Table 3.3). Vrp1p has also been shown to interact with Las17p, yeast homologue of human WASP. Thus the interaction between WIP and Las17p was analyzed but no interaction between these two proteins was detected using yeast two hybrid assay (Table 3.3). Thus the lack of inability of WIP to suppress the Actin patch polarization defect could be due to the lack of interaction with Las17p.

Table 3.3 Interaction assessed by yeast two hybrid between WIP and Profilin or SH3 domain-containing proteins.

<table>
<thead>
<tr>
<th>AD</th>
<th>BD-WIP</th>
</tr>
</thead>
<tbody>
<tr>
<td>AD-Hofl</td>
<td>+</td>
</tr>
<tr>
<td>AD-Myo5</td>
<td>+/-</td>
</tr>
<tr>
<td>AD-Bzz1</td>
<td>+</td>
</tr>
<tr>
<td>AD-Mti1</td>
<td>+</td>
</tr>
<tr>
<td>AD-Myo3</td>
<td>+</td>
</tr>
<tr>
<td>AD-Rvs167</td>
<td>+</td>
</tr>
<tr>
<td>AD-WASP</td>
<td>+</td>
</tr>
<tr>
<td>AD-Las17p</td>
<td>-</td>
</tr>
</tbody>
</table>

AD-WASP indicate that full length of the proteins was expressed as fusion proteins of activation domain of Gal4p transcription factor. The others indicate that SH3 domains of these proteins were expressed as fusion proteins of activation domain of Gal4p transcription factor.
3.1.6 Localization is crucial for WIP's ability to suppress the growth defect

3.1.6.1 Residues 1-317 of WIP is essential for restoring growth defect

Deletion of N-terminal 50 amino acids abolished the activity of WIP in *S.cerevisiae*. In order to identify the minimum functional fragment of WIP which can suppress the growth defect of *vrp1Δ* strain we carried out deletion analysis of WIP from the C-terminus. In order to keep the stability of WIP's truncates, each deletion was constructed as GST fusions and expressed under the transcriptional control of VRP1 promoter from a high copy plasmid in *vrp1Δ* strain. Cells of *vrp1Δ* strain expressing such GST fusion proteins were analyzed for its growth at high temperature before carrying out further deletion (Table 3.4).

Table 3.4 Deletion analysis of WIP suppressing growth defect in *vrp1Δ* strain

<table>
<thead>
<tr>
<th>Truncates</th>
<th>Localization</th>
<th>Growth at 37°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>WIP1-503</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>WIP1-403</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>WIP1-317</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>WIP1-291</td>
<td>+/-</td>
<td>-</td>
</tr>
<tr>
<td>WIP1-266</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>WIP1-215</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

All truncates are expressed as GST-tagged proteins from high copy plasmid under the control of VRP1 promoter. Amino acids residues 1-317 are essential for suppressing growth defect in *vrp1Δ* strain, and residues 292-316 is crucial for the proper localization of WIP to cortical patches in *vrp1Δ* strain.
The first two deletion constructs, WIP\(_{1-403}\)-GST and WIP\(_{1-317}\)-GST were able to suppress the growth defect of \(vrp1\Delta\) strain as \(vrp1\Delta\) cells expressing either of the truncate proteins were able to grow at 37°C (Table 3.4). Further deletion constructs, WIP\(_{1-215}\)-GST was made and tested in \(vrp1\Delta\) strain. It was found that this construct was unable to correct the growth defect of \(vrp1\Delta\) strain. In order to further delineate the functional domains, another deletion construct, WIP\(_{1-266}\)-GST was made and this construct was also unable to suppress the growth defect (Table 3.4). Similarly WIP\(_{1-291}\)-GST was also unable to suppress the growth defect of \(vrp1\Delta\) strain, but WIP\(_{1-317}\)-GST suppressed the growth defect indicating that the residues 1-317 of WIP was essential for suppress growth defect. The inability of WIP\(_{1-291}\)-GST to suppress the growth defect is probably not due to poor expression as WIP\(_{1-291}\)-GST is expressed comparably with WIP\(_{1-317}\)-GST (Figure 3.11).

![Western blot](image.png)

Figure 3.11 Western blot for \(vrp1\Delta\) cells expressing WIP’s truncates as GST fusion proteins from high copy plasmid.

Cells of \(vrp1\Delta\) strain expressing WIP truncate GST fusion proteins from high copy plasmid were lysed and the cell lysate was assessed by electrophoresis. GST fusion proteins were detected by GST antibody and total protein was evaluated by Anti-Hexokinase.
3.1.6.2 WIP<sub>1-291</sub> and WIP<sub>1-266</sub> are non-functional due to lack of localization

Our previous data revealed that WIP<sub>51-503</sub> localizes to the cortical patches in vrpl<sup>A</sup> strain (data not shown) indicating that the V domain is not essential for the localization of WIP. Thus a localization motif was speculated to be located in the C-terminal of WIP. WIP<sub>1-291-GST</sub> expressed from high copy plasmid does not suppress the growth defect of vrpl<sup>A</sup> strain. This could be due to defects in localization.

To verify the localization of WIP truncates, DNA encoding GFP was fused to the 3' end of WIP truncates and the fusion proteins were expressed from low copy plasmid in vrpl<sup>A</sup> cells. Compared with WIP-GFP and WIP<sub>1-317-GFP</sub>, WIP<sub>1-291-GFP</sub> and WIP<sub>1-266-GFP</sub> localizes poorly to cortical patches in vrpl<sup>A</sup> strain (Figure 3.12). This suggests that the fragment WIP<sub>292-316</sub> is crucial for the proper localization of WIP, and the localization to cortical patches of WIP is essential for its ability to suppress the growth defect of vrpl<sup>A</sup> strain at high temperature.
Figure 3.12 Localization of WIP truncates in vrplA strain
WIP1-503, WIP1-403 and WIP1-317 localizes to cortical patches well while WIP1-291 and WIP1-266 localize poorly. Bar, 5μm.

3.1.6.3 Artificial localization enhances the ability of WIP truncates to suppress the growth defect

In order to determine that whether the loss of WIP’s ability to suppress growth defect is due to lack of localization signal, CAAX box was fused to DNA encoding WIP-GST truncates and WIP1-291-GST-CAAX and WIP1-266-GST-CAAX were expressed from high copy plasmid in vrplA strain. Transformant vrplA cells expressing WIP1-291-GST-CAAX grew at 37°C while WIP1-266-GST-CAAX grew marginally (Table 3.5, Figure 3.13). This suggests that the artificial localization signal enhances the ability of WIP truncates to suppress the growth defect of vrplA strain.

This also consolidates the previous conclusion that loss of ability of WIP’s C-terminal deletion truncates to suppress the growth defect is due to the poor localization.
The last 100 amino acid residues of WIP at C-terminus were shown to be essential for the interaction with WASP (Ramesh, et al., 1997). Our results indicates that WIP1-317 is the minimal functional fragment for suppressing the growth defect of vrplA strain, and WIP1-266 is the minimal fragment for suppressing growth defect if supplemented with a localization motif. Therefore, WASP binding region of WIP is not crucial for WIP’s ability to suppress the growth defect in vrplA S. cerevisiae.

Table 3.5 Membrane localization enhances the ability of WIP truncates to suppress the growth defect of vrplA strain

<table>
<thead>
<tr>
<th>Temperature Sensitivity Assay in vrplA strain</th>
<th>-GST</th>
<th>-GST-CAAX</th>
</tr>
</thead>
<tbody>
<tr>
<td>WIP1-503</td>
<td>+</td>
<td>ND</td>
</tr>
<tr>
<td>WIP1-403</td>
<td>+</td>
<td>ND</td>
</tr>
<tr>
<td>WIP1-317</td>
<td>+</td>
<td>ND</td>
</tr>
<tr>
<td>WIP1-291</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>WIP1-266</td>
<td>-</td>
<td>+/-</td>
</tr>
<tr>
<td>WIP1-215</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Figure 3.13 Western blot for WIP C-terminal deletion truncates WIP1-291 and WIP1-266. Cells of vrplA strain expressing WIP truncate GST fusion proteins from high copy plasmid were lysed and the cell lysate was assessed by electrophoresis. GST fusion proteins were detected by GST antibody and total protein was evaluated by Anti-Hexokinase.
3.2 Functional analysis of WIRE in \textit{vrp1A} strain

3.2.1 WIRE expressed from high copy plasmid suppresses the growth and endocytosis defect of \textit{vrp1A} strain

WIRE is a member of mammalian Verprolin family of proteins and exhibits the highest homology with WIP. It is predicted that WIRE has similar functions as WIP in actin cytoskeleton remodeling (Aspenstrom, 2005). As stated before, \textit{vrp1A} yeast strain has actin cytoskeletal defects such as loss of endocytosis, depolarization of actin patches, and lack of growth at 37°C. In the previous section, WIP was shown to be the functional homologue of Vrp1p and able to suppress the growth and endocytosis defects of \textit{vrp1A} strain. Therefore, an interesting question is whether the other member of Verprolin family of proteins, WIRE, is also functional homologue of Vrp1p. The research strategy used to identify the function of WIRE is similar to that of WIP.

3.2.1.1 WIRE expressed from low copy plasmid does not suppress growth, endocytosis or actin patches polarization defect of \textit{vrp1A} strain

In order to express WIRE in \textit{vrp1A} strain (AMY88), we amplified the DNA encoding WIRE using PCR and placed the gene under the transcriptional regulation of VRP1 promoter. This construct was introduced into a low copy plasmid Yeplac111. This plasmid was introduced into \textit{vrp1A} strain by lithium acetate transformation and WIRE was expressed as a GST fusion protein since fusion of GST has been known to
stabilize ectopic proteins which are unstable in host cells (Yang, et al., 2005). The transformants were analyzed for growth at high temperature. WIRE-GST expressed from a low copy plasmid was unable to suppress the growth, endocytosis or actin patches defect of this strain (Figure 3.14, Figure 3.15). This lack of suppression could be due to several reasons, including poor localization and poor expression of WIRE in yeast.

Addition of CAAX box motif to N-Vrp1p, the yeast homologues of WIRE, has been shown to enhance the localization to cell membrane and activity of N-Vrp1p in vrplA (Thanabalu and Munn, 2001). WIRE-GST was fused with CAAX motif at the C-terminus and introduced into vrplA cells. Transformant cells expressing WIP-GST-CAAX from low copy plasmid grew marginally indicating that addition of CAAX marginally improves the activity of WIRE to suppress the growth defect, but not to suppress endocytosis or actin patches depolarization defect in vrplA cells (Figure 3.14, Figure 3.15). Western blot indicated that WIRE is expressed in a comparable level and quite stable in yeast cells (Figure 3.14 B).
Figure 3.14 Expression of WIRE from low copy plasmid does not suppress growth defect of vrp1A strain

A) Cells of vrp1A strain expressing WIRE-GST or WIRE-GST-CAAX fusion proteins from low copy plasmid, or WIRE-GST from high copy plasmid, were streaked on YPUAD plate and incubated at 37°C for 3 days before the cell growth was assessed.

B) Cells expressing fusion proteins were inoculated in YPUAD, and lysed when the OD<sub>600</sub> reached 0.5. The cell lysate were analyzed by SDS-PAGE and fusion proteins were detected by anti-GST. Anti-Hexokinase was used to indicate the level of total proteins.
Figure 3.15 Expression of WIRE from low copy plasmid does not suppress endocytosis or actin patches polarization defect of *vpr1A* strain

A) Actin staining of *vpr1A* cells expressing WIRE-GST or WIRE-GST-CAAX from low copy plasmid. Cells of exponential phase were fixed using formaldehyde and permeabilized by Triton X-100. Cells were then stained by rhodamine or Alexa fluorescence 488 conjugated phalloidin.

B) Endocytosis assay of *vpr1A* cells expressing WIRE-GST or WIRE-GST-CAAX fusion proteins from low copy plasmid. Cells of exponential phase were re-inoculated with YPUAD including Lucifer Yellow dye for one hour before viewing under the fluorescence microscope. Bar, 5 μm.
3.2.1.2 WIRE expressed from high copy plasmid suppresses the defects in growth and endocytosis, but not the actin patches polarization

In order to enhance the expression of WIRE, we placed the WIRE under the transcriptional regulation of VRP1 promoter on YEplac181, a high copy plasmid. This plasmid was then introduced into *vrp1A* strain and the transformants were analyzed for growth at high temperature. The *vrp1A* cell transformants grew at 37°C (Figure 3.16 A) suggesting that WIRE expressed from a high copy plasmid is able to suppress the growth defect of *vrp1A* strain. The *vrp1A* cell transformants were analysed for fluid phase endocytosis and actin patch polarization. Expression of WIRE from a high copy plasmid suppresses the endocytosis defect but not the actin patch polarization defect of *vrp1A* strain (Figure 3.16 A and B).

It was reported that the ability to suppress the actin patches polarization defect was co-related with the ability to grow on high salt (1M NaCl) YPD plate (Thanabalu and Munn, 2001). The cells expressing WIRE from high copy plasmid were streaked on YPUAD plate with 1 M NaCl and incubated in 24°C for 5 days. These cells were not able to grow (data not shown) confirming that WIRE expressed from high copy plasmid is not able to suppress the defect in actin patches polarization.
Figure 3.16 WIRE-GST expressed form high copy plasmid suppresses endocytosis but not actin patches polarization defect of \textit{vrp1A} strain

A) \textit{vrp1A} cells expressing WIRE-GST fusion proteins from a high copy plasmid was inoculated in YPUAD. Cells of exponential phase were re-inoculated with fresh YPUAD including Lucifer Yellow dye for one hour before viewing under the fluorescence microscope.

B) \textit{vrp1A} cells expressing WIRE-GST from high copy plasmid was inoculated in YPUAD. Cells of exponential phase were fixed using formaldehyde and permeabilized by Triton X-100. Cells were then stained by rhodamine or Alexa fluorescence 488 conjugated phallolidin. Bar, 5\,\mu m.
3.2.2 WIRE localizes to cortical actin patches

3.2.2.1 WIRE localizes to cortical patches

In order to check the localization of WIRE in yeast cells, WIRE was visualized in \textit{vrp1A} strain by fusing the GFP protein to the C-terminus of WIRE. This WIRE-GFP construct was introduced into \textit{vrp1A} strain on a low copy plasmid and the transformants were viewed under a fluorescent microscope. WIRE-GFP localizes to cortical patches in \textit{vrp1A} strain compared with GFP which showed diffused cytoplasmic staining (Figure 3.17).

![GFP and WIRE-GFP localization](image)

Figure 3.17 WIRE localizes to cortical patches in \textit{vrp1A} strain

Cells of \textit{vrp1A} strain expressing WIRE-GFP from low copy plasmid at exponential phase were viewed under fluorescence microscope. Bar, 5\textmu m.
3.2.2.2 WIRE localizes to actin patches

As mentioned above, WIRE was localized to cortical patches in \textit{vrp1A} strain. In order to check whether WIRE was localized to actin patches, WIRE-GFP and Arc40p-RFP were expressed in \textit{vrp1A} strain. Arc40p is one of the seven subunits of Arp2/3 complex, an actin uncleator which localizes at the actin patches. Cells with expression of both WIRE-GFP and Arc40-RFP were viewed under fluorescence microscopy using GFP and RFP filter respectively (Figure 3.18). Since there is no interaction between WIRE and Arc40p assessed by yeast two hybrid assay (data not shown), therefore the co-localization of patches indicates that WIRE is localized to cortical actin patches.

![WIRE-GFP and Arc40-RFP localization](image)

Figure 3.18 WIRE co-localizes with Arc40p in \textit{vrp1A} strain
Cells of \textit{vrp1A} strain expressing both WIRE-GFP and Arc40p-RFP from low copy plasmid were grown to exponential growth phase and viewed under fluorescence microscope. Bar, 5 \textmu m.
3.2.3 WIRE interacts with actin but not Las17p

Yeast two hybrid assay was carried out to verify the interaction between WIRE and actin. WIRE was cloned into pACT2 with activation domain, DNA encoding actin was cloned into pAS2-1 with binding domain and both of them were co-transformed into PJ69-4A. The yeast transformants expressing AD-WIRE and BD-Actin were able to grow on SD-Trp-Leu-His plates showing that WIRE interacts with actin (Figure 3.19).

In order to check whether the human WIRE interacts with Las17p, the yeast homologues of its interacting partner WASP, WIRE was cloned into pACT2 with activation domain, DNA encoding Las17p was cloned into pAS2-1 with binding domain and both of these two plasmids were co-transformed into PJ69-4A. The yeast transformants expressing AD-WIRE and BD-Las17p were selected on SD(-Trp-Leu) plate and streaked on SD(-Trp-Leu-His) plate to check for growth. The transformants did not grow on SD(-Trp-Leu-His) plate indicating that even though Las17p is the yeast homologues of WASP, WIRE does not interact with Las17p as assessed by yeast two hybrid assay (Figure 3.19).
Figure 3.19 WIRE interacts with actin, but not Las17p assessed by yeast two hybrid assay

WIRE was cloned into pACT2 plasmid, and AD-WIRE was expressed in PJ69-4A. Actin/Las17 was cloned into pAS2-1 and BD-Actin/Las17p was expressed in PJ69-4A. PJ69-4A cells expressing both AD-WIRE and BD-Actin/Las17p were streaked on SD(-Trp-Leu-His) plate and incubated at 30°C for 3-5 days.

