The Role of Sla1p in linking actin dynamics to the endocytic machinery in *Saccharomyces cerevisiae*.

A Thesis Submitted to the

Faculty of Biomedical and Life Sciences

For the Degree of
Doctor of Philosophy

By
Derek Warren

Department of Biochemistry and Molecular Biology
The University of Glasgow

January 2002

Acknowledgements.

Thanks to everyone who has helped me survive my time in the lab, especially to the original crew of Hilary, Tommy, Campbell and Cath before we moved and became the Davidson massive. I would like to thank my supervisor, Kathryn Ayscough, for her support, guidance and a lot of tolerance, especially after all the Arsenal induced hangovers.

Special thanks to my parents for their continued support and encouragement. I would also like to say a huge thank you to my patient fiancée Alison for allowing me to remain a financially crippled student for the last four years. I would like to thank Alison for all her support during the course of my PhD and for allowing me to vent my stress at Dundee football matches.

Last but not least I would like to thank Arsenal football club. Although they have won no silverware during my PhD, they now play the type of football that is a joy to watch although it would probably give George Graham a heart attack.

Abstract.

Sla1p is a protein required for cortical actin patch structure and organisation in budding yeast. An association with the endocytic machinery has also been shown by immunoprecipitation of Sla1p with EH domain containing proteins, End3p and Pan1p. Here we use immunofluorescence to demonstrate that localisation of Sla1p to the cell cortex requires its C-terminal repeat region and also End3p. We show that cells either lacking *SLA1* or expressing the *sla1ΔCt* mutant display defects in both fluid phase and receptor mediated endocytosis, suggesting that Sla1p may be part of the endocytic machinery.

This study also demonstrates modification of Sla1p by the Actin Regulating Kinases, Ark1p/Prk1p and the yeast protein phosphatase-1 Glc7p. Immunofluorescence microscopy revealed that the association of Sla1p with cortical actin patches is regulated by the interactions of the Ark1p/Prk1p kinases. We also provide evidence for the existence of multiple isoforms of Sla1p *in vivo*.

Finally, using the yeast 2-hybrid system, we identified Ycl034wp and Ynr065cp as interacting partners of the central region of Sla1p. Sequence analysis of Ycl034wp predicted this protein contained a VHS and a GAT domain. These domains have been identified in a number of proteins involved in vesicle trafficking. The mammalian homologues of Ynr065cp (Sortilin and Sorl-1) have also been implicated in vesicle trafficking. This indicates that the central region of Sla1p interacts with proteins involved in the transport of vesicles.

Taken together, the data presented here allows us to predict a dynamic model where Sla1p is able to interact with proteins involved in actin dynamics, the endocytic machinery and

also proteins involved in vesicle trafficking. Association of these different sets of machinery may be regulated by cycles of phosphorylation and dephosphorylation. This allows Sla1p to function as an adaptor protein, bringing proteins involved in different stages of the endocytic pathway into close proximity and allowing efficient endocytosis.

Table of Contents.

Figures.

Tables

Abbreviations.

LAT-A	Latrunculin-A
5-FOA	5-Fluoro-orotic acid
A	Alanine
I	Isoleucine
Q	Glutamine
T	Threonine
L	Leucine
G	Glycine
X	Any amino acid
PCR	Polymerase Chain Reaction
G-actin	Monomeric actin
F-actin	Filamentous actin
CH domain	Calponin Homology domain
SH3 domain	Src Homology-3 domain
EH domain	Epsin homology domain
AP-2 complex	Adaptor protein-2 complex
U	Units
UV	Ultra violet
GST	Glutathione S Transferase
URA	Uracil
LEU	Leucine
TRP	Tryptophan.

Chapter 1.

Introduction.

1.1 Actin.

Actin is ubiquitously expressed in eukaryotic cells and is required for a wide range of cellular processes including endocytosis, exocytosis, cell motility, cell polarity and cytokinesis. Its importance to the functions of cells is reflected in the very high level of sequence conservation throughout eukaryotes. For example, there are no amino acid substitutions between chicken skeletal muscle actin and human skeletal muscle actin (Sheterline *et al*, 1995) and yeast actin shares 88 % amino acid identity with human skeletal muscle actin. The high degree of sequence conservation suggests that actin is a protein which has essential cellular functions in all eukaryotes.

Two pools of actin exist in cells, monomeric G-actin and filamentous F-actin. The pool of F-actin is the 'functional form' of actin, as most of the known biological functions of actin require it to be in the filamentous form. The pool of G-actin may act as a store of actin to allow rapid polymerisation of F-actin structures when required. Unlike mammalian cells, yeast do not contain a large pool of G-actin and most of the actin is present in the filamentous form in these cells. G-actin has been measured at about 0.015 ng/μg of total cell protein and the total actin measured at 1.5 ng/μg of total cell protein. This means that G-actin represents only about 1 % of the total yeast actin (Karpova *et al*, 1995; Karpova *et al*, 1998). The reason for yeast cells lacking a large pool of G-actin is most probably because these cells are non-motile, so require less actin in the monomeric actin to allow rapid assembly of new actin structures.

Many proteins control actin polymerisation and depolymerisation within cells. These include proteins that sever or cap actin filaments, nucleate polymerisation, cross-link and stabilise actin filaments (for review see Ayscough, 1998). Others bind to G-actin, maintaining up to 50% of cytoplasmic actin as monomers (Korn, 1982) and are able to respond to signals by releasing G-actin and allowing its polymerisation (Lassing and Lindberg, 1985). Filamentous actin is found in a variety of supramolecular assemblies such as networks, foci and bundles. The two pools of actin are highly dynamic and change size and distribution throughout development and differentiation.

The atomic structure of monomeric actin complexed with DNAse I (Kabsch *et al*, 1990), gelsolin segment-1 (McLaughlin *et al*, 1993) and profilin (Schutt *et al*, 1993) have been determined. The 375-residue polypeptide is arranged into two domains, each of which are comprised of two subdomains (Figure 1.1). The large domains are organised to form a hinged molecule containing a deep cleft into which the actin essential cofactors, ATP/ADP and divalent cations bind (Kabsch *et al*, 1990). Although a number of high resolution crystal structures have been reported for complexes of G-actin with actin binding proteins, currently there are only models for the actin filament structural. F-actin can be induced to form orientated arrays that can be used to generate low-resolution structural information by fibre diffraction. These models predict that actin filaments are dimers of two protofilaments that form a helical structure and contain uniformly orientated actin molecules (Holmes *et al*, 1993). They are polar structures with two structurally and biochemically different ends, a slow growing pointed end and a faster growing barbed end.

1.2 Polymerisation of actin.

Polymerisation of actin in vitro requires ATP as well as monovalent (K^+) and divalent (Ca^{2+} or Mg^{2+}) cations. Initally there is a lag phase, as new filaments are nucleated, and then

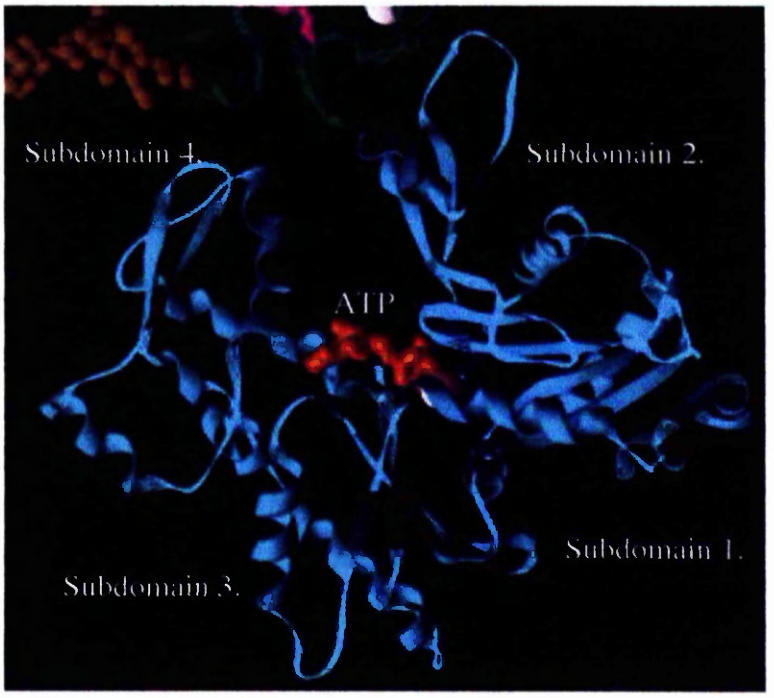
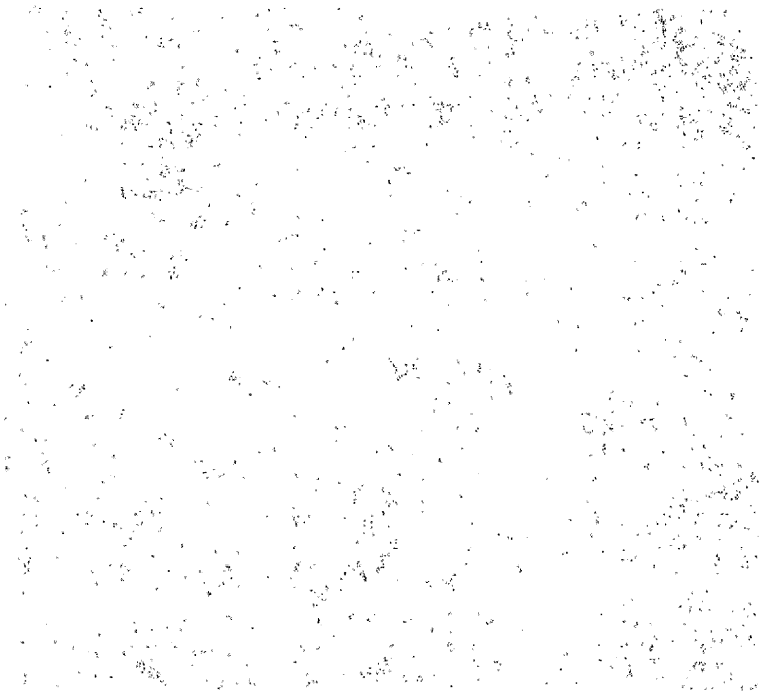


Figure 1.1. The atomic structure of G-actin complexed with DNAse I. ATP and the calcium ion are located in the cleft between the two large domains. Adapted from SwissProt NCBI (http://www.ebi.ac.uk/swissprot/).



a rapid polymerisation phase, as short filaments elongate. Actin filaments form by the non-covalent association of actin monomers that contain ATP in their nucleotide-binding cleft. After incorporation of an actin monomer into the filament, ATP is hydrolysed to ADP and this hydrolysis is accompanied by a conformational change (Lorenz *et al*, 1993; Tirion *et al*, 1995).

The critical property of actin is that the filaments are polarised and dynamic. The nature of the filament is such that ATP-actin molecules are added continually to the barbed end whereas ADP-actin is lost continually from the pointed end of a filament. This process requires an energy input via ATP hydrolysis and is thought to contribute to the rapid exchange of subunits of the actin filaments observed in cells.

1.3 Using yeast to test predictions from structural information.

Information generated from atomic structure of actin has allowed prediction of the roles of various actin domains. Residues that surround the cleft region are likely to be involved in binding or hydrolysis of the nucleotide. Other regions of actin are potentially involved in making essential contacts for filament formation or for interactions with actin binding proteins. Due to the high degree of sequence and structural similarities between budding yeast actin and actin from higher eukaryotes, yeast have proven to be a powerful tool allowing the predictions from these studies to be tested.

A systematic mutational scan of *ACT1*, in which charged residues were replaced by alanine (Wertman *et al*, 1992) has been particularly valuable. Characterisation of the alleles generated has provided insights into functions and interactions of actin and has been used to map the binding sites for actin binding proteins (Honts *et al*, 1994; Belmont *et al*, 1998; 1999) and actin associating drugs. For example, residues important for binding of the

adenine nucleotide were proposed, based on the atomic structure (Kabsch *et al*, 1990). A point mutation in one of these residues (Q137A) has been shown to disrupt the hydrogen bonding to the ATP molecule. This mutant displayed a variety of phenotypes including slow actin filament turnover, slow fluid phase endocytosis and defects in their actin organisation (Belmont *et al*, 1998; 1999).

A study carried out by Honts and others mapped the Sac6p (fimbrin) interacting site on the actin molecule. They identified eight *act1* alleles that showed suppression with *sac6* mutant alleles. Sequence analysis revealed all mutations altered residues that cluster to a small domain of the actin crystal structure. Biochemical analysis demonstrated defects in the ability of several mutant actins to bind Sac6p and a reduction in the Sac6p-induced cross-linking of mutant actin filaments (Honts *et al*, 1994).

More recently the Latrunculin-A (LAT-A) binding site has been mapped on the actin molecule. LAT-A can disrupt the actin cytoskeleton in yeast cells (Ayscough *et al*, 1997). This has enabled the identification of point mutations in the actin gene that cause cells to become resistant to the effects of the drug. Sequence analysis of the mutations revealed that they were all clustered close to the nucleotide-binding site of the actin crystal structure (Ayscough *et al*, 1997). The crystal structure of actin bound to LAT-A has been resolved and indicates that LAT-A may act by interfering with conformational changes that are necessary for polymeriastion (Morton *et al*, 2000). When the LAT-A binding site is occupied, ATP is clamped within the actin filament and the filament is stablised (Morton *et al*, 2000).

1.4 The actin cytoskeleton of budding yeast.

In yeast filamentous actin primarily exists in cortical patches and cytoplasmic cables. The cortical patches are discrete F-actin rich structures and the cables are comprised of long bundles of F-actin. Both forms of F-actin become polarised in a cell-cycle dependent manner (Figure 1.2) (for review see Pruyne and Bretscher, 2000).

At some point during the cell cycle a predetermined bud site is selected. Cortical actin patches concentrate at this site and form a ring structure while cables run parallel along the mother-bud axis. As the bud emerges, cortical actin patches are located in the bud and cables remain parallel to the mother-bud axis, extending from the mother cell to the newly forming bud. Patches and cables redistribute randomly between the mother and bud while an F-actin cytokinetic ring assembles at the bud neck, contracts and finally disassembles. After cytokinesis both cortical patches and cytoplasmic cables re-polarise to the former bud neck to allow synthesis of cell wall between the two cells.

1.5 Actin binding proteins in yeast.

The organisation of the budding yeast actin cytoskeleton changes through the cell cycle and also in response to external signals, such as yeast pheromones (for review see Pruyne and Bretsher, 2000). This demonstrates the requirement for cells to remodel the actin cytoskeleton in response to both intracellular and extracellular signals. Actin binding proteins allow cells to regulate actin dynamics and control the assembly/disassembly of actin monomers into higher order structures (figure 1.3). Much of our understanding about the functions of actin binding proteins has come about from biochemical studies that have described how these proteins modify the state of actin filaments *in vitro*. It is also important to determine the functions of actin binding proteins *in vivo*. Due to the similarities that exist between the actin cytoskeleton in budding yeast and higher

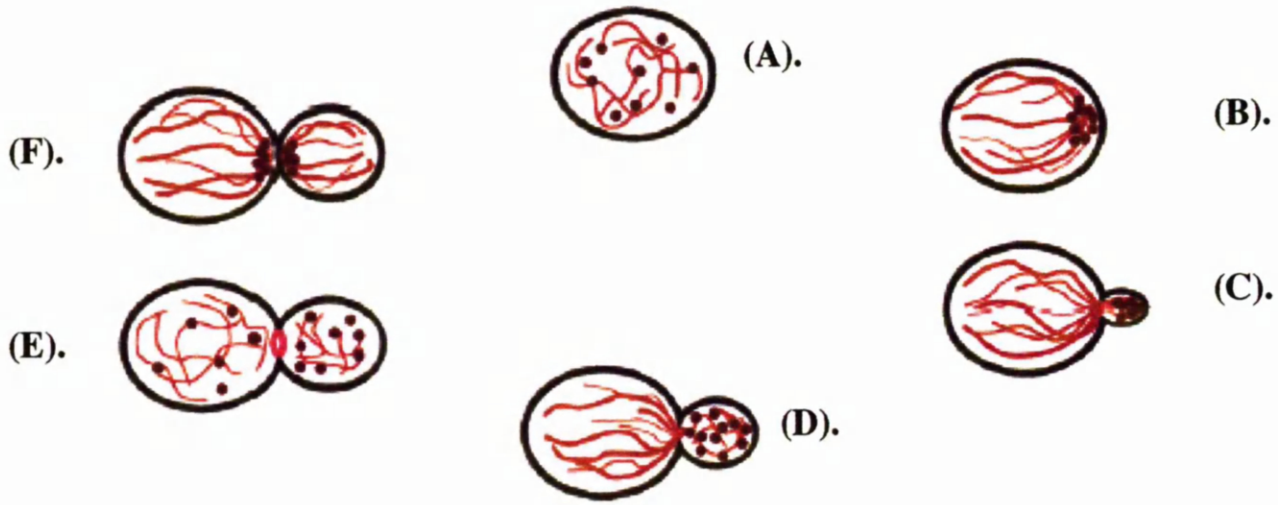


Figure 1.2 The yeast actin cytoskeleton through the cell cycle. (A) Cells in mid G1 phase grow isotropically. Cortical actin patches (Black dots) are distributed randomly through the cell cortex and cytoplasmic actin cables (Red lines) have random orientations. (B) In late G1 phase, cortical actin patches polarised to a predetermine site at the cell cortex and cables orient toward this site. (C) As the bud emerges cortical actin patches move into the bud while cables extend from the bud to the mother cell (D) and the bud continues to grow. (E) Patches and cables redistribute randomly between the mother cell and the bud and an F-actin ring assembles at the bud neck, contracts and then disassembles. (F) After cytokinesis both cortical actin patches and cytoplasmic actin cables repolarise to the former bud neck to allow synthesis of cell wall between the two cells. Adapted from Pruyne and Bretscher, 2000.

eukaryotes, yeast have proved to be a useful tool in studying the functions of these proteins *in vivo* (for review see Ayscough, 1998). A wide variety of actin binding proteins have been identified that allow cells to regulate the organisation of the actin cytoskeleton and some of these will be discussed in the following section.

1.5.1 Profilin.

Profilin is a small actin binding protein that has been identified in a wide variety of organisms (yeast, *Drosophila* and mammalian cells) (Christensen *et al*, 1996; Rothkegel *et al*, 1996). Although profilins from different organisms show moderate sequence conservation, 22% between plant and mammalian profilins, they have been demonstrated to complement each other. For example, plant profilin microinjected into mammalian cells was able to functionally substitute for endogenous mammalian profilin (Rothkegel *et al*, 1996). Originally profilin was thought to sequester actin monomers (Carlsson *et al*, 1977), however the principle role of profilin is now thought to be in regulating actin dynamics. The current idea is that profilin binds to a ADP-actin monomer to form a profilactin complex. Profilin then promotes exchange of ATP for ADP on actin (Mockrin and Korn, 1980; Goldschmidt-Clermont *et al*, 1991), allowing rapid assembly at the barbed ends of actin filaments (Pantaloni and Carlier, 1993; Kang *et al*, 1999). Biochemical studies using yeast profilin revealed that profilin inhibits hydrolysis of ATP by monomeric actin (Haarer *et al*, 1990). Yeast cells lacking profilin grow extremely slowly, become large and round, lack dectable actin cables and no longer polarise their cortical actin patches (Haarer *et al*, 1990). All of the evidence suggests that profilin serves to promote polymerisation of F-actin structures.

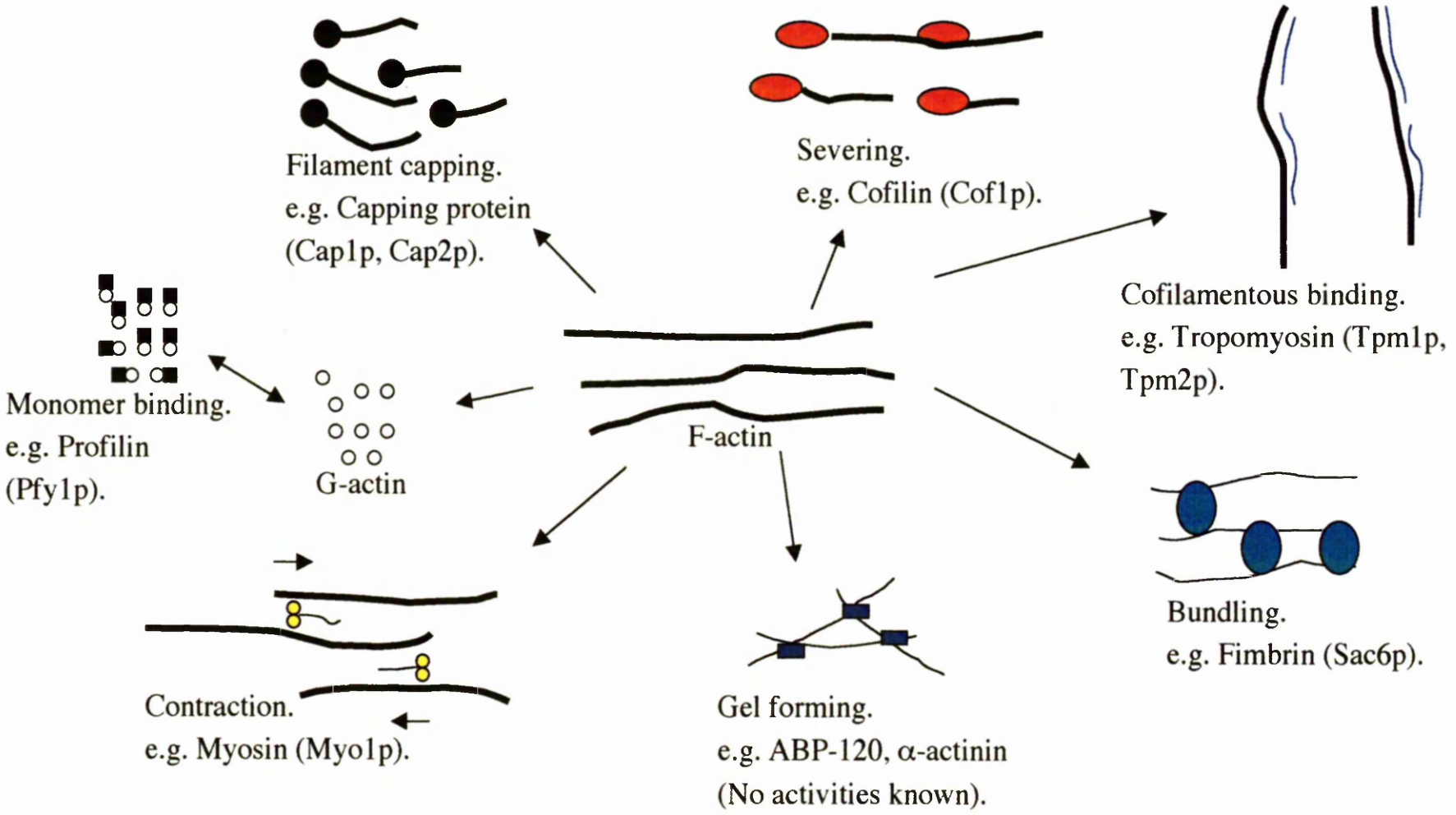


Figure 1.3 Activities of actin-binding proteins allow regulation of the actin cytoskeleton. Examples of yeast homologues of these actin-binding proteins are shown in brackets. Adapted from Ayscough, 1998.

1.5.2 The Cofilin/ADF family members.

The cofilin/ADF family members have been shown to bind both actin monomers and actin filaments *in vitro* and stimulate actin filament disassembly (Carlier *et al*, 1997). The association between cofilin and actin is inhibited by either phosphoinositides (Yonezawa *et al*, 1989, 1991) or phosphorylation (Morgon *et al*, 1993). The actin depolymerisation activity of cofilin is regulated in a pH dependent manner, being favoured in an alkaline environment (Yonezawa *et al*, 1985). Yeast cofilin is essential for the viability of yeast cells (Iida *et al*, 1993; Moon *et al*, 1993), demonstrating the importance of disassembly of the actin filament network. Studies using partial loss of function alleles in the yeast cofilin gene (*COF1*) demonstrated that rapid cycles of actin filament assembly and disassembly depends on cofilin and that the F-actin binding and depolymerisation activity are the essential cofilin functions (Moon *et al*, 1993; Lappalainen *et al*, 1997).

1.5.3 Tropomyosins.

Tropomyosins are elongated shaped proteins that bind along the length of actin filaments, spanning several actin monomers (Ayscough *et al*, 1998). Tropomyosins share a moderate sequence homology, for example yeast Tpm1p shares a 20% amino acid identity with human TpmB. Deletion of the two tropomyosin encoding genes (*TPM1* and *TPM2*) from budding yeast is lethal (Drees *et al*, 1995). However, cells containing the temperature sensitive tropomyosin double mutant (*tpm1-2 tpm2Δ*) contain no detectable cytoplasmic cables and have an aberrant cell morphology at the non-permissive temperature, suggesting that tropomyosin serves to stabilise actin filaments (Karpova *et al*, 1998). Subsitution of residues proposed to be involved in actin's interaction with tropomyosin for alanine was lethal when expressed as the sole source of actin in cells (Lorenz *et al*, 1995). These data support the hypothesis that tropomyosins stabilise actin filaments by interacting with several actin subunits along the length of an actin filament.