### 3.2.4 The V domain is crucial for WIRE to suppress growth and endocytosis defect of vrplA strain

It was reported that Vrp1p interacts with actin through residues 1-70 (V domain) (Vaduva, et al., 1997) and WIP has a V domain with the actin binding motif (Ramesh, et al., 1997). Our previous data and report of Vaduva showed that this V domain and the actin binding motif of WIP is essential for WIP’s activity in S.cerevisiae (Vaduva, et al., 1999). In order to address the role of V domain in WIRE, V domain (N-terminal 1-52 residues) of WIRE was deleted and WIRE\textsubscript{53-441} (WIRE\textsubscript{AV}) was expressed as a GST fusion protein from high copy plasmid. vrplA cells expressing WIRE\textsubscript{53-441}-GST did not grow at 37°C or uptake Lucifer yellow, suggesting that WIRE\textsubscript{53-441} is not able to suppress either of the defects in growth or endocytosis (Figure 3.20). This indicates that the V domain is essential for WIRE to suppress high temperature growth defect and endocytosis defect of vrplA strain.
Figure 3.20 V domain of WIRE is essential for suppression of growth and endocytosis defect in vrplA strain.

A) Transformants of vrplA cells expressing WIRE-GST or WIRE_{53-441}-GST from high copy plasmid were streaked on YPUAD plate and incubated at 37°C for 3 days before growth was assessed.

B) Cells of exponential phase were inoculated with YPUAD including Lucifer Yellow for one hour before viewing under microscope.

C) Cells of exponential phase were lysed and fusion proteins from cell lysate were detected by anti-GST after electrophoresis and proteins transference. Bar, 5 μm.
3.2.5 Deletion of V domain abolishes the interaction with actin

In order to check the relation between the loss of function of WIRE\textsubscript{53-441} (WIRE\textsubscript{AV}) and its interaction with actin, WIRE\textsubscript{53-441} was cloned into pACT2 (activation domain) and DNA encoding Actin was cloned into pAS2-1 (DNA binding domain), then both of these recombinant plasmids were co-transformed into pJ69-4A strain. Transformants expressing both AD-WIRE\textsubscript{53-441} and BD-Actin were not able to grow on SD(-Trp-Leu-His) plate indicating that WIRE\textsubscript{53-441} does not interact with actin (Table 3.6). This suggests that WIRE’s interaction with actin is mediated by the V domain and that this domain is crucial for activity of WIRE in \textit{S.cerevisiae}. This also indicates that the activity of WIRE to suppress growth defect and endocytosis defect is mediated by the interaction with actin.

3.2.6 KLKK motif is essential for the suppression of growth and endocytosis defects

As mentioned before, the verprolin family of proteins have a conserved actin binding motif "KLKK" (Aspenstrom, 2005). To address whether “KLKK” motif is important for the function of V domain, we mutated the K\textsubscript{49}LKK\textsubscript{52} motif of WIRE to A\textsubscript{49}AAA\textsubscript{52} (named as 4A) using site directed mutagenesis. WIRE\textsuperscript{4A} mutant on a high copy plasmid was introduced into \textit{vrplA} strain and expressed as a GST fusion protein under the transcriptional regulation of \textit{VRPl} promoter. The transformant \textit{vrplA} cells expressing WIRE\textsuperscript{4A}-GST were unable to grow at 37°C or take up Lucifer yellow dye (Figure 3.21), suggesting that the KLKK motif is crucial for WIRE to suppress the
Figure 3.21 KLKK motif is crucial for WIRE to suppress growth and endocytosis defects in vrplA strain.

A) Growth at high temperature. Transformants of vrplA cells expressing WIRE-GST or WIRE4A-GST from high copy plasmid were streaked on YPUAD plate and incubated at 37°C for 3 days before growth was assessed.

B) Cells of exponential phase were inoculated with YPUAD including Lucifer Yellow for one hour before viewing under microscope.

C) Cells of exponential phase were lysed and fusion proteins from cell lysate were detected by anti-GST after electrophoresis and proteins transference. Bar, 5 μm.
growth defect at high temperature and endocytosis defect. Unlike WIP^{4A} which suppresses the growth defect at high temperature, WIRE^{4A} does not restore the growth at all. Combine the results of both WIRE and WIP, it is indicated that “KLKK” motif is more crucial for WIRE than WIP to suppress growth defect and endocytosis defect of vrpIΔ strain.

3.2.7 KLKK motif of WIRE mediates interaction with actin

Mutating KLKK motif to AAAA abolished the function of WIRE in suppressing the growth defect at evaluate temperature and endocytosis defect. Data also showed that WIRE interacts with monomer actin though the V domain. This in turn raises the interesting question whether the interaction of WIRE with actin was mediated by the KLKK motif and whether the loss of function for WIRE to suppress the defect was caused by the loss of actin binding. In order to determine this, DNA fragment encoding WIRE^{4A} was cloned into pACT2 and introduced into pJ69-4A together with pBD-Actin. Transformants were selected by SD-Trp-Leu plate and re-streaked on SD(-Trp-Leu-His) plate to check the growth. Transformants expressing AD-WIRE^{4A} did not grow on SD-Trp-Leu-His plate (Table 3.6) indicating that KLKK mutation of WIRE abolishes the interaction with actin. This also verified that the KLKK motif is the unique actin binding site of WIRE and that the interaction with actin is essential for WIRE to suppress the growth and endocytosis defects.
Table 3.6 Interaction with actin of WIRE is co-related with its ability to suppress the growth and endocytosis defects of \( vrp1 \Delta \) strain

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<th>Growth at 37°C</th>
<th>Endocytosis</th>
<th>Actin Interaction</th>
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<td>WIRE</td>
<td>+</td>
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<tr>
<td>WIRE(^{4A})</td>
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<td>WIRE(_{53-441})</td>
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For endocytosis and growth assay at high temperature, all the above mutant constructs were expressed as a GST fusion proteins from high copy plasmid under the control of VRP1 promoter in \( vrp1 \Delta \) strain.

3.3 Summary for WIP and WIRE

\( S. \text{cerevisiae} \ vrp1 \Delta \) strain exhibits defective growth at high temperature, defective endocytosis and actin patches polarization due to abnormal organization of actin cytoskeleton. WIP and WIRE are mammalian homologues of Vrplp. Both WIRE and WIP localize to actin patches in \( vrp1 \Delta \) strain. Expression of WIP or WIRE in \( vrp1 \Delta \) strain suppresses both the growth and endocytic defects of this strain without correcting the actin patch polarization defect. Both WIP and WIRE require the WH2/V domain for suppressing the growth and endocytosis defect. However, the KLKK motif of WIRE, but not WIP, is crucial for the interaction with actin. The actin interaction with both WIP and WIRE are critical for the suppression of growth and endocytosis defects of \( vrp1 \Delta \) strain. The KLKK motif and N-terminal proline rich region of WIP play a redundant role in the interaction with actin and suppression of growth and endocytosis defects while the KLKK motif of WIRE is critical for both its
activity and interaction with actin. Therefore there is correlation between the proteins' ability to interaction with actin and the ability to suppress the growth defect of vrplA strain.
4 Functional analysis of CR16 in \textit{vrp1A} strain

CR16 was initially identified as a glucocorticoid-regulated gene expressed in subpopulations of neurons in the brain, including the hippocampus (Weiler, \textit{et al.}, 1996). The subsequent analysis revealed that CR16 forms a complex with N-WASP, a central actin cytoskeleton regulator, and binds with both G-actin and F-actin (Ho, \textit{et al.}, 2001). Interaction with actin monomers and WASP-family proteins are key features shared with all the members of Verprolin proteins. CR16 shares approximately 25\% identity with WIP, the first identified mammalian member of Verprolin family of proteins (Ho, \textit{et al.}, 2001). Therefore CR16 is proposed to be a functional homologue of WIP and WIRE, and a mammalian member of Verprolin family of proteins. The CR16 open reading frame encodes a 45 kDa protein containing 32\% proline. The western blot shows a protein doublet of 68 and 72 kDa on SDS PAGE from hippocampal extracts. However, when hippocampal extracts are electrophoresed on non-denaturing polyacrylamide gels, the CR16 protein migrates as a 48 kDa protein that better correlates with the calculated size. Sequence alignment reveals 12 sequence homologies to the SH3 binding domain consensus sequence XPXXPPP. In addition, CR16 has at least 36 copies of the PXXP motif, which is contained in all known SH3 binding domains. In order to determine whether CR16 is a functional homologue of Vrp1p, we expressed the CR16 under the control of VRP1 promoter using both centromeric (low copy number) and 2 \mu plasmids (high copy number) in \textit{S. cerevisiae vrp1A} strain (AMY88). Since it has been reported that
Functional analysis of CR16

deletion of the WH2/V domain lead to loss of protein stability (Vaduva, et al., 1999), and that translation fusion with GST has been shown to stabilize proteins, we expressed CR16 with a C-terminal GST fusion in S. cerevisiae.

4.1 Expression of CR16 suppresses both growth and endocytosis defect of vrp1Δ strain

4.1.1 Expression of CR16 suppresses growth defect of vrp1Δ strain

CR16 expressed from all three constructs, low copy plasmid, high copy plasmid and high copy GST fusion plasmid, suppressed the growth defect of vrp1Δ strain to varying degree, with the expression from a low copy plasmid having the weakest suppression and the CR16-GST expressed from a 2 μ plasmid (high copy plasmid) having the highest activity (Figure 4.1). The observed suppression of growth defect was also seen in liquid culture (Figure 4.1). Thus CR16 is a functional homologue of Vrp1p and GST fusion stabilized the protein in yeast. Further mutational analysis was done using the GST fusion construct.
Functional analysis of CR16

Figure 4.1 Expression of CR16 suppress growth defect of \( vrplA \) strain

A) Transformant \( vrplA \) cells expressing CR16 or CR16-GST from high copy or low copy plasmid were streaked on YPUAD plate and incubated at 37°C or 24°C for 3 days.

B) Transformant \( vrplA \) cells expressing CR16 or CR16-GST from high copy or low copy plasmid were inoculated in YPUAD for overnight and diluted to \( OD_{600} \) 0.05. Cells were inoculated in fresh YPUAD media and the value of \( OD_{600} \) was measured at a time interval of one hour for 12 hours.

4.1.2 Expression of CR16 suppresses endocytosis defect of \( vrplA \) strain

We assessed the ability of CR16 to correct the endocytic defect of \( vrplA \) by carrying out fluid phase endocytosis assay using exponentially growing cultures of the
Functional analysis of CR16

transformants. The transformants expressing CR16 from both low copy and high copy plasmid, and CR16-GST expressed from high copy plasmid were able to take up Lucifer Yellow dye at 24°C but the strain expressing only GST did not take up LY (Figure 4.2) indicating that CR16 restore the endocytosis of vrplΔ strain.

![Image of GST and CR16-GST with LY and DIC](image)

Figure 4.2 Expression of CR16 suppress endocytosis defect of vrplΔ strain
Endocytosis assay of vrplΔ cells expressing CR16-GST fusion proteins from high copy plasmid. Cells of exponential phase were re-inoculated in YPUAD including Lucifer Yellow dye for one hour before viewed under the fluorescence microscope. Bar, 5 μm.

4.1.3 Expression of CR16 did not restore actin patches polarization

We also analyzed the ability of CR16 to correct the actin patch polarization defect of vrplΔ. The actin patches in vrplΔ cells expressing CR16-GST were still depolarized (Figure 4.3) suggesting that CR16 corrected the endocytic growth defect without correcting the actin patch polarization defect. We have previously found that the ability of S. cerevisiae cells to grow on plates with 1M NaCl correlated well with the
presence of a polarized actin cytoskeleton (Thanabalu and Munn, 2001). *S. cerevisiae* \( *{vrp1A} \) cells expressing CR16-GST were not able to grow on YPUAD plates with 1 M NaCl (data not shown) consistent with the lack of actin patch polarization.

**Figure 4.3** Expression of CR16 does not suppress actin patches polarization defect of \( *{vrp1A} \) strain

Actin staining of \( *{vrp1A} \) cells expressing CR16-GST from high copy plasmid. Cells of exponential phase were fixed using formaldehyde and permeabilized by Triton X-100. Cells were then stained with Alexa fluorescence 488 conjugated phalloidin. Bar, 5 \( \mu \)m.

### 4.1.4 CR16 localized to cortical patches in \( *{vrp1A} \) strain

In order to analyze the subcellular localization of CR16, we expressed CR16-GFP fusion construct in \( *{vrp1A} \) cells and the GFP signal was analyzed using live cell imaging. CR16-GFP was found to localize to cortical patches compared to the diffused cytoplasmic staining observed in cells expressing just GFP (Figure 4.4 A). Since Vrp1p has been shown to localize to cortical actin patches (Vaduva, *et al.*, 1997), we checked if CR16-GFP also localized with actin patches by introducing plasmid expressing Arc40p-RFP (Red Fluorescent Protein) into cells expressing CR16-GFP.
Arc40p, a subunit of the Arp2/3 complex has been shown to localize to cortical actin patches (Winter, et al., 1999). Cells expressing CR16-GFP and Arc40p-RFP were viewed using fluorescent microscopy. Some of the CR16-GFP patches were found to co-localize with Arc40p-RFP, suggesting that CR16-GFP localizes to cortical actin patches (Figure 4.4 B). In order to verify whether there is interaction between Arc40p and CR16, CR16 was cloned into pACT2 and fused with DNA activation domain. Arc40p was cloned into pAS2-1 and fused with DNA binding domain. These two plasmids were co-transformed into PJ69-4A and transformants were selected on SD(-Trp-Leu) plates. The streaked cells of transformants did not grow on SD(-Trp-Leu-His) indicating that CR16 does not interact with Acr40p (data not shown). This suggests that the co-localization observed is not due to interaction between CR16 and Arc40p. Therefore CR16 localizes to actin patches.
Figure 4.4 CR16 localizes to cortical actin patches in *vrp1A* strain

A) CR16 localizes to cortical patches. CR16-GFP was expressed in *vrp1A* strain. Transformant cells expressing CR16-GFP were viewed under GFP filter of fluorescence microscope.

B) CR16 co-localizes with Arc40p to cortical actin patches. CR16-GFP was co-expressed with Arc40p-RFP in *vrp1A* strain. Transformant cells expressing both CR16-GFP and Arc40p-RFP were viewed under fluorescence microscope. Bar, 5 μm.
4.1.5 Addition of CAAX motif enhance the activity of CR16 in suppressing the growth defect of \textit{vrp1A} strain

As shown in the previous section, localization is essential for WIP’s ability to suppress the growth defect of \textit{vrp1A} strain. Addition of CAAX motif at the C-terminus recovers the activity of WIP_{1-219}-GST to suppress the growth defect. In order to test whether enhanced localization through CAAX motif can improve the activity of CR16, the CAAX box was fused to the C-terminus of CR16-GST, and expressed from low copy plasmid in \textit{vrp1A} strain. Transformants expressing CR16-GST-CAAX or CR16-GST were streaked on YPUAD plate and incubated at 37°C for 3 days. Cells expressing CR16 from low copy plasmid were used as a control. The growth of cells expressing CR16 or CR16-GST from low copy plasmid was comparable. Growth of cells expressing CR16-GST-CAAX from low copy plasmid is better than that of CR16 or CR16-GST (Figure 4.5), indicating that addition of CAAX motif enhance the activity of CR16-GST to suppress the growth defect of \textit{vrp1A} strain. It also suggests that enhanced localization improves the ability of CR16 in suppressing the growth defect of \textit{vrp1A} strain.
4.2 V domain of CR16 is essential for suppression of growth defect

As discussed before, the V domains of WIP and WIRE are essential for these mammalian proteins to suppress the growth and endocytosis defects of *vrplA* strains.

In order to determine whether the V domain of CR16 is essential for its activity, DNA fragment without V domain (CR16_61-485) was amplified by PCR and fused with GST.

**4.2.1 V domain of CR16 is essential for suppression of growth defect but not for the endocytosis defect**

*S. cerevisiae* *vrplA* cells expressing CR16_61-485-GST (CR16ΔV-GST) from high copy plasmid were not able to grow at 37°C as assessed by both colony formation on agar media and liquid culture at 37°C (Figure 4.7 A and B). However *vrplA* cells
expressing CR16_{61-485}-GST was still able to take up Lucifer Yellow (Figure 4.7 A). To analyze the protein expression and stability, the transformants expressing CR16_{61-485}-GST were lysed and cell lysate was analysed by western blot. Anti-GST antibodies detected a band for CR16_{61-485}-GST (CR16ΔV) indicating that CR16_{61-485}-GST is expressed at a level comparable to that of the full length CR16-GST (Figure 4.7B). Therefore the inability of CR16_{61-485}-GST to suppress the growth defect of vrp1A strain is not due to poor stability of this protein, but due to the loss of V domain.

Figure 4.6 V domain of CR16 is essential for suppression of growth defect
A) Transformants expressing CR16-GST, CR16ΔA-GST or CR16_{61-485}-GST from high copy plasmid were streaked on YPUAD plate and incubated at 37 °C for 3 days.
B) Transformants were inoculated in YPUAD for overnight and diluted to OD_{600} 0.05. Cells were inoculated in fresh YPUAD media and the value of OD_{600} was measured at a time interval of one hour.
Figure 4.7 V domain of CR16 is not crucial for suppression of endocytosis defect
A) Cells of exponential phase were re-inoculated in YPUAD including Lucifer Yellow dye for one hour before viewed under the fluorescence microscope.
B) Cells were incubated in YPUAD, and lysed when the OD600 reached 0.5. the cell lysate was analysed by western blot. Fusion proteins were detected using anti-GST. Anti-Hexokinase was used to indicate the level of total proteins. Bar, 5 μm.

4.2.2 V domain is not essential for the localization of CR16

The inability of CR1661-485-GST to suppress the growth defect of vrp1Δ strain could be due to loss of localization of CR16. In order to analyze the localization of CR1661-485, DNA encoding CR1661-485 was fused to the N-terminus of GFP then introduced and expressed from low copy plasmid in AMY88. Low copy plasmid was
chosen for the testing of localization because fluorescence light of GFP expressed from low copy plasmid has appropriate brightness which is easy to be detected by the fluorescence microscopy used for this study. CR1661-485-GFP localizes to cortical patches in vrplΔ strain while cells expressing GFP exhibits a diffused cytoplasm signal (Figure 4.8A). CR1661-485-GFP and Arc40p-RFP were co-expressed in vrplΔ strain (AMY88) to check for co-localization. Arc40p is one of the seven subunits of Arp2/3 complex which is an actin nucleator and localizes to actin patches in yeast cells (Winter, et al., 1999). Viewed under the fluorescence microscopy, some patches of CR1661-485-GFP under GFP filter overlaps with patches of Arc40p-RFP under RFP filter (Figure 4.8 B), indicating that CR1661-485 co-localizes with Arc40p to actin patches in vrplΔ strain. Western blot of vrplΔ cells lysate expressing CR1661-485-GST from high copy plasmid showed that expression of CR1661-485-GST was comparable with that of CR16-GST (Figure 4.7 B). Thus the inability of CR1661-485 to suppress of growth defect of vrplΔ by is not due to poor expression or lack of localization to actin patches.
Figure 4.8 V domain is not essential for the localization of CR16
A) Localization assay. Transformant *vrp1A* cells of exponential phase expressing CR16-GFP or CR16<sub>61-485</sub>-GFP were viewed under fluorescence microscope.
B) Co-localization assay. Transformant *vrp1A* cells of exponential phase co-expressing CR16-GFP or CR16<sub>61-485</sub>-GFP together with Arc40p-RFP were viewed under fluorescence microscope. Bar, 5 μm.
4.3 Actin binding motif, RLRK is essential for actin binding but not for suppressing the growth or endocytosis defect

The WH2/V domain of WIP and Vrp1p mediates the interaction with actin through the actin binding motif KLKK located in the WH2/V domain (Vaduva, et al., 1999). Mutations of the KLKK motif or deletion of the V domain abolished WIP's ability to suppress the growth defect of vrp1Δ strain (Vaduva, et al., 1999). Both WIRE and WIP have a KLKK motif in the WH2/V domain, similar to that of Vrp1p, while CR16 has a RLRK motif. CR16 interacts with both G-actin and F-actin (Ho, et al., 2001).

4.3.1 RLRK actin binding site is essential for the interaction with actin

In order to determine if the RLRK motif mediates the interaction with actin, the 58RLRK61 residues of this motif were mutated to 58AAAA61 (CR164A) by site directed mutagenesis. The interaction of CR16 or CR164A with actin was examined using yeast two hybrid assay. DNA encoding CR16 and CR164A (with amino acid substitution) were cloned into pACT2 to generate pAD-CR16 and pAD-CR164A respectively and introduced into pJ69-4A cells harboring plasmid expressing BD-Actin (Thanabalu and Munn, 2001). Transformants expressing AD-CR16, but not AD-CR164A, together with BD-actin grew on SD(-Trp-Leu-His) plates, indicating that CR16 interacts with actin as assessed by yeast two hybrid assay, while CR164A does not interact with actin (Table 4.1). This suggests that the RLRK motif of CR16 mediates the interaction with actin and that probably there are no other additional actin binding motifs in CR16.
Table 4.1 RLRK motif of CR16 is crucial for the interaction with actin

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<th>BD</th>
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<tr>
<td>AD-CR16</td>
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<td>AD-CR16&lt;sub&gt;61-485&lt;/sub&gt;</td>
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4.3.2 RLRK motif is not crucial for suppression of growth or endocytosis defect

The previous data showed that the actin binding motif of WIRE, KLKK motif, is crucial for the interaction with actin, and crucial for the suppression of growth and endocytosis defect of <i>vrlA</i> strain. Thus it raises an interesting question whether the actin binding motif is also crucial for WIRE's homologue, CR16, to suppress the growth and endocytosis defect of <i>vrlA</i> strain. In order to determine this, CR16<sup>4A</sup>-GST was expressed in <i>vrlA</i> cells from high copy plasmid to analyze the role of RLRK motif in suppressing the growth and endocytosis defect of <i>vrlA</i> strain. Cell expressing CR16<sup>4A</sup>-GST were still able to grow at 37°C as can be assessed by the plate assay (Figure 4.9A) suggesting that the actin binding motif is not critical for CR16 to suppress the growth defect of <i>vrlA</i> cells. Similarly expression of the CR16<sup>4A</sup>-GST mutant protein was able to correct the endocytosis defect of this strain (Figure 4.9B). These results are in contrast to the observation that mutating the KLKK
to AAAA in WIRE abolished the ability of WIRE to suppress the growth defect of
\(vrp1A\) strain (Section 3.2.6). This suggests that the actin binding motif in CR16 has a
functional redundant domain/motif in CR16 located within the V domain.

4.3.3 RLRK motif is not essential for localization to cortical actin
patches

The previous data showed that CR16 co-localizes with Acr40p to actin patches. Thus
it raises the question that whether the localization to actin patches of CR16 is
correlated with its ability to interact with actin. In order to determine this, CR16\(^{4A}\) was
fused to the N-terminus of GFP and then expressed from low copy plasmid in \(vrp1A\)
strain. CR16\(^{4A}\)-GFP localizes to cortical patches (data not shown). Arc40-RFP was
co-expressed with CR16\(^{4A}\)-GFP in \(vrp1A\) strain and viewed under the GFP filter and
RFP filter respectively. CR16\(^{4A}\)-GFP localizes to cortical patches and some of the
CR16\(^{4A}\)-GFP patches were found to be overlapped with Arc40p-RFP patches (data not
shown) suggesting that RLRK motif is not essential for CR16 to localize to cortical
actin patches.
Functional analysis of CR16

Figure 4.9 RLRK motif is not crucial for suppression of growth or endocytosis defect

A) Transformant *wrl* cells were streaked on YPUAD plate and incubated at 37°C or 24°C for 3 days.

B) Cells were re-inoculated in YPUAD including Lucifer Yellow dye for one hour before viewed under the fluorescence microscope.

C) Fusion proteins were detected using anti-GST. Anti-Hexokinase was used to indicate the level of total proteins. Bar, 5 μm.
4.4 Proline rich sequence at N-terminal plays a redundant role to the actin binding motif

Deletion of the V-domain showed that the V domain of CR16 is essential for the suppression of the growth defects of *vrp1Δ* strain (Figure 4.6) but mutation of the RLRK motif to AAAA did not abolish CR16's ability to suppress the growth defects (Figure 4.8) suggesting that there are other motifs within the V domain which have activities redundant with that of the RLRK motif. We compared the sequences of V domain of WIP, WIRE with that of CR16 and found that the WH2/V domain of CR16 is very rich in proline as compared to the other verprolin family members. In order to determine whether these additional proline residues are the functional redundant motif, deletion mutants were constructed which effectively removed most of the proline rich region in the V domain. A mutant combining mutation at actin binding motif (RLRK to AAAA) and deletion of N-terminal proline rich region were also constructed. The above constructs were introduced into *vrp1Δ* strain and expressed as GST fusion proteins from high copy plasmid to analyze the ability to suppress the growth and endocytosis defect of this strain.

4.4.1 Proline rich region is not critical for the suppression of growth or endocytosis defect

Result of temperature sensitivity assay showed that *vrp1Δ* cells expressing GST fusion proteins of CR16_{31-485} grew at 37°C indicating that the proline rich region at
N-terminus of CR16 is not essential for the suppression of growth or endocytosis defect of \textit{vrpI}Δ strain (Figure 4.10 A, B and C).

\textbf{4.4.2 Proline rich region plays a redundant role to the actin binding motif for the suppression of growth defect}

CR16 suppresses the growth defect of \textit{vrpI}Δ strain even without the N-terminal proline rich region. This could be due to the presence of the actin binding motif. Similar to our observation with WIP, the proline rich sequence deletion was combined with that of actin binding site mutation. GST fusion protein of CR16\textsuperscript{4A}_{31-485} was expressed from high copy plasmid in \textit{vrpI}Δ strain to check for the suppression of growth defects. Result showed that CR16\textsuperscript{4A}_{31-485}-GST is not able to suppress the growth defect (Figure 4.10 A and B, Table 4.2), indicating that combination of proline rich region deletion with the site mutation at RLRK motif abolishes the suppression of growth defect of \textit{vrpI}Δ strain even though proteins were expressed at comparable levels to CR16-GST (Figure 4.10 A and B, Figure 4.11, Table 4.2). This suggests that proline rich region at N-terminus of CR16 plays a redundant role with the actin binding motif for the suppression of growth defect of \textit{vrpI}Δ strain.

In order to narrow down the essential proline rich region of CR16 at the N-terminus which plays the redundant role in suppression of growth defect in \textit{vrpI}Δ strain, CR16\textsubscript{11-485}, CR16\textsuperscript{4A}\textsubscript{11-485}, CR16\textsubscript{16-485} and CR16\textsuperscript{4A}\textsubscript{18-485} were constructed and expressed as GST fusion proteins from high copy plasmid in \textit{vrpI}Δ strain. Transformant cells
expressing CR16\textsubscript{11-485}-GST or CR16\textsubscript{18-485}-GST grew at 37°C while cells expressing CR16\textsuperscript{AA}\textsubscript{11-485}-GST or CR16\textsuperscript{AA}\textsubscript{18-485}-GST did not (Table 4.2), indicating that the first 10 residues of N-terminal CR16 plays redundant role with the actin binding motif in suppressing growth and endocytosis defects.

Figure 4.10 N-terminal proline rich region and actin binding motif play redundant roles in suppressing growth defect of \textit{vrp1A} strain

A) Transformants expressing CR16-GST, CR16\textsubscript{31-485}-GST or CR16\textsubscript{31-485}-GST from high copy plasmid were streaked on YPUAD plate and incubated at 37°C for 3 days

B) Transformants were inoculated in YPUAD for overnight and diluted to OD\textsubscript{600} 0.05. Cells were inoculated in fresh YPUAD media and the value of OD\textsubscript{600} was measured at a time interval of one hour.
Figure 4.11 N-terminal proline rich region and actin binding motif play redundant roles in suppressing endocytosis defect of vrpΔ strain

A) Cells of exponential phase were re-inoculated in YPUAD including Lucifer Yellow dye for one hour before viewed under the fluorescence microscope.

B) Cells were incubated in YPUAD, and lysed when the OD$_{600}$ reached 0.5. the cell lysate was analysed by western blot. Fusion proteins were detected using anti-GST. Anti-Hexokinase was used to indicate the level of total proteins. Bar, 5μm.
Table 4.2 N-terminal proline rich region of CR16 and actin binding motif play redundant role for suppressing growth defect of \textit{vrp1A} strain

<table>
<thead>
<tr>
<th></th>
<th>Growth at 37°C</th>
<th>Actin interaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>CR16</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>CR16\textsuperscript{4A}</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>CR16\textsubscript{11-485}</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>CR16\textsuperscript{4A11-485}</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CR16\textsubscript{18-485}</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>CR16\textsuperscript{4A18-485}</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CR16\textsubscript{31-485}</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>CR16\textsuperscript{4A31-485}</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CR16\textsubscript{61-485}</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Transformant cells of \textit{vrp1A} strain expressing the above CR16 mutants as GST fusion proteins from high copy plasmid were assessed for the growth at high temperature. The interaction between the above CR16 mutants and Actin was assessed using yeast two hybrid.

4.5 Redundant role of proline rich region and actin binding motif was further verified by CR16-WIRE chimera molecules

Data from previous section (section 4.8) reveals that the V domain of CR16 is essential for the suppression of growth defect of \textit{vrp1A} strain. However, we noticed that the CR16\textsubscript{61-485} expressed from high copy plasmid has trace activity to restore the growth at high temperature and comparable activity to restore the endocytosis of \textit{vrp1A} strain. This is in contrast with the results from WIP and WIRE. Since the
WIRE53-441 (WIREΔV) has lost the ability to suppress the growth or endocytosis defects, but still able to localize to cortical patches, it can be used as a non-functional fragment analyze the effect from other regions of CR16.

4.5.1 Proline rich region and actin binding motif of CR16 play redundant role in chimera molecules to suppress the growth and endocytosis defect

We made a series of fusion constructs of V domain of CR16 (CR161-63, CR1611-63, CR1631-63, CR164A1-63, CR16D1-63, and CR16D4A31-63) and WIRE53-441 (WIREΔV), to determine whether fusing the V domain of CR16 would improve the activity of WIRE53-441 to suppress the defects of vrplΔ strain. CR161-63-WIRE53-441-GST, CR164A1-63-WIRE53-441-GST, CR1611-63-WIRE53-441-GST and CR1631-63-WIRE53-441-GST expressed form high copy plasmid were able to suppress the growth defect of vrplΔ strain while CR16D4A1-63-WIRE53-441-GST or CR16D4A31-63-WIRE53-441-GST were not able to suppress the growth defect of vrplΔ strain (Figure 4.12 and Table 4.3). Thus the fusion chimeras have similar activities to CR16. It is clear that the combination of proline rich region deletion and actin binding motif mutation abolishes the function for chimera molecules to suppress the growth or endocytosis defect. This suggests that the V-domain of CR16 contains two functionally redundant regions, the actin binding motif and a proline rich sequence. Further more, the WH2/V domain of CR16 may have additional activities compared to those of WIP and WIRE.
Figure 4.12 Proline rich region and actin binding motif of CR16 play redundant role for chimera molecules to suppress the growth defect

Table 4.3 Suppression of growth defect for chimera molecules in \textit{vrpIA} strain

<table>
<thead>
<tr>
<th>High copy GST fusion</th>
<th>Growth at 37°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>CR16\textsubscript{1-63}</td>
<td>-</td>
</tr>
<tr>
<td>CR16\textsubscript{1-63}-WIRE\textsubscript{53-441}</td>
<td>+</td>
</tr>
<tr>
<td>CR16\textsubscript{4A-1-63}-WIRE\textsubscript{53-441}</td>
<td>+</td>
</tr>
<tr>
<td>CR16\textsubscript{11-63}-WIRE\textsubscript{53-441}</td>
<td>+</td>
</tr>
<tr>
<td>CR16\textsubscript{4A-11-63}-WIRE\textsubscript{53-441}</td>
<td>-</td>
</tr>
<tr>
<td>CR16\textsubscript{51-63}-WIRE\textsubscript{53-441}</td>
<td>+</td>
</tr>
<tr>
<td>CR16\textsubscript{4A-31-63}-WIRE\textsubscript{53-441}</td>
<td>-</td>
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</tbody>
</table>

4.5.2 V domain of CR16 enhances the activity of WIP and WIRE to suppress growth and endocytosis defect

V domain of verprolin proteins are crucial for carrying out important cellular functions such as suppression of growth and endocytosis defect. But the various
members of verprolin family of proteins behave slightly differently. WIRE lost the ability to suppress the defects of \( wrlA \) strain once it lost the actin interaction mediated by KLKK motif. Similarly, WIP lost the ability when it lost actin interaction mediated by either KKLKK motif or proline rich region. CR16 is unique that it kept full activity even through lost the actin interaction, and that proline rich region in N-terminal played redundant role with actin binding motif to suppress cellular defects. Thus it is possible that the V domain of CR16 has higher activity than those of WIP or WIRE.

In order to verify this hypothesis, we fused V domain of CR16 to the N-terminus of WIP, WIRE, WIP_{51-503} or WIRE_{53-441}, then expressed the chimera molecules as GST fusion proteins from low copy or high copy plasmids in \( wrlA \) strains (Table 4.4). Transformants cells expressing CR16_{1-63}-WIP_{1-503}-GST, CR16_{1-63}-WIP_{51-503}-GST, CR16_{1-63}-WIRE_{1-441}-GST, or CR16_{1-63}-WIRE_{53-441}-GST from low copy plasmid suppressed the growth and endocytosis defect, while \( wrlA \) cells expressing WIP or WIRE from low copy plasmid did not suppress. Transformants expressing CR16_{1-63}-WIP_{51-503}-GST or CR16_{1-63}-WIRE_{53-441}-GST from high copy plasmid suppressed the growth and endocytosis defect while \( wrlA \) cells expressing WIP_{51-503}-GST or WIRE_{53-441}-GST did not suppress. Further more, fusing V domain of CR16 to high copy WIP or WIRE enhanced the suppression of defect of \( wrlA \) cells (Table 4.4). It suggests that the V domain of CR16 contains two functionally redundant motifs, the actin binding motif and a proline rich region. Therefore the V
domain of CR16 may have additional activities compared to those of WIP or WIRE, to suppress growth and endocytosis defects in \textit{vrplA} strain.

Table 4.4 V domain of CR16 enhances the activity to suppress growth defect in \textit{Vrpld} strains

<table>
<thead>
<tr>
<th></th>
<th>Growth at 37°C</th>
<th>Endocytosis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(LC) GST</td>
<td>(HC) GST</td>
</tr>
<tr>
<td>CR1-63</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>WIP</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>CR1-63-WIP</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>WIP(51-503)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CR1-63-WIP(51-503)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>WIRE</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>CR1-63-WIRE(53-441)</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>WIRE(53-441)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CR1-63-WIRE(53-441)</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

All the above constructs were expressed as GST fusion proteins in \textit{vrplA} strain from high copy or low copy plasmid. The growth and endocytosis of \textit{vrplA} cells expressing one of the above GST fusion proteins were assessed.