1.5.4 Capping Proteins.

Capping proteins are ubiquitously expressed heterodimeric actin-binding proteins that contain a moderate to a high degree of sequence homology. For example yeast Cap1p shares a 27% amino acid identity with human CAPZA whereas yeast Cap2p shares a 46% amino acid identity with human CAPZB. Capping proteins have been shown to nucleate actin polymerisation and to control filament length *in vitro* (for review see Schafer and Cooper, 1995). The budding yeast Cap1p and Cap2p have been demonstrated to bind the barbed end of actin filaments preventing the addition or removal of actin monomers (Amatruda *et al*, 1992). Deletion of the genes encoding yeast capping proteins results in cells containing lower amounts of F-actin (Karpova *et al*, 1995). This data indicates roles of capping proteins in stabilising F-actin structures.

1.5.5 Actin Bundling Proteins.

Actin bundling proteins are abundant throughout the eukaryotic kingdom and examples include fimbrin, α-actinin and spectrin (for review see Dubreuil, 1991). Each of these proteins contain at least one actin-filament binding domain, composed of two Calponin Homology (CH) domains. These actin-binding domains all display a similar structure, however the length and flexibility of the spacer sequences separating the actin binding domains is different in each of these proteins (Figure 1.4) (Dubreuil, 1991). These differences determine the different properties of these actin-bundling proteins. Budding yeast contain just one known actin bundling protein Sac6p (yeast fimbrin). Cells lacking *SAC6* contain abnormal actin structures, are defective in cell morphogenesis and also endocytosis (Adams *et al*, 1991; Singer-Kruger and Ferro-Novick, 1997). This suggests that actin bundling proteins serve to strengthen the actively growing regions of cells.

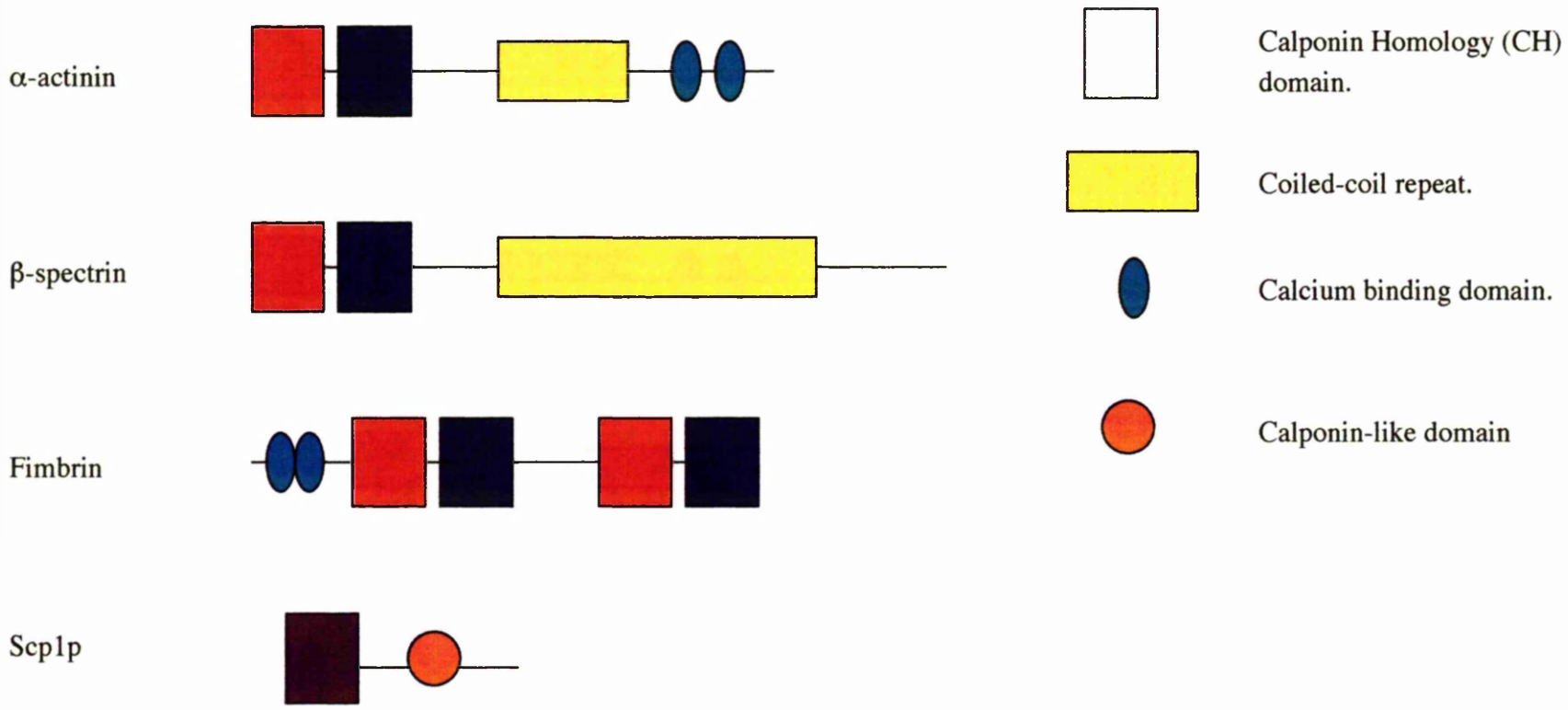


Figure 1.4 Schematic representation of Calponin Homology domain-containing proteins.

1.5.6 Myosins.

All of the actin filament motor proteins identified to date belong to the myosin family. Myosins contain a conserved motor domain but other domains vary from myosin to myosin and determine the specific role of the molecule in the cell. Myosins can be divided into different subfamilies, the most abundant being the myosin II subfamily. Members of this subfamily contain two motor head domains and a long rod shaped tail. Each of these two head domains possesses an ATPase and a motor activity. Smaller myosins are also found throughout evolution, the best characterised being the myosin I subfamily. These smaller myosins are thought to be primitive myosins from which the myosin II subfamily has evolved.

Budding yeast contain five different myosins that have been demonstrated to fulfil and variety of cellular roles. Myo3p and Myo5p are class I myosins that are involved in the organisation of the actin cytoskeleton (Goodson *et al*, 1996; Lechler *et al*, 2000). Both Myo3p and Myo5p have been reported to interact with Las17p and the Arp2/3p complex (Evangelista *et al*, 2000; Lechler *et al*, 2000). This may allow these myosins to spatially and temporally regulate the processes of actin dynamics and vesicle transport in yeast cells. Myo1p belongs to the class II myosins and has been demonstrated to play roles in maintaining the cell structure (Brown, 1997) and cytokinesis (Lippincott and Li, 1998). Myo2p and Myo4p are class V myosins and are involved in vesicle transport (Mazzoni *et al,* 1993) and cell polarity (Santos *et al*, 1997; Long *et al*, 1997).

1.5.7 Differences between yeast and mammalian actin-binding proteins.

Although yeast cells contain many actin-binding proteins that have homologues in mammalian cells, they do not contain homologues of every mammalian actin binding protein. For example budding yeast cells contain no known proteins possessing a gel

forming activity similar to that of α-actinin found in mammalian cells. α-actinin belongs to a family of proteins possessing CH (Calponin Homology) domains (Figure 1.4) (Dubreuil, 1991). Budding yeast contain three actin-binding proteins belonging to this family (Sac6p, Scp1p and IQGAP). However mammalian cells display a much wider variety of proteins containing these domains. This may represent the ancestery of this family in that yeast cells contain the earliest forms of this family and mammalian cells have evolved a wider number of proteins belonging to this family.

1.6 Actin cytoskeleton disrupting drugs.

The actions of the actin-cytoskeleton modifying drugs Latrunculin-A (LAT-A) and cytochalasin-D and phalloidin have been elucidated. Actin cytoskeleton disrupting drugs have been useful in allowing researchers to disrupt the normal functions of actin and examine the consequences.

1.6.1 Phalloidin.

Phalloidin is an example of a drug that stabilises actin filaments. It has been shown to bind actin filaments more tightly than actin monomers (Estes *et al*, 1981) preventing actin filament depolymerisation and shifting the equilibrium from monomer toward filament by lowering the critical concentration for polymerisation by 10 to 30 fold (Estes *et al*, 1981; Faulstich *et al*, 1977). Phalloidin has proved a useful tool in fluorescence microscopy, when coupled to a fluorescent molecule it allows visualisation of a cells actin cytoskeleton (Adams and Pringle, 1991).

1.6.2 Latrunculin-A (LAT-A).

LAT-A is an actin cytoskeleton-disrupting drug that sequesters actin monomers and prevents them from taking part in the rapid cycles of actin assembly/disassembly

(Ayscough *et al*, 1997). The effects of LAT-A are rapid and reversible making it a useful laboratory tool. LAT-A has recently been shown to alter the actin-monomer subunit interface preventing its polymerisation into F-actin structures (Morton *et al*, 2000). LAT-A is capable of disrupting the yeast actin cytoskeleton and has allowed researchers to study the yeast actin cytoskeleton *in vivo* (Ayscough *et al*, 1997).

1.6.3 Cytochalasin-D.

Cytochalasin-D is thought to have multiple effects on actin but overall also results in F-actin disruption (for review see Cooper, 1987). One effect that has been determined is that cytochalasin-D binds to the barbed end of actin filaments in a similar way to capping proteins (Flanagan and Lin, 1980; Brown and Spudich, 1981). Binding of cytochalasin-D to actin filaments inhibits both association and disassociation of actin monomers from the barbed end (Flanagan and Lin, 1980; Brown and Spudich, 1981). Cytochalasin-D also binds to actin monomers. Studies have shown that this drug increases the rate of spontaneous actin polymerisation (Tellam and Frieden, 1982) and to increases the rate of ATP hydrolysis by actin monomers (Brenner and Korn, 1981).

1.7 Nucleation of actin polymerisation.

Actin polymerisation requires nucleation either by generating an actin trimer or from the end of an existing filament. Formation of the actin trimer is the rate-limiting step in actin polymerisation (Cooper *et al*, 1983). The Arp2/3 complex has emerged as a strong candidate for nucleating actin filament assembly (Figure 1.5). The Arp2/3 complex consists of seven subunits, two of which are the actin related proteins (Arp) Arp2p and Arp3p. Much of our understanding of this complex has been focused on Arp2p and Arp3p. As both of these proteins are structurally similar to actin, the Arp2/3 complex is structurally well suited to serve as an actin filament nucleator (Kelleher *et al*, 1995). Through

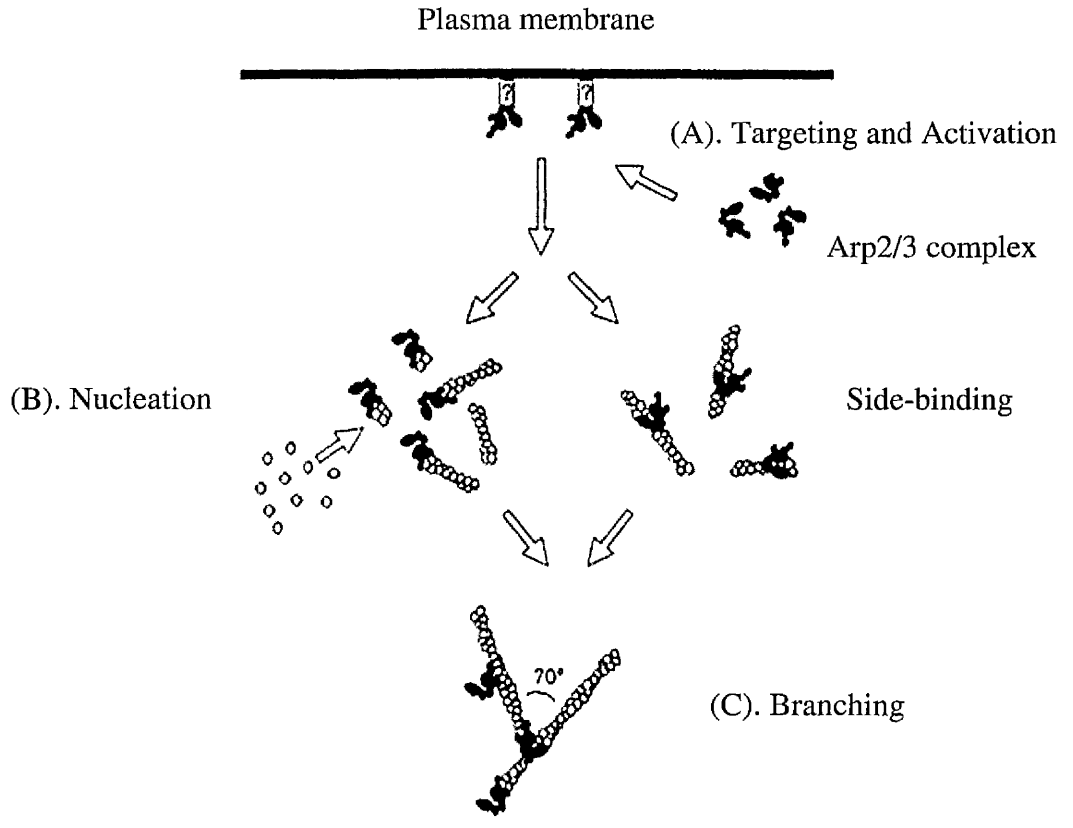


Figure 1.5 Arp2/3 complex binds to the sides of actin filaments, nucleating actin polymerisation and forming a branched actin filament network. (A) The Arp2/3 complex is targeted to and activated at sites of new actin polymerisation. The Wiscott-Aldrich syndrome protein (WASP) family proteins, that bind directly to the Arp2/3 complex, are strong candidates for activators. (B) Once in place the Arp2/3 complex initiates nucleation of new actin filaments. (C). The Arp2/3 complex can also bind to the sides of existing filaments to cause side branching resulting in a branched actin filament network. Adapted from Machesky and Gould, 1999.

structural modelling, it has been predicted that at the barbed end an Arp2p/Arp3p heterodimer interacts with the pointed end of actin molecules to nucleate filament growth toward the barbed end (Kelleher *et al*, 1995).

The Arp2/3 complex has been proposed to mimic the fast growing barbed end of an actin filament allowing the Arp2/3 complex to nucleate actin assembly, cap the slow growing end and cross-linking actin filaments (Mullins *et al*, 1998). This method of actin nucleation produces a branched actin network at regions of rapid actin dynamics (Mullins *et al*, 1997), for example at the leading edge of motile cells or cortical actin patches in budding yeast (Figure 1.5).

Sequence identification of the subunits of the Arp2/3 complex has demonstrated that they are highly conserved throughout eukaryotic cells (Welch *et al*, 1997). Yeast serves as an excellent genetic system to study the *in vivo* function of the Arp2/3 complex. In budding yeast, Arp2p and Arp3p are components of cortical actin patches (Moreau *et al*, 1996; Winter *et al*, 1997). Both Arp2p and Arp3p function to maintain the proper organisation of the cortical actin cytoskeleton and Arp3p is required for actin patch motility (Moreau *et al*, 1996; Winter *et al*, 1997).

1.8 Activation of the Arp2/3 complex by the Wiscott-Aldrich syndrom protein family.

Purified Arp2/3 complex only weakly activates actin nucleation. However this activity is dramatically enhanced by the addition of WASP (Wiscott-Aldrich syndrom protein) family proteins (Machesky and Insall, 1998). WASP family proteins share several different functional domains (Figure1.6). WASP homology domain 1 (WH1), an N-terminal domain

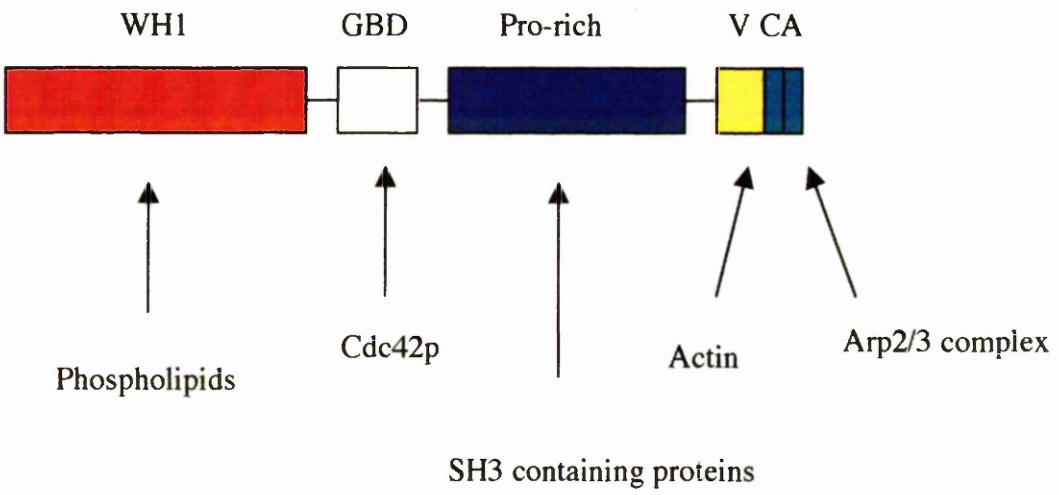


Figure 1.6 Functional domain structure of WASP family proteins. WASP homology domain-1 (WH1) interacts with phopholipids. A central GTPase binding domain (GBD), is lacking in the yeast WASP homologue Las17p, interacts with activated Cdc42p. Downstream of the GBD a proline rich region interacts with SH3 domain containing proteins. At the C-terminus of WASP family members a V domain interacts with G-actin and a CA domain interacts with the Arp2/3 complex.

that contains sequence homology to pleckstrin homology (PH) domains, has been shown to interact with phospholipids *in vitro* (Miki *et al*, 1996; Imai *et al*, 1999). Both WASP and N-WASP contain a GTPase binding domain that interacts with Cdc42p (Kolluri *et al*, 1996; Symons *et al*, 1996), a small GTP binding protein that is involved in regulation of actin cytoskeleton rearrangements (for review see Nobes and Hall, 1995).

Downstream of the WASP GTPase binding domain exists a proline rich region that interacts with the SH3 domains of several signalling proteins (Miki *et al*, 1996). Towards the C-terminus of the protein exists a V domain that binds to G-actin and a CA domain that interacts with the Arp2/3 comlex (Miki and Takenawa, 1998; Machesky *et al*, 1999; Rohatgi *et al*, 1999). The presence of different functional regions of WASP family proteins suggests that it functions as a molecular scaffold that links various signalling pathways to actin dynamics. The yeast WASP-like protein Las17p/Bee1p (Li, 1997) contains all the functional regions mentioned above except for the GTPase binding domain that interacts with Cdc42p (Symons *et al*, 1996).

The C-terminal region of WASP family members enhances the nucleation activity of the Arp2/3 complex (Machesky *et al*, 1999). Las17p has been demonstrated to interact with the Arp2/3 complex, and the acidic domain of Las17p is necessary for this association. Las17p also stimulates the nucleating activity of the Arp2/3 complex *in vitro* and both the V and acidic domains are required for the optimal stimulation of the Arp2/3 complex (Winter *et al*. 1999).

1.9 Identification of *SLA1*.

One of the first actin binding proteins identified in yeast was Actin Binding Protein-1 (Abp1p). Abp1p was identified as an actin binding protein by affinity chromatography

(Drubin *et al*, 1988). It was localised to the cortical actin patches of yeast cells, and its overexpression was shown to depolarise the cortical actin cytoskeleton. Deletion of *ABP1* had no obvious phenotype indicating functional redundancy exists between Abp1p and other unknown yeast proteins.

Recent work has however demonstrated that Abp1p interacts with and stimulates the nucleating activity of the Arp2/3p complex in yeast cells (Goode *et al*, 2001). Abp1p contains two acidic sequences that are required for activation of the Arp2/3p complex *in vitro* (Goode *et al*, 2001). The mammalian homologue of Abp1p, mAbp1, has been demonstrated, by coimmunoprecipitation and colocalisation, to interact with dynamin *in vivo* (Kessels *et al*, 2001). This may suggest that Abp1p links the actin cytoskeleton to endocytosis (Kessels *et al*, 2001).

To gain more of an insight into the function of Abp1p a synthetic lethal screen was performed to identify mutations that caused cells to become dependent on the expression of functional Abp1p for viability (Holtzman *et al*, 1993). Three genes were identified as being essential in cells lacking functional Abp1p. These were *SLA1* (Synthetic Lethal with *ABP1*-1), *SLA2* and *SAC6* (yeast fimbrin).

Sla1p contains an interesting set of structural characteristics (Figure 1.7). Three Src Homology-3 (SH3) domains are located in its N-terminal third (Figure). In a variety of organisms SH3 domains have been shown to mediate protein-protein interactions between signalling and cytoskeletal proteins (Ren *et al*, 1993; Yu *et al*, 1994). Its central region contains two domains that share the highest level of sequence conservation between Sla1p and its *S.pombe* homologue (Holtzman *et al*, 1993; Ayscough *et al*, 1999), and its C-

Figure 1.6 Schematic representation of the domain structure of Sla1p. Sla1p contains 3 SH3 domains in it N-terminal third. Located in the central region are two domains sharing the highest sequence similarity with other Sla1p homologues. The C-terminal region of Sla1p is comprised of repeats rich in glutamine, proline, glycine and threonine.

terminal third is comprised of repeat motifs with an approximate consensus LxxQxTGGxxxPQ (Holtzman *et al*, 1993; Ayscough *et al*, 1999).

1.10 Phenotypes of *Δsla1* cells.

Deletion of *SLA1* caused cells to become temperature sensitive, have an aberrant cortical actin cytoskeleton organisation and to depend on the expression of *ABP1* (Holtzman *et al*, 1993). More recent studies have shown that the region of Sla1p required for normal actin organisation is different from the region required to rescue the Abp1p dependent phenotype (Ayscough *et al*, 1999).

Studies using plasmid borne domain deletions of *SLA1* revealed that Sla1p lacking approximately two thirds of its C-terminal repeat region contained a relatively normal cortical actin cytoskeleton organisation but cells required the expression of *ABP1*. Cells expressing *SLA1* lacking the GAP1+SH3#3 region (Figure 1.7) had an aberrant cortical actin cytoskeleton organisation but were viable in the absence of functional *ABP1* (Ayscough *et al*, 1999). These data suggest that Sla1p has more than one function and that these are attributable to separable domains.

1.11 Interactions of Sla1p.

Sla1p has been reported to interact with an increasing number of proteins. These interactions have been determined by a number of approaches including genetic, 2-hybrid and coimmunoprecipitation. Most of these proteins can be divided into two groups, either they are actin binding proteins or proteins involved in endocytosis. This section will concentrate on the reported physical interactions of Sla1p.

1.11.1 Interactions with actin binding proteins.

It has been demonstrated, by immunoprecipitation and GST pull-downs, that Las17p and Abp1p interact with Sla1p (Li, 1997; Warren *et al*, in press). Also during the course of these studies, others in the Ayscough lab demonstrated that Sla2p interacts with the GAP1+SH3#3 region of Sla1p in the yeast 2-hybrid system (Gourlay *et al*, submitted). This interaction was shown to be direct using purified proteins *in vitro* (Gourlay *et al*, submitted).

SLA2 is a nonessential gene that is required for the organisation of the yeast cortical actin cytoskeleton, growth at higher temperatures, cell morphogenesis (Holtzman *et al*, 1993) and the internalisation step of endocytosis (Raths *et al*, 1993; Wesp *et al*, 1997). All Sla2p-related proteins share a similar arrangement of three predicted coiled-coil domains, a proline rich region and a C-terminal talin homology domain. Talin is a protein found in focal adhesion plaques and the talin-homology domain of Sla2p has been shown to bind actin *in vitro* (McCann and Craig, 1997). The central coiled-coil domain of Sla2p has an endocytic function that is redundant with the function of Abp1p (Wesp *et al*, 1997).

1.11.2 Interactions with components of the endocytic machinery.

The C-terminal repeat region of Sla1p has been shown by yeast 2-hybrid and immunoprecipitation to interact with the Pan1p/End3p complex (Tang *et al*, 2000). The Sla1p/Pan1p/End3p trimeric complex (Figure 1.8) is proposed to function in the process of endocytosis (Tang and Cai, 2000). Pan1p and End3p are yeast proteins that contain Epsin Homology (EH) domains. EH domains are protein-protein interaction modules and have been shown to interact with the NPFxD motif (Slacini *et al*, 1997), a motif involved in ubiquitin-independent endocytosis (Tan *et al*, 1996; Wendland *et al*, 1998). Both Pan1p

and End3p also participate in the regulation of the cortical actin cytoskeleton (Benedetti *et al*, 1994, Tang and Cai, 1996, Tang *et al*, 1997).

Pan1p contains two long repeats (LR1 and LR2) in its N-terminal region, each containing an EH domain and multiple copies of the LxxQxTG repeat that are also present in the C-terminal repeat region of Sla1p. The LR2 region of Pan1p is essential for viability and has been shown to interact with End3p (Tang *et al*, 1997). End3p contains an EH domain in its N-terminal region and two sets of C-terminal repeats. (Benedetti *et al*,1993; Raths *et al*, 1993). Others had demonstrated an interaction between the LR2 region of Pan1p and the C-terminal repeat region of End3p (Figure 1.8) before we began this work (Tang *et al*, 1997). During the course of these studies Sla1p was shown to interact with the LR1 region of Pan1p and the EH domain of End3p via its C-terminal repeat region (Tang *et al*, 2000). In addition, other studies demonstrated Pan1p has been shown to contain motifs that are phosphorylated by Prk1p and Ark1p. These studies demonstrated that Prk1p phosphorylates the LxxQxTG motifs present in LR1 and LR2 of Pan1p. Binding of End3p to Pan1p prevented the phosphorylation of Pan1p by Prk1p (Zeng and Cai, 1999). Sla1p contains 5 of these LxxQxTG repeats and also a further 9 repeats with the QxTG consensus within its C-terminal repeat region and studies *in vitro* using these Sla1p repeats fused to GST revealed they were a good substrate for Prk1p (Tang *et al*, 2000).