4.6 V domain of CR16 does not suppress growth or endocytosis defect of \textit{vrplA} strain

The results from previous section show that V domain is important for the function and interaction of CR16 with actin. It is not clear whether the V domain alone can suppress the growth defect of \textit{vrplA} strain. Therefore, we constructed new clones that express the V domain of CR16 in cells so that the function of V domain will not be
affected by other regions beyond. CR16_{1-63}, CR16_{11-63}, CR16_{31-63}, CR16^{4A}_{1-63}, CR16^{4A}_{11-63}, and CR16^{4A}_{31-63} were fused to the GST and expressed from YCplac181 (high copy) plasmid to test the ability in suppressing cellular defects of \textit{vrp1A} strain. The same mutants were also cloned into pACT2 for testing the interaction with actin or SH3 domain-containing proteins.

GST fusion molecules of V domain of CR16 (CR16_{1-63}-GST) expressed from high copy plasmid in \textit{vrp1A} strain did not suppress the growth or endocytosis defect (data not shown). Since CR16_{61-485} expressed from high copy plasmid restores endocytosis, it suggests that the functional domains which are essential for suppressing endocytosis defect are located in CR16_{61-485}. Since either CR16_{1-63} or CR16_{61-485} does not restore the growth at high temperature, it suggests that the V domain is crucial for suppressing growth defect.

Since the CR16_{63-485} localizes to cortical patches in \textit{vrp1A} strain indicating that the localization signal is located in C-terminal of CR16. CR16_{1-63} does not suppress the growth defect might be due to lack of localization. CR16_{1-63} is a fragment consisting of 63 residues, and it does not suppress growth defect also might due to lack of stability in vivo. Therefore CR16_{1-63} was fused to the N-terminus of GST to enhance the protein stability and fused to N-terminus of CAAX to enhance the localization. However, none of CR16_{1-63}-GST, CR16_{1-63}-CAAX, or CR16_{1-63}-GST-CAAX expressed from high copy plasmid suppressed the growth or endocytosis defect (data
Functional analysis of CR16

not shown), indicating that the V domain requires sequence located in the CR16_63-485 for suppressing growth defects of vrplΔ strain.

4.7 Interaction between CR16 and SH3 domain-containing proteins of S. cerevisiae

Vrp1p, the first identified member of verprolin family of proteins, has been shown to interact with a number of cytoskeletal proteins such as Las17p (Li, 1997), Myo3p (Evangelista, et al., 2000), Myo5p (Goodson, et al., 1996), Rvs167p (Balguerie, et al., 1999), Bzzlp (Soulard, et al., 2002) and Mti1p (Mochida, et al., 2002). The proline rich sequence in WIRE has been shown to interact with profilin (Aspenstrom, 2002). Thus we checked for the interaction between these proteins and CR16 or CR16 mutants using yeast two hybrid.

Two sets of constructs were made for yeast two hybrid assay. The first sets included pAD-Myo3, pAD-Myo5, pAD-Bzz1, pAD-Hof1, pAD-Profilin, pBD-CR16 series and pBD-V domain series of CR16. The other set of new constructions included pAD-V domain series of CR16. Any one clone of DNA activation domain fusions and one clone of DNA Binding Doman fusions from each set were selected and co-transformed into PJ69-4A. Transformants were selected by SD(-Trp-Leu) plate and streaked on SD(-Trp-Leu-His) to check for growth. Result was listed in Table 4.5. No interaction with Profilin, Rvs167p or Mti1p was detected. From the results of yeast
two hybrid assay, we can make up the interaction map. For CR16, the Myo3p binding site was located in residues 1-30, Hof1p binding site was located in residues 30-62, Myo5p and bzz1p binding sites were located in residues 63-485 (Figure 4.13).

Table 4.5 Interaction of CR16 and its mutants assessed using yeast two hybrid assay

<table>
<thead>
<tr>
<th></th>
<th>AD-Myo3p</th>
<th>AD-Myo5p</th>
<th>AD-Bzz1p</th>
<th>AD-Hof1p</th>
<th>AD-Proflin</th>
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<td>BD-CR161-485</td>
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<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
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<td>+</td>
<td>+</td>
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<td>-</td>
</tr>
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<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
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<td>BD-CR164A31-485</td>
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<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
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<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
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</tr>
<tr>
<td>BD-CR161-63</td>
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<td>-</td>
<td>-</td>
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</tr>
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<td>BD-CR164A1-63</td>
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<tr>
<td>AD-CR164A31-63</td>
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</tr>
</tbody>
</table>
Figure 4.13 A possible interaction map of CR16 with SH3 domain-containing proteins

4.8 Summary for CR16

Expression of CR16 in vrp1Δ strain suppresses both the growth defect and endocytic defect of this strain without correcting the actin patch polarization defect. CR16 requires the WH2/V domain for suppressing the growth defect. However the actin binding of CR16 is not critical for the suppression of growth defect of vrp1Δ strain. Mutational analysis of CR16 indicates that the proline rich region and actin binding motif in the WH2/V domain of CR16 play a redundant role in suppressing the growth defect of vrp1Δ strain, unlike WIRE where the actin binding motif is critical for activity. Therefore the WH2/V domain of the mammalian verprolin family members may have different activities in vivo.
5 Functional analysis of Vrp1p

Vrp1p has two functional modules: N-terminal peptide, Vrp1p1-364 and C-terminal peptide, Vrp1p364-817 (Thanabalu and Munn, 2001). Both Vrp1p1-364 and Vrp1p364-817 have been shown to restore growth at restrictive temperature to vrplΔ strain. Vrp1p364-817, but not Vrp1p1-364, was able to correct the actin patch polarization defect of vrplΔ strain. Both Vrp1p1-364 and Vrp1p364-817 bind to actin monomers (Thanabalu and Munn, 2001, Thanabalu, et al., 2007, Vaduva, et al., 1997) while Vrp1p364-817 in addition binds to Las17p, the yeast homologue of mammalian WASP (Karpova, et al., 1998, Naqvi, et al., 1998). The localization of Vrp1p364-817 to cortical actin patches is dependent on Las17p (Thanabalu and Munn, 2001) and deleting the Las17p binding domain (LBD) of Vrp1p364-817 leads to complete loss of activity of Vrp1p364-760 in actin patch polarization. Interaction with actin monomers and WASP-family proteins are key features shared with all the members of verprolin proteins.

5.1 LBD is critical for Vrp1p’s role in actin patch polarization

It has been reported that Vrp1p364-817 can complement both growth and actin patches polarization defects of the vrplΔ strain (Thanabalu & Munn 2001) and the restoration of actin patch polarization depends on the presence of Las17p binding domain (LBD, Vrp1p760-817) as deletion of LBD results in a peptide Vrp1p364-760 which is unable to complement either the growth defect or actin patch polarization defect (Thanabalu et
Functional analysis of Vrp1p

al., 2007). However it is not clear whether the LBD is required for the ability of full length Vrp1p1-817 to complement the actin patch polarization defect. In order to determine the role of LBD for the full length we made a deletion construct expressing Vrp1p1-760 under the control of VRP1 promoter on a low copy plasmid. Expression of Vrp1p1-760 restored growth at high temperature in both solid and liquid media (Figure 5.1 A and B), and restored endocytosis but not actin patch polarization at both 24°C and 37°C (Figure 5.2 A and B).

In order to analyze the localization of Vrp1p1-760, DNA encoding GFP was fused to the C-terminal of Vrp1p1-760 and Vrp1p1-817-GFP respectively and expressed from low copy plasmid under the transcriptional control of VRP1 promoter in vrp1Δ strain. Vrp1p1-817-GFP localizes to cortical patches while Vrp1p1-760-GFP shows a diffused cytoplasmic staining similar to the cells expressing GFP (Figure 5.3 A). In order to analyze the nature of cortical patches, Arc40p-RFP was co-expressed in vrp1Δ cells which are expressing Vrp1p1-817-GFP. Arc40p is one of the seven subunits of Arp2/3 complex which is a nucleator of actin and localizes to actin patches (Winter, et al., 1999). The co-localization of Vrp1p1-817-GFP and Arc40p-RFP shows that some patches of Vrp1p1-817-GFP overlaps with patches of Arc40p-RFP indicating that Vrp1p1-817-GFP localizes to cortical actin patches (data not shown). The loss of localization to cortical patches or the inability to correct the actin patches polarization defect is not due poor expression of Vrp1p1-760 (Figure 5.3 B)
Functional analysis of Vrplp

Figure 5.1 LBD is not essential for Vrplp to restore growth at high temperature

A) *vrp1Δ* cells expressing Vrplp or Vrplp<sub>1-760</sub> from low copy plasmid were streaked on YPUAD plate and incubated at 24°C or 37°C respectively. The cell growth at high temperature was assessed after 3 days.

B) Overnight culture of *vrp1Δ* cells expressing Vrplp or Vrplp<sub>1-760</sub> from low copy plasmid were diluted to OD<sub>600</sub> 0.05. Cells were inoculated in fresh YPUAD media and the value of OD<sub>600</sub> was read every hour.
Figure 5.2 LBD is essential for Vrp1p to restore actin patches polarization.

A) Exponentially growing \textit{vrp1A} cells expressing Vrp1p or mutants were incubated in YPUAD with Lucifer yellow for 1 hour before being viewed under fluorescence microscope.

B) Exponentially growing \textit{vrp1A} cells expressing Vrp1p or Vrp1p\textsubscript{1,760} from low copy plasmid were fixed using formaldehyde and permeabilized using Triton X-100. Cells were stained by Alexa fluorescence 488 conjugated phalloidin. Bar, 5 \textmu m.
Figure 5.3 Las17p binding domain is essential for Vrp1p to localizes to actin patches

A) Vrp1p-GFP or Vrp1p_{1-760}-GFP was expressed in vrp1A strain from low copy plasmid. Transformant cells of exponential phase were viewed using fluorescence microscope under GFP filter.

B) Vrp1p-GFP or Vrp1p_{1-760}-GFP was introduced into vrp1A strain and expressed from low copy plasmid. Cells expressing fusion proteins were inoculated in YPUAD, and lysed when the OD_{600} reached 0.5. Western blot was applied to the cell lysate. Fusion proteins were detected by anti-GFP. Anti-Hexokinase was used to indicate the level of total proteins. Bar, 5 μm.
5.2 Vrp1p1-364-LBD localizes efficiently to cortical actin patches

Vrp1p1-364 compliments the growth defect of vrpIA strain without correcting the actin patch polarization defect of the strain and adding a CAAX motif to Vrp1p1-364 enhanced the activity of Vrp1p1-364 without correcting the actin patch polarization defect (Thanabalu and Munn, 2001). In order to check whether LBD can enhance the cortical actin patch localization of Vrp1p1-364, GFP was fused to the C-terminus of Vrp1p1-364-LBD760-817 and Vrp1p1-364 respectively, and fusion proteins were expressed from low copy plasmid under the control of VRP1 promoter in vrpIA strain. The GFP signal was analyzed using live cell imaging. Vrp1p1-364-LBD760-817-GFP localizes to cortical patches while Vrp1p1-364-GFP showed a diffused cytoplasmic staining in vrpIA strain (Figure 5.4 A). In order to check whether the cortical patches are actin patches, Arc40p-RFP was expressed together with either Vrp1p1-364-GFP or Vrp1p1-364-LBD760-817-GFP and the patches were analyzed under RFP and GFP filter using live cell imaging. In vrpIA strain, Vrp1p1-364-LBD760-817-GFP localizes to cortical actin patches (Figure 5.4 B) which partially overlaps with cortical actin patches, while Vrp1p1-364-GFP shows a diffused cytoplasmic staining. However this is probably due to inefficient localization rather than lack of localization, as N-Vrp1p1-364 can restore growth at 37°C (Thanabalu and Munn, 2001).
Functional analysis of Vrp1p

Figure 5.4 N-Vrp1p-LBD localizes efficiently to cortical actin patches
A) Localization of GFP tagged N-Vrp1p and N-Vrp1p-LBD in vrplA cells. Transformants of vrplA cells expressing N-Vrp1p-GFP or N-Vrp1p-LBD-GFP were viewed under GFP filter.
B) Co-localization of GFP tagged N-Vrp1p-LBD with Arc40p-RFP in vrplA cells. vrplA cells co-expressing N-Vrp1p-GFP or N-Vrp1p-LBD-GFP with Arc40p-RFP were viewed under GFP or RFP filter.
C) Western blot of GFP tagged N-Vrp1p and N-Vrp1p-LBD. Transformants of vrplA cells expressing N-Vrp1p-GFP or N-Vrp1p-LBD-GFP were inoculated in YPUAD, and lysed when the OD600 reached 0.5. the cell lysate was analysed by western blot. Fusion proteins were detected using anti-GFP. Anti-Hexokinase was used to indicate the level of total proteins. Bar, 5 μm.

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5.3 Vrplp1-364-LBD corrects actin patch polarization defect of vrplΔ strain

Vrplp1-364-LBD was expressed under the control of VRPI promoter from low copy plasmid in vrplΔ strain to analyze the activity of Vrplp1-364 and LBD fusion protein. Expression of Vrplp1-364-LBD complements the temperature sensitive growth and endocytosis defect of vrplΔ strain more efficiently than Vrplp1-364 or Vrplp1-364-CAAX (Figure 5.5 A and B, Figure 5.6 A). In addition Vrplp1-364-LBD is also able to correct the actin patch polarization defect of vrplΔ strain unlike Vrplp1-364 or N-Vrplp1-364-CAAX at both 24°C and 37°C (Figure 5.6 B).

Previous report found a correlation between growth on 1M NaCl and actin patch polarization (Thanabalu and Munn, 2001). Thus we checked the growth of vrplΔ transformants on YPUAD plate with 1M NaCl, and found that cells expressing Vrplp1-364-LBD fusion protein are able to grow on 1M NaCl YPUAD plates while cells expressing Vrplp1-364 or Vrplp1-364-CAAX are not able to grow on YPUAD plates with 1M NaCl (data not shown). This could be either due to enhanced localization or increased expression of Vrplp1-364 caused by fusion to LBD. In order to determine between the two possibilities, Vrplp1-364-LBD and Vrplp1-364 were fused to N-terminal of GFP respectively and GFP fusion proteins were expressed from low copy plasmid in vrplΔ strain. Western blot was carried out using total cell extract. The western blot showed that both Vrplp1-364 and Vrplp1-364-LBD are expressed at comparable levels (Figure 5.4 C). Therefore, the better localization of Vrplp1-364-LBD
to cortical patches might be the reason why Vrp1p1-364-LBD restores the growth at high temperature more efficiently than Vrp1p1-364 or Vrp1p1-364-CAAX.

Figure 5.5 Vrp1p1-363 corrects growth defect of vrp1Δ strain
A) Growth at high temperature of vrp1Δ cells expressing N-Vrp1p1-364, N-Vrp1p1-364-LBD or N-Vrp1p1-364-CAAX from low copy plasmid.
B) Growth curve at high temperature of vrp1Δ cells expressing N-Vrp1p1-364, N-Vrp1p1-364-LBD or N-Vrp1p1-364-CAAX from low copy plasmid.
Figure 5.6 Vrp1p_{1,364}-LBD corrects actin patches polarization defect of \textit{vrplA} strain

A) Endocytosis of \textit{vrplA} cells expressing N-Vrp1p_{1,364}, N-Vrp1p_{1,364}-LBD or N-Vrp1p_{1,364}-CAAX.

B) Actin staining of \textit{vrplA} cells expressing N-Vrp1p_{1,364}, N-Vrp1p_{1,364}-LBD or N-Vrp1p_{1,364}-CAAX.
5.4 Actin binding domain is essential for actin patch polarization

5.4.1 Vrp1p_{1-70} and Vrp1p_{270-364} play redundant roles in actin patches polarization

Vrp1p interacts with actin through residues 1-70 which contain a V domain (Verprolin homology domain, also named as WASP homology 2 domain, WH2) including a single actin binding motif, KLKKAET (Vaduva, et al., 1997). Vrp1p_{270-364} was also reported to interact with actin which was verified by yeast two hybrid assay (Thanabalu and Munn, 2001), indicating the existence of a second actin binding motif. Vrp1p_{1-364} does not restore actin patches polarization of \( vrp1A \) strain, while fusion of LBD (Las17p Binding Domain, Vrp1p residues 760-817) to the C-terminus of Vrp1p_{1-364} rescues the ability to restore actin patches polarization. In order to determine whether both of these two actin binding regions located in Vrp1p_{1-364} are required for actin patch polarization or cell growth at elevated temperature, four deletion constructs were constructed and expressed under the control of VRP1 promoter from a low copy plasmid in \( vrp1A \) strain. These constructs are Vrp1p_{1-364}-LBD, Vrp1p_{70-364}-LBD, Vrp1p_{1-270}-LBD and Vrp1p_{70-270}-LBD (Figure 5.7).

Transformants of \( vrp1A \) cells expressing any one of the four fusion proteins grew on YPUAD plate at 37°C indicating that all the four fusion proteins are able to restore
the growth at 37°C of *vrp1Δ* strain to variety degree (Figure 5.8 A and B). This probably due to the LBD, which as a GST fusion can rescue the growth of *vrp1Δ* cells at 37°C (Thanabalu, *et al.*, 2007). Endocytosis assay showed that all fusion proteins restores endocytosis, and actin staining assay showed that both of Vrp1p<sub>70-364</sub>-LBD and Vrp1p<sub>1-270</sub>-LBD are able to restore actin patch polarization of *vrp1Δ* strain just like Vrp1p<sub>1-364</sub>-LBD. However *vrp1Δ* cells expressing Vrp1p<sub>70-270</sub>-LBD still exhibits depolarized actin patches (Figure 5.9 A and B). All the four chimera constructs were fused to N-terminal of GFP and expressed in *vrp1Δ* strain respectively. The expression of these various fusion proteins was checked by western blot using anti-GFP antibodies and found that all the fusion proteins is expressed reasonably well (Figure 5.8 C) suggesting that the observed difference in ability to restore actin patches polarization is not due to difference in protein expression. Thus it is concluded that the actin binding regions, Vrp1p<sub>1-70</sub> and Vrp1p<sub>270-364</sub>, play a redundant role in actin patches polarization.
It was in the context of changing population demographic landscape and decline of Sultanate in Moro territories that a power and identity vacuum existed. The space was filled through a reconnecting with the dormant Islamic consciousness that made possible through the return of Islamic scholars from the Middle East Islamic Universities. Chief among them was Ustadz Hashim Salamae an Al-Azhar alumnus. These scholars not only brought new political thinking but also the Middle East culture. Among the early signs of their influence were the replacement of traditional local Muslim dresses by a Middle East fashioned dresses. Customary rituals perceived deviant to Islam were prohibited. The Ustadz (religious scholar) slowly replaced the Pandita (Traditional Muslim preacher). Political and religious thinking began to echo contemporary Islamic thinkers in the Middle East, Hashim Salamat admitted drawing...

Figure 5.8 Vrp1p_{1-70} and Vrp1p_{270-364} region play redundant roles in contribution of growth at high temperature
A) Growth at 37°C of vrp1Δ strain expressing N-Vrp1p-LBD truncates from low copy plasmid.
B) Growth curve at 37°C of vrp1Δ strain expressing N-Vrp1p-LBD truncates from low copy plasmid.
C) Western blot of cell lysate from vrp1Δ strain expressing N-Vrp1p-LBD and its truncates from low copy plasmid.
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Figure 5.9 Vrp1p_{1-70} and Vrp1p_{270-364} region play redundant roles in contribution of endocytosis and actin patches polarization
A). Endocytosis of vrp1Δ strain expressing N-Vrp1p-LBD and its truncates.
B) F-actin staining of vrp1Δ strain expressing N-Vrp1p-LBD and its truncates for actin patches polarization assay. Bar, 5 μm.
It is also possible that the deletion may have affected the folding of some other domains which are critical for actin patch polarization and plays redundant roles with WH2/V domain of Vrp1p.

5.4.2 Identification of the second actin binding motif of Vrp1p_{1-364}

Vrp1p_{1-364} has a well characterized actin binding domain, the WH2/V domain (Vrp1p_{1-70}) and have the second actin binding domain in Vrp1p_{1-364} (Thanabal and Munn 2001). Yeast two hybrid assay showed the interaction between Vrp1p_{270-364} and actin indicating the prospective actin binding site located in residues 270-364. The well known actin binding sites of Verprolin family proteins are composed by positive charged residues such as KKLKK (WIP), KLKK (WIRE) and RLRK (CR16) indicating that lysine (K) and arginine (R) mediate the actin binding. Thus the putative actin binding site was sought in Vrp1p_{270-364} by searching for consecutive positive charged residues.

R_{282}R_{283} are two consecutive arginines that might mediate actin binding. In order to verify the actin interaction mediated by this RR motif, R_{282}R_{283} were mutated to A_{282}A_{283} by site directed mutagenesis. Both Vrp1p_{270-364} and Vrp1p^{A282A283}_{270-364} were cloned into pACT2 respectively while yeast actin was cloned into pAS2-1. pAD-Vrp1p^{A282A283}_{270-364} or pAD-Vrp1p_{270-364} were co-transformed with pBD-Actin
into PJ69-4A. PJ69-4A transformant cells expressing AD-Vrp1p_{270-364} and BD-Actin grew on SD(-Trp-Leu-His) plate while transformant cells expressing AD-Vrp1p^{A282A283}_{270-364} and BD-Actin did not grow (Figure 5.10) suggesting that Vrp1p_{270-364} but not Vrp1p^{A282A283}_{270-364}, interacts with actin. This indicates that R_{282}R_{283} is an actin binding site mediating the actin interaction of Vrp1p_{270-364}.

Figure 5.10 Identification of the second actin binding site of N-Vrp1p PJ69-4A transformant cells expressing AD-Vrp1p_{270-364}/Vrp1p^{A282A283}_{270-364} and BD-Actin were streaked on SD(-Trp-Leu-His) plates and incubated in 30°C for 5 days.

5.5 Actin binding and proline rich motif of Vrp1p play redundant roles

KLKKAET motif of Vrp1p at N-terminus is essential for interaction with actin. Mutation of K_{45}K_{46} to A_{45}A_{46} abolishes the interaction between Vrp1p_{1-70} and actin indicating that the KLKKAET is an actin binding site (Thanabalu and Munn, 2001, Vaduva, et al., 1997). Alignment of Vrp1p and CR16 shows a similar proline rich region at the N-terminal. The previous data on CR16 showed that proline rich region
and actin binding site of CR16 play redundant roles in suppression of growth defect in *vrp1Δ* strain. This raises an important question: Does the proline rich region and actin binding site of Vrp1p play redundant roles in suppressing actin patches polarization defect and growth defect of *vrp1Δ* strain?

### 5.5.1 Actin binding motif KLKK and proline rich region of Vrp1p play redundant role in cells growth at elevated temperature

Since the actin binding motif KLKKAET in WH2/V domain has been well characterised, the attention was focused on this domain. Vrp1p1-270 and Vrp1p^A45A46^1-270 (A45A46 indicates mutating K45K46 to A45A46) were expressed in *vrp1Δ* strain to analyze the importance of actin binding motif for cell viability at elevated temperature. Not much difference was found between Vrp1p1-270 and Vrp1^A45A46^1-270 (Figure 5.11 A) suggesting that although the actin binding domain is important, the actin binding motif is redundant with other motifs.

In order to determine whether the proline rich region at the N-terminal of Vrp1p is correlated with actin binding motif in restoring the growth of *vrp1Δ* strain at high temperature, another two clones comprising Vrp1p23-270 (deleting the proline rich region of Vrp1p) and Vrp1p^A45A46^23-270 (deleting proline rich region and mutating actin binding site) were constructed. The fragments were cloned into low copy plasmid, transformed into *vrp1Δ* cells and expressed under the control of VRP1 promoter. Transformant cells were selected on SD-Leu plates, re-streaked onto YPUAD plates
and incubated in 37° C. The vrplΔ cells expressing Vrp1p23-270 grew well while cells expressing Vrp1pA45A4623-270 or Vrp1p70-270 did not (Figure 5.11 A) indicating that either deletion of proline rich region or mutation of actin binding site does not abolish the ability to complement the growth defect of vrplΔ strain while combining the two mutations abolishes the ability to complement the growth defect. Endocytosis assay showed that expression of either Vrp1pA45A461-270 or Vrp1p23-270 restores endocytosis of vrplΔ strain (Figure 5.11 B). Therefore the proline rich region and actin binding site KLKKAET in N-terminus of Vrp1p play redundant role in complementing the growth and endocytosis defects of vrplΔ strain.
It was in the context of changing population demographic landscape and decline of Sultanate in Moro territories that a power and identity vacuum existed. The space was filled through a reconnecting with the dormant Islamic consciousness that made possible through the return of Islamic scholars from the Middle East Islamic Universities. Chief among them was Ustadz Hashim Salamae, an Al-Azhar alumnus.

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5.5.2 Actin binding motif KLKK and proline rich region of Vrp1p play redundant role in actin patches polarization

It has been reported that the Vrp1p364-817, but not Vrp1p1-364, restores polarization of cortical actin patches in vrplΔ strain (Thanabal and Munn, 2001). The previous data of this research showed that additional LBD to C-terminal of Vrp1p1-364 or Vrp1p1-270 confers the ability to restore actin patches polarization of vrplΔ strain. This also suggests that the Las17p interaction plays a role in actin patches polarization. The experiments with CR16 showed that the proline rich sequence located in the WH2/V domain of CR16 is redundant with the actin binding motif inside the WH2/V domain. Therefore it raises the hypothesis that proline rich region and actin binding site of Vrp1p1-270 might play a similar redundant role for Vrp1p1-270 to restore actin patches polarization if fused with additional Las17p binding domain.

In order to verify this hypothesis, Vrp1p760-817 (Las17p binding domain) was fused to the C-terminal of Vrp1p23-270, Vrp1pA45A46 \(_{1-270}\) and Vrp1pA45A46 \(_{23-270}\) respectively. Vrp1p23-270-LBD, Vrp1pA45A46 \(_{1-270}\)-LBD and Vrp1pA45A46 \(_{23-270}\)-LBD were transformed and expressed respectively in vrplΔ strain from low copy plasmid. Growth at high temperature and polarization of actin patches of these transformant vrplΔ cells were analyzed. Vrp1p1-270-LBD, Vrp1pA45A46 \(_{1-270}\)-LBD and Vrp1p23-270-LBD are able to correct the growth, endocytosis and actin patch polarization defects of the strain while Vrp1pA45A46 \(_{23-270}\)-LBD is able to correct the growth and endocytosis defect but not able to correct the actin patch polarization defect (Figure 5.12, Figure 5.13) indicating
Figure 5.12 Neither actin binding KLKKAET motif nor proline rich region of Vrp1p is crucial for cell growth at elevated temperature
A) Growth at 37°C of vrp1Δ cells expressing Vrp1p1-270-LBD or its mutants. Cells were streaked on YPUAD plates and incubated in 30°C for 3 days.
B) Growth curve at 37°C of vrp1Δ cells expressing Vrp1p1-270-LBD and its mutants. Cells from exponential phase were diluted to OD_{600} 0.05 and inoculated in fresh YPUAD media. The OD_{600} was measured at 1 hour interval for 10 hours.
C) Western blot of vrp1Δ cells expressing Vrp1p1-270-LBD and its mutants.
Figure 5.13 Actin binding motif KLKKAET and proline rich region of Vrp1p play redundant role in actin patches polarization
A) Endocytosis of *vrp1Δ* cells expressing Vrp1p_{1-270}-LBD or its mutants.
B) F-actin staining of *vrp1Δ* cells expressing Vrp1p_{1-270}-LBD or its mutants for actin patches polarization assay. Bar, 5 μm.
that either deletion of proline rich region or mutation of actin binding site is not crucial for the restoration of actin patches polarization, while combination of the above deletion and mutation abolishes actin patch polarization. This suggests that proline rich region and actin binding site of Vrp1p at N-terminus play redundant role in restoring actin patches polarization of \textit{vrp1A} strain.

5.6 Vrp1p-las17p interaction is essential for actin patch polarization

Vrp1p\textsubscript{364-817}, like Vrp1p\textsubscript{1-817}, co-localizes with cortical actin patches and restores actin patch polarization in \textit{vrp1A} strain (Thanabal and Munn, 2001). This localization to cortical patches requires Las17p. The last 57 residues at C-terminus of Vrp1p (Vrp1p\textsubscript{760-817}) was reported to be essential for the interaction with Las17p (Thanabal and Munn, 2001). Thus, we want to identify the Las17 binding site in C-terminus of Vrp1p, and question whether the interaction with Las17 is crucial for the restoring actin patches polarization.

5.6.1 Deletion analysis from the C-terminus of Vrp1p\textsubscript{364-817}

The C-terminal region of Vrp1p was aligned with the mammalian Verprolin (WIP, WIRE/WICH and CR16) proteins. The alignment indicates that the residues \textit{YPS}\textsubscript{803} and K\textsubscript{799} are highly conserved among the four members of the verprolin family (Figure 5.14). Point mutation constructs, \textit{YPS}\textsubscript{803} to \textit{AAA}\textsubscript{803}, K\textsubscript{799} to A\textsubscript{799} and combination of point mutation of \textit{YPS}\textsubscript{803} and K\textsubscript{799}, were constructed, transformed
and expressed in \textit{vrp1A} strain. Transformant \textit{vrp1A} cells expressing any one of the above truncates could grow at 37°C indicating that none of the mutations abolishes the activity of Vrp1p\textsubscript{364-817} in cell viability at high temperature (data not shown). This suggests that none of the motifs above is essential for complementing the growth defect at high temperature.

Deletion of the Las17p binding domain (LBD) from Vrp1p or Vrp1p\textsubscript{364-817} abolishes its ability to correct actin patch polarization defect of \textit{vrp1A} strain, while deletion of Las17p binding domain abolishes Vrp1p\textsubscript{364-817}'s ability, but not that of Vrp1p\textsubscript{1-817}, to restore the growth of \textit{vrp1A} strain at high temperature. This is due to the inability of Vrp1p\textsubscript{364-760} to localize to cortical patches which can be corrected by the addition of CAAX motif. However Vrp1p\textsubscript{364-760-CAAX does not restore cortical actin patch polarization in \textit{vrp1A} strain (Thanabalu, \textit{et al.}, 2007). It is possible that deletion of LBD\textsubscript{760-817} from Vrp1p\textsubscript{364-817} could have caused some defect in the tertiary structure of the protein, and/or affected interaction with other unidentified proteins important for actin patches polarization. In order to characterize the role of Vrp1p-Las17p interaction in actin patch polarization, amino acid residues composing Las17p binding motif must be identified. The last 35 amino acids of Vrp1p is sufficient to mediate
interaction with Las17p (Madania, et al., 1999). Thus we carried out a deletion analysis of the C-terminal domain, deleting 10 amino acid residues at a time and expressed the mutants in \textit{v}r\textit{p}1\textit{A} strain from low copy plasmid.

Expression of \textit{v}r\textit{p}1\textit{p}_{364-817}, \textit{v}r\textit{p}1\textit{p}_{364-807}, or \textit{v}r\textit{p}1\textit{p}_{364-797} enables \textit{v}r\textit{p}1\textit{A} cells growing at 37°C while expression of \textit{v}r\textit{p}1\textit{p}_{364-787} does not (Figure 5.16 A), indicating that amino acids 787-797 are crucial for the activity of \textit{v}r\textit{p}1\textit{p}_{364-817}. Endocytosis assay showed that expression of \textit{v}r\textit{p}1\textit{p}_{364-817}, \textit{v}r\textit{p}1\textit{p}_{364-807}, or \textit{v}r\textit{p}1\textit{p}_{364-797} restores endocytosis while expression of \textit{v}r\textit{p}1\textit{p}_{364-787} does not (Figure 5.15 B). Actin patches polarization assay showed that \textit{v}r\textit{p}1\textit{A} cells expressing \textit{v}r\textit{p}1\textit{p}_{364-817}, \textit{v}r\textit{p}1\textit{p}_{364-807}, or \textit{v}r\textit{p}1\textit{p}_{364-797} restores actin patches polarization while cells expressing \textit{v}r\textit{p}1\textit{p}_{364-787} does not, indicating that amino acids 787-797 are crucial for the activity of \textit{v}r\textit{p}1\textit{p}_{364-817} to complement the actin patches polarization defect (Figure 5.16).

In order to check the localization of the four C-terminal \textit{v}r\textit{p}1\textit{p} deletion truncates, GFP was fused to the C-terminal of the above truncates respectively, and GFP fusion proteins were expressed in \textit{v}r\textit{p}1\textit{A} strain and visualized under fluorescence microscope. \textit{v}r\textit{p}1\textit{p}_{364-817}-GFP and \textit{v}r\textit{p}1\textit{p}_{364-807}-GFP exhibit strong localization to cortical patches while \textit{v}r\textit{p}1\textit{p}_{364-797}-GFP exhibits weak localization. \textit{v}r\textit{p}1\textit{p}_{364-787}-GFP shows a defused cytoplasmic staining (Figure 5.17 A). Western blot shows that the proteins are expressed in a comparable level (Figure 5.17 B). This suggests that deletion of C-terminal of \textit{v}r\textit{p}1\textit{p}_{364-817} abolishes its ability to restore growth at 37°C and actin
patches polarization not due to lack of protein stability, but due to the lack of localization to cortical patches.
Figure 5.15 C-terminal of \text{Vrp1p}_{364-817} is crucial for restoring the growth and endocytosis at 37°C
A) Growth at 37°C of \text{vrp1A} cells expressing N-\text{Vrp1p}_{364-817} series truncates from low copy plasmid. Cells were streaked on \text{YPUAD} and incubated in 37°C for 3 days.
B) Endocytosis of \text{vrp1A} cells expressing N-\text{Vrp1p}_{364-817} series truncates. Bar, 5 µm.
Figure 5.16 C-terminal of Vrp1p is crucial for restoring the actin patches polarization of vrp1A strain
Actin staining of vrp1A cells expressing N-Vrp1p series truncates from low copy plasmid. Bar, 5 µm.
Figure 5.17 C-terminus of Vrp1p is crucial for localization to cortical patches

A) Localization of vrplA cells expressing GFP fusion protein of N-Vrp1p364-817 series truncates from low copy plasmid.