1.12 Roles for actin dynamics in membrane trafficking

One of the most obvious phenotypes when yeast actin-binding proteins are mutated is that cells become large and round, suggesting that membrane trafficking is defective when the actin cytoskeleton is disrupted. The first evidence for this arose from studies using conditional actin mutants that displayed a partial defect in the secretion of invertase (Novick and Botstein, 1985). Further evidence was demonstrated from studies using

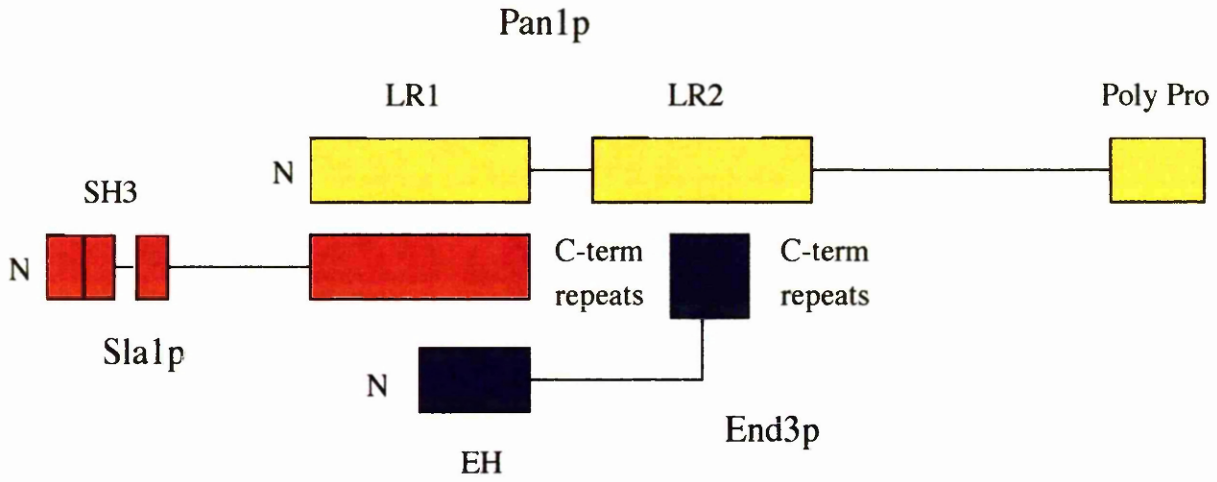


Figure 1.8 Schematic representation of interactions between Pan1p, End3p and Sla1p. The second Long Repeat (LR1) of Pan1p interacts with the C-terminal repeat regions of End3p. Sla1p interacts with the first Long Repeat (LR2) of Pan1p and the N-terminal EH domain of End3p via its C-terminal repeat region. (Adapted from Tang *et al*, 2000).

mutants affecting either actin cytoskeleton components or regulators of actin cytoskeletal polarity. For example mutations in either Cdc42p, its exchange factor Cdc24p or the actin binding protein Sac6p resulted in cells that displayed reduced polarity of the actin cytoskeleton and showed inappropriate growth of the mother cells resulting in abnormally large cells (Sloat and Pringle, 1978; Adams *et al*; 1990; Adams *et al*, 1990). Fimbrin has also been demonstrated to be required for endocytosis in yeast cells, implicating the actin cytoskeleton in endocytosis as well as exocytosis (Kubler and Riezman, 1993).

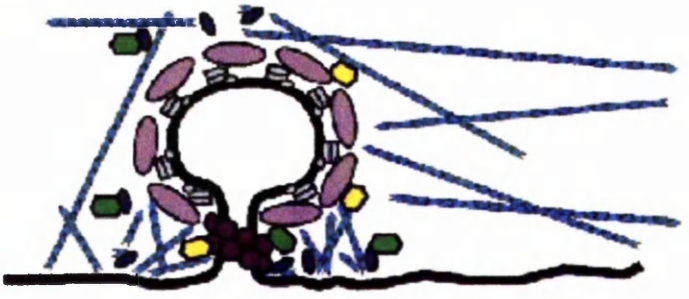
Clathrin-dependent receptor mediated endocytosis is the best characterised endocytic pathway in both yeast and mammalian cells. In this pathway, plasma membrane-localised receptors bind to their ligand and then associate with the heterotetrameric adaptor complex AP-2. AP-2 recruits and polymerises clathrin, ultimately leading to the formation of clathrin coated vesicles. These vesicles are then uncoated and the adaptors and clathrin are recycled. The uncoated vesicle then enters the early endosome (for review see Schmid, 1997).

Several functional roles for actin in the endocytic process have been proposed (Figure 1.9). The actin cytoskeleton might provide a force allowing vesicles to bud or mediate vesicle transport or fusion events (Lamaze *et al*, 1997; Merrifield *et al*, 1999). Alternatively the actin cytoskeleton may provide a scaffold at the plasma membrane for the endocytic machinery (Wendland *et al*, 1998; Gaidarov *et al*, 1999). Evidence suggests that endocytosis is closely linked to and is dependent upon actin dynamics. Treatment of cells with the actin monomer monomer sequestering drug LAT-A depolymerises the actin cytoskeleton and inhibits receptor mediated endocytosis in both yeast (Ayscough and Drubin, 1998) and mammalian cells (Lamaze *et al*, 1997). Mutations in a large number of

(A).

(B).

(C).



(D).

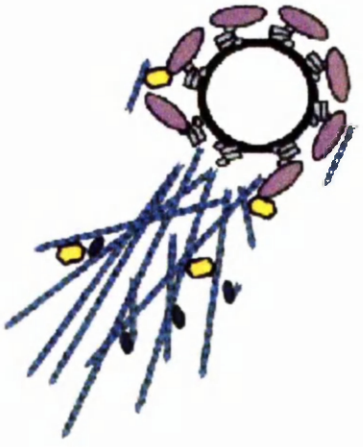


Figure 1.9
Potential roles for the actin cytoskeleton in endocytosis. (A) The vesicle budding site may be marked by proteins attached to filamentous actin allowing endocytosis to begin. Cytoskeletal structures may act as a scaffold for the endocytic machinery and provides support for the deformation and invagination of the plasma membrane. (C) The cortical actin barrier underlying the vesicle may need to be disassembled and actin polymerisation may provide force, driving membrane fission during endocytic vesicle formation. (D) Actin polymerisation may promote the movement of the newly formed endocytic vesicle into the cytoplasm. Adapted from Qualmann *et al*, 2000.

yeast cortical actin patch associated proteins, such as Pan1p, End3p, Sla2p, also produce defects in both endocytosis and cortical actin cytoskeleton organisation.

Many of the mammalian homologues of the actin patch associated proteins have been identified to be associated with clathrin coated pits. The mammalian homologue of Sla2p is mHIP1p (huntingtin interacting protein-1). Recent studies have demonstrated that mHIP1p associates with clathrin coated pits and vesicles (Engqvist-Goldstein *et al*, 1999). mHIP1p has been shown to interact with clathrin and the AP-2 complex, and has been proposed to participate in the assembly of the clathrin latice which gradually drives the invagination of clathrin coated pits (Waelter *et al*, 2001).

Eps15p, the mammalian homologue of Pan1p, has also been shown to be associate with clathrin coated pits (Benmerah *et al*, 1995) via an interaction with the AP-2 adaptor complex (Delft *et al*, 1997). More recently, the Eps15 interaction with AP-2 has been shown to be required for assembly of clathrin-coated pits and for endocytosis (Benmerah *et al*, 1998; 1999). As mentioned earlier the mammalian homologue of Abp1p, mAbp1, has been reported to interact with dynamin, a GTPase that functions in endocytosis. Recent studies have demonstrated that both Pan1p and Abp1p can interact with, and stimulate the nucleating activity of the Arp2/3 complex (Duncan *et al*, 2001; Goode *et al*, 2001). Pan1p, Abp1p and their mammalian homologues have been demonstrated to interact with other proteins involved in endocytosis. This would suggest that endocytosis is directly linked to actin dynamics in yeast and that disruption of the actin cytoskeleton also disrupts endocytosis. This is supported by the observations that mutants of cortical actin patch components disrupt both endocytosis and the cortical actin cytoskeleton.

1.13 Aims of this project.

Sla1p is required for proper organisation of the cortical actin patches. Previous studies using a plasmid borne *SLA1* mutant lacking approximately two thirds of the C-terminal repeat region demonstrated that this region was required to rescue the Abp1p-dependent phenotype associated with the *SLA1* knockout. To further characterise the roles and interactions of the C-terminal repeat region of Slap, we constructed yeast strains expressing *SLA1* that is lacking the entire C-terminal repeat region.

During the course of this work, the C-terminal repeat region of Sla1p was shown to contain motifs that are a potential target for the Actin Regulating Kinases Ark1p and Prk1p (Tang *et al*, 2000). These kinases could therefore potentially regulate the functions of Sla1p within cells. This project aimed to further our understanding of the possible regulation of Sla1p function, and its roles in regulating both organisation of the actin cytoskeleton and endocytosis at the cell cortex.

The final aim of this project was to identify interacting partners for the central region of Sla1p, using the yeast 2-hybrid system. To date little is known about this region of Sla1p even though it contains the two highest regions of sequence similarity between the known homologues of Sla1p. This may help us to elucidate further the functions of Sla1p and could shed light on how Sla1p functions are conserved through evolution.

Chapter 2.

Materials and Methods.

2.1 Materials.

All chemicals used in this study were obtained from BDH/Merck or Sigma unless over-wise stated.

2.2 Yeast Strains, Plasmids and Oligonucleotides.

All yeast strains, plasmids and oligonucleotides generated and used during the course of this study are listed in tables 2.1, 2.2 and 2.3 respectively.

2.3 Molecular Biology Techniques.

2.3.1 Restriction enzyme digestion of DNA.

All enzymes used in this study were from New England Biolabs and digests were carried out according to the manufacturer's instructions, using the buffers supplied. Digests were typically performed in a final volume of 20 μl, using 1-2 U of enzyme per 10 μl reaction. When performing double digests where enzymes required similar conditions, the DNA was digested simultaneously with both enzymes according to the manufacturer's instructions. Most digests were carried out at 37°C for 3 hours unless otherwise recommended by the manufacturer.

Table 2.1 Yeast strains used.

KAY	Genotype	Origin.
302	MATα, *trp1-1, leu2-3,112, lys2-801, his3-Δ200, ura3-52*	DDY852
300	MATa, *trp1-1, leu2-3,112, his3-Δ200, ura3-52, Δsla1::URA3*	Ayscough *et al*, 1999
301	MATα, *trp1-1, leu2-3,112, his3-Δ200, ura3-52,* Δsla1::URA3	Ayscough *et al*, 1999
303	KAY302 + *SLA1-9xmyc::TRP1*	Ayscough *et al*, 1999
369	KAY302 + *sla1ΔCt::TRP1*	Warren *et al*, In press
370	MATa, *trp1-1, leu2-3,112, lys2-801, his3-Δ200, ura3-52, sla1ΔCt::TRP*	This study
363	KAY302 + *sla1ΔCt-9xmyc::TRP1*	Warren *et al*, In press
364	KAY302 + *sla1ΔCt-3xHA::TRP1*	This study
397	KAY302 + *SLA1-GFP::HIS3*	This study
476	KAY303 + *END3-3xHA::HIS3*	This study
355	KAY302 + *SLA1-3xHA::TRP1*	This study
116	MATa, *leu2-3,112, his3Δ200, ura3-52, bar1*	Benedetti *et al*, 1994
117	KAY116 + integrated *end3-1*	Benedetti *et al*, 1994
462	MATa, *end3-1, leu2-3,112, his3Δ200, ura3-52, SLA1-GFP::HIS3*	Warren *et al*, In press

599	KAY302 + *Δabp1::HIS3*	This study
600	KAY599 + pKA11	This study
406	KAY600 + *sla1ΔCt::TRP1*	This study
376	MATa, *leu2-3,112, his3Δ200, ura3-52, lys2-801, Δark1::HIS3*	Cope *et al*, 2000
381	MATa, *leu2-3,112, his3Δ200, ura3-52, lys2-801, Δprk1::LEU2*	Cope *et al*, 2000
463	MATa, *trp1-1, leu2-3,112, his3Δ200, ura3-52, lys2-801, Δark1::HIS3*	This study
464	MATα, *trp1-1, leu2-3,112, his3Δ200, ura3-52, lys2-801,* Δprk1::LEU2	This study
456	MATa, *trp1-1, leu2-3,112, his3Δ200, ura3-52, lys2-801, Δark1::HIS3, Δprk1::LEU2*	This study
393	MATa, *trp1-1, leu2-3,112, his3Δ200, ura3-52, lys2-801, Δark1::HIS3, SLA1-9xmyc::TRP1*	This study
394	MATα, *trp1-1, leu2-3,112, his3Δ200, ura3-51, lys2-801, Δprk1::LEU2,* SLA1-9xmyc::TRP1	This study
405	MATα, *trp1-1, leu2-3,112, his3Δ200, ura3-52, lys2-801, Δark1::HIS3, Δprk1::LEU2, SLA1-9xmyc::TRP*	This study
465	KAY405 + pJC232 (pEG-KT + *PRK1*)	This study
466	KAY405 + pJC233 (pEG-KT + Kinase dead *prk1*)	This study
467	MATa, *trp1-1, leu2-3,112, his3Δ200, ura3-52, lys2-801, Δark1::HIS3, sla1ΔCt::TRP1*	This study
468	MATα, *trp1-1, leu2-3,112, his3Δ200, ura3-52, lys2-801, Δprk1::LEU2, sla1ΔCt::TRP1*	This study

469	MATa, *trp1-1, leu2-3,112, his3Δ200, ura3-52, lys2-801, Δark1::HIS3, Δprk1::LEU2*	This study
455	MATa, *ade2-1, his3-11, leu2-3,112, trp1-1, ura3-1 can1-100, ssd1-d2, GAL+*	W303
460	KAY455 + *glc7::LEU2, trp1::glc7-13::TRP1*	
438	MATa, *trp1-901, leu2-3,112, ura3-52, his3-200, gal4Δ, gal80Δ,, LYS2::GAL1$_{UAS}$-GAL1$_{TATA}$-HIS3, GAL2$_{UAS}$-GAL2$_{TATA}$ -ADE2*	James *et al*, 1996
581	KAY438 + pDW1	This study
582	KAY581 + pKA	This study
568	KAY438 + pKA + pKA	This study
480	MATa/α, *his3Δ1/his3Δ1, leu2Δ1/leu2Δ1, ura3Δ/ura3Δ, met15/MET15, LYS2/lys2Δ.*	Research (RG) Genetics B4743
447	MATα, *his3-1, leu2Δ1, ura3Δ, met15, LYS2*	RGBY4742
479	KAY480 + *Δycl034w::Kanmx/Δycl034w::Kanmx*	RG 33441
465	MATa, *his3Δ1, leu2Δ1, ura3Δ, Δycl034w::Kanmx*	This study
515	MATα, *his3Δ1, leu2Δ1, ura3Δ, Δycl034w::Kanmx*	This study
510.1	KAY302 + *Δysc84::HIS3*	Hilary Dewar, unpublished data
516	MATα, *his3Δ, leu2Δ, ura3Δ, Δycl023w::Kanmx, Δysc84::HIS3*	This study
126	MATa, *his3-Δ200, leu2-3,112, ura3-52, Δabp1::LEU2*	Ayscough *et al*, 1997
406	MATα, *his3-Δ200, leu2-3,112, ura3-52, Δabp1::LEU2*	DDY322
466	MATa , *his3Δ, leu2Δ, ura3Δ, Δycl034w::Kanmx, Δabp1::LEU2*	This study
467	MATa , *his3Δ, leu2Δ, ura3Δ, Δycl034w::Kanmx, Δsla1::URA3*	This study

111	MATa, *ura3-52, lys2-801, ade2-101, trp1-Δ63, his3-Δ200, leu2-Δ1, fus1 LACZ::URA3.*	DK499
316	KAY111 + *STE2-9xmyc::TRP1.*	Warren *et al*, In press
391	KAY316 + *Δsla1::URA3.*	Warren *et al*, In press

yDW	Genotype	Origin
83	KAY581 + pGAD-*YCL034w* insert	This study
104	KAY581 + pGAD-*YCL034w* insert	This study
106	KAY581 + pGAD-*YCL034w* insert	This study
107	KAY581 + pGAD-*YCL034w* insert	This study
108	KAY581 + pGAD-*YCL034w* insert	This study
110	KAY581 + pGAD-*YCL034w* insert	This study
109	KAY581 + pGAD-*YSC84* fragment	This study
101	KAY581 + pGAD-*YDR128w* fragment	This study
88	KAY581 + pGAD-*YNR065c* fragment	This study
39	KAY581 + pGAD-*YNR065c* fragment	This study
97	KAY581 + pGAD-*ZRT3* fragment	This study
100	KAY581 + pGAD-*ZRT3* fragment	This study
111	KAY581 + pGAD-*YPR171w* fragment	This study
38	KAY581 + pGAD-*ADH* fragment	This study
60	KAY581 + pGAD-*ADH* fragment	This study
82	KAY581 + pGAD-*ADH* fragment	This study
99	KAY581 + pGAD-*ADH* fragment	This study
102	KAY581 + pGAD-*ADH* fragment	This study

58	KAY581+ pGAD-*GAL4* fragment	This study
59	KAY581 + pGAD-*GAL4* fragment	This study

Table 2.2 Oligonucleotides used in these studies.

oKA	Sequence	Description
13	CATTATTGGGACTGAGAACG	*SLA1-tag* C-terminal check (5')
14	CCATTGGATCCATTCAAAACC	*SLA1-tag* C-terminal check (3')
407	TGCTGCTCCGGTTTCATCTGCTCCGGTTTC ATCTGCTCCCGCTCCATTGGATCCATTCAA TCCGGTTCTGCTGCTAG	Tagging and deleting C-terminal repeats of *SLA1* (5')
408	GTAATCATTGGCATCATCACAAAGCCAGTA GATAAGGGTAAGATATTGTTGCCACCGGTT CCTCGAGGCCAGAAGAC	Tagging and deleting C-terminal repeats of *SLA1* (3')
83	GCTTGGACTGTTAAACCGGC	*sla1ΔCt-tag::TRP1* (5') check
84	GTGGCAATTGGTATTAGGCC	*sla1ΔCt-tag::TRP1* (3') check
112	ATCTATAAACAAACAATCGCATAACCGCAC GTATATACACGCACACACCTATCAATCACA CGGATCCCCGGGTTAATTA	*Δabp1::HIS3* (5')
113	TACAAAAGCTTTAACGTCTCTGTAAGTATTT TTTTACGTAAGAATAATATAATAGCATGAGA ATTCGAGCTCGTTTAAA	*Δabp1::HIS3* (3')
130	ACCATTGATGTATTTCTGTA	*Δabp1::HIS3* 5' check
131	ACCATTGATGTATTTCTGTA	*Δabp1::HIS3* 3' check
148	CGTGCAAGATTCCGAATACCG	5' YEp13 sequencing
152	ATATAGGCGCCAGCAACCGCA	3' YEp13 sequencing
153	ATACCACTACAATGGATGATG	pGAD (5') sequencing
154	AGATGGGCATTAATTCTAGTC	pGAD (3') sequencing
182	ACATCATCATCGGAAGAGAG	pGBD (5') sequencing

oKA	Sequence	Description
183	AATGAAAGAAATTGAGATGG	pGBD (3') sequencing
184	CGTCGTGGATCCGACCAAAGATTTCCCCAA	*SLA1 HD1-HD2* region (5')
185	CGTCGTCTGCAGGACCCATCATTCGCCTTA	*SLA1 HD1-HD2* region (3')
456	CACGAATTGCAAGCATTACAAGCAGAAAT CAATGCTGCAGCCGCTGCAGCTGCACGGA TCCCCGGGTTAATTAA	HA tagging *END3* (5')
457	GTGAAATAACAAACAGTAAATATTACACA TTCATGTACATAAAATTAATTATCGGAATT CGACCTCGTTTAAAC	HA tagging *END3* (3')
204	GAGTTCCGATAGCAGTGGCAG	*END3-HA* (5') check
205	GTATACACCACACCGTTACTG	*END3-HA* (3') check
111	ACGATCTCAAACCCTTTCGGTGATCATAA CAAAGCTGCAGCCGCTGCAGCTGCACGG ATCCCCGGGTTAATTAA	Tagging *YCL034w* + 7xAla linker (5')
112	GGATTAAATGTACTATATATATATATATAT ATATGTGTGTATGCATACGTACATACTGAA TTCGAGGTTTAAAC	Tagging *YCL034w* (3')
226	CCAGGCAAGGGCGATCAGAAG	*YCL034w-tag* (5') check
227	CTGGAGCCATCACATATCATC	*YCL034w-tag* (3') check

Table 2.3 Plasmids used in these studies.

Plasmid	Description	Origin
pWZV86	For *TRP1* marked gene deletions	K. Nasmyth
pWZV87	For C-terminal 9xmyc tagging (*TRP1* marked)	K. Nasmyth
pWZV89	For C-terminal 3xHA tagging (*TRP1* marked)	K. Nasmyth
pFA6-*HIS3*Mx6	For *HIS3* marked gene deletions	Longtine *et al*, 1998
pFA6-3x*HA*-*HIS3*MX6	For C-terminal 3xHA tagging (*HIS3* marked)	Longtine *et al*, 1998
pFA6-13x*myc*-*TRP1*	For C-terminal 13xmyc tagging (*TRP1* marked)	Longtine *et al* 1998
pGBDU-C3	2-hybrid binding domain plasmid	James *et al*, 1996
pGADU-C1	2-hybrid activation domain plasmid	James *et al*, 1996
pDW1	pGBDU-C3 + HD1-HD2 region of *SLA1*	This study
pDW38	pGAD + *ADH* fragment	This study
pDW39	pGAD + *YNR065c* fragment	This study
pDW58	pGAD + *GAL4* fragment	This study
pDW59	pGAD + *GAL4* fragment	This study
pDW60	pGAD + *ADH* fragment	This study
pDW82	pGAD + *ADH* fragment	This study
pDW83	pGAD + *YCL034w* fragment	This study
pDW88	pGAD + *YNR065c* fragment	This study

pDW97	pGAD + *ZRT3* fragment	This study
pDW99	pGAD + *ADH* fragment	This study
pDW100	pGAD + *ZRT3* fragment	This study
pDW101	pGAD + *YDR128w* fragment	This study
pDW102	pGAD + *ADH* fragment	This study
pDW104	pGAD + *YCL034w* fragment	This study
pDW106	pGAD + *YCL034w* fragment	This study
pDW107	pGAD + *YCL034w* fragment	This study
pDW108	pGAD + *YCL034w* fragment	This study
pDW109	pGAD + *YSC84* fragment	This study
pDW110	pGAD + *YCL034w* fragment	This study
pDW111	pGAD + *YPR171w* fragment	This study
pJC232	pEG-KT + *PRK1*	Cope *et al*, 1999
pJC233	pEG-KT + Kinase dead *prk1*	Cope *et al*, 1999
pKA1	pRS316 + *SLA1*	Ayscough *et al*, 1999
pKA11	pRS316 + *ABP1*	Ayscough *et al*, 1999

2.3.2 Removal of the terminal 5' phosphate groups using calf intestinal alkaline phosphatase (C.I.P.).

A ninth volume of 10 X C.I.P. Buffer (NEB) and 0.5 U of C.I.P. were added to the digest. Samples were incubated at 37°C for 30 minutes and then run on an agarose gel and were purified as described in following sections.

2.3.3 Extraction of DNA from an Agarose Gel.

Extraction of DNA from agarose gels was performed using a QIAEXII kit™ (QIAGEN). The DNA to be extracted was run on a 0.8% agarose gel (see section 2.2.6) and viewed by illuminating the gel with a UV source. The DNA band was cut out of the gel, and the gel slice was placed in a 1.5 ml microfuge tube. 3 volumes of Buffer QX1™ to 1 volume of gel slice was added to the tube along with 10 μl of resuspended QIAEX II™. Tubes were incubated at 50°C for 10 minutes to solubilise the agarose and allows the QIAEX II™ particles to bind the DNA. The sample was then centrifuged at top speed in a bench top microfuge for 30 seconds and the supernatant was carefully removed with a pipette. The pellet was washed once with 500 μl of Buffer QX1™ and twice with 500 μl of Buffer PE. Tubes were centrifuged at top speed in a bench top microfuge for 30 seconds after each wash. The pellet was then air-dried for 15 minutes, DNA was eluted from the QIAEX II™ particles by resuspending the pellet in 20 μl of sterile water and incubating at room temperature for 5 minutes. Tubes were centrifuged for 30 seconds and the supernatant was placed in a fresh microfuge tube and stored at –20°C until required.