B) Western blot of cell lysate from vrplA cells expressing GFP fusion protein of N-Vrp1p364-817 series truncates from low copy plasmid. Bar, 5 μm.
5.6.2 MPKPR motif of Vrp1p<sub>364-817</sub> is essential for growth at high temperature and actin patches polarization

It has been previously shown that the amino acids 408-412 (408FPAPE412) of WIRE/WICH are essential for interaction with WASP (Aspenstrom, 2004). The region of Vrp1p which matches with these five amino acids falls within 788-792 and is represented in Vrp1p by 788MPKPR792. These five amino acids were mutated to alanines (788MPKPR792 to 788AAAAA792, labeled as 5A) by site directed mutagenesis. Expression of Vrp1p<sup>5A</sup><sub>364-817</sub> from low copy plasmid abolishes the ability of this peptide to complement the growth defect of <i>vrp1Δ</i> strain (Figure 5.18 A and B). This mutation also abolishes the ability of Vrp1<sub>364-817</sub> to complement endocytosis or actin patches polarization defect (Figure 5.19). Western blot showed that the mutant protein is expressed at a comparable level, indicating that the loss of ability of Vrp1<sup>5A</sup><sub>364-817</sub> to complement growth and actin patches polarization defect is not due to poor expression (Figure 5.18 C).
Figure 5.18 MPKPR motif is essential for restoring the growth at high temperature

A) Growth at high temperature of vrplA cells expressing Vrp1p364-817, Vrp1p364-817 with MPKPR mutation or CAAX fusions from low copy plasmid.

B) Growth curve of vrplA cells expressing Vrp1p364-817, Vrp1p364-817 with MPKPR mutation or CAAX fusions from low copy plasmid.

C) Western blot of vrplA cells expressing Vrp1p364-817 or its mutant. GFP fusion proteins of Vrp1p5A_{364-817} was expressed and detected using anti-GFP antibody. Anti-Hexokinase antibody was used to indicate the level of total proteins.
Figure 5.19 MPKPR motif is essential for restoring endocytosis and actin patches polarization

A) Endocytosis of vrplA cells expressing Vrp1p364-817 or its mutant.
B) Actin staining of vrplA cells expressing Vrp1p364-817, Vrp1p364-817 with MPKPR mutation or CAAX fusions from low copy plasmid. Bar, 5 μm.
5.6.3 MPKPR motif of Vrp1p364-817 is essential for localization in vrp1Δ strain

Now it raises an interesting question that whether the loss of function for Vrp1p5A364-817 to complement either growth defect or actin patches polarization defect is due to lack of localization. Therefore, in order to determine this, GFP was fused to the C-terminus of Vrp1p5A364-817 and GFP fusion protein was expressed in vrp1Δ strain. Fluorescence microscopy images showed that Vrp1p364-817-GFP localizes to cortical patches while Vrp1p5A364-817-GFP lost the localization and displayed a defused cytoplasmic staining (Figure 5.20). Western blot showed that Vrp1p5A364-817-GFP and Vrp1p364-817-GFP are expressed in a comparable level, indicating that the loss of localization for Vrp1p5A364-817 is not due to poor expression or stability of protein (Figure 5.18 C). This suggests that the loss of ability of Vrp1p5A364-817 to restore growth at high temperature and actin patches polarization is due to the lack of localization to cortical patches.
5.6.4 Localization of Vrp1p\textsuperscript{5A}_{364-817} to cortex restores viability at high temperature

Addition of a CAAX box to the C-terminus has been used to confer covalent lipid modification and strong cortical localization to proteins which do not normally localize to the cortex (Golsteyn, \textit{et al.}, 1997). Therefore the CAAX box (CIIC) of \textit{S. cerevisiae} Ras1p (Powers, \textit{et al.}, 1984) was fused to the C-terminus of Vrp1p\textsuperscript{5A}_{364-817} and Vrp1p\textsuperscript{5A}_{364-817}-GFP respectively, and CAAX tagged proteins were expressed in \textit{vrp1Δ} strain from low copy plasmid. Fluorescence images showed that Vrp1p\textsuperscript{5A}_{364-817}-GFP-CAAX localizes to cortex (Figure 5.20).

Figure 5.20 MPKPR motif is essential for Vrp1p\textsubscript{364-817} to localizes to cortical patches Localization of \textit{vrp1Δ} cells expressing Vrp1p\textsubscript{364-817}-GFP series from low copy plasmid.
This raises an interesting question if artificially directing Vrp1p_{364-817}^{SA} to the cortex mediated by CAAX box restores the functions of Vrp1p_{364-817}^{SA}. Thus the growth at high temperature and actin patches polarization of vrplΔ cells expressing Vrp1p_{364-817}^{SA}-CAAX was checked. Transformants of vrplΔ cells expressing Vrp1p_{364-817}^{SA}-CAAX from low copy plasmid grew at 37°C. However the actin patches were still depolarized (Figure 5.19 B). This confirms that the localization of Vrp1p_{364-817}^{SA} is essential for its function in restoring growth at high temperature and specific localization to cortical patches is essential for restoring actin patches polarization in vrplΔ strain.

5.6.5 MPKPR motif of Vrp1p_{364-817} is crucial for the interaction with Las17p

F_{408}PAPE_{412} motif of WIRE/WICH has been reported to be essential for interaction with WASP (Aspenstrom, 2004). Alignment between WIRE and Vrp1p shows that M_{788}PKPR_{792} motif of Vrp1p is the homologue sequence of WIRE’s FPAPE motif. Therefore it was suspected that MPKPR motif of Vrp1p is crucial for the interaction with Las17p which is essential for localization of Vrp1p_{364-817} to cortical patches and essential for the function of Vrp1p_{364-817} to restore cell growth at elevated temperature or actin patches polarization.

Las17p_{1-206} is the region which mediates the interaction of Las17p to Vrp1p (Naqvi, et
al., 1998). Yeast two hybrid assay showed that PJ69-4A cells expressing both AD-Vrp1p$^{SA}_{364-817}$ and BD-Las17p$^{1-206}$ do not grow on SD(-Trp-Leu-His) plates, while cells expressing AD-Vrp1p$_{364-817}$ and BD-Las1p$_{71-206}$ are able to grow on SD(-Trp-Leu-His) plates (Figure 5.21). This indicates that the interaction of Vrp1p$_{364-817}$ with Las17p requires the MPKPR motif of Vrp1p. Thus the conclusion could be drawn that Las17p interaction mediated by MPKPR motif is crucial for Vrp1p$_{364-817}$ to localize to cortical patches, to complement the growth defect and actin patches polarization defect of vrplA strain.

![Figure 5.21 MPKPR motif is crucial for the Interaction with Las17p](image)

**Figure 5.21** MPKPR motif is crucial for the Interaction with Las17p
Yeast two hybrid assay for assessing the interaction between Vrp1p$_{364-817}$ and Las17p mediated by MPKPR motif.

### 5.7 Summary for Vrp1p

The actin cytoskeleton in *S.cerevisiae* consists of cortical actin patches and cables. The cortical actin patches are polarized correlating with directed growth of the cells.
Both Vrp1p (yeast WIP) and Las17p (yeast WASP) play an important role in actin patch polarization, endocytosis and growth at elevated temperature as loss of Vrp1p or Las17p leads to defects in endocytosis, actin patch polarization and growth at elevated temperature. Vrp1p\textsubscript{1-364} can support growth of \textit{vrp1}\textit{Δ} cells at high temperature while Vrp1p\textsubscript{364-817} can support growth of \textit{vrp1}\textit{Δ} cells at high temperature as well as actin patch polarization. A second actin binding motif located in Vrp1p\textsubscript{1-364} was identified and either one of Vrp1p\textsubscript{1-364}'s two actin binding domains is sufficient for actin patch polarization in the presence of Las17p binding domain. Although the actin binding domain is critical for actin patch polarization, the actin binding motif plays redundant roles with the proline rich sequence located inside the WH2/V domain of Vrp1p to restore cell growth at high temperature and actin patches polarization. Las17p binding domain located at Vrp1p\textsubscript{760-817} is critical for Vrp1p's role in actin patch polarization, and fusing the Las17p binding domain to C-terminal of Vrp1p\textsubscript{1-364} enable Vrp1p\textsubscript{1-364} to restore actin patch polarization. The Las17p biding domain is essential for proper localization of Vrp1p\textsubscript{364-817} and for complement of growth and actin patches polarization defects. Fusing CAAX tag to C-terminal of Vrp1p\textsuperscript{5A}\textsubscript{-364-817} enables it to localize to cortex. Vrp1p\textsuperscript{5A}\textsubscript{-364-817-CAAX fully restores cell viability at high temperature, but not actin patches polarization. Therefore, the Vrp1p-Las17p interaction mediated by MPKPR motif is crucial for Vrp1p\textsubscript{364-817}'s localization and for complement of cell growth and actin patches polarization defects.
6 Discussion

6.1 Functional analysis of mammalian Verprolin proteins using *S. cerevisiae*

Eukaryotic cells maintain their shape and size due to the presence of cytoskeleton made up of microfilament, microtubules and intermediate filaments. Rapid reorganization and remodeling of actin cytoskeleton in these cells regulate crucial cellular processes such as endocytosis, cytokinesis, cell polarization, and cell morphogenesis. These cellular processes are crucial for cell adhesion, motility, metastasis, chemotaxis, immune response and wound healing. Most of these cellular processes are dependent on localized assembly and disassembly of actin filaments and are regulated by the activities of around 20-30 highly conserved actin-associated proteins. These molecular regulators control the temporal and spatial assembly of actin structures and regulate the turnover of actin filaments so that cells alter their actin cytoskeleton in response to both internal and external signals. The assembly of diverse actin structures is monitored by a highly conserved mechanism in both invertebrates and vertebrates, even in the simple unicellular organism, *Saccharomyces cerevisiae* (Moseley and Goode, 2006). The actin cytoskeleton in yeast cells consists of cortical actin patches, actin cables and a contractile actin ring (Adams and Pringle, 1984). The cortical actin patches are polarized towards nascent bud sites and are postulated to be the sites of endocytosis. Actin cables are crucial for cell polarity, and
cooperate with contractile actin ring which is essential for cytokinesis (Mabuchi, 2006).

Rapid actin polymerization provides the force to drive the reorganization of actin cytoskeleton. In eukaryotic cells the Arp2/3 complex and formins promote actin polymerization (Pollard, 2007). The rate limiting step in actin polymerization is the formation of a nucleus made up of three actin monomers (Higgs and Pollard, 2001). The Arp2/3 complex accelerates the formation of nucleus by providing two actin related proteins, Arp2 and Arp3 (Mullins, et al., 1998). The activity of Arp2/3 complex in mammalian cells is regulated by the WASP family of proteins including WASP, N-WASP, WAVE-1, -2, -3 and in yeast by the WASP homologue Las17p. The WASP and N-WASP are central players in the regulation of F-actin nucleation and promote actin polymerization by activating the Arp2/3 complex (Stradal, et al., 2004). WASP family of proteins have been shown to interact with the mammalian verprolin family of proteins: WIP, CR16 and WIRE/WICH (Aspenstrom, 2002, Ho, et al., 2001, Kato, et al., 2002, Ramesh, et al., 1997). However, the role of mammalian verprolin family of proteins in regulating WASP and N-WASP is not clear (Aspenstrom, 2005). Further more, though the verprolin proteins are identified as WASP interacting partners, they have other functions that are independent of WASP family proteins (Anton and Jones, 2006).

A distinct feature of the verprolin (Vrp1p, WIP, WIRE, and CR16) family members is
the presence of a V domain (also referred to as WH2 domain) at the N-terminal (Aspenstrom, 2005) and a WASP/N-WASP/Las17p binding domain at the C-terminal. Though the members of mammalian verprolin proteins are sequence homologues, the question that has not been addressed is whether all the three mammalian verprolin proteins play the same role? *S.cerevisiae* has a single verprolin, Vrp1p (Donnelly, *et al.*, 1993) and a single WASP homologue, Las17p (Li, 1997) while mammalian cells have at least three verprolin proteins and two WASP family members which can interact with verprolin proteins which potentially can generate six different combinations of protein complexes. The *S.cerevisiae* proteins, Vrp1p and Las17p form a complex (Naqvi, *et al.*, 1998), similar to its mammalian counterparts, WIP complexed with WASP (Ramesh, *et al.*, 1997), WIRE complexed with N-WASP (Kato, *et al.*, 2002) and CR16 complexed with N-WASP (Ho, *et al.*, 2001). *S.cerevisiae* *vrp1A* or *las17A* cells exhibit defects in endocytosis, actin patches polarization and growth at elevated temperature (Li, 1997, Munn, *et al.*, 1995). Expression of WIP suppresses the growth defects of both *vrp1-I* and *vrp1A* mutant strain and the endocytosis defect of *vrp1-I* strain (Vaduva, *et al.*, 1999), indicating that WIP and Vrp1p are functional homologues. In this study, *S.cerevisiae* *vrp1A* strain was used to analyze the functional characteristics of the mammalian verprolin proteins, WIP, WIRE/WICH and CR16. The result from this study shows that WIP, WIRE and CR16 are functional homologues of Vrp1p. Therefore the simple unicellular organism, *vrp1A* strain of budding yeast, should provide a simple and useful tool for identifying and characterizing the functional domain of mammalian verprolin proteins.
6.2 Mammalian Verprolin proteins suppress cellular defects of yeast *vrp1 Δ* strain

6.2.1 Expression of mammalian Verprolin suppress growth defect of *vrp1 Δ* strain

*S. cerevisiae vrplΔ* cells are not viable at elevated temperature (Donnelly, *et al.*, 1993). Actin structures such as actin cables that extend along the mother-bud axis, are required for polarized growth (Pruyne, *et al.*, 1998), cell viability at high temperature and especially cytokinesis suggesting that the inviability of *vrp1 Δ* cells could be due to defects in the actin cytoskeleton (Donnelly, *et al.*, 1993, Munn, *et al.*, 1995). Actin polymerization is essential for the formation of actin cables and provides force to promote the actin patches distribution (Moseley and Goode, 2006) and Vrplp has been proposed to promote actin actin polymerization through the acidic domain of Myo5p and Myo5p (Geli, *et al.*, 2000). WIP is the first well characterised member of Verprolin family of proteins, and Vrplp is the yeast homologue of WIP (Vaduva, *et al.*, 1999). As the name implies, WASP interacting protein, WIP binds to WASP and decrease the ability of WASP to active Arp2/3 complex to initiate actin polymerization mediated by Cdc42 (Konno, *et al.*, 2007, Martinez-Quiles, *et al.*, 2001). WIP has also been proposed to reduce the rate of actin polymerization and depolymerization by stabilizing F-actin (Martinez-Quiles, *et al.*, 2001).
Western blot shows that the protein stability of CR16 and some of its truncate are not comparable to those of WIP or WIRE. The GST tag has been shown to be able to stabilize proteins. Thus expressing GST fusions of CR16 or its truncate in yeast cells makes it easy to eliminate the possibility that proteins lose their function due to instability. In order to test the function of mammalian verprolins, WIP, WIRE or CR16 was expressed as GST fusion proteins from low copy plasmid under the control of VRP1 promoter in *S. cerevisiae* *vrp1α* strain. Expression of CR16 from low copy plasmid suppresses the growth defect of *vrp1α* strain while WIP or WIRE does not (Figure 3.3, Figure 3.14, Figure 4.1). This indicates that CR16 has higher activity than WIP or WIRE in suppressing the growth defect of *vrp1α* strain though it is expressed from a low copy plasmid. Sequence alignment shows that CR16 has the highest homology with Vrp1p (Figure 6.1) among the three members of Verprolin family of proteins, and this might be a possible reason for the high activity of CR16 compared to WIP and WIRE in the ability to suppress the growth defect of *vrp1α* strain.

The inability of WIP or WIRE to suppress the growth defect of *vrp1α* strain could be due to low expression or low activity when expressed from low copy plasmid. Therefore WIP or WIRE was expressed as GST fusion proteins from high copy plasmid in *vrp1α* strain. The expression of WIP or WIRE from high copy plasmid suppressed the growth defect of *vrp1α* strain (Figure 3.3, Figure 3.14). This suggests that WIP and WIRE are also functional homologues of Vrp1p though their intrinsic activity in suppressing the growth defect is lower compared to that of CR16. This
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could be due to the lower sequence homology between Vrplp and WIP/WIRE which reflects the evolutionary distance from Vrplp.

![Sequence alignment of Verprolin family of proteins.](image)

Figure 6.1 Sequence alignment of Verprolin family of proteins. Sequence of gene is analyzed using Vector NTI 9.0.

6.2.2 Expression of mammalian Verprolin suppresses the endocytosis defect of *vrp1A* strain

Endocytosis is an important cellular process through which the plasma membrane invaginates and produces a vesicle which can fuse with endosomes (Engqvist-Goldstein and Drubin, 2003, Smythe and Ayscough, 2006). Endocytosis is involved in recycling of plasma membrane and down-regulation of cell surface receptors (Drubin, *et al.*, 2005). Actin localizes to the cortical region of the cell just below the plasma membrane (Smythe and Ayscough, 2006). The pinching off of the newly formed vesicles after invagination is promoted by the physical force generated by actin polymerization (Merrifield, 2004). Verprolin proteins, complexed with WASP family of proteins, are involved in actin polymerization catalyzed by Arp2/3 complex (Goley and Welch, 2006). *S.cerevisiae vrp1A* strain is defective in endocytosis (Munn, *et al.*, 1995) suggesting that WIP, WIRE and CR16 could also be involved in endocytosis.
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*S. cerevisiae* *vrp1A* strain expressing CR16 form low copy plasmid can uptake Lucifer yellow (data not shown) indicating that CR16 can suppress the endocytosis defect while expression of WIP or WIRE from low copy plasmid does not (Figure 3.4, Figure 3.15). This inability of WIP or WIRE is due to the low expression from a low copy number plasmid since both WIP and WIRE expressed from high copy plasmid suppress the endocytosis defect of *vrp1A* strain (Figure 3.4, Figure 3.15). Why does the suppression of endocytosis require expression of WIP or WIRE from high copy plasmid? The sequence alignment of the four verprolin proteins shows that the sequence homology between WIP or WIRE and Vrp1p is lower than that between CR16 and Vrp1p. This might be the reason why CR16 expressing from low copy plasmid has sufficient activity to suppress the endocytosis defect of *vrp1A* strain while WIP or WIRE expressed from low copy plasmid does not suppress the endocytosis defect. CR16 has 30.7% prolines in its sequence and thus it can potentially interact with higher number of molecules of Myo3p or Myo5p. Myo3 and Myo5p play redundant roles in endocytosis (Mooseker and Cheney, 1995) and they require a protein with V/WH2 domain to promote actin polymerization (Geli, *et al.*, 2000).

6.2.3 Expression of Vrp1p restore actin patches polarization of *vrp1A* strain

Actin cytoskeleton in *Saccharomyces cerevisiae* is composed of actin cables and actin patches (Adams and Pringle, 1984, Kilmartin and Adams, 1984). The distribution of both patches and cables changes dramatically during the cell cycle (Chowdhury, *et al.*, 1992). Actin filaments are found in actin cables going through the cytoplasm
(Mulholland, et al., 1994) and in cortical patches associated with invaginations of the plasma membrane which are postulated to be the site of endocytosis. During the cell cycle the distribution of actin patches are regulated (Lew and Reed, 1995). Actin patches polarize into small emerging buds, then disperse in the mother and large bud during mitosis phase (Winsor and Schiebel, 1997). Actin patches re-polarize to the neck region of the budding cells before the ending of mitosis indicating that the re-polarized actin patches might function in cytokinesis (Winsor and Schiebel, 1997). Therefore the proper polarization of actin patches is involved in multiple cellular processes such as endocytosis, cell viability at high temperature and cytokinesis.

Expression of WIP, WIRE or CR16 does not suppress the actin patches polarization defect of vrplA strain (Figure 3.3, Figure 3.16, Figure 4.3) while expression of Vrp1p complements the actin patch polarization (Figure 5.2). It was reported that expression of C-Vrp1p, like full length Vrp1p, restores actin patches polarization (Thanabalu and Munn, 2001). Why did the mammalian Verprolin proteins fail to suppress actin patches polarization defect of S.cerevisiae vrplA strain? Maybe this is because the mammalian proteins do not interact with other proteins which are important for actin patch polarization. Expression of any one of the three mammalian proteins (WIP, WIRE or CR16) can suppress the growth defect at 37°C and the endocytosis defect of vrplA strain without correcting the actin patch polarization defect. Thus all the three mammalian verprolin proteins have activities similar to N-Vrp1p1-364 (Thanabalu and Munn, 2001), which is able to suppress the growth defect of vrplA strain without
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correcting the actin patch polarization defect. This suggests that the activities of verprolin required for growth at elevated temperature are independent of the role of Vrp1p in cortical actin patch polarization. Yeast two hybrid assay was used to check for interaction between the mammalian proteins (CR16, WIP, WIRE) and the *S.cerevisiae* proteins (SH3 domain of Myo3p, Myo5p, Hof1p, Rvs167; actin, Las17p). All the three mammalian proteins interact with actin but did not interact with Las17p just like the N-Vrp1p1-364 which suggests that Vrp1p-Las17p interaction is critical for actin patch polarization and that correction of different defects requires either different activities or different levels of activity.

### 6.3 V domain is critical for suppressing the growth and endocytosis defect

Verprolin proteins are characterized by the presence of a V/WH2 domain at the N-terminal and a WASP/N-WASP binding domain at the C-terminal (Aspenstrom, 2005). The V domain of verprolin proteins probably plays an important role in the function of these proteins. Sequence analysis reveals that the V domain is composed of a proline rich region and an actin binding motif. The proline rich region at N-terminal contains one or more putative profilin binding motifs. Profilin is an actin monomer binding protein which promotes actin polymerization by enhancing the exchange of ADP for ATP in G-Actin molecules (Goldschmidt-Clermont, *et al.*, 1991, Mockrin and Korn, 1980). The actin binding motif is presented as KLKK among the consensus of sequence RXALLXdIXkGXkLkK (Adams and Pringle, 1984, Kilmartin
and Adams, 1984). However CR16 has RLRK as the actin binding motif. Based on sequence homology, a second actin binding domain was postulated to be present downstream of the V domain at the N-terminal (Aspenstrom, 2005). In this study, the V/WH2 domain is defined as WIP1-50, WIRE1-53 and CR161-63. Thus all the activities of the mammalian proteins are probably due to the interaction between actin and the V/WH2 domain at the N-terminus.

Though expression of WIP, WIRE or CR16 as GST fusion proteins from high copy plasmid suppresses the growth defect of wrp1Δ strain, expression of WIP51-503 (WIPΔV), WIRE53-441 (WIREΔV) or CR1663-485 (CR16ΔV) does not suppress growth defect. In the endocytosis assay, wrp1Δ cells expressing CR1663-485 were able to take up Lucifer yellow dye while those expressing WIP51-503 or WIRE53-441 does not (Figure 3.6, Figure 3.20, Figure 4.7). This suggests that the three mammalian verprolin proteins suppress the endocytosis defect either by different mechanisms or CR16 has more than one domain which can rescue the endocytosis defect of wrp1Δ strain. Thus the V domain of WIP and WIRE is essential for suppression of the endocytosis defect while V domain of CR16 is not essential.

All the three mammalian members of verprolin family expressed from high copy plasmid suppress the actin related cellular defects such as defect of growth at high temperature and defect of endocytosis, however, when expressed from low copy plasmid, the protein which has the ability to suppress this cellular defect is CR16, but
not WIP or WIRE. Why CR16 is unique in its activity? Is it because of its unique V domain? To answer this question, DNA encoding V domain of CR16 was fused to the N-terminus of DNA encoding WIP or WIRE. The chimera proteins of CR161-63-WIP or CR161-63-WIRE show an enhanced activity in suppressing the growth and endocytosis defect of vrplΔ strain compared with WIP or WIRE. Both CR161-63-WIP1.503 and CR161-63-WIRE1.441 expressed from low copy plasmid suppress the growth and endocytosis defect while neither of WIP nor WIRE expressed from low copy plasmid does. This enhanced activity of the chimera proteins might be due to the presence of two V domains, or because of the unique V domain of CR16. In order to clarify this question, DNA encoding V domain of CR16 was fused to the N-terminus of WIP51.503 (WIPΔV) or WIRE53-441 (WIREΔV) and chimera proteins of CR161-63-WIP51-503 and CR161-63-WIRE53-441 were expressed from low copy plasmid. Transformants of vrplΔ cells expressing either CR161-63-WIP51-503 or CR161-63-WIRE53-441 from low copy plasmid grow at 37°C indicating that chimera proteins have enhanced activity even in the absence of V domain of WIP or WIRE. This suggests that the V domain of CR16 is unique and has higher activity to suppress endocytosis and growth defect of vrplΔ strain.

Why is the V domain of verprolin protein essential for suppressing actin related cellular defects, such as endocytosis defect and growth defect at elevated temperature? The V domain is essential for verprolin proteins to function in suppression of cellular defect of vrplΔ strain and this might be due to the interaction with actin mediated by
the V domain. Why does the expression of the mammalian verprolin proteins suppress endocytosis and growth defect instead of actin patches polarization defect? Is it because of the insufficient contribution from V domain? Vrp1p\textsubscript{364-817} has been shown to restore the growth at high temperature and actin patches polarization of \textit{vrp1A} strain (Thanabalu and Munn, 2001). However, mutation of the Las17p binding motif abolishes Vrp1p\textsubscript{364-817}'s localization to cortical patches, and abolishes the ability to restore the actin patches polarization and growth at high temperature. Addition of localization signal such as CAAX helps to restore localization and growth defect, but does not help to restore actin patches polarization (Figure 5.18). This suggests that the Las17p binding is essential for Vrp1p\textsubscript{364-817} to suppress the cellular defects, especially actin patches polarization defect of \textit{vrp1A} strain. CR16, WIP or WIRE does not interact with Las17p as assessed by yeast two hybrid assay. Therefore, the mammalian verprolin proteins do not suppress the actin patches polarization defect of \textit{vrp1A} strain, probably because of the lack of interaction with Las17p.

It has been reported that the Vrp1p\textsubscript{364-817} restores the actin patches polarization defect of \textit{vrp1A} strain, and localizes to cortical actin patches dependent on Las17p (Thanabalu and Munn, 2001). Mutation of MPKPR motif of Vrp1p\textsubscript{364-817} abolishes its interaction with Las17p, abolishes the localization to cortical patches, and abolishes the suppression of actin patches polarization defect (Figure 5.11 and Figure 5.12). This suggests that interaction with Las17p is important for Vrp1p\textsubscript{364-817} to localize to cortical patches and restore actin patches polarization. Therefore Las17p might play a
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role for mammalian verprolin proteins (WIP, WIRE and CR16) to restore the actin patches polarization in \( vrplA \) strain. Though WIP/WIRE/CR16 can localize to cortical patches independant of Las17p, the patches where WIP/WIRE/CR16 localizes might be different from those patches where Las17p localizes. Therefore WIP/WIRE/CR16 does not localize to correct location due to the lack of interaction with Las17p. This might be one of the possible reasons why WIP/WIRE/CR16 does not have sufficient activity to suppress the actin patches polarization defect in the absence of interaction with Las17p in \( vrplA \) strain. Fusion of the Las17p binding domain of Vrp1p, Vrp1p_{760-817} to N-Vrp1p_{1-364} allowed the fusion protein to correct the actin patch polarization defect of \( vrplA \) strain. The LBD fusion leads to the localization of the two proteins to the same cortical actin patches probably leading to the polarization of cortical actin patches.

### 6.4 KLKK motif and actin interaction

The V domain is critical for the ability of all the three mammalian proteins to suppress the growth defect of \( vrplA \) strain. Mutational analysis reveals that the interaction with actin mediated by V domain is critical for the activity of WIP. Mutating the KKLKK motif to AALAA or deleting proline rich region does not abolish the interaction with actin, but combining mutation of KKLKK motif with deletion of proline rich region, abolishes the interaction with actin. This suggests that the proline rich region and KKLKK motif of WIP can mediate interaction with actin. It has been reported that
WIP interact with profilin through the ABM-2 motif (Purich and Southwick, 1997). Therefore it is possible that proline rich region play a role in the interaction with actin mediated by profilin.

On the other hand, mutating the RLRK motif of CR16 to AAAA abolishes the interaction with actin assessed by yeast two hybrid assay, suggesting that CR16 has only one actin binding motif though CR16 has more proline rich region compared to WIP. However, the mutation of RLRK motif of CR16 does not abolish its ability to suppress the growth or endocytosis defect of vrplA strain. Deletion of the N-terminal proline rich region does not abolishes CR16’s ability to suppress growth or endocytosis defect. Combining deletion of proline rich region and mutation of RLRK motif abolishes CR16’s activity in suppressing growth defect of vrplA strain. This suggests that the proline rich region and actin binding motif play a redundant role in suppressing the growth defect (Figure 4.10).

In contrast to WIP or CR16, mutating the KLKK motif of WIRE to AAAA abolishes its interaction with actin and abolishes WIRE’s activity. This suggests that WIRE interacts with actin only through the actin binding motif. This indicates that the interaction with actin of WIRE is essential and correlated with its function in actin related cellular process. Conclusively, the interaction with actin of Verprolin proteins plays an important role in their function with different mechanism. In future work, WIP1-50, WIRE1-53 and CR161-63 would be expressed in yeast respectively, and the
interactin between these domains and actin would be further assessed using yeast two hybrid.

6.5 Roles of proline rich region

6.5.1 Proline rich region of N-terminal plays redundant roles with actin binding motif

The N-terminal proline rich region of verprolin proteins contains one or several motifs which are implicated in binding of profilin, an actin monomer binding protein. However profilin interaction with the V/WH2 domain of CR16, WIRE or WIP could not be detected by yeast two hybrid assay and GST pulls down assays. It is possible that the interaction is very transient. Profilin promotes actin polymerization by promoting the conversion of ADP-actin to ATP-actin. Therefore the verprolin proteins might regulate actin polymerization through binding with profilin. The putative profilin binding motif (also named as ABM-2 motif) is characterized as a consensus sequence of XPPPPP, in which X can be one of the following amino acids: glycine, alanine, leucine or serine (Purich and Southwick, 1997). WIP and WIRE have been shown to bind with profilin (Aspenstrom, 2002, Ramesh, et al., 1997) and interaction of WIRE to profilin is mediated by its N-terminal 1-95 residues (Aspenstrom, 2002).

In this study, functional analysis reveals that either CR16^{4A}-GST or CR16_{31-485}-GST suppresses the growth defects while CR16^{4A}_{31-485}-GST does not, indicating that the N-terminal proline rich region (amino acid residues 1-31) in the V domain of CR16
plays a redundant role with the RLRK actin binding motif. Mutation of this RLRK motif abolishes its interaction with actin. Similarly, either WIP$^{4A}$-GST or WIP$_{14,503}$-GST suppresses the growth and endocytosis defect while WIP$^{4A}_{14,503}$-GST does not, indicating that the proline rich region (amino acid residues 1-13) in the V domain of WIP plays a redundant role with its actin binding motif, KKLKK motif. But in contrast to CR16, mutation of WIP’s KKLKK motif does not abolish its interaction with actin. Similar results were observed with N-Vrp1p, Vrp1p$_{22-270}$ or Vrp1p$_{A45A46}^{1-270}$ restores growth of vrplA cells at high temperature, while Vrp1p$_{A45A46}^{23-270}$ does not, suggesting that the N-terminal proline rich region of Vrp1p (amino acid residues 1-22) plays a redundant role with the actin binding motif.

Similarly, either Vrp1p$_{23-270}$-LBD or Vrp1p$_{A45A46}^{1-270}$-LBD restores the actin patches polarization, while Vrp1p$_{A45A46}^{23-270}$-LBD does not; indicating that the proline rich region of N-terminal Vrp1p plays a redundant role with the actin binding motif in restoring the actin patches polarization. Therefore, in conclusion, the N-terminal proline rich region and actin binding motif of verprolin proteins (except WIRE) play a redundant role in suppressing the actin related cellular defects such as growth, endocytosis or actin patches polarization defect.

Why do the verprolin proteins need the redundant presence of proline rich region and actin binding motif? The reason might be in the requirement of co-operation between mammalian verprolin proteins and WASP/N-WASP. WASP/N-WASP binds to and activates Arp2/3 complex through its VCA region (for N-WASP is VVCA region) at
the C-terminal. The VCA/VVCA region comprises of three characteristic domains, a
verprolin homology domain (V domain), a cofilin like domain (C domain) and an
acidic domain (A domain). Thus unlike verprolin proteins, WASP/N-WASP has a V
domain at the C-terminal domain. The C and A domains have been suggested to bind
to Arp2p or Arp3p (Rodal, et al., 2005), which are subunits of Arp2/3 complex and
share sequence homology with actin. Therefore, as a monomeric actin binding domain,
the V domain of verprolin protein might contribute to a high concentration of actin
monomers. Profilin contributes to the conversion of ADP-actin to ATP-actin, and
ATP-actin is polymerized more rapidly than ADP-actin (Mockrin and Korn, 1980).
Thus, the presence of either actin binding sites or N-terminal proline rich region
would lead to a high concentration of ATP-actin which is to be added to the barbed
end of actin filament, and hence to regulate the actin polymerization. In this study,
WIP is expressed in \textit{vrp1A} strain in the presence of Las17p, the yeast homologues of
WASP. When the mammalian WIP is expressed in \textit{vrp1A} strain, the cooperation
between WIP and Las17p might be similar with, but not as sufficient as that of Vrp1p
and Las17p, as WIP does not physically interact with Las17p as assessed by yeast two
hybrid assay. This might be the reason why mammalian verprolin proteins do not
suppress the actin patches polarization defect of \textit{vrp1A} strain, and why WIP and CR16
need at least either N-terminal proline rich region or actin binding motif to suppress
growth or endocytosis defects of \textit{vrp1A} strain. Absence of both actin binding motif
and proline rich region leads to a loss of function for WIP to suppress the cellular
defects of \textit{vrp1A} strain.
Second Wave of Islam - Islamic Revival and the re-assertion of Moro Identity

It was in the context of changing population demographic landscape and decline of Sultanate in Moro territories that a power and identity vacuum existed. The space was filled through a reconnecting with the dormant Islamic consciousness that made possible through the return of Islamic scholars from the Middle East Islamic Universities. Chief among them was Ustadz Hashim Salamae an Al-Azhar alumnus. These scholars not only brought new political thinking but also the Middle East culture. Among the early signs of their influence were the replacement of traditional local Muslim dresses by a Middle East fashioned dresses. Customary rituals perceived deviant to Islam were prohibited. The Ustadz (religious scholar) slowly replaced the Pandita (Traditional Muslim preacher). Political and religious thinking began to echo contemporary Islamic thinkers in the Middle East, Hashim Salamat admitted drawing...
interaction as well as the suppression of growth and endocytosis defects of \textit{wrlA} strain. Why does suppression of cellular growth defect by WIRE correlate with the binding to actin? Co-immunoprecipitation assay reveals that profilin binds to N-terminal truncate, WIRE\textsubscript{1-95}, but not bind to full length WIRE (Aspenstrom, 2002). This indicates that the profilin binding site located at WIRE\textsubscript{1-95} might be masked or blocked by other regions of WIRE\textsubscript{96-441} in a three dimensional manner. Further question that should be addressed is: why only the profilin binding motif of WIRE, but not WIP or CR16, is masked? We analyzed the sequence of all the three mammalian verprolin proteins and found that the profilin binding motif (ABM-2 motif), is APPPPP in WIP and CR16, while it is GPPPPP in WIRE (Figure 6.3). Glycine is the smallest amino acid among the 20 types of amino acids. Alanine is also quite small, but has an extra methyl group. Therefore it is possible that the folding of GPPPPP motif of WIRE might be quite tight such that the profilin binding site is masked inside the fragment WIRE\textsubscript{95-441}, while the folding of APPPPP motif of WIP might be quite open due to the bigger size of alanine so that the profilin binding site is exposed outside. This might be the reason why WIRE loses the interaction with profilin while WIRE\textsubscript{1-95} interacts with profilin.