DNA ligation.

DNA ligation was performed using a Rapid DNA ligation kit (Roche Diagnostics). DNA was diluted 1:5 in DNA Dilution Buffer. 10 μl of 2 X DNA Ligase Buffer was added along with 1 μl of DNA Ligase. The contents were mixed well and incubated at room temperature for 5 minutes. Tubes were stored at –20°C until required for transformation into bacterial cells.

2.3.5 Amplification of DNA using the polymerase chain reaction.

The polymerase chain reaction (PCR) is a method used to amplify specific DNA fragments using specific oligonucleotide primers complementary in sequence to the regions flanking the sequence of interest. PCR was used on several occasions during this project, the method described here was a general PCR method used to amplify sequences from plasmids designed to allow deletion or tagging of genes within the yeast genome.

All PCR carried out during this study was achieved using the Bioline reagents. For each PCR reaction the following were mixed in 0.5 ml microfuge tubes: 1 μM primer 1, 1 μM primer 2, 200 nM dNTP's, 1 X *Taq* reaction buffer, 1.5 mM $MgCl_2$, 1 μl template DNA and water to 99.5 μl. The components were mixed gently and microfuged briefly then 5 U of *Taq* polymerase was added to each reaction individually. The PCR occurred over 30 cycles of amplification in a MWG Biotech Primus PCR machine, each involving steps of 94°C (1 minute), 52°C (2 minute), 72°C (2 minutes). A final elongation step was used at the end of the amplification cycles by incubating at 72°C for 5 minutes. The resulting product was electrophoresed on an agarose gel to check that the amplification had worked.

2.3.6 Electrophoresis of DNA using agarose gels.

DNA fragments were routinely separated and visualised using flat bed agarose gel electrophoresis. Typically a 0.8% of the agarose in 1 x TAE (10 mM Tris-HCl pH 7.5, 1 mM EDTA pH7.5) solution was used but the percentage could be varied to bias separation of smaller or larger band fragments. An agarose gel was made by melting the agarose solution and allowing it to cool to about 50°C. 50 ml of this solution was then decanted into a falcon tube and 2 μl of a 5 mg/ml stock of ethidium bromide was added. The solution was then poured into a casting tray containing a comb with the required number of teeth. After the gel had set, the DNA was mixed with 6 x gel loading buffer (0.25% Bromophenol Blue, 0.25% Xylene Cyanol FF, 30% glycerol in water) and then loaded into the wells of the gel. Samples were electrophoresed in 1 x TAE at between 80 and 120 V until the bromophenol blue of the loading buffer was at the bottom of the gel. The DNA was then visualised by illuminating the gel with an UV light source.

2.3.7 Bacterial media.

2xYT 1.6% tryptone

1% yeast extract

0.5% NaCl

Supplement with 2% agar for solid media.

M9 media 0.675% Na_2HPO_4

0.3% KH_2PO_4

0.1% ammonium chloride

0.05% NaCl

0.035% do leucine mix

Supplement with 2% agar for solid media

Autoclave

Allow to cool to 50°C and then add:

5 mM $MgSO_4$

0.25 mM $CaCl_2$

100 μg/ml ampicillin

2% glucose

2.3.8 Preparation of Calcium Competent DH5α cells.

An overnight culture of DH5α cells was diluted 1:200 into 100 ml of 2xYT liquid. Cultures were then incubated at 37°C for approximately 2 hours until OD_{600}=0.5-0.6. The culture was then split into falcon tubes and placed on ice for 10 minutes. Cells were harvested by centrifugation in an Heaerus megafuge at 600 *g* for 5 minutes at 4°C. The cells pellet was resuspended in 50 ml of ice cold 100 mM $CaCl_2$. Tubes were incubated on ice for a further 30 minutes. Cells were again harvested by centrifugation and the cell pellet was resuspended in 5 ml $CaCl_2$ + 15% glycerol. 100 μl aliquots were snap frozen in liquid nitrogen and stored at –80°C.

2.3.9 Transformation of Calcium Competent DH5α cells.

Calcium competent cells were removed from the –80°C freezer and thawed on ice. 40 μl of cells were placed into a fresh tube and 1 μl of DNA was added. Tubes were incubated on

ice for 30 minutes and heat shocked at 42°C for 40 seconds. 200 μl of 2xYT (1.6% tryptone, 1% yeast extract and 0.5% NaCl) was then added to tubes and the culture was incubated at 37°C for 1 hour without shaking. Cells were then spread onto plates containing the appropriate antibiotic (most usually 100 μg/ml ampicillin) and incubated overnight at 37°C.

2.3.10 Plasmid DNA purification (Miniprep).

Plasmid DNA was purified using the QIAGEN™ QIAprep spin kit™. Cells from a 2 ml overnight bacterial culture were harvested by centrifugation at top speed in bench top microfuge. The cell pellet was resuspended in 250 μl of Buffer P1™ and 250 μl of Buffer P2™ was added to tubes. Tubes were then inverted 6 times and incubated at room temperature for 5 minutes. 350 μl of Buffer N3™ was added and the tubes were again inverted 6 times. Tubes were centrifuged at top speed in a bench top microfuge for 10 minutes. The supernatant was then poured into a QIAprep spin column™ in a 2 ml collection tube and then tubes were spun at top speed for 1 minute. The flow through was discarded and the QIAprep spin column™ was washed with 750 μl of Buffer PE and centrifuging at top speed in a bench top microfuge for 1 minute. The flow through was again discarded and tubes were centrifuged for an additional 1 minute to remove residual wash buffer. The QIAprep column™ was placed in a fresh 1.5 ml microfuge tube and DNA was eluted from the column by adding 30 μl of sterile water, incubating at room temperature for 1 minute and centrifuging at top speed for 1 minute.

2.4 Yeast Methods.

2.4.1 Yeast media.

YPAD

1% yeast extract

2% peptone

0.002% adenine

2% glucose

For solid media supplement with 2% agar.

Drop-out media

0.67% nitrogen base without amino acids

0.2% drop-out mix

2% glucose

For solid media supplement with 2% agar.

sporulation media

1% potassium acetate

2% agar

5-FOA media

0.67% nitrogen base (Difco)

0.2% drop-out uracil mix

2% glucose

0.1% 5-FOA (Melford Laboratories)

2% agar.

2.4.2 Mating Yeast Cells.

Cells of the opposite mating type were patched onto each other and allowed to grow at 30°C for 1-2 days.

2.4.3 Sporulation and Tetrad Analysis.

Diploid strains to be sporulated were grown on YPAD plates overnight at 30°C. Cells were patched onto a sporulation plate and incubated at 30°C for up to five days (generally three days). The plate was monitored daily to check development of four spored asci. When these were plentiful, cells were taken from the plate using a toothpick and were resuspended in 100 μl filter sterilised 0.1 M potassium phosphate buffer + 0.5 mg/ml 100T zymolyase (ICN Biomedical) and incubated for 10 minutes at room temperature. Once digested, spores were struck onto YPAD plates, placed under a micromanipulator (Singer) with the needle beneath and facing up toward the cells on the plate. Looking down the eye-piece of the micromanipulator, the region of spread cells was located and tetrads isolated and dissected into rows of four spores on the YPAD plate using the x and y co-ordinates. When sufficient spores were isolated the plate was incubated at 30°C until the spores germinated and became visible. The number of viable spores was noted and they were patched onto YPAD or selective plates to test for genetic markers.

2.4.4 Mating Type Determination.

Mating type was determined by crossing with both tester strains (KAY30 MATa *his1*; and KAY 31 MATα *his1*). Cells were patched onto plates lacking histidine and grown at 30°C overnight. Only haploid cells that have mated to form diploid cells were able to grow on these plates.

2.4.5 Halo assay for Latrunculin-A Sensitivity.

A 2 ml culture was grown in YPAD liquid media at 30°C overnight. 2 mls of fresh YPAD liquid was inoculated with 10 μl of the overnight culture. 1% agar was melted and cooled to 50°C before adding to the liquid cell mix. The mix was immediately poured onto a YPAD plate and the agar was allowed to set. Once set, four sterile antibiotic assay discs were placed on top of the agar. 10 μl of dilutions from a 50 mM stock of LAT-A (Santa Cruz) was added to the discs (0, 1, 2, 5 mM). Plates were incubated for 2 days at 30°C until halos were visible in the lawn of cells. Relative sensitivity was calculated by measuring the diameter of halo formed and plotting a graph of halo diameter against the log[LAT-A]. Halo sizes for a concentration of 12 mM LAT-A were calculated. These values were plotted on a graph as a ratio of the wild-type halo size.

2.4.6 Yeast Transformations.

Cells were grown to mid log phase $OD_{600} = 0.5$. 15 mls of cells were harvested at 600 *g*, washed once in 0.1 M TE (10 mM Tris-HCl pH 7.5, 1 mM EDTA pH 7.5) and once in 0.1 M Lithium acetate/ 0.1 M TE. Cells were then resuspended in 100 μl 0.1 M Lithium acetate/ TE. 15 μl of 10 mg/ml herring sperm DNA boiled for 15 minutes was added along with 0.1-1 μg of transforming DNA. The mix was then vortexed briefly, 700 μl 40 % PEG4000 in 0.1 M lithium acetate/ TE added and vortexed again. The transformation was incubated at room temperature for 90 minutes on a rotator then heat shocked at 42°C for 15 minutes. Cells were spun down in a microfuge and resuspended in 100 μl of sterile water and plated on selective plates.

2.4.7 'Knock in' Strategy of Generating Integrated Gene Deletion or Tagged Strains.

A set of plasmids exist that allow genes within the yeast genome to be easily tagged or deleted (Longtine *et al*, 1998). These plasmids allow amplification of a tag or stop codon and selection marker by PCR (see section 2.2.5) using oligonucleotides that have been designed with regions of homology to a specific site in the yeast genome. Transformation of this PCR product into yeast cells (see section 2.3.5) allow its incorporation into the specific site in the yeast genome via homologous recombination. The transformation mixture is spread onto plates lacking certain nutrients, allowing selection of transformed cells.

2.4.8 Screening for Integrated Tags or Gene Deletions by Colony PCR.

After transformants have grown into colonies they were struck out onto YPAD plates and incubated at 30°C overnight. The next day, a single colony was picked using a toothpick and resuspended in 20 μl of SPZ Buffer (1.2 M sorbitol, 0.1 M Potassium Phosphate buffer pH7.5 and 2.5 mg/ml zymolyase (ICN Biomedicals)). Cell suspensions were incubated at 37°C for 30 minutes and boiled for 5 minutes. This crude prep was used as the template in a standard PCR (see section 2.2.5) that used oligonucleotides that were designed to the regions flanking the insert region. This PCR would either generate a wild type band or a larger integration band. PCR products were visualised by running on an agarose gel (see section 2.2.6).

2.4.9 Temperature Sensitivity.

A single colony of cells was picked using a toothpick and was resupended in 100 μl of water in a well of a frogging pond. Cells were frogged out onto YPAD plates and

incubated at either 30°C or 37°C for 1-2 days to assess if a strain has a temperature sensitive defect.

2.4.10 Measuring Cell Number and Generation Times.

An overnight culture was used to inoculate 100 ml of cells to a cell density of about $5x10^5$ cells/ml. Cells were grown for 1.5 hours at 26°C in a shaking incubator. The culture was split into 2 x 50 ml cultures and then incubated at 26°C and 37°C for 30 minutes. A 500 μl sample was removed and sonicated for 10 seconds. The sample was diluted 1:200 with Casyton™ buffer and then the cell number was measured in the Schärfe system CASY 1™. This was repeated at various time points.

2.4.11 Screen to Identify Genes That When Overexpressed Can Rescue the *ABP1* Dependent Phenotype of *sla1ΔCt* Mutant Cells.

KAY599 cells were transformed with pKA11 (*URA* marked) (see section 2.3.5) and were then crossed with cells expressing *sla1ΔCt* (KAY369) to generate a strain lacking *ABP1* and expressing the *sla1ΔCt* mutation that were viable due to expression of functional *ABP1* from pKA11. This strain was then transformed with 20 μg of a YEp13 (*LEU* marked) expression library using the yeast 2-hybrid library transformation protocol (see section 2.4.3). Cells were spread onto plates containing media lacking uracil and leucine and incubated for about 3 days at 30°C. Transformed cells were then replica plated onto plates media lacking leucine and containing 0.1% 5-FOA (5-Flurooorotic acid, Melford laboratories) to select against the *URA* marked plasmid expressing functional *ABP1* (pKA11). This reagent is toxic to yeast cells expressing the *URA* genes. For cells to grow in the presence of 5-FOA they must therefore lose the *URA* marked plasmid, so only inserts capable of rescuing the lethal phenotype of these cells will produce a viable colony. These plates were incubated at 30°C for one week.

After a week incubation of these plates colonies were clearly visible. 150 colonies of varying sizes were picked. The library plasmid was then rescued from these cells (see section 2.4.5) and PCR was used to screen for plasmids containing *ABP1* or *SLA1* inserts. Of the 150 colonies only 9 were sequenced.

2.4.12 Iodination of the MFN5 peptide.

The MFN5 peptide (Siegal *et al*, 1999) was iodinated with chloramine T as desribed in (Olah *et al*, 1994). Briefly, the MFN5 peptide was resuspended in 0.3M sodium dihydrogn phosphate, pH7.5, to a concentration of 0.5 mg/ml. To this 1.5 mCi of ^{125}I was added, followed by 100 μl of a 1 mg/ml chloramine T solution. After 4 minutes at room temperature , the reaction was stopped by addition of 150 μl of a 0.5 mg/ml sodium metabisulfate solution. The entire solution was passed through a ? column to remove unbound iodine. Fractions were collected in centrifuge tubes and their specific radioactivity was determined using a COBRA™ Auto-Gamma counter (Packard).

2.4.13 α-factor uptake assays.

YPAD plates were spread with 200 μl ($2x10^6$ cells) from an overnight culture of either KAY316 or KAY391 cells. Plates were incubated overnight at 30°C. Cells were resuspended in 10 ml YPAD + 1M sorbitol and centrifuged at 500 *g* for 5 minutes to remove budded cells. The supernatant was decanted into a fresh tube and cells were harvested by centrifugation. Cells were resuspended in 2 ml ice cold YPAD to a concentration of $5x10^8$ cells/ml.

α-factor internalisation assays were performed essentially as described previously (Dulic *et al*, 1991). Approximate $8x10^7$ counts/minute ^{125}I-MFN5 was added to the 2 ml cell suspension on ice. ^{125}I-MFN5 peptide was allowed to bind to cells on ice for one hour.

Unbound MFN5 peptide was removed by centrifugation and cells were allowed to initiate peptide internalisation by adding 2 ml prewarmed YPAD and incubating at 30°C. At various time points, tubes were vortexed briefly and 100 μl cell aliquots were removed and washed in 1ml pH1.1 or pH6, 0.1M potassium phosphate buffer. The amount of cell associated radioactivity after each wash was determined by gamma counting in a COBRA™ Auto-Gamma counter (Packard). The amount of α-factor peptide internalised is expressed as a ratio of pH1.1-resistant to pH6-resistant radioactivity. To determine the fold increase in uptake, the pH1.1/pH6 ratio was divided by the starting value in each case.

2.5 Yeast 2-Hybrid Protocol.

2.5.1 Construction of Bait Plasmid pGBDU-HD1+HD2.

The bait plasmid to be used in the yeast 2-hybrid screen was constructed by subcloning the central region, containing HD1 and HD2, of *SLA1* into the pGBDU-C3 vector. To achieve this, the central region (nucleotides 1461-2192) of *SLA1* was amplified by PCR, using Biolone Taq, from pKA1. The primers used were designed so that Pst1 (oKA184) and BamH1 (oKA185) restriction sites flank the region of interest. The amplified region central region was then subcloned into the Pst1/BamH1 restriction sites of pGBDU-C3. This created an in-frame fusion of the central region of *SLA1* to the *GAL4* DNA binding domain on the bait vector (pDW1). pDW1 was sequenced from the 5' and 3' ends using oligonucleotides oKA182 and oKA183 respectively which bind pGBDU-C3 regions flanking the insert.

2.5.2 Testing pDW1 for Self-Activation.

In order to check that the pDW1 plasmid did not self-activate transcription of the reporter genes, 2 μg of the plasmid was co-transformed into KAY438 with 2 μg of empty activation

plasmid (pGAD-C1). The cells were first grown on plates selecting for both plasmids and then transferred onto plates selecting for the expression of reporter genes.

For a negative control yeast cells were co-transformed with empty pGBDUC-3 (binding domain plasmid) and empty pGAD-C1 (empty activation domain plasmid). These cells were struck onto plates selecting for both plasmid and then transferred to plates selecting for expression of the reporter genes as before.

2.5.3 2-Hybrid Library Transformation.

pDW1 was transformed into KAY438 as described in section 2.2.5 . This strain (KAY581) was then grown overnight in liquid media lacking uracil at 30°C. This culture was diluted into 100 mls and incubated in a 30°C shaking incubator until the cell density was approximately 2x 10^7 cells/ml. Cells were harvested by centrifugation at 600 *g* for 4 minutes. The cell pellet was washed in sterile water and then centrifuged as before. The following was added to the cell pellet: 360 μl 1 M lithium actetate; 2.4 ml 50 % PEG4000; 50 μl (boiled 15 minutes and then put on ice) herring sperm DNA (15 mg/ml); 20 μg library DNA from pGADC-1/2/3 and 680 μl sterile distilled water. The mixture was then vortexed for one minute, incubated at 30°C for 30 minutes and then heat shocked at 42°C for 30 minutes with gentle mixing every 5 minutes. Cells were harvested by centrifugation at 600 *g* for 4 minutes and the resulting cell pellet was resuspended in 3.6 ml sterile distilled water. The cell suspension was spread onto 8 x 140mm diameter selective media (lacking uracil and leucine) plates to select for the bait (URA) and the prey (LEU) plasmids. Cells were allowed to grow for two days at 30°C.

After two days growth at 30°C the transformation had grown to confluence. Cells were replica plated onto plates to select for expression of the reporter genes,the media lacked uracil, leucine, histadine and adenine (amino acid drop-out mix from BIO 101) , using Whatman paper to transfer cells onto the 8 x 140 mm plates.

2.5.4 Elimination of False Positives.

To remove as many false positives from the library screen before the plasmids were rescued or sequenced, strains were struck onto plates that lacked adenine but contained 0.1% 5-FOA (5-Fluoroorotic acid, Melford laboratories). This reagent is toxic to cells expressing the *URA* genes. For cells to grow in the presence of 5-FOA they must therefore lose the bait (URA marked) plasmid. Any cells that are able to grow on this media are false positives because the media is selecting for cells expressing one of the reporter genes (adenine), these cells are activating their reporter genes but only contain the prey plasmid.

2.5.5 Extraction of Yeast 2-Hybrid Library Plasmids.

Yeast cells were grown overnight in liquid media lacking uracil and leucine. Cells were harvested from 1 ml of overnight culture by centrifugation at 5000 rpm for one minute in a bench top microfuge. The cell pellet was resuspended in 500 μl Buffer S (10 mM potassium phosphate buffer pH 7.2; 10 mM EDTA; 50 mM β-mercaptoethanol and 50 μg/ml zymolyase) and incubated at 37°C for 30 minutes. Next 100 μl of Lysis Buffer (25 mM Tris-HCl pH 7.5; 25 mM EDTA; 2.5 % (*w/v*) SDS) was added to the cells and the cells were mixed by vortexing. Cells were incubated for a further 30 minutes at 65°C before the addition of 166 μl 3M potassium acetate (pH 5.5). Cell suspensions were then incubated on

ice for 10 minutes before centrifugation at top speed for 10 minutes in a bench top microfuge at 4°C. The supernants were transferred to fresh tubes on ice containing 800 μl of 100 % ethanol and left for 10 minutes. DNA was pelleted by centrifugation at top speed for 10 minutes in a bench top micrafuge at 4°C, washed in 70 % ethanol and allowed to air dry. The pellet was then resuspended in 40 μl sterile distilled water. These plasmid preparations contain both the *URA3* and *the LEU2* marked 2-hybrid plasmids. To select for the *LEU2* marked (library) plasmids, these preparations were transformed into electrocompetent KC8 cells, which lack a functional *LEUB* gene. This *E.coli* strain contains a *leuB* mutation, which allows for the direct selection of the yeast *LEU2* gene carried on the library plasmids.

2.5.6 Preparation of Electro-Competent KC8 Cells.

A 2 ml overnight culture of KC8 cells was used to inoculate 400 ml of 2 x YT media. Cells were incubated in a 37°C shaking incubator and grown to an approximate OD_{600}= 0.5-0.6. Cells were cooled on ice for 30 minutes and the harvested by centrifugation at 3000 *g* for 10 minutes at 4°C. The cells pellet was resuspended in 50 ml ice-cold sterile water, mixed and the centrifuged as before. This washing step was repeated again. The cell pellet was then resuspended in 20 ml of ice cold 10 % glycerol, cells were harvested by centrifugation. The cell pellet was resuspended in an equal volume of ice cold 10 % glycerol. Cells were then aliquoted (50 μl/transformation) into microcentrifuge tubes and frozen on dry ice. Cells were stored at –80°C until needed.

2.5.7 Electroporation of KC8 Cells with Yeast 2-Hybrid Plasmid Preparations.

Cells were electroporated with the 2-hybrid plasmid preparations, incubated at 37°C for 30 minutes in 2 x YT, before plating onto M9 plates lacking leucine that allow for the selection of *LEU2* marked library plasmids. Plates were incubated overnight at 37°C.

Colonies were picked and grown in a small volume (2 ml) of 2 x YT + 100 μg/ml ampicillin. Plasmids were extracted from the KC8 cells using the Qiagen miniprep kit (see section 2.1.9) and transformed into chemically competent DH5α cells (see section 2.1.8). This subsequent transformation allowed stocks of the plasmids recovered to be stored as DH5α cells are better at maintaining plasmids.

2.5.8 Checking the Rescued Plasmids Activate Expression of the Reporter Genes.

To check that the activation plasmids had been rescued from the yeast cells and the activation was real, the rescued library plasmids were retransformed into KAY582. Each plasmid was checked that it was able to activate expression of the reporter genes in combination with the bait plasmid pDW1, by growing on media lacking adenine and histidine.

2.6 Protein Methods.

2.6.1 Whole cell yeast extracts.

Cells were grown in liquid media overnight in a shaking incubator at 30°C. Cells were harvested from 1.5 mls of the overnight culture by centrifugation in a bench top microfuge at 3000 *g* for one minute. 100 μls of acid washed glass beads were added to the pellet along with 25 μls of 2 x SDS sample buffer (62.5 mM Tris-HCl (pH 6.8), 20 % glycerol, 2 % SDS, 0.0025 % bromophenol blue). Cells were then boiled for three minutes and vortexed for one minute. Another 100 μls of 2 x SDS sample buffer was added and the protein sample was vortexed briefly. Before the protein is separated on a SDS gel the sample was boiled for an additional one minute.

2.6.2 Protein preps by grinding in liquid nitrogen.

An overnight culture of cells was diluted into 100 mls of the appropriate growth media and grown to an OD_{600}=1.0. Cells were harvested by centrifuging at 3000 *g* for 5 minutes. The cell pellet was resuspended in 1 ml of the appropriate buffer and frozen in liquid nitrogen for 10 minutes. Cells were the ground in the presence of liquid nitrogen, using a pestal and mortar. The cell extract was collected into 1.5 ml eppendorf tubes and stored at –80°C until required.

2.6.3 Immunoprecipitation procedures.

Yeast cells were lysed by liquid nitrogen-grinding method in IP buffer (0.5 mM KCl, 2 mM EDTA, 25 mM HEPES (pH 7.5), 0.5 % Triton X-100) freshly supplemented with 1 mM PMSF and aqueous phase protease inhibitors. The cells lysate was centrifuged at 100,000 *g* for 20 minutes at 4°C in a TLA100 rotor (Beckman™). 500 μl of the high-speed

supernatant was pre-cleared by adding 2 μl 50 mM Latrunculin-A and 10 μl mouse IgG-agarose conjugate (Santa Cruz, USA). Samples were incubated at 4°C for one hour with mixing. The agarose-conjugated antibody was removed by centrifugation at 3000 rpm for 4 minutes at 4°C. The pre-cleared supernatant was transferred to a fresh tube and 20 μl of mouse anti-HA agarose conjugated antibody (Santa Cruz, USA) was added. Samples were incubated at 4°C for one hour with mixing. Antibody bound proteins were harvested by centrifugation at 3000 *g* for 4 minutes at 4°C. Beads were washed three times in IP buffer.

2.6.4 Treatment of immunoprecipitated proteins with λ-phosphatase.

After washing, beads were resuspended in IP buffer and treated with λ-phosphatase (New England Biolabs) for 30 minutes at 37°C. Proteins were eluted from the beads by addition of 40 μl 2 x SDS sample buffer and then boiling for one minute.

2.6.5 Fractionation of yeast cell extracts.

Cell extracts for sucrose gradient fractionation were prepared by the liquid nitrogen-grinding method in UBT (50 mM KHEPES, pH 7.5, 100 mM KCl, 3 mM $MgCl_2$, 1 mM EGTA, 0.5 % Triton X-100) freshly supplemented with 1 mM PMSF and aqueous phase protease inhibitors. The lysate was thawed at 4°C and spun at 3000 *g* for 4 minutes at 4°C to clear unlysed cells. The supernatant was then spun at 100,000 *g* for 20 minutes at 4°C to obtain a high-speed supernatant to load onto the sucrose gradient.

2.6.6 Sucrose gradients

Linear sucrose gradients (11 ml of 3-30%) were prepared in Ultra-Clear tubes (Beckman, USA). Samples (500 μl) of yeast extract were layered on top. The gradients were spun at 35,000 rpm in an SW41 rotor for 18 hours at 4°C. Fractions (400 μl) were removed from

the bottom of the gradient and assayed on SDS-PAGE. Immunoblotting was used to determine the position of various proteins.

2.6.7 SDS-PAGE electrophoresis.

The appropriate percentage of acrylamide gel was poured according to the recipes below.

Separating gel:

	7.5 %	10 %	12 %
Sterile water	4.10 mls	3.44 mls	2.69 mls
30 % acrylamide, 0.8 % bisacrylamide (37.5:1)	2.25 mls	3.00 mls	3.75 mls
Bottom Buffer (1.5 M Tris-HCl (pH 8.8), 0.4 % SDS)	2.25 mls	2.25 mls	2.25 mls
10 % ammonium persulfate	31 µls	31 µls	31 µls
TEMED	5 µls	5 µls	5 µls

5 % Stacking gel:

Sterile water	1.71 mls
30 % acrylamide, 0.8 % bisacrylamide	0.50 mls
Top buffer (0.5 M Tris-HCl (pH 6.8), 0.4 % SDS)	0.75 mls
10 % ammonium persulfate	35 µls
TEMED	3.5 µls

Gels were run in 1 x Running buffer (0.025 M Tris, 0.192 M Glycine, 0.1 % SDS, pH 8.6) at 120 volts for approximately 1.5 hours, until the dye front has run off the bottom of the gel.

2.6.8 Western blotting.

Proteins were separated by SDS-PAGE electrophoresis as described above. The PVDF membrane and 3 mm filter paper were cut to size. The PVDF was soaked in methanol while the filter paper and two Scotchbrite pads were soaked in ice-cold blotting buffer (10 mM CAPS, 10 % methanol, pH 11). The blot sandwich was assembled keeping everything wet and care was taken to exclude air bubbles. The sandwich was then placed in the blotting apparatus, with the membrane toward the anode. An ice pack was placed in the apparatus to keep the transfer cool and blotting was performed at 400 mAmps for one hour.

2.6.9 Western blot detection using Enhanced Chemi-Luminescence (ECL).

Proteins are blotted as described above. The wet membrane was blocked at room temperature for about 30 minutes in 5 % milk powder in 1 % TBST (1 x TBS + 0.2 % Tween-20). The membrane was then rinse briefly in 1 x TBST and was then placed in a plastic bag with 3 mls of blocking solution plus primary antibody (concentrations as described in the text). The membrane was incubated in the presence of primary antibody for either one hour at room temperature with shaking or overnight at 4°C with shaking. The blot was then removed from the plastic bag and washed with 1 x TBST briefly followed by one 15 minute and two 5 minute washes in 1 x TBST. The membrane was placed in a plastic bag and incubated with blocking solution containing horseradish peroxidase conjugated secondary antibody (usually 5000 x dilution) for between 30 minutes and one hour at room temperature with shaking. The membrane was removed from the plastic bag and washed as before but with an additional 5 minute wash in 1 x TBST. In the dark room equal volumes of developing reagents (Solution I : 250 mM luminol (3-aminophthalhydrazide from Fluka No 09253) in DMSO, 90 mM p-coumaric acid in DMSO, 0.1 M Tris-HCL pH 8.5; Solution II: hydrogen peroxide, 0.1 M Tris-HCl pH 8.5)

were mixed and the membrane was incubated in the developing solution for at least one minute. The solutions were then drained off and the membrane wrapped in cling-film. The membrane was then exposed to X-ray film (Konica) in a cassette and the film was developed in an automatic developer.

2.6.10 Western blot detection using Alkaline Phosphatase.

The blotted membrane is probed with antibodies as above except the secondary antibody is an alkaline phosphatase conjugated antibody (cocentrations as described in the text). After incubation with secondary antibody, the membrane was washed as above. The membrane was then developed in 10 mls of developing solution (100 mM NaCl, 5 mM $MgCl_2$, 100 mM Tris (pH 9.5)) containing 132 μls of NBT (nitro blue tetrazolium) stock (0.5 g in 10 ml 50 % DMF) and 66 μls BCIP (5-bromo-4-chloro-3-indolyl phosphate ρ-toluidine salt) stock (0.25 g in 10 ml 100 % DMF). After sufficient colour development the reagents were washed off with water and the membrane was air-dried.

2.6.11 2-Dimensional gel electrophoresis.

Whole cell extracts were prepared in Rehydration Buffer (9 M Urea, 2% CHAPS, 0.2%/ 1:200 Ampholine (pH 3-10), 2.8 mg DTT). Whole cells extracts were centrifuged at 3000 *g* for 5 minutes at 4°C in a bench top microfuge. A few grains of bromophenol blue were added to the supernatant and 500 μl of extract was placed into a well of the rehydration tray. A Drystrip (pH 3-10, 18 cm, Amersham Pharmacia) was carefully placed face down into the whole cell extract, excluding any air bubbles. The Drystrip was then covered in 3 ml of Drystrip Cover Fluid (Amersham Pharmacia) and the gel strips were allowed to rehydrate overnight at 4°C. Hydrated drystrips were placed on the flat bed of a Multiphor II system (Amersham Pharmacia) and the first dimension was run under the following conditions: STEP1 500 V, 2 mA, 5 W, for 1 minute; STEP2 3500 V, 2 mA, 5 W, for 1.5

hours; STEP3 3500 V, 2 mA, 5 W, for 6.5 hours. Before running the second phase, the Drystrip was equilibrated by soaking in SDS sample buffer for 30 minutes. The Drystrip was then placed on top of a 3-15% SDS separating gradient gel, encased in 1% agarose and run through the gel at 10 mAmps with cooling overnight. Western blotting was then performed, HA-tagged proteins were detected by probing with Rabbit αHA (1:1000) and αRabbit HRP (1:5000).

2.7 Microscopy methods.

2.7.1 Live cell microscopy methods.

2.7.1.1 Lucifer Yellow staining of vacuoles.

Cells were grown to an OD_{600} = 0.25. 0.5 ml cells harvested in a bench top microfuge and resuspended in 30 μl YPAD liquid. 10 μl of 40 mg/ml Lucifer yellow was added and incubated at room temperature for one hour in the dark. Cells were washed three times in ice cold succinate/azide buffer and resuspended in 10 μl of buffer and kept on ice until viewed with an Olympus microscope BX-60 fluorescence microscope containing an 100W mercury lamp and an Olympus 100 X Plan-NeoFluar oil immersion objective. Images were captured using a Roper Scientific Micromax 140E cooled CCD camera using IP lab software (Scanalytics, Fairfax, VA) on an Apple Macintosh G4 computer.

2.7.1.2 Visualisation of GFP tagged proteins.

A 10 μl aliquot of a mid-log phase OD_{600}= 0.2-0.4 was removed and placed on a glass slide. A coverslip was place over the liquid and GFP tagged proteins were visualised on the fluorescence microscope.

2.7.2 Fixed cell microscopy methods.

2.7.2.1 Indirect Immunofluorescence

Mid-log cultures were fixed in 5 % final volume formaldehyde solution for between 30-60 minutes. Cells were then harvested in a bench top microfuge and washed twice in KPi + sobitol (1.2 M sorbitol, 0.1 M Potassium phosphate buffer, pH 7.5). Cells were resuspended in KPi sorbitol buffer. 1 μl of β-mercaptoethanol and 20 μl 1 mg/ml 100T zymolyase was added. Tubes were incubated at 37°C for 35 minutes. 20 μl of the cell suspension was placed onto a well of a slide that had been lined with poly-l-lysine. Cells were allowed to settle for 10 minutes, then aspirated off gently. The well was covered with 0.1 % SDS solution for a maximum of 1 minute and then washed 10 times with PBS + 0.1 % BSA. Next 20 μl of PBS/BSA + primary antibody was placed on the well. The slide was incubated for 1 hour in the dark. The well was washed 10 times with PBS/BSA, then incubated for 1 hour covered in PBS/BSA + secondary antibody. The well was washed again 10 times with PBS/BSA then a drop of phenylenediamine mounting solution (1 mg/ml p-phenylenediamine, 22.5 ng/ml 4'6' Diamidino 2-phenylindole dihydrochloride (DAPI) in Glycerol) was placed onto each well. A cover slip was placed over the wells and sealed with nail polish around the edges.

2.7.2.2 Rhodamine phalloidin staining.

Cells were grown to an OD_{600}=0.2-0.4, fixed in 5 % final volume of formaldehyde solution for one hour at room temperature and then harvested in a microfuge. The cell pellet was washed three times in 0.1 M PBS/0.1 M BSA/0.1 % triton and then resuspended in 40 μl of 0.1 M PBS/0.1 M BSA/1 % triton plus 5 μl Rhodamine phalloidin. Cells were incubated at room temperature for 30 minutes in the dark, washed three times with 0.1 M PBS/0.1 M BSA and resuspended in 100 μl of the above. Cells were placed in poly-l-lysine coated

wells allowed to settle for 10 minutes, washed twice with 0.1 M PBS/0.1 M BSA and then mounted with a drop of mounting solution before the cover slip was sealed with nail polish. Slides were then viewed on the fluorescence microscope.

2.7.2.3 Electron Microscopy.

Cells were grown to early log phase (OD_{600}=0.4 – 0.6) and harvested at 3000 *g* for 5 minutes. The cell pellet was rinsed in 1.5 ml of fixative (0.04 M potassium phosphate buffer (pH 6.7), 1 M sorbitol, 3 % formaldehyde, 0.5 % glutaraldehyde (EM grade, Polysciences), 1 mM $Mgcl_2$, 1 mM $CaCl_2$, final pH should be between 6.5 and 7). Resuspend pellet in Fixative and incubate at room temperature for two minutes. Cells were washed three times with 50 mM potassium phosphate buffer (pH 7.4) and then incubated in 4 % potassium permanganate for four hours. After this, cells were washed four times with sterile water. 2 % filter sterilised uranyl actetate was added and cells were incubated overnight at 4°C, with mixing. In the next step cells were dehydrated, by washing them in increasing concentrations of ethanol. Cells were washed twice with 50 % ethanol over 10 minutes and then twice in 70 % ethanol over 10 minutes. Next cells were washed in 80 %, 90 %, 95 % ethanol for 5 minutes each and then three times in 100 % ethanol over 30 minutes. 50 % White Resin (Agar scientific, Cambridge, UK) in ethanol was added and cells were incubated at room temperature for two hours with mixing. Cells were harvested by centrifugation and then resuspended in 100 % White Resin and incubated at room temperature overnight with mixing. Cells were harvested by centrifugation and the tube was filled to the top with 100 % White Resin. Tubes were incubated at 60°C overnight. Samples are now ready to be sliced and stained. Sections were prepared on an ultramicrotome. Sections were silver in interference colour, corresponding to a thickness of between 50 – 70 nm. The thinnest sections provide the best resolution.

Chapter 3.
Characteriastion of the *sla1ΔCt* Mutant and the Role of Sla1p in Endocytosis.

3.1 Background.

Sla1p has been demonstrated to be required for the proper organisation of the yeast cortical actin cytoskeleton (Holtzman *et al*, 1993; Ayscough *et al*, 1999). A previous study using plasmid borne domain deletion mutants of *SLA1* indicated that the C-terminal repeat region of Sla1p was required to rescue the Abp1p-dependent phenotype but not the actin phenotype associated with the *Δsla1* deletion (Ayscough *et al*, 1999). However, the *sla1ΔCt* mutant used in this study did not lack the entire C-terminal repeat region, only approximately two thirds of this region was deleted.

To elucidate further the functions of the C-terminal repeat region of Sla1p, an integrated *sla1ΔCt* mutant was constructed that lacked the entire C-terminal repeat region. This chapter concentrates upon the phenotypes that are associated with this mutant.

3.2 Results.

3.2.1 Construction of an integrated *sla1ΔCt* mutant.

The 'knock-in' strategy (figure 3.1) was used to construct a strain lacking the region encoding for the entire C-terminal repeat region of *SLA1* from the genome (see Materials and Methods section 2.4.7 and Longtine *et al*, 1998). Briefly, primers designed to the

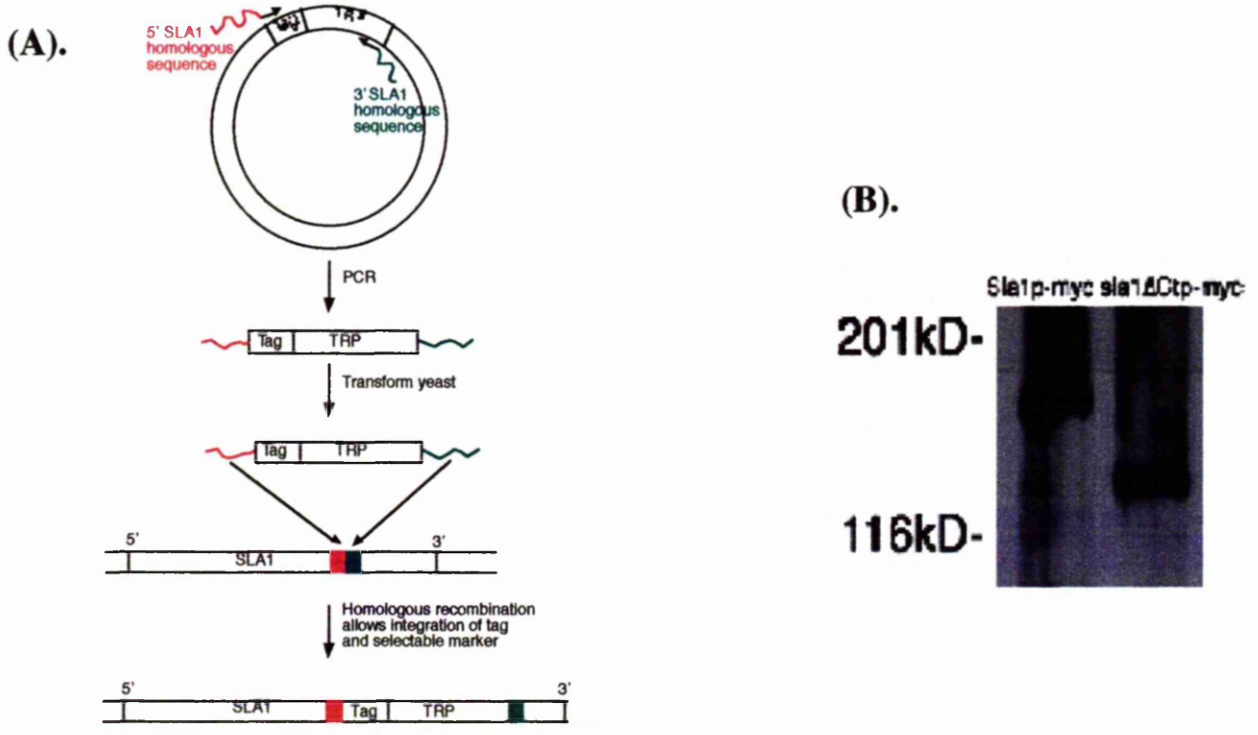


Figure 3.1 Construction and expression of an integrated *sla1ΔCt* mutant. (A) The 'knock-in' strategy was used to construct a strain lacking the entire C-terminal repeat region of *SLA1*. Primers are designed to the region of interest and are used to amplify a tag and stop codon. This product was transformed into yeast cells and integration into the genome occurs via homologous recombination. (B) Immunoblotting was carried out to detect Sla1p-myc and sla1ΔCtp-myc from whole cells extracts. Membranes were probed with rabbit anti-myc (Santa Cruz) primary and anti-rabbit-HRP (Sigma) secondary antibodies. Myc-tagged proteins were detected by ECL.

region of interest are used in amplify a tag and stop codon from a set of plasmids (Longtine *et al*, 1998). The PCR product is transformed into yeast and insertion of the PCR product into the genome occurs via homologous recombination. Colonies are picked and screened to check that the PCR product has integrated into the correct site in the genome by colony PCR (see Materials and Methods section 2.4.8). Western blotting is then performed to check for expression of tagged proteins. As shown in figure 3.1, the *sla1ΔCt-myc* mutant is expressed in the yeast cells.

3.2.2 Temperature sensitivity and Growth.

Previous studies have shown that *SLA1* null cells are temperature sensitive (Holtzman *et al*, 1993). To test whether cells expressing *sla1ΔCt* are also temperature sensitive, cells were incubated at 30 and 37°C on YPAD plates. As reported previously, cells lacking *SLA1* were inviable at 37°C. Cells expressing *sla1ΔCt* were viable at 37°C (Figure 3.2a), showing that cells lacking the C-terminal repeat region of Sla1p are not inviable at high temperatures. However, cells expressing *sla1ΔCt* appeared to grow at a slower rate at 37°C on plates compared to wild type cells.

To study the rates of growth in liquid media, growth curves were performed. Cells were grown in YPAD liquid media at 30 and 37°C and samples were removed at two hour time points to determine the cell number in each culture (Figure 3.2b). At 30°C cells expressing *sla1ΔCt* grew at about the same rate as wild-type cells, both having a doubling time of about 2 hours. At 37°C wild type cells had a doubling time of about 2 hours, compared to cells lacking the C-terminal repeat region of Sla1p with a doubling time of about 4.5 hours. Although these cells are able to grow at high temperatures, they do have a slow growth defect at 37°C.

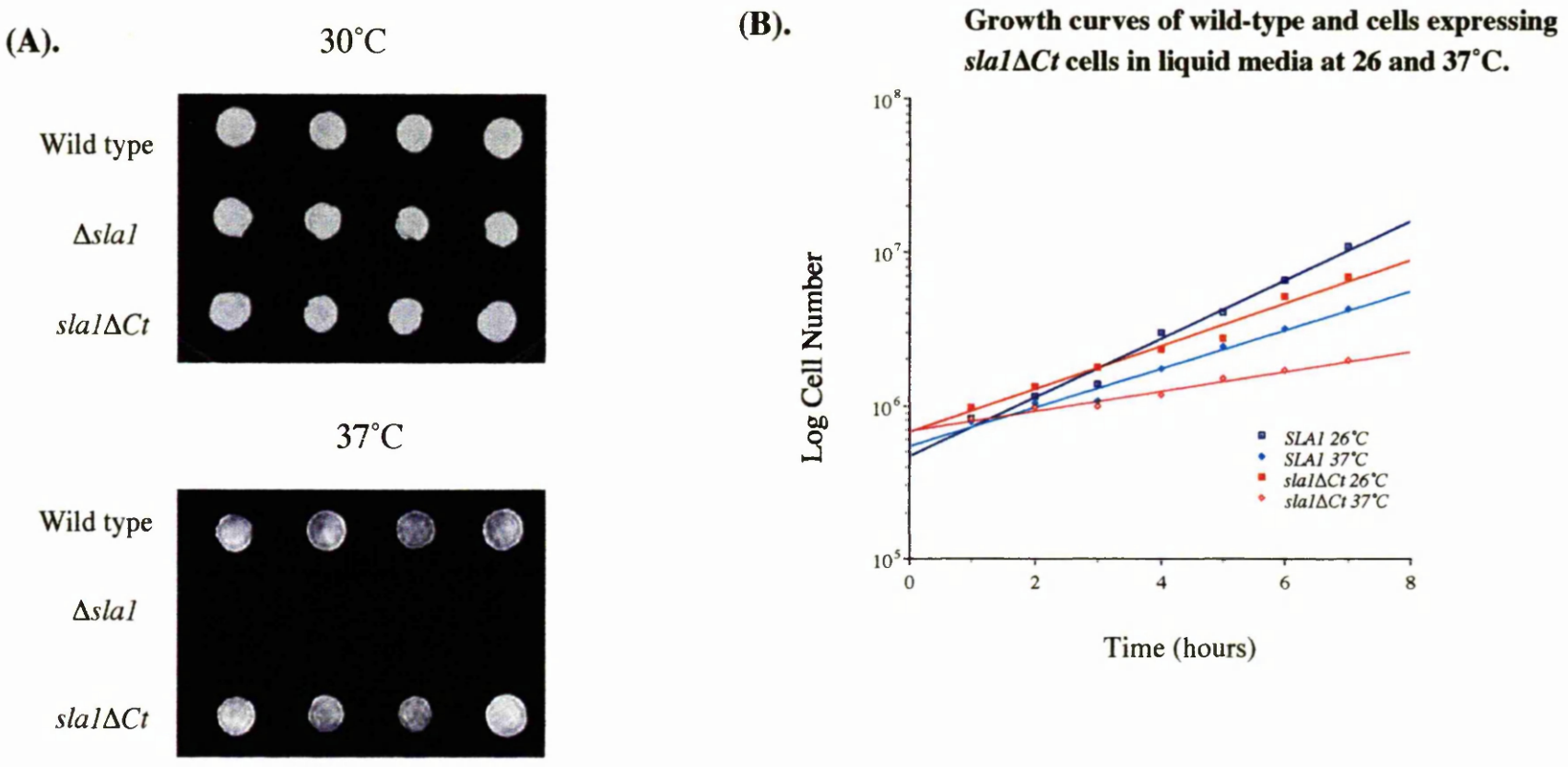


Figure 3.2. Effect of the *sla1ΔCt* mutation on cells growth. (A) Cells expressing *sla1ΔCt* are viable at 37°C. Wild type (KAY302), Δ*sla1* (KAY300) and *sla1ΔCt* (KAY369) cells were patched onto rich YPAD plates and incubated at 30 and 37°C. (B) Cells expressing *sla1ΔCt* have a slow growth defect at 37°C. The growth rates of wild type (KAY302) and *sla1ΔCt* (KAY369) cells were determined by taking aliquots from an actively growing culture every hour and measuring the cell number in the aliquot.

3.2.3 Cells expressing *sla1ΔCt* have a relatively normal cortical actin organisation.

The actin organisation of yeast cells can be examined by fluorescence microscopy using rhodamine phalloidin, a specific stain for filamentous actin (See Methods and Materials section 2.7.2.2 and Pringle *et al,* 1989). Wild type cells contain small, punctate cortical actin patches that become highly polarised to the growing bud (Figure 3.3a). As reported previously cells lacking *SLA1* (Figure 3.3b) contain fewer, larger chunks of actin that are poorly polarised (Holtzman *et al*, 1993). Cells expressing *sla1ΔCt* contain a relatively normal cortical actin cytoskeleton (Figure 3.3c). Most cells contain small, punctate patches that become highly polarised. However in about a quarter of these cells a larger chunk of actin is also observed in cells containing small punctate patches.

3.2.4 Effect of Latrunculin-A treatment on the actin cytoskeleton.

Previous studies have shown that Latrunculin-A (LAT-A) is an actin cytoskeleton-disrupting drug that binds to actin monomers preventing the assembly step in a rapid cycle of assembly and disassembly (Ayscough *et al*, 1997, Morton *et al*, 2000). Cells lacking *SLA1* have been shown to have a reduced sensitivity to Latrunculin-A in halo assays compared to wild-type cells, suggesting a reduction in actin dynamics caused by the deletion (Ayscough *et al*, 1997). Halo assays were performed to examine the effect of the *sla1ΔCt* mutation on cells sensitivity to LAT-A. Cells expressing *sla1ΔCt* demonstrated a smaller halo size then wild-type cells (Figure 3.4a), indicating that these cells are less sensitive to the effects of LAT-A. Figure 3.4b shows the apparent relative sensitivity of each strain to the effects of LAT-A (see materials and methods section and Reneke *et al*, 1988).