Figure 6.3 Alignment of V domains from verprolin proteins Profilin binding motif (ABM-2 motif), represents APPPPP in CR16 and WIP, while represents GPPPPP in WIRE. Actin binding motif represents RLRK in CR16, while represents KKLKK in WIP and KLKK in WIRE.
6.5.2 Proline rich region binding to SH3 domain containing proteins

Verprolin proteins are highly proline rich and the proline rich regions are postulated to mediate the binding to profilin as well as SH3 domain containing proteins, such as Myo3p, Myo5p, Hoflp and Bzzlp. Though proline rich region at the N-terminal of WIRE has been shown to interact with profilin (Aspenstrom, 2002), no interaction between the proline rich region and profilin could be detected using yeast two hybrid assay and GST pull down assay in this study. Vrplp, the yeast verprolin, has been reported to bind with SH3 domains of Myo3p, Myo5p and Hoflp (Anderson, et al., 1998, Naqvi, et al., 2001). WIP and WIRE have been shown to bind with SH3 domain-containing proteins Nck and cortactin (Anton, et al., 1998, Aspenstrom, 2002, Kinley, et al., 2003). Thus the proline rich region of verprolin proteins might play roles in the regulation of actin cytoskeleton mediated by SH3 domain-containing proteins.

Both Myo3p and Myo5p are un-conventional type I myosins. They have an acidic domain (A domain) which activates Arp2/3 complex (Evangelista, et al., 2000, Lechler, et al., 2000, Tanaka and Matsui, 2001). Myo3p and Myo5p are functionally redundant in endocytosis (Mooseker and Cheney, 1995). Las17p presents an acidic domain that constitutively activates Arp2/3 complex (Rodal, et al., 2005). The acidic domain of Myo3p, Myo5p and Las17p are redundant for their functions in the growth of the cells at 37°C, as deletion of any two acidic domains from the three molecules
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has no effects on the cells while deletion of all the three domains abolishes the growth of yeast cells at 37°C (Evangelista, et al., 2000, Lechler, et al., 2000). Though Myo3p and Myo5p have the acidic domains to activate Arp2/3 complex, they require the presence of V domain (WH2 domain) from V domain containing proteins such as verprolin proteins and Las17p. It has been reported that in co-operation with the V domain of Vrp1p, Myo3p and Myo5p promote actin polymerization by activating the Arp2/3 complex (Geli, et al., 2000, Lechler, et al., 2001). Therefore the V domain of mammalian verprolin proteins might plays similar roles in actin polymerization in yeast cells by co-operating with acidic domain of Myo3p or Myo5p.

Using yeast two hybrid analysis we found that the interaction of CR16 with Myo3p is mediate by CR16 N-terminal proline rich region and the SH3 domain of Myo3p. We also found that the interaction of CR16 with Myo5p is mediated by the internal proline rich region and SH3 domain of Myo5p (section 4.6). It has been reported that cortactin, another SH3 domain containing proteins without V domain, has been postulated to form a complex with WIP to activate Arp2/3 complex (Kinley, et al., 2003). Thus similarly, it is possible that the N-terminal and internal proline rich sequence of CR16 function through Myo3p and Myo5p. Though Myo3p and Myo5p lack an actin binding V domain, it might promote actin polymerization by forming a complex with V domain of mammalian verprolin proteins which provides actin to acidic domain of Myo3p/Myo5p in yeast vrplA strain. However this raises an interesting question: since activation of Arp2/3 complex requires supply of actin, how
CR16\textsuperscript{AA} which has mutation of actin binding motif in V domain and does not interact with actin, mediates activation of Arp2/3 complex? It has been reported that the tail domain of Myo3 and Myo5 can interact with actin through the tail homology domain located next to the SH3 domain (Geli, et al., 2000) when the actin is required for CR16/Myo3p complex to active Arp2/3 complex. The actin interaction mediated by Myo3/5p might be the explanation as to why CR16\textsuperscript{AA} still functions in vivo after the RLRK motif has been mutated.

6.6 Localization of Verprolin proteins is essential for their functions

6.6.1 Verprolin proteins localize to cortical patches

In live cells, proteins need to localize specifically to carry out their functions after being synthesized. Therefore analyzing the location sites of verprolin proteins will lead to a better understanding of their roles in regulation and reorganization of actin cytoskeleton. Four GFP tagged verprolin proteins, WIP-GFP, WIRE-GFP, CR16-GFP (This study) and Vrp1p-GFP (Thanabalu and Munn, 2001) were visualized under the fluorescence microscope. They all localize to cortical patches in \textit{vrp1A} strain. Arc40p is a subunit of Arp2/3 complex which is a nucleator for actin polymerization and localizes to actin patches. Arc40p-RFP co-localizes with the four GFP labeled verprolin proteins, indicating that the verprolin proteins localizes to actin patches.

6.6.2 Localization of Verprolin proteins is essential for suppressing cell defect

Most of the proteins in vivo have specific location to carry out their functions and the
localization after synthesis is crucial for their functions. In this study, deletion analysis shows that WIP\(_{1,317}\)-GST suppresses the growth defect while WIP\(_{1,291}\)-GST does not. The loss of function is due to the lack of sufficient localization to cortical actin patches as WIP\(_{1,291}\)-GFP shows a weaker localization (Figure 3.12, Table 3.4) compared with that of WIP\(_{1,317}\)-GFP. This indicates that the localization of WIP to cortical actin patches is essential for its activity to suppress the growth defect of \(vprp1A\) strain. Consistent with this, WIP\(_{1,291}\)-GST-CAAX suppresses the growth defect while WIP\(_{1,291}\)-GST does not (Table 3.5), indicating that the artificial localization to cortex restores WIP\(_{1,291}\)'s activity in suppressing the growth defect. This also confirms that the localization of WIP is essential for its function. Thus WIP has a membrane localization signal and it is probably located between residues 266-317 since WIP\(_{1,317}\)-GFP but not WIP\(_{1,266}\)-GFP localizes sufficiently to cortical patches. The V/WH2 domain of all the three mammalian proteins (CR16, WIRE, WIP) is not needed for the localization to cortical actin patches even though the actin binding motif is located in the V/WH2 domain. The three mammalian proteins localize to cortical actin patches and not to the plasma membrane suggesting that the membrane in these patches might be enriched for certain lipids compared to the plasma membrane in general.

Vrp1p restores the actin patches polarization of \(vprp1A\) strain while Vrp1p\(_{1,760}\) does not. Vrp1p-GFP localizes to cortical patches while Vrp1p\(_{1,760}\)-GFP does not, indicating that the fragment Vrp1p\(_{760-817}\). Las17p binding domain, is essential for Vrp1p's
localization and the localization is essential for its function in restoring actin patches polarization of \( vrplA \) strain. \( Vrpl_{p1-364}\)-LBD but not \( Vrpl_{p1-364} \), restores actin patches polarization, and \( Vrpl_{p1-364}\)-LBD-GFP but not \( Vrpl_{p1-364} \)-GFP, localizes to cortical patches. This suggests that Las17p binding domain is essential for localization of \( Vrpl_{p1-364} \) and the localization is essential for restoring actin patches polarization. Similarly, \( Vrpl_{364-817} \) but not \( Vrpl^{5A}_{364-817} \), restores the actin patches polarization or growth at high temperature, and \( Vrpl_{364-817}\)-GFP but not \( Vrpl^{5A}_{364-817} \)-GFP localizes to cortical patches, indicating that the Las17p binding site, MPKPR motif, is essential for \( Vrpl_{364-817} \)’s localization to cortical patches, and essential for the function in restoring actin patches polarization and growth at high temperature.

However, it is not clear whether the loss of function of \( Vrpl_{364-817} \) is due to lack of Las17p binding or lack of localization. Targeting the mutant protein to the cell cortex by fusing the CAAX motif, \( Vrpl^{5A}_{364-817}\)-CAAX restores growth at high temperature but not actin patches polarization, indicating that the localization to cell cortex rescue’s \( Vrpl_{364-817} \)’s function in growth at elevated temperature without rescuing the actin patch polarization defect of \( vrplA \) cells. Therefore, in conclusion, the localization of \( Vrpl \) to cortical patches mediated through Las17p, is essential for its ability to restore actin patches polarization but not for growth at high temperature.

6.7 Further directions

1) N-terminal Proline rich region of WIP and KLKK motif plays redundant roles in the interaction with actin, and roles in suppressing both growth and endocytosis
Discussion

defects. The interaction with actin is essential for and correlated with suppressing these two cellular defects. How does the proline rich region work in mediating WIP interacting with actin? It has been proposed that the N-terminal proline rich region of WIP mediates the interaction with Profilin. Is the interaction between WIP and actin mediated by Profilin or KLKK? Both yeast two hybrid assay and GST pull down assay using WIP proline rich region or KLKK motif will enable us to answer this question.

2) Unlike WIP, the RLRK motif of CR16 is essential for the interaction with actin, but not for the suppression of growth or endocytosis defects. The data suggests that the proline rich region in V domain plays redundant role with the RLRK motif. Similar to WIP, this proline rich region could mediate the interaction with actin. Both yeast two hybrid assay and GST pull down assay using CR16 proline rich region or RLRK motif will enable us to determine the interaction between CR16 and actin is mediated by Profilin or RLRK. The mapping of interaction between CR16 and SH3 domain-containing proteins shows that Myo3p binding site is located in the N-terminal Proline rich region. The N-terminal proline rich region plays redundant roles with RLRK actin binding site to suppress the growth defect. What is the role of Myo3p is suppressing the growth defect of vrplI strain? Identification of amino acid residues in the N-terminal of CR16 which are essential for binding with Myo3p will allow us to analyze the role of Myo3p-CR16 interaction in suppressing the growth defect of vrplI strain.
3) Vrp1p<sub>364-817</sub> restores both growth at high temperature and actin patches polarization while Vrp1p<sub>1-364</sub> restores only growth due to the lack of interaction of Las17p. Vrp1p<sup>5A</sup><sub>364-817</sub> does not restore the growth or actin patches polarization due to the lack of cortical localization, while Vrp1p<sub>364-817</sub> does in the presence of its Las17p binding site, MPKPR motif. Las17p is an essential binding partner of Vrp1p, and plays multiple roles in function not only the suppression of growth defect at high temperature, but also suppression of actin patches polarization defect, since Vrp1p<sup>5A</sup><sub>364-817</sub>-CAAX restores only the growth at high temperature, but not actin patches polarization. This suggests that Las17p functions not only for the localization of Vrp1p, but also contributes restoring the actin patch polarization. How does Vrp1p and Las17p cooperate in restoring the actin patches polarization defect of Vrp1<sub>Δ</sub> strain? It is possible that Vrp1p stabilize Las17p and promote actin polymerization through the VCA domain of Las17p. An vitro actin polymerization assay using purified Las17p and Vrp1p peptides will allow us to determine the role of Vrp1p in Las17p mediated actin polymerization.

Further research towards the above three directions would provide an explanation how the Verprolin proteins work in yeast cells, and lead to a better understanding how the actin cytoskeleton is organized and remodeled.
6.8 Summary

In summary, four proteins of verprolin family were analyzed in this study. The three mammalian verprolin proteins, WIP, WIRE and CR16 are able to suppress the growth and endocytosis, but not actin patches polarization defect of *vrplA* strain. Therefore they are functional homologues of Vrp1p. The V domain at the N-terminal of WIP and WIRE is essential for the function to suppress the growth and endocytosis defects while the V domain of CR16 is essential for growth at high temperature, but not essential for endocytosis. V domains of all the three verprolin proteins are essential for mediating interaction with actin. It is postulated that there is a conserved actin binding motif inside the V domain. The RLRK motif of CR16 and KLKK motif of WIRE are essential for the interaction with actin while KKLKK motif of WIP is not essential for the actin interaction. Though both actin binding motifs of CR16 and WIRE are essential for the interaction with actin, only KLKK motif of WIRE is essential for its function in suppressing cellular defect of *vrplA* strain. The proline rich region of WIP and CR16 play a redundant role with their actin binding sites in suppressing the cellular defect of *vrplA* strain. Similarly, the N-terminal proline rich region and actin binding site of Vrp1p1-270 play a redundant role in restoring the growth at high temperature. Further more, the two actin binding domain of Vrp1p1-364-LBD, Vrp1p1-70-LBD or Vrp1p271-364-LBD, play a redundant role for restoring the actin patches polarization. In a different manner, WIRE interacts with
actin mediated by the KLKK motif, and the interaction is correlated with the function of WIRE. All the three mammalian verprolin proteins localize to cortical actin patches and the localization of WIP is crucial for its function. Similarly, both Vrp1p and Vrp1p<sub>364-817</sub> localize to cortical patches mediated by the interaction with Las17p, and the localization is essential for their ability in restoring the actin patches polarization. Therefore, though all the verprolin proteins present a conserved V domain at the N-terminal and a WASP/N-WASP/Las17p binding domain at the C-terminal, they carry out their functions in an approximately same, but specifically different manner.
References


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Rimm, I.J. and Rappeport, J.M., Bone marrow transplantation for the


Vaduva, G., Martin, N.C., and Hopper, A.K., Actin-binding verprolin is a polarity


Publications


4) **Meng, L.,** Rajmohan, R., Yu, S., and Thanabalu, T., Vrp1p-Las17p interaction is essential for actin patch polarization, (in preparation).

5) Rajmohan, R., **Meng, L.,** Thanabalu, T., Las17p-Vrp1p interaction is essential for growth at 37 °C in the absence of Arp2/3 activation domain, (in preparation).
Appendix

List of plasmid constructs used for this study.

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### Table of Mutagenesis Sites

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*This study indicates the source of the mutagenesis studies.*
Islamic Revival and the re-assertion of Moro Identity

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YCplac111 with full length WIP(site directed mutation from K44KLKK48 to A44KLAA48 )

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YCplac111 with WIP(residues 1-403) - (E)GFP

This study

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YCplac111 with WIP(residues 1-317) - (E)GFP

This study

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This study

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This study

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This study

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This study

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YCplac111 with WIP4A(residues 1-266) - (E)GFP

This study

pHG-WIP

YCplac181 with full length of WIP-GFP

This study

pHG-WIP2A

YCplac181 with full length WIP2A-GFP (mutagenesis K44KLKK48 to K44KLAA48 )

This study

pHG-WIP4A

YCplac181 with full length WIP4A-GFP (site directed mutation K44KLKK48 to A44KLAA48 )

This study

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| pHG-CR164A18-485 | YEplac181 with CR16-GFP (18-485) 4A | This study |
| pHG-CR164A31-485 | YEplac181 with CR16-GFP (31-485) 4A | This study |
| pHG-CR164A47-485 | YEplac181 with CR16-GFP (47-485) 4A | This study |
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| pEG-CR16 | YCplac111 with CR16-EGFP full length | This study |
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| pEG-CR16D7 | YCplac111 with CR16-EGFP without Exon7 | This study |
| pEG-CR164A | YCplac111 with CR16-4A-EGFP (1-485) | This study |
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| pEG-CR164A47-485 | YCplac111 with CR16-EGFP (47-485) 4A | This study |
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| pACR164A | pACT2 with CR164A (1-485) mutagenesis R58A59A60A61 to A58A59A60A61 | This study |
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| pACR164A47-485 | pACT2 with CR16(47-485) 4A | This study |
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| pBCR1631-485 | pBAS2-1 with CR16 (31-485) | This study |
| pBCR1647-485 | pBAS2-1 with CR16 (47-485) | This study |

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**Appendix**

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Among the possible through the return of Islamic scholars from the Middle East Islamic was filled through a reconnecting with the dormant Islamic consciousness that made signs of their influence were the replacement of traditional local (Traditional Muslim preacher). Political and religious thinking began to echo to Islam were prohibited. The Ustadz (religious scholar) slowly replaced the Pandita

**Appendix**

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Among the signs of their influence were the replacement of traditional local Muslim dresses by a Middle East fashioned dresses. Customary rituals perceived deviant possible through the return of Islamic scholars from the Middle East Islamic Sultanate in Moro territories that a power and identity vacuum existed. The space was filled through a reconnecting with the dormant Islamic consciousness that made religious thinking began to echo contemporary Islamic thinkers in the Middle East, Hashim Salamat admitted drawing to Islam were prohibited. The Ustadz (religious scholar) slowly replaced the Pandita

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### Appendix

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