(A). Wild type **(B).** Δ*sla1* **(C).** *sla1*Δ*Ct*

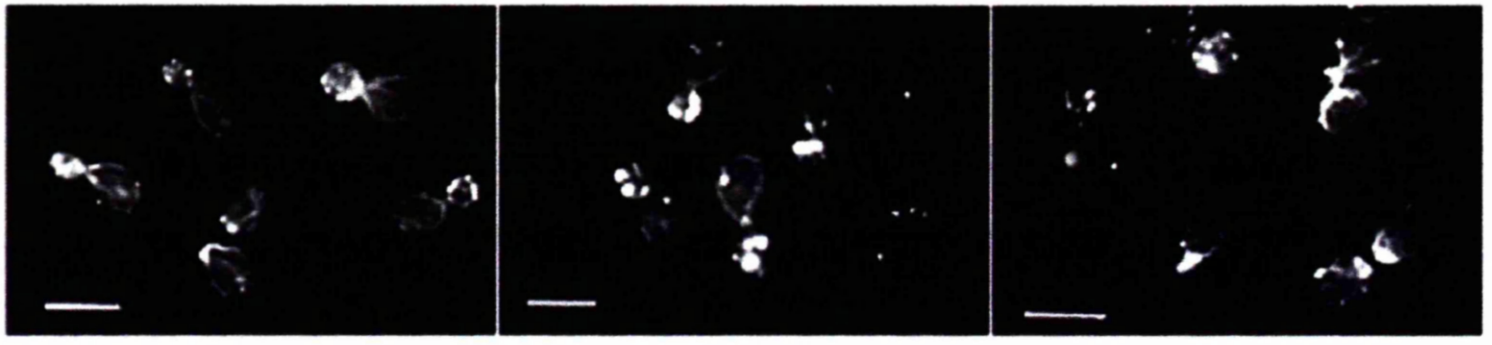


Figure 3.3. Effect of the *sla1*Δ*Ct* mutation on the organisation of the cortical actin cytoskeleton. (A) Rhodamine phalloidin, a specific stain for filamentous actin, was used to visualise the actin cytoskeleton of (A) wild type (KAY302), (B) Δ*sla1* (KAY300) and (C) *sla1*Δ*Ct* (KAY369) cells by fluorescence microscopy. Bar 10 μm

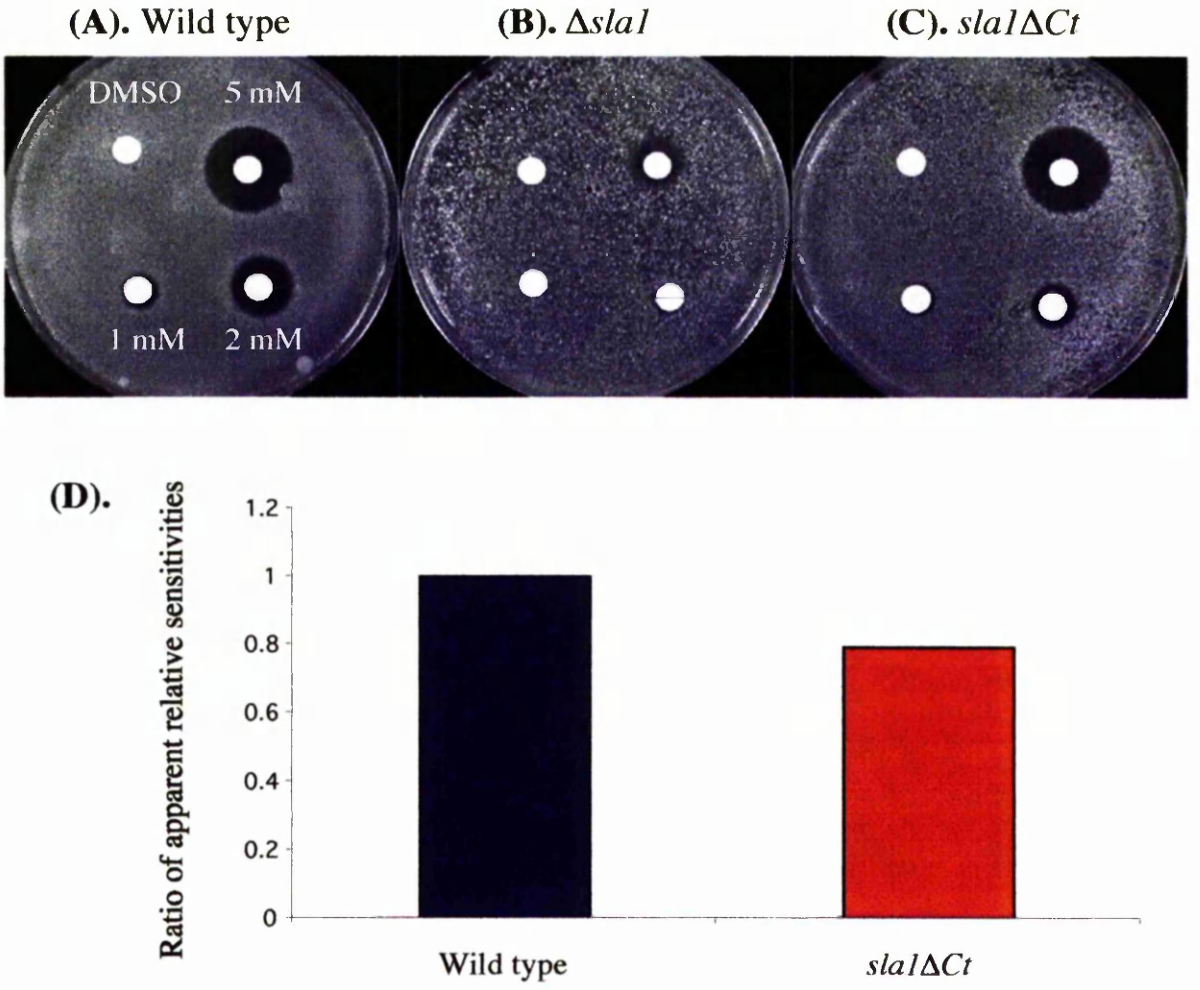


Figure 3.4. Effect of the *sla1ΔCt* mutation on the sensitivity of cells to Latrunculin-A treatment. Halo assays were performed to assess the sensitivity of cells expressing *sla1ΔCt* to LAT-A. Concentrations measured 1 mM, 2 mM and 5 mM. (A) Wild type (KAY302), (B) Δ*sla1* (KAY300) and (C) *sla1ΔCt* (KAY369) cells. (D) Comparison of the apparent relative sensitivity of wild type (KAY302) and *sla1ΔCt* (KAY369) cells to the effects of LAT-A (See Materials and Methods section and Reneke *et al*, 1988).

3.2.5 Cells expressing *sla1ΔCt* require expression of functional *ABP1* for viability.

Sla1p was originally identified in a synthetic lethal screen with *ABP1* (Holtzman *et al*, 1993). Studies with plasmid borne Sla1p lacking approximately two thirds of the C-terminal repeat region demonstrated that this region was required to rescue the Abp1p dependence of cells (Ayscough *et al*, 1999). To test whether cells expressing the integrated *sla1ΔCt* mutant, lacking the entire C-terminal repeat region, are Abp1p dependent a strain lacking *ABP1* was crossed with the *sla1ΔCt* mutant. The resulting diploid cells were sporulated and the tetrads dissected. The possible outcomes from this cross are shown in Figure 3.5a.

If cells expressing *sla1ΔCt* are not Abp1p dependent then all progeny from this cross will be viable as is the case when cells lacking *ABP1* are crossed with wild type cells (Figure 3.5.b). As shown in figure 3.5.b most tetrads contained at least one of spore that failed to grow into a colony on YPAD plates suggesting that cells expressing *sla1ΔCt* are Abp1p dependent. To clarify which combinations of mutations are viable cells were plated onto the appropriate selective media to select for the specific mutations. Figure 3.5.c shows a representative tetrad from which only three spores grew into colonies. None of the viable colonies from the *Δabp1/sla1ΔCt* cross were able to grow on both selection plates suggesting that cells expressing *sla1ΔCt* depend on the expression of a functional *ABP1* gene for viability.

3.2.6 Cells expressing *sla1ΔCt* contain aberrant cell wall morphology.

Cells lacking *SLA1* have been shown to contain aberrant cell wall morphology. These cells contain a thick cell wall around the mother cells probably due to the aberrant localisation of Rho1p, the regulatory subunit of β-glucan synthase in these cells (Ayscough *et al*, 1999).

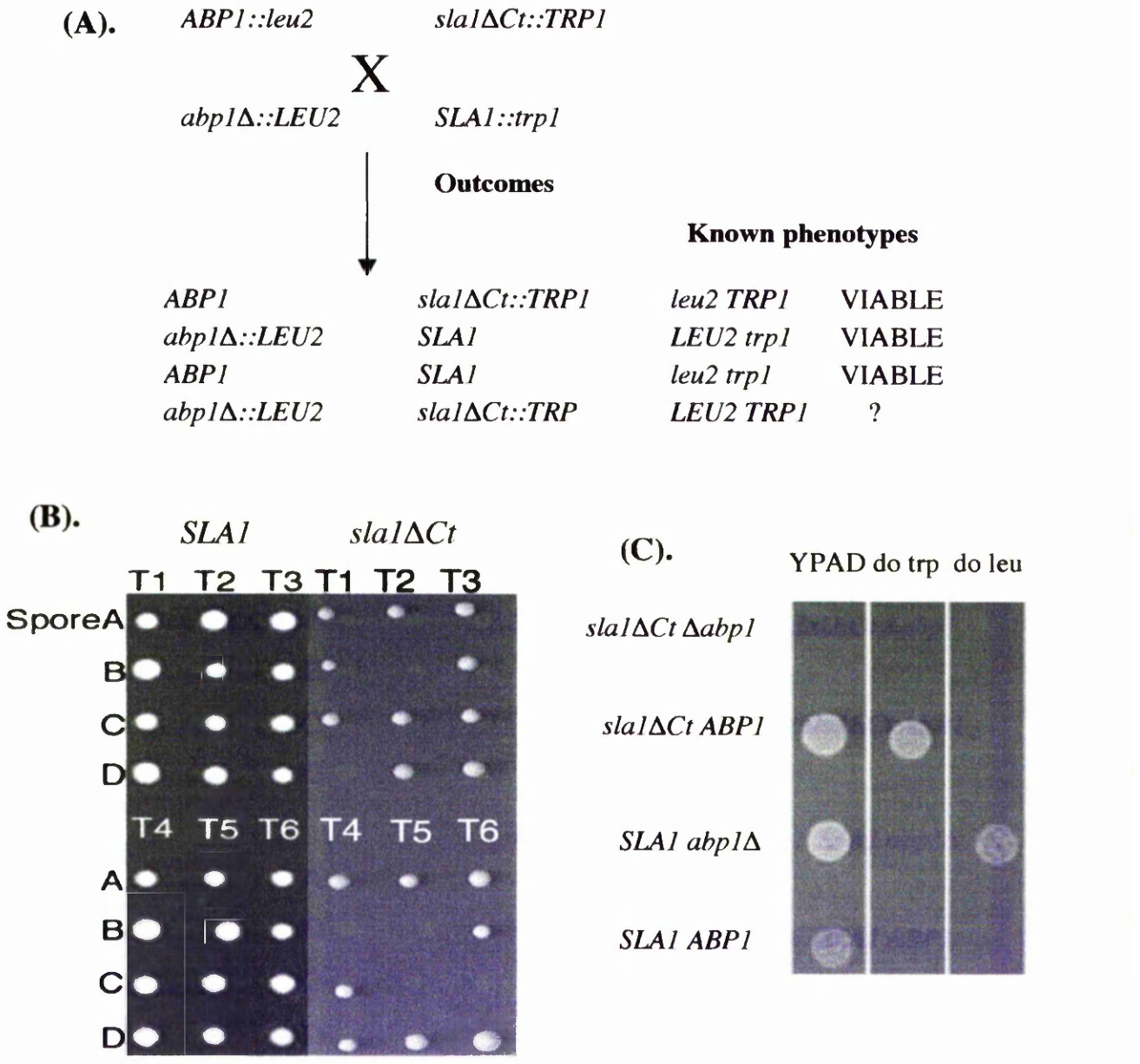


Figure 3.5. Abp1p dependence of cells expressing *sla1ΔCt*. (A) Possible outcomes of a cross between cells lacking *ABP1* (KAY126) and cells expressing *sla1ΔCt* (KAY369). Haploid cells were crossed and the resulting diploid cells were sporulated. (B) Tetrads were then dissected and spores allowed too grow on YPAD plates at 30°C to observe how many were viable from each tetrad. All viable colonies were patched onto the appropriate selective media to determine which combination of mutations were viable. (C) representative tetrad patched onto appropriate selection plates.

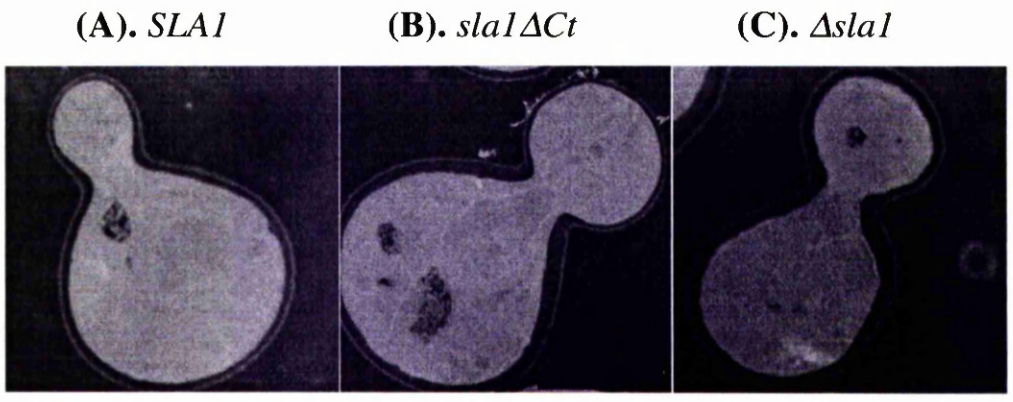


Figure 3.6. *Δsla1* null cells and cells expressing *sla1ΔCt* have thick cell walls. (A) Wild type (KAY302), (B) *Δsla1ΔCt* (KAY369) and (C) *Δsla1* (KAY300) cells were grown to log phase and then fixed and processed for electron microscopy using potassium permanganate fixation to enhance visualisation of the cell wall and membranes.

In order to ascertain if cells expressing *sla1ΔCt* contain thick cell walls, electron microscopy was used to observe the yeast cell wall (see Methods and Materials section 2.7.2.3). As reported previously cells lacking *SLA1* contained a thick cell wall around the mother cell. A thick cell wall was also observed around the mother cell of cells expressing *sla1ΔCt* (Figure 3.6).

3.2.7 The C-terminal repeat region of Sla1p is required to localise the protein to the cell cortex.

Sla1p has been shown to localise to the cell cortex in a patch structure that partially overlaps with cortical actin patches, about 50% of the Sla1p patches colocalised with actin patches (Warren *et al*, in press). To study the importance of the C-terminal repeat region in targeting Sla1p to the cell cortex, a myc tagged form of *sla1ΔCt* was generated.

The localisation of sla1ΔCtp-myc was observed by indirect fluorescence microscopy (see Methods and Materials section 2.7.2.1; Ayscough and Drubin, 1998). Sla1p-myc localised to the sites of cell growth in small patch structures as was reported in earlier studies (Figure 3.7a). Sla1ΔCtp-myc did not show any punctate cortical staining indicating that the C-terminal repeat region of Sla1p is required for its localisation to the cell cortex (Figure 3.7b).

3.2.8 Sla1p localisation to the cell cortex is mediated by an interaction with End3p.

The Sla1p C-terminal repeat region has recently been shown to interact in immunoprecipitations with the Pan1p/End3p complex (Tang *et al*, 2000). As Sla1p lacking this region does not localise to the cell cortex it would suggest that the interaction between

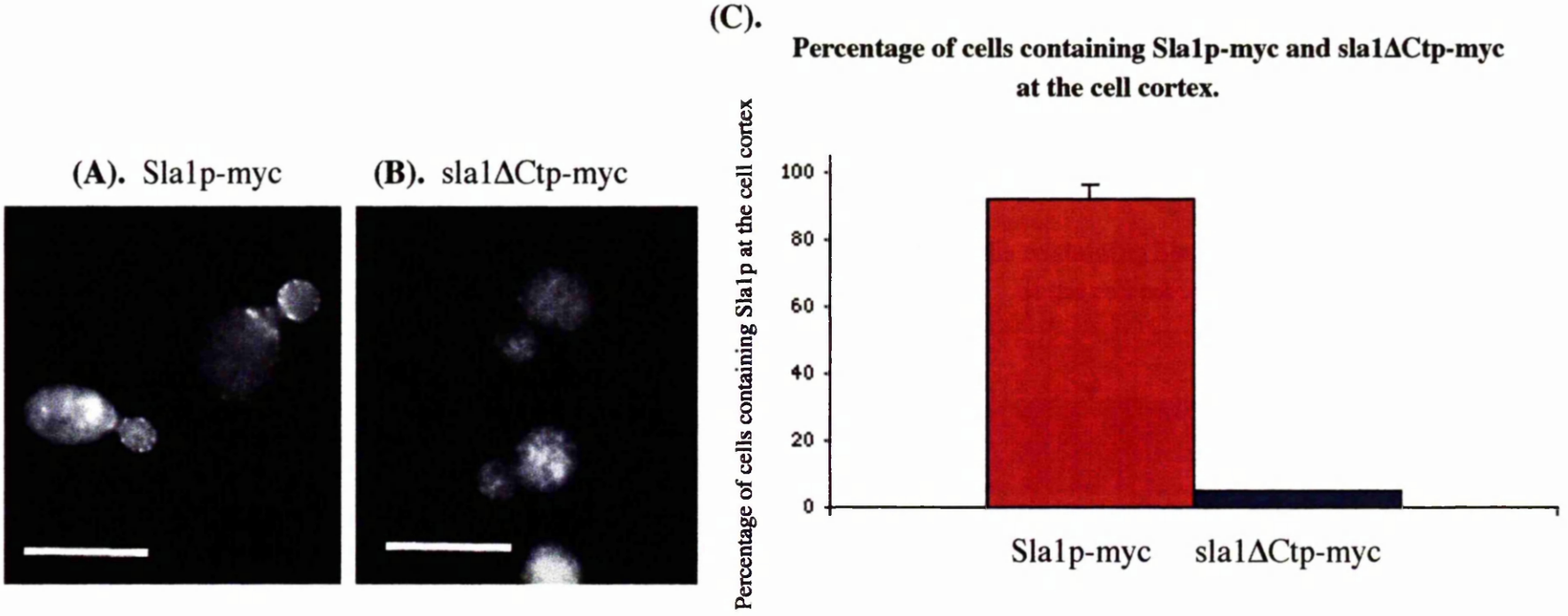


Figure 3.7. The C-terminal repeat region of Sla1p is required for its localisation to the cell cortex. (A) Sla1p-myc (KAY303) and (B) sla1ΔCtp-myc (KAY363) was localised by indirect immunofluorescence microscopy using Rabbit anti-myc A14 (1:100) and anti Rabbit-Cy3 (1:200) antibodies. (C) Counts of cells containing cortical patches of Sla1p-myc or sla1ΔCtp-myc. Bar 10 μm.

the C-terminal repeat region and this complex are required to localise Sla1p to the cell cortex. To investigate the importance of End3p for the localisation of Sla1p, a strain containing the *end3-1* temperature sensitive allele (Benedetti *et al*, 1994) and GFP tagged *SLA1* was constructed.

At the permissive temperature both wild type and *end3-1* cells had a normal cortical actin organisation and Sla1p-GFP was organised into small punctate patches that became polarised through the cell cycle. After wild type cells were incubated at the 37°C for two hours the cortical actin organisation and the Sla1p-GFP localisation remained the same as at 29°C. However, after two hours incubation at the non-permissive temperature, *end3-1* cells contained an aberrant cortical actin organisation (Figure 3.8). In these cells there were fewer cortical chunks of actin that were poorly polarised. These cells had also lost Sla1p-GFP from the cell cortex and instead contained a diffuse cytoplasmic staining demonstrating that functional End3p is required to localise Sla1p to the cell cortex. More recent studies have now also demonstrated that cells lacking *END3* also contain a diffuse Sla1p-GFP localisation and an aberrant cortical actin cytoskeleton (Warren *et al*, in press). This confirms that End3p is required for localisation of Sla1p to the cell cortex.

In order to ascertain whether Sla1p-GFP had been lost from the cell cortex in *end3-1* mutant cells at the non-permissive temperature due to protein degradation, cells were incubated at the non-permissive temperature for two hours and western blotting was performed to detect Sla1p-GFP in whole cell extracts. Full length Sla1p-GFP was detected in whole cell extracts of both wild type and *end3-1* cells after a shift to the non-permissive temperature (data not shown), indicating that Sla1p-GFP is lost from the cell cortex in *end3-1* cells at the non-permissive temperature and is not degraded.

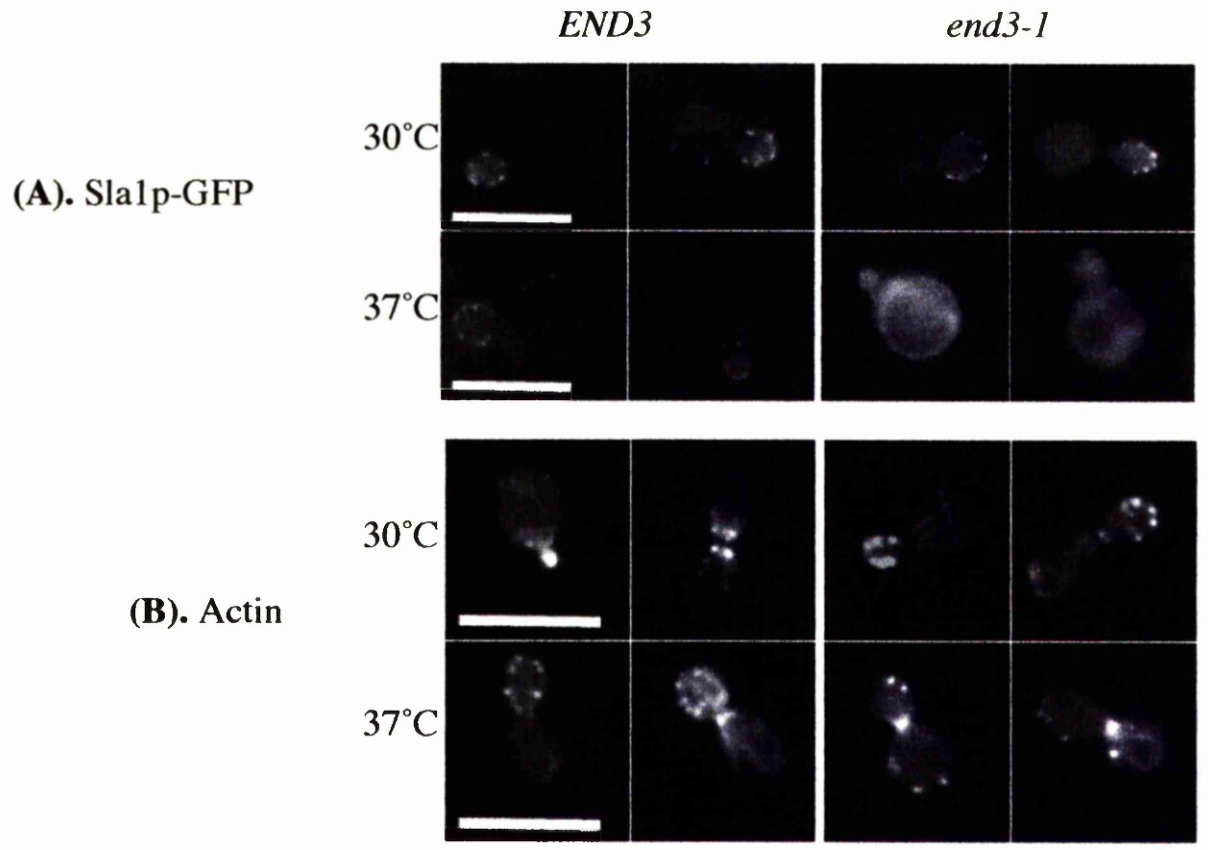


Figure 3.8 End3p is required for localisation of Sla1p to the cell cortex and for proper organisation of the cortical actin cytoskeleton. (A) Localisation of Sla1p-GFP was observed in wild type (KAY397) and *end3-1* (KAY462) cells by direct fluorescence microscopy before and after a 2 hour incubation at the non-permissive temperature. (B) The cortical actin cytoskeleton was observed in fixed cells by rhodamine phalloidin staining before and after a 2 hour incubation at the non-permissive temperature. Bar 10 μm

3.2.9 Cells lacking *SLA1* or the C-terminal repeat region have defects in fluid phase endocytosis.

Pan1p and End3p have roles in endocytosis (Tang and Cai, 1996, Benedetti *et al*, 1994, Tang *et al*, 1997) and interactions between Sla1p and End3p are required for the cortical localisation of Sla1p. To investigate the effect of *SLA1* mutants on fluid phase endocytosis Lucifer yellow was used. Wild type cells, *Δsla1* cells or cells expressing the *sla1ΔCt* mutant were grown to mid log phase before being assayed for their ability to endocytose Lucifer yellow. Lucifer yellow is a small fluorescent molecule that is used as a marker for fluid-phase endocytosis in yeast (see Methods and Materials 2.7.1.1; Dulic *et al*, 1991).

In wild type cells, Lucifer yellow accumulates in the vacuole and can be visualised by fluorescence microscopy. Cells either lacking *SLA1* or expressing *sla1ΔCt* are defective in Lucifer yellow uptake to the vacuole. About 45% of *SLA1* null cells and 40% of cells expressing *sla1ΔCt* did not contain Lucifer yellow in their vacuoles (Figure 3.9). Cells that contained vacuolar Lucifer yellow had a reduced relative intensity of vacuolar staining when compared to wild type cells. Both these observations suggest that fluid phase endocytosis is defective in *SLA1* mutant cells.

3.2.10 Cells lacking *SLA1* are defective in the internalisation step of endocytosis.

Cells either lacking *SLA1* or expressing *sla1ΔCt* are defective in the accumulation of Lucifer yellow in their vacuoles. This could be due to either a defect in the internalisation step of endocytosis or defects in the movement of internalised vesicles. To determine if these cells display a defect in the internalisation step of endocytosis, iodinated α-factor peptide was used (Materials and Methods section 2.4.13 and Siegel *et al*, 1999).

(A).

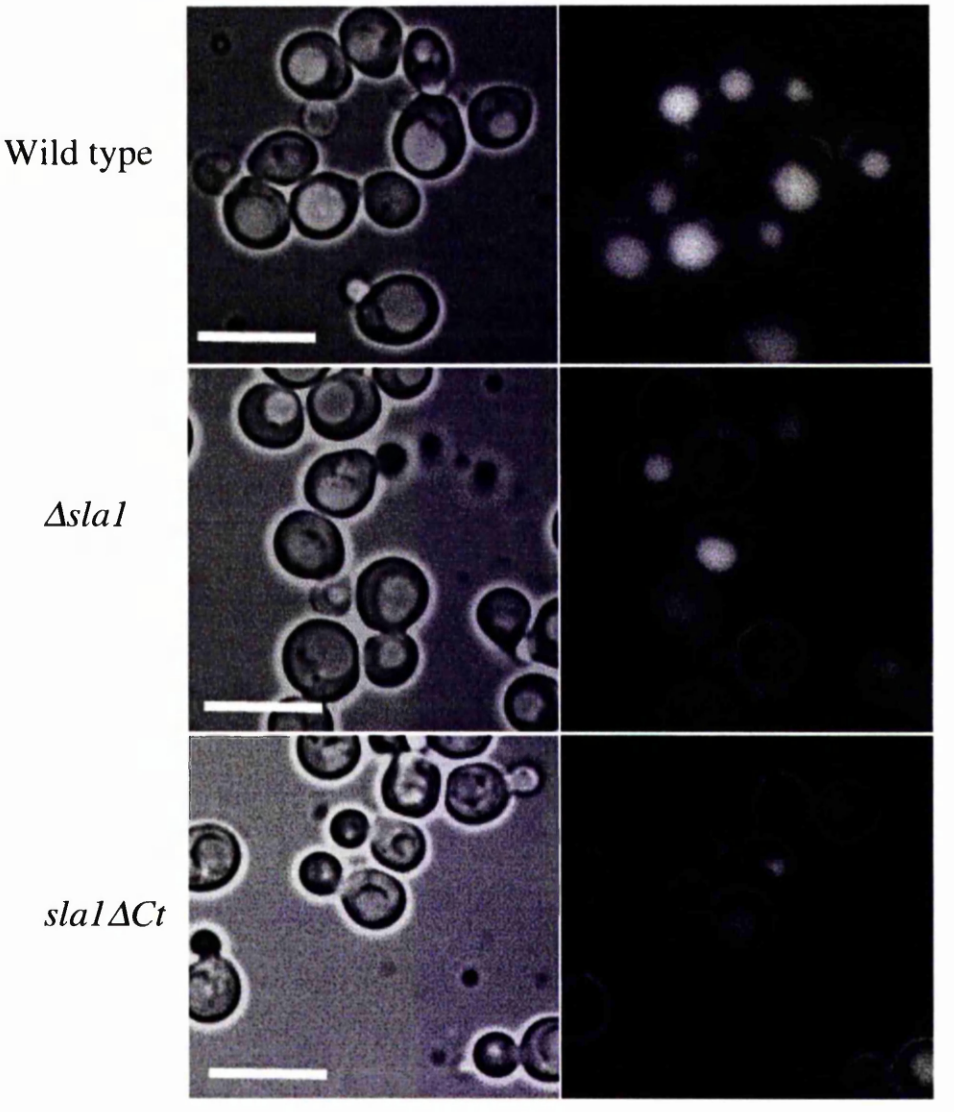
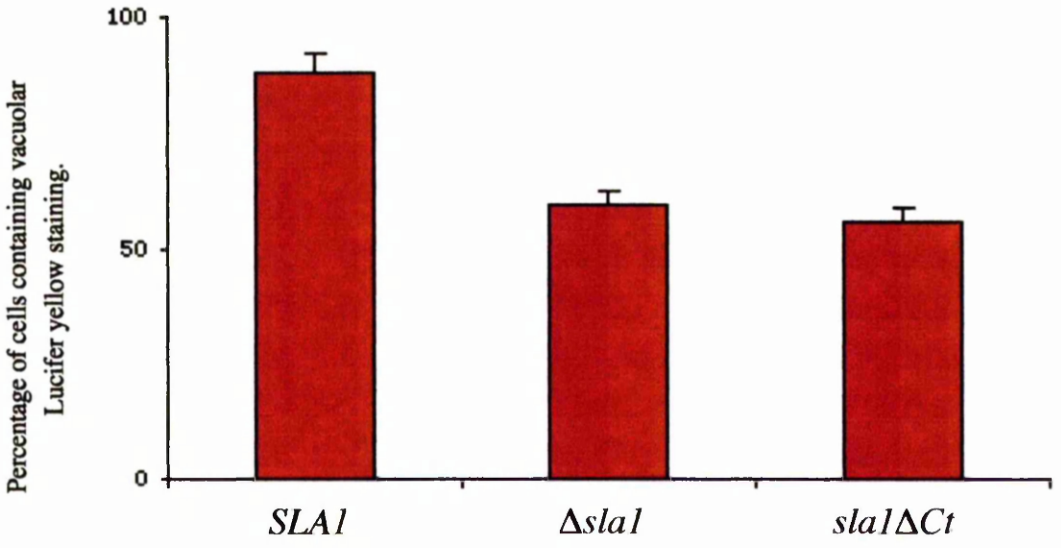


Figure 3.9 Effects of the *slaΔCt* mutation upon fluid phase endocytosis. (A) Actively growing wild type (KAY302), *Δsla1* (KAY300) and *sla1ΔCt* (KAY369) cells were incubated in the presence of Lucifer yellow for 1 hour. Lucifer yellow uptake was then visualised by fluorescence microscopy. Bar 10 μm.

(B). **Percentage of cells containing vacuolar Lucifer yellow staining.**



(C). **Relative intensity of vacuolar Lucifer yellow staining.**

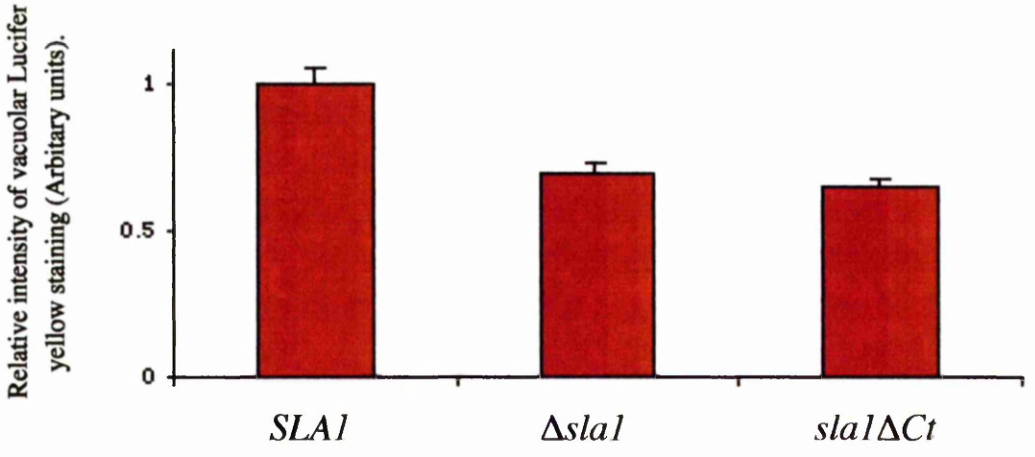


Figure 3.9 Cells lacking the C-terminal repeat region of Sla1p are defective in fluid-phase endocytosis. (B) Cell counts of cells containing Lucifer yellow in their vacuoles. (C) Intensity of vacuolar Lucifer yellow staining in the cells containing Lucifer yellow in their vacuoles.

Wild-type cells show a rapid uptake of the iodinated α-factor peptide that peaks at about 10 minutes (figure 3.10a) after beginning the pulse chase experiment. These cells show a 3-fold increase (figure 3.10b) in the internalised peptide after 10 minutes. *Δsla1* cells show a slight uptake of the peptide that peaks at around 10 minutes (figure 3.10a) after beginning the pulse-chase experiment. These cells show a much higher starting value for peptide that could not be removed by a pH1.1 wash (figure 3.10a). This may be due to either the peptide 'sticking' to the thick cell wall or due to an increase in the number of dead cells in the *Δsla1* samples. Although an increase in the internalised peptide is observed in *Δsla1* cells the amount taken up is much less than by wild-type cells (figure 3.10b). This data demonstrates that *Δsla1* cells have defects in the internalisation step of endocytosis, however there is not a complete block of internalisation as an increase in the internalised peptide is observed.

3.2.11 Cortical actin patch components have different but overlapping cell fractionation patterns.

Sla1p has been shown to interact with a number of different proteins involved in actin dynamics and endocytosis. The C-terminal repeat region interacts with the Pan1p/End3p complex (Tang *et al*, 2000) and Sla1p has also been shown to coimmunoprecipitate with Las17p (Rong Li, 1997), Sla2p and Abp1p (Warren *et al*, in press). To determine if these proteins are found in the same complex or are present in different complexes, whole cell extracts were spun through a 3-30 % sucrose gradient (see Methods and Materials section 2.6.6; Yang *et al*, 1999). Fractions were collected from the bottom of the gradient and western blotting was performed to detect which fractions contained different proteins.

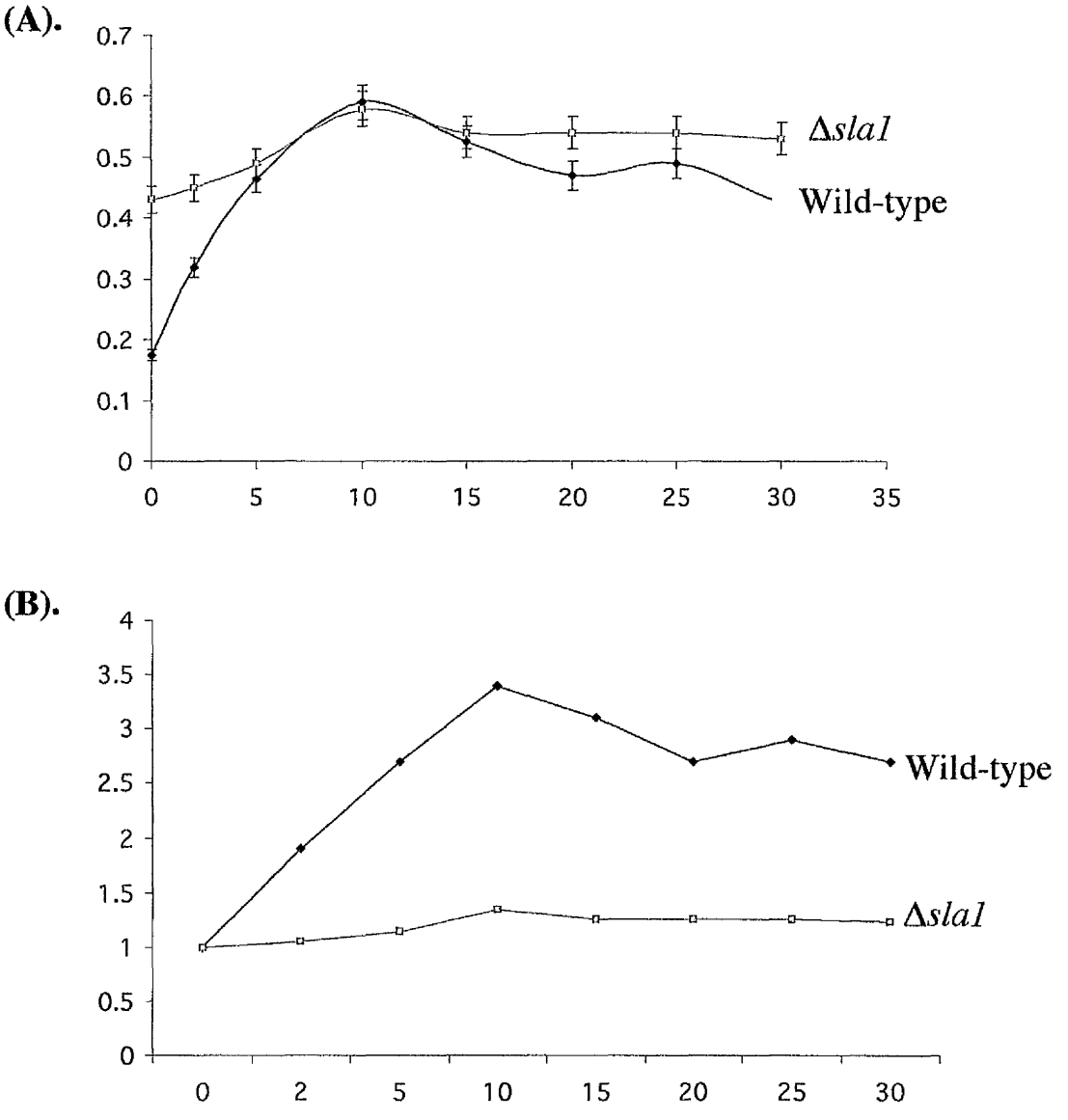


Figure 3.10. The effects of deletion of *SLA1* on the internalisation step of endocytosis. Wild type and Δ*sla1* cells were assayed by the pulse chase protocol (see Materials and Methods section 2.4.13) at 30°C. Cells were incubated on YPAD plates overnight, resuspended and harvested by centrifugation. Iodinated α-factor peptide was allowed to bind for one hour. Unbound peptide was removed washing in prewarmed YPAD. (A) Ratio of pH1.1/pH6 wash at different time points. (B) Fold activation compared to starting values.

Abp1p and Sla2p displayed similar fractionation patterns (Figure 3.11) to those described in earlier studies (Yang *et al*, 1999). Only fraction 7 contained all four of the cortical actin patch associated proteins observed (figure 3.11). Other fractions contained different combinations of these proteins. For example, fraction 5 contained only Abp1p and Sla1p whereas fraction 8 contained Sla1p, End3p and Sla2p (figure 3.11). This data demonstrates that cortical actin patch associated proteins contain a varied but overlapping fractionation pattern.

To ascertain whether these proteins are actually present in the same complex in these fractions, immunoprecipitations were performed. Unfortunately this proved unsuccessful, probably due to the high sucrose concentrations present in the fractions interfering with the antibody binding.

3.2.12 Screen to identify genes that when overexpressed can rescue the Abp1p-dependent phenotype of the *sla1ΔCt* mutant.

Cells lacking *ABP1* and expressing the *sla1ΔCt* mutation are inviable, suggesting that Abp1p and the C-terminal repeat region of Sla1p contain a functional redundancy. To elucidate this possible functional redundancy further, the yeast 2-hybrd system was utilised to attempt to identify interacting partners for the C-terminal repeat region of Sla1p. However, this technique was unsuccessful as the C-terminal repeat region of Sla1p fused to the *GAL4* binding domain plasmid self-activated in the yeast 2-hybrid system (data not shown). This observation has since been supported by data from other studies (Tang *et al*, 2000).

Next, a screen to identify genes that when overexpressed could rescue the Abp1p-dependent phenotype was performed (figure 3.12). A strain containing both the *Δabp1* and

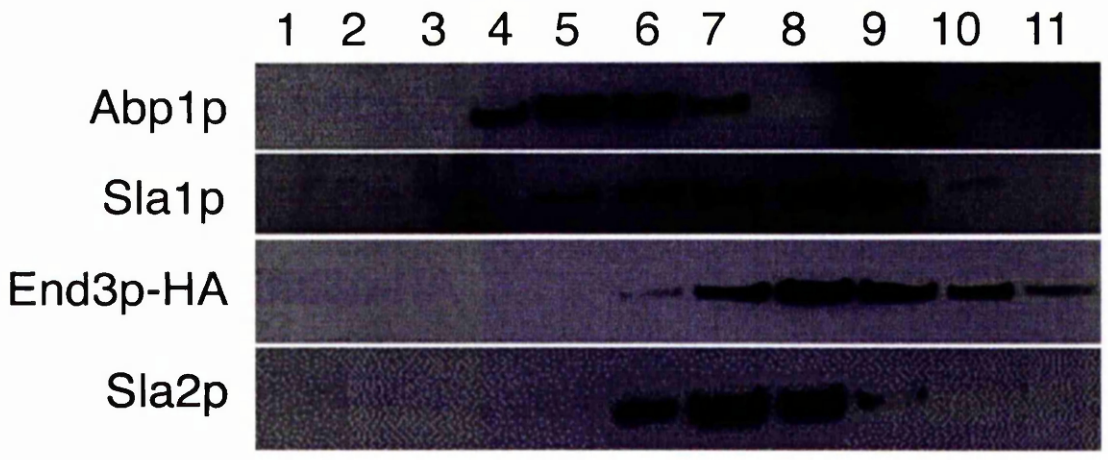


Figure 3.11 Fractionation of cortical actin patch associated proteins. Whole cell extracts of KAY476 *(SLA1-myc END3-HA)* were fractionated on linear 3-30% sucrose gradients. Fractions were collected from the bottom of the gradient. Western blotting was performed to identify which fractions contained various actin patch components.

the *sla1ΔCt* mutations that contained a plasmid expressing full length *ABP1*, allowing these cells to be viable, was constructed. After transforming these cells with a YEp13 yeast genomic library, the plasmid containing *ABP1* was selecting against by replica plating transformed cells onto media containing 5-Fluoro orotic acid (5-FOA) (See Materials and Methods section 2.4.12). 150 colonies that were able on media containing 5-FOA were picked and screened for the presence of *SLA1* or *ABP1* inserts by PCR, 116 contained *ABP1* and 25 contained *SLA1*. The 9 remaining plasmids were sequenced in order to elucidate the identity of their inserts. Unfortunately all 9 inserts were identified as fragments of *ABP1*.

3.3 Discussion.

Sla1p is required for the proper organisation of the cortical actin cytoskeleton in budding yeast (Holtzman *et al*, 1993). Studies using plasmid borne domain deletions of Sla1p have shown that the domain required too rescue the aberrant actin cytoskeleton organisation and the temperature sensitive phenotype is different to the domain required to rescue the Abp1p-dependent phenotype associated with the knockout. These studies identified the C-terminal repeat region to be required to rescue the Abp1p-dependent phenotype but was dispensable for the aberrant actin and temperature sensitive phenotypes (Ayscough *et al*, 1999). To examine if this is true when the truncated *SLA1* was expressed at wild-type levels, an integrated *sla1ΔCt* mutant was constructed. Expression of integrated *sla1ΔCt* rescued the temperature sensitivity associated with the knockout, cells displayed a relatively normal cortical actin cytoskeleton organisation but were still Abp1p-dependent.

Localisation of myc tag truncated Sla1p revealed that the C-terminal repeat region of Sla1p is required for its interaction with the cell cortex. Recent studies have demonstrated an interaction between the C-terminal repeat region of Sla1p and the N-terminus of End3p and

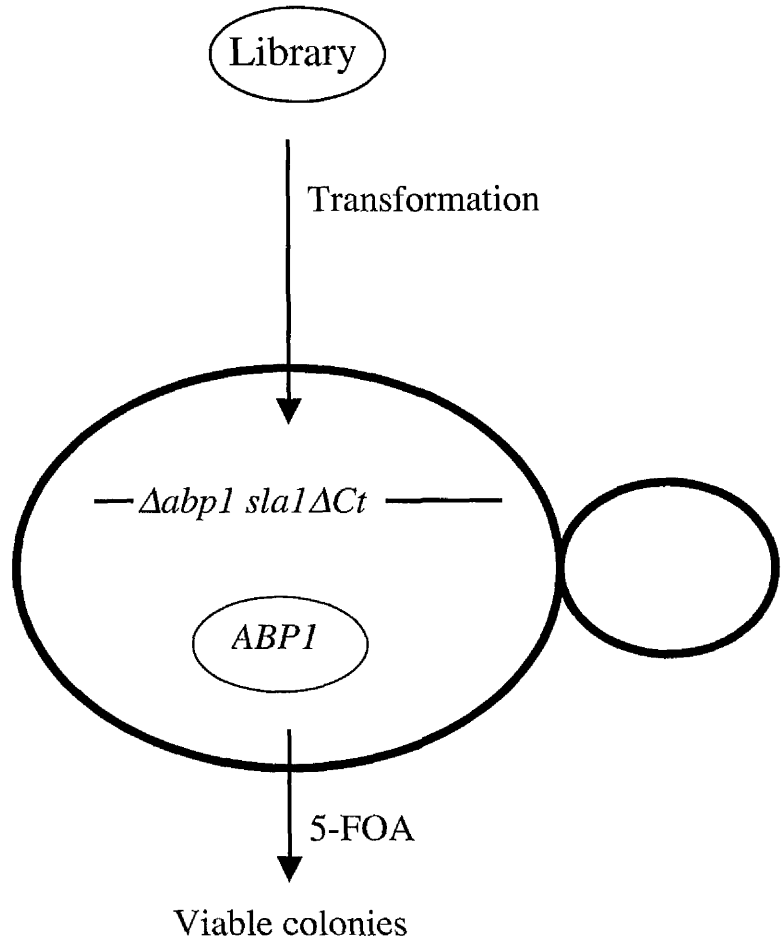


Figure 3.12 Schematic representation of the screen to identify genes that when overexpressed can rescue the Abp1p-dependent phenotype of cells expressing the *sla1ΔCt* mutation. A strain was constructed containing both the *Δabp1* and *sla1ΔCt* mutations. Viability was maintained by expression of *ABP1* from a *URA3* marked plasmid. These cells were transformed with a *LEU2* marked YEp13 yeast genomic library. Colonies were replica plated onto media containing 5-FOA, which is toxic to cells expressing the *URA* genes so for cells to grow they must lose the *URA3* marked (*ABP1*) plasmid. Only inserts capable of rescuing the lethal phenotype will produce a viable colony in the presence of 5-FOA.

also with the LR1 region of Pan1p (Tang *et al*, 2000), proteins previously shown to be involved in endocytosis (Benedetti *et al*, 1994; Tang and Cai, 1996; Tang *et al*, 1997). Using a temperature sensitive allele of *END3* we were able to demonstrate that functional End3p is also required for the cortical localisation of Sla1p suggesting that the interaction between the C-terminal repeat region of Sla1p and End3p is required for mediating the cortical localisation of Sla1p.

This trimeric complex of Sla1p/End3p/Pan1p has proposed functions in endocytosis (Tang *et al*, 2000). Mutations in both *PAN1* and *END3* have been demonstrated to impair endocytosis (Benedetti *et al*, 1994; Tang and Cai, 1996; Tang *et al*, 1997). In support of this trimeric complex functioning in endocytosis, cells expressing *sla1ΔCt* were partially defective in fluid phase endocytosis. As there is not a complete block in endocytosis in these cells this indicates that either only some of the endocytic pathways have been blocked by this mutation or that endocytosis is still occurring but the whole process is proceeding less efficiently.

Our data presented here also suggests that cortical patch proteins exist in different complexes at the cell cortex. Cell fractionation experiments indicated that Sla1p, Sla2p, End3p and Abp1p display different but overlapping fractionation distributions. This data is also supported by the recent observation that Sla1p and Abp1p have largely separate localisation patterns that occasionally colocalise (Warren *et al*, in press). This may suggest that different complexes exist at the cell cortex and these can assemble and disassemble allowing different cellular functions to be fulfilled. Future work to investigate if these proteins do exist in different complexes would be to use *SLA1* null cell extracts in the cellular fractionation experiments to investigate if the fraction pattern changes. If Sla1p is

acting as a molecular scaffold protein then you would expect the fractionation patterns to alter when the protein is missing.

Chapter 4.

Possible Phosphorylation and Regulation of Sla1p.

4.1 Background.

Sla1p contains a core repeat motif of LxxQxTGGxxxPQ in its C-terminus. This repeat region has been proposed to contain residues that are targets of the Actin Regulating Kinases Ark1p and Prk1p (Zeng and Cai, 1999). Studies carried out during the course of this work using the yeast protein Pan1p revealed that the threonine residue in the LxxQxTG motif is phosphorylated by Prk1p, suggesting Prk1p phosphorylation regulates Pan1p (Zeng and Cai, 1999). *In vitro* mutagenesis also revealed that the glycine and glutamic acid residues present in this motif are essential for is phosphorylation by Prk1p, however, the requirement for the leucine residue within this motif has not been addressed (Zeng and Cai, 1999).

Earlier studies have also demonstrated that Pan1p forms a complex with End3p (Tang *et al*, 1997). End3p binds to Pan1p at the LR2 region where several of the LxxQxTG repeats are present *in vivo*. However, *in vitro* kinase assays revealed that binding of End3p to Pan1p prevents the phosphorylation of these motifs (Zeng and Cai, 1999), indicating a role for phosphorylation in the assembly/disassembly of protein complexes at the cell cortex. Sla1p contains 5 exact copies of this motif and also another 9 motifs with the QxTG consensus within its C-terminal region. When this region of Sla1p was fused to GST it was shown to be as good a substrate for Prk1p as the Pan1p repeats in vitro (Zeng and Cai, 1999).

A number of protein phosphatases have also been reported to interact with cortical patch components. Most notably, Sla1p and Pan1p have been shown to interact with the yeast protein phosphatase-1 (PP1) Glc7p in the yeast 2-hybrid system (Tu *et al*, 1996; Venturi *et al*, 2000; Uetz *et al*, 2000). Glc7p has been implicated in a wide range of cellular processes and has recently been shown to localise to the presumptive bud site and to remain localised to the bud neck during the yeast cell cycle (Bloecher and Tatchell, 2000). This protein phosphatase is therefore in a position to interact with cortical patch associated proteins. Sla1p has also been demonstrated to interact with two serine/threonine phosphatases known as Ppz1p and Ppz2p (Venturi *et al*, 2000). These phosphatases are related to Glc7p and have been localised to the nucleus (Clotet *et al*, 1996). Other studies have shown that Ppz1p and Ppz2p are involved in signal transduction and the yeast cells stress response (Posas *et al*, 1995).

In this chapter we provide evidence suggesting that Sla1p is phosphorylated in vivo and that this phosphorylation may be in part due to the functions of the Actin Regulating Kinases Ark1p and Prk1p. Preliminary data also indicates that Sla1p may be dephosphorylation by the yeast protein phosphatase-1 Glc7p. These data allow us to suggest that the functions of Sla1p are regulated by cycles of phosphorylation and dephosphorylation.

4.2 Results.

4.2.1 Sla1p is phosphorylated *in vivo*.

Previous studies have suggested that the threonine residue in the C-terminal repeat motif of Sla1p is a potential target for the Actin Regulating Kinases, Ark1p and Prk1p (Zeng and

Cai, 1999). To investigate whether Sla1p is phosphorylated, HA tagged full length Sla1p and HA-tagged Sla1ΔCtp were immunoprecipitated from whole cell extracts. Samples were then treated with Lambda phosphatase, a general serine/threonine phosphatase, and western blotting was performed to observe whether there was a size difference between phosphatase treated and untreated Sla1p.

A mobility shift is observed between phosphatase treated and untreated samples for both full length Sla1p (Figure 4.1a) and sla1ΔCtp (Figure 4.1b). Sla1p had a molecular weight of about 144.5 kDa and this decreased to about 134.9 kDa after phosphatase treatment, suggesting that Sla1p has been dephosphorylated. A mobility shift is also observed in samples lacking the C-terminal repeat region, sla1ΔCtp before phosphatase treatment had a molecular weight of about 111.4 kDa that was reduced to about 106.7 kDa after treatment. This data indicates that Sla1p is also phosphorylated at sites outside the C-terminal repeat region. This data suggests that Sla1p is phosphorylated in cells.

4.2.2 The Actin Regulating Kinases Ark1p and Prk1p potentially phosphorylate Sla1p.

If Ark1p and Prk1p phosphorylate the threonine residue in the C-terminal repeat region of Sla1p, Sla1p from cells lacking *ARK1* and *PRK1* should have a different mobility compared to Sla1p from wild type cells. To test this whole cell extracts were prepared from wild type and *Δark1Δprk1*, cells and western blotting was performed to observe if a mobility shift existed.

A small though reproducible mobility shift is observed between Sla1p from wild-type cells and from cells lacking *ARK1* and *PRK1* (Figure 4.2). Sla1p from cells lacking both *ARK1* and *PRK1* had an increased mobility compared to Sla1p from wild-type cells, suggesting

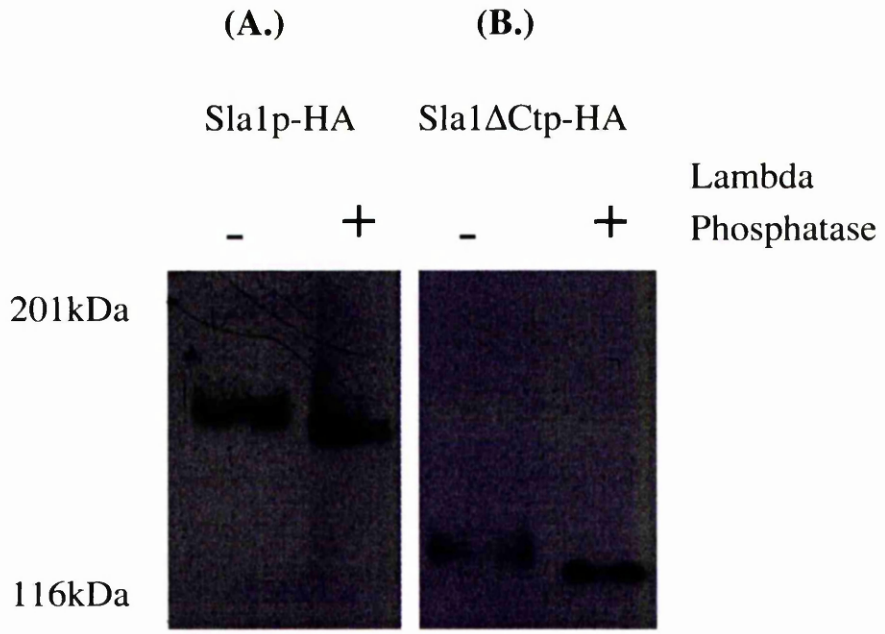


Figure 4.1 Phosphorylation of Sla1p. (A) Sla1p-HA (KAY355) and (B) sla1ΔCtp-HA (KAY364) were immunoprecipitated using agarose conjugated anti-HA antibody. Immunoprecipitated proteins were then treated with lambda phosphatase for 30 minutes. Proteins were separated on a 7.5% SDS gel and western blotting was performed. HA tagged proteins were detected by ECL after probing with Rabbit anti-HA (1:1000) and anti-Rabbit HRP (1:5000). Sla1p from wild-type extracts had a molecular weight of about 144.5 kDa, after phosphatase treatment its molecular weight was about 134.9 kDa and sla1ΔCtp from wild type extracts had a molecular weight of about 111.4 kDa, after phosphatase treatment this was reduced to about 106.7 kDa.

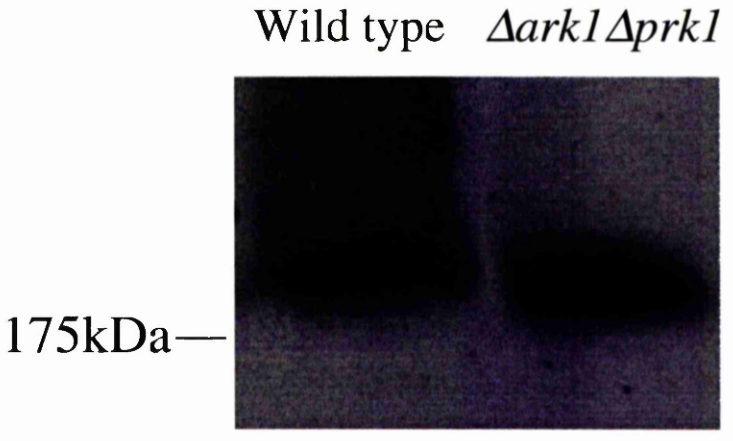


Figure 4.2 Potential regulation of Sla1p by the Actin Regulating Kinases Ark1p/Prk1p. To investigate if the Actin Regulating Kinases phosphorylate Sla1p, whole cell extracts from wild type (KAY302) and *Δark1Δprk1* (KAY379) cells were analysed by immunoblotting for Sla1p. Sla1p was detected by ECL after probing membranes with Rabbit anti-Sla1p,1:100, Campbell Gourlay and Kathryn Ayscough) and anti-Rabbit-HRP, 1:5000, (Sigma) antibodies. A mobility shift was observed between Sla1p from wild type cells, with a molecular weight of about 198.6 kDa, and from *Δark1Δprk1* cells, with a molecular weight of about 192.2 kDa. This suggests that Sla1p is phosphorylated by Ark1p and Prk1p.

that it has not been modified to the same extent as Sla1p from the wild type cells. Wild-type Sla1p had a molecular weight of about 198.6 kDa whereas Sla1p from cell extract lacking *ARK1* and *PRK1* had a reduced molecular weight of about 192.8 kDa. It was also noticed that Sla1p from cells lacking *ARK1* and *PRK1* formed a tighter band when compared to Sla1p from wild type cells, this may also indicate that the Sla1p from these cells is lacking some modifications. The reduction in the molecular weight of Sla1p form cells lacking *ARK1* and *PRK1*, about 6 kDa), was not as large as the shift observed after Lambda phosphatase treatment of Sla1p, about 10 kDa, (Figure 4.2a), also suggesting that Sla1p is also targeted for phosphorylation by other kinases.

4.2.3 Ark1p and Prk1p are required for proper localisation of Sla1p.

Recent studies have revealed that the cortical actin cytoskeleton is aberrant in cells lacking both *ARK1* and *PRK1* (Cope *et al*, 1999). In these cells most of the F-actin is localised to a single, large clump. This clump contains the cortical actin patch associated proteins Abp1p, Sla2p and Sac6p (Cope *et al*, 1999). The aberrant actin organisation in the *Δark1Δprk1* cells is rescued by expression of *PRK1* from a low copy number plasmid but is not rescued by the expression of a kinase dead form of *PRK1* (Cope *et al*, 1999). This suggests that kinase activity is important for proper cortical actin cytoskeleton organisation. To assess the effect of Ark1p and Prk1p on the localisation of Sla1p, mutants containing single *Δark1* or *Δprk1* deletions and myc tagged *SLA1* were constructed. As has been previously reported, these cells contained a normal cortical actin cytoskeleton organisation and Sla1p-myc localised to small punctate patches that are polarised to the buds of cells (Ayscough *et al*, 1999).

In cells lacking both *ARK1* and *PRK1* Sla1p colocalises exclusively to the actin clump. This aberrant localisation of Sla1p is not rescued by a plasmid borne kinase dead *PRK1*

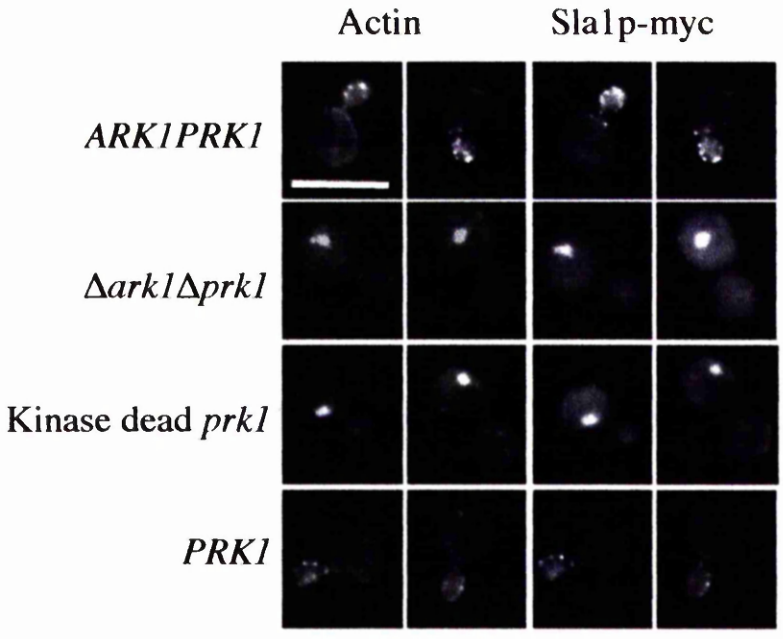


Figure 4.3 Sla1p requires the Actin Regulating Kinases Ark1p/Prk1p for its proper localisation. Sla1p-myc and actin were localised in wild type (KAY303) and Δ*ark1*Δ*prk1* (KAY405) cells by indirect fluorescence microscopy. Myc tagged proteins were localised by incubating cells with rabbit anti-myc A14 (1:100) and then anti-rabbit Alexa (1:200) antibodies and actin was localised by rhodamine phalloidin. Cells lacking the Actin Regulating Kinases contained an aberrant cortical actin organisation that was not rescued by expression of a kinase dead form of *PRK1* from a low copy number plasmid (KAY466) but was rescued by expression of functional *PRK1* expressed from a low copy number plasmid (KAY465). This indicates that phosphorylation by Ark1p/Prk1p is required for proper localisation of Sla1p. Bar 10μm

gene, but is rescued by functional *PRK1* expressed on a low copy number plasmid (Figure 4.3), indicating that phosphorylation is important for proper localisation of these Sla1p.

4.2.4 Genetic interactions between *SLA1* and the Actin Regulating Kinases *ARK1/PRK1*.

As the potential targets of the Actin Regulating Kinases are the threonine residues within each C-terminal repeat of Sla1p (Zeng and Cai, 1999), we wanted to ascertain the effect of the deletion of *ARK1* and *PRK1* upon the localisation of sla1ΔCtp-myc. To investigate this we set out to construct a strain lacking *ARK1* and *PRK1*, and also expressing the sla1ΔCtp-myc. However, it proved impossible to generate haploid cells containing all three of these mutations, suggesting that this combination may be lethal to cells. To determine whether this was the case, *Δark1sla1ΔCt-myc* cells were crossed with *Δprk1sla1ΔCt-myc* cells. Diploid cells were sporulated and the tetrads dissected. Dissected tetrads were grown on rich (YPAD) plates at 30°C, however, not all of the spores were viable. The lack of four viable colonies indicated that certain combinations of mutations were causing lethality.

All viable colonies were transferred to synthetic media, allowing selection of the auxotrophic genes that mark the mutations. Figure 4.4 shows a representative tetrad patched onto the selection plates. None of the viable colonies contained all three of the *sla1ΔCt-myc, Δark1* and *Δprk1* mutations, indicating that this combination is lethal to cells. The *sla1ΔCt-myc* mutation is synthetic lethal with the *Δark1Δprk1* mutations. We have subsequently shown that deletion of *SLA1* is also synthetically lethal with the *Δark1Δprk1* mutations (data not shown).

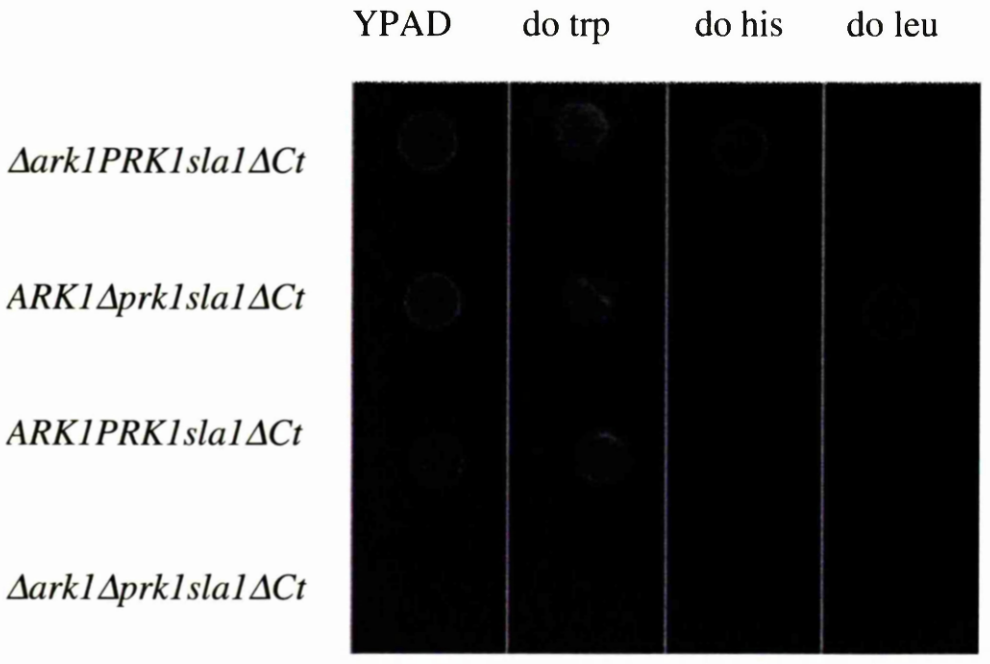


Figure 4.4 The *sla1ΔCt* mutation is synthetic lethal in combination with the *Δark1Δprk1* mutations. Strains containing the *Δark1::HIS3sla1ΔCt::TRP1* (KAY393) mutations were crossed with cells containing the *Δprk1::LEU2sla1ΔCt::TRP1* (KAY394) mutations. Diploid cells were sporulated on minimal sporulation plates and the resulting tetrads were dissected onto YPAD plates and spores were allowed to grow at 30°C. All viable colonies were transferred to selection plates to identify which combination of mutations were lethal.

4.2.5 Multiple isoforms of Sla1p exist in yeast cells.

The data presented so far in this chapter indicates that Sla1p is phosphorylated in yeast cells. This suggests that multiple isoforms of Sla1p may exist. To investigate whether this is the case, whole cell extracts of a strain expressing a HA-tagged form of Sla1p were analysed by 2-D gel electrophoresis (See Materials and Methods section 2.6.11). Using this technique we were able to detect four isoforms of Sla1p (Figure 4.5). The largest population of Sla1p is toward the positive end of the drystrip, suggesting that this population of Sla1p is phosphorylated.

In order to ascertain if any of these isoforms of Sla1p were phosphorylated, whole cell extracts were treated with λ-phosphatase before analysis by 2-D gel electrophoresis. However, this proved to be unsuccessful as Sla1p was degraded during the phosphatase treatment, and no full length Sla1p was detected. As Sla1p isolated from cells lacking *ARK1* and *PRK1* displayed a mobility shift when analysed by SDS-PAGE, we decided to investigate whether any of the isoforms where missing from whole cell extracts prepared from these cells. This also proved to be unsuccessful, as the amount of Sla1p present in whole cell extracts prepared from these cells was very low. This may have been due to the aberrant actin cytoskeleton arrangement in these cells. These cells contain a single clump of actin, to which Sla1p colocalises. Whole cell extracts have to be prepared in a buffer containing a low ionic strength for 2-D gel electrophoresis, as the first dimension separates proteins due to their charge. These conditions may not have solubilised the proteins contained in the clump of actin, resulting in the loss of these proteins from the extract during centrifugation.

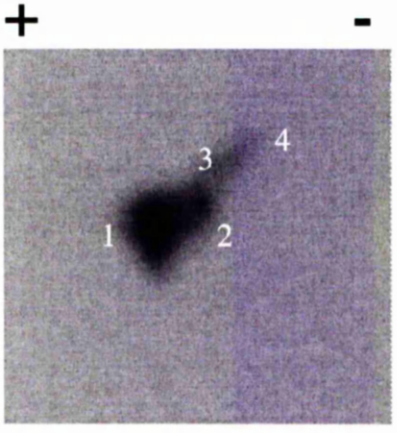


Figure 4.5 Multiple isoforms of Sla1p exist in yeast cells. Whole cell extracts of yeast expressing a HA tagged form of Sla1p were analysed by 2-D gel electrophoresis. Western blotting was performed and membranes were probed with Rabbit-anti HA, 1:1000, (Santa Cruz) and anti-Rabbit-HRP, 1:5000, (sigma) antibodies. Sla1p-HA was detected by ECL. This technique detected at least four different isoforms of Sla1p (numbered 1-4).

4.2.6 Sla1p is potentially dephosphorylated by yeast protein phosphatase-1 (PP1) Glc7p.

Sla1p contains a variety of potential phosphorylation sites within its sequence and Sla1p from cells lacking the actin regulating kinases has an increased mobility on an SDS-PAGE gel suggesting these kinases are among those that modify Sla1p. To allow regulation of the functions of Sla1p in the cell, Sla1p may therefore be a target for protein phosphatases. One candidate protein phosphatase is the yeast PP1 Glc7p.

GLC7 is an essential gene in yeast cells so cannot be deleted from the genome. However a set of temperature sensitive point mutants exist, most of which have cell cycle defects (Mike Stark, personal communication). One mutant that does not display a cell cycle defect is the *glc7-13* allele (Mike Stark, personal communication). To observe if *GLC7* mutants had any effect on Sla1p we decided to use the *glc7-13* allele. Whole cell extracts of wild type and *glc7-13* cells were prepared from cells grown at the permissive temperature. Western blotting was performed to determine if a mobility shift existed between Sla1p from the two extracts, as shown in figure 4.6. A mobility shift is observed for Sla1p in the two cell extracts. Sla1p in the *glc7-13* extract has a decreased mobility suggesting it is slightly larger than that of Sla1p in the wild type extract. Sla1p from wild type extracts had a molecular weight of about 212 kDa whereas Sla1p from extracts containing the *glc7-13* allele had a molecular weight of about 258.8 kDa. This indicates that Sla1p may be hyperphosphorylated in the *glc7-13* mutant (Figure 4.6).

4.3 Discussion.

Within the C-terminal repeat region of Sla1p exist 5 exact copies of these repeats and also present are a further 9 motifs with a QxTG consensus, suggesting Sla1p is a likely target for the Prk1p kinase. Sla1p contains potential phosphorylation sites for other kinases, for

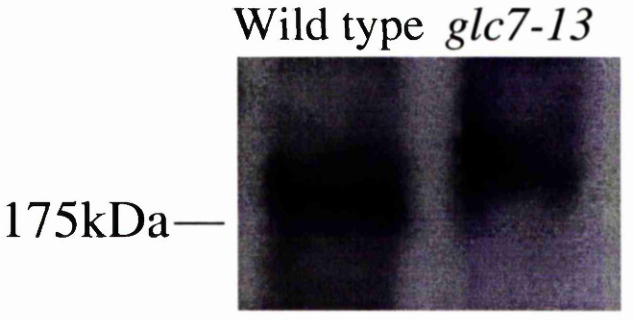


Figure 4.6 Potential dephosphorylation of Sla1p by yeast protein phosphatase-1 Glc7p. To assess if Sla1p is targeted by Glc7p whole cell extracts of wild type (KAY455) and *glc7-13* (KAY460) cells were analysed by immunoblotting for Sla1p. Sla1p was detected by ECL after probing with rabbit anti-Sla1p (1:100) and anti-rabbit-HRP (1:5000) antibodies. A mobility shift was observed between Sla1p from wild type, with a molecular weight of about 212 kDa, and *glc7-13*, with a molecular weight of about 258 kDa, cells suggesting that Glc7p facilitates modification of Sla1p.

example cAMP dependent protein kinase, that lie outside the C-terminal repeat region, however the LxxQxTG motifs are the only phosphorylation motif identified in the C-terminal repeat region. This may imply that this region of Sla1p is specifically targeted for phosphorylation by Ark1p /Prk1p, allowing control of the functions of Sla1p. In this study we were able to demonstrate that Sla1p is phosphorylated and that the Actin Regulating Kinases Ark1p and Prk1p contribute to this.

In this study we have demonstrated that in cells lacking both of the Actin Regulating Kinases Ark1p and Prk1p there is a complete co-localisation of Sla1p with cortical actin structures. Studies that were undertaken during the course of this work have demonstrated that this is not the case in wild type cells. Analysis of Sla1p-YFP and Abp1p-CFP simultaneously expressed in live cells has revealed that these proteins are found in largely separate cortical actin populations that occasionally co-localise (Warren *et al*, in press). This suggests that phosphorylation of the LxxQxTG motifs in Sla1p and Pan1p by Ark1p/Prk1p in cells may be required to cause dissociation of cortical actin structures. We have also presented data showing the importance of phosphorylation in the disassembly of Sla1p containing actin structures. Expression of a kinase dead form of the Prk1p protein in cells lacking *ARK1* and *PRK1* does not cause the dissociation of Sla1p from actin, demonstrating the importance of phosphorylation in the disassociation of cortical complexes.

If phosphorylation is important for the dissociation of complexes at the cell cortex then it is likely that these proteins undergo dephosphorylation to allow them to associate to form new complexes. Sla1p has been demonstrated to interact with members of the protein phosphatase-1 family, that include Glc7p, Ppz1p and Ppz2p (Tu *et al*, 1996; Venturi *et al*,

2000). In support of this we have also provided evidence that Glc7p may dephosphorylate Sla1p.

Future work would include *in vitro* kinase assays demonstrating that Ark1p and Prk1p are capable of phosphorylating a purified, bacterially expressed C-terminal repeat region fragment of Sla1p. A kinase-dead form of prk1p could be used as a negative control for this assay. Other future studies may focus on the phosphorylation sites that lie outside of the C-terminal repeat region of Sla1p. These phosphorylation sites may also allow cells to control the association of Sla1p with other binding partners. This may involve looking at the effects of deletions of other kinases, for example cAMP dependent protein kinase, that potentially target Sla1p upon the actin cytoskeleton.

Chapter 5.
Identification and Charaterisation of Sla1p Interacting Proteins.

5.1 Background.

Sla1p is comprised of a multidomain structure and has been shown to interact with an increasing number of proteins. During the course of this study Sla1p was shown to interact with the Pan1p/End3p complex via its C-terminal repeat region (Tang *et al*, 2000). In addition Sla1p has recently been shown to interact with Sla2p and Ysc84p via its GAP1+SH3#3 region (Hilary Dewar, unpublished data) and also with Abp1p and Las17p, the yeast WASP homologue (Rong Li, 1997; Warren *et al*, in press).

The yeast 2-hybrid approach has been widely used to identify interactions between proteins. This system utilises two plasmid borne gene fusions, that are cotransformed into a host yeast strain containing inducible reporter genes (see Methods and Materials; James *et al*, 1996). The protein of interest (BAIT) is encoded as a gene fusion to a DNA binding domain of the *GAL4* protein. A second protein or a library of proteins (PREY) is encoded as a gene fusion to a transcription activation domain. Interaction between the two proteins results in localisation of the transcription activation domain to the DNA of the host strain, activating transcription of the adjacent reporter genes generating a phenotypic signal (Figure 5.1).

An initial aim of this work was to identify interacting partners of the C-terminal repeat region of Sla1p, to do this we used the yeast 2-hybrid system. However, as mentioned in chapter 3, this approach was unsuccessful as this region of Sla1p fused to the *GAL4* binding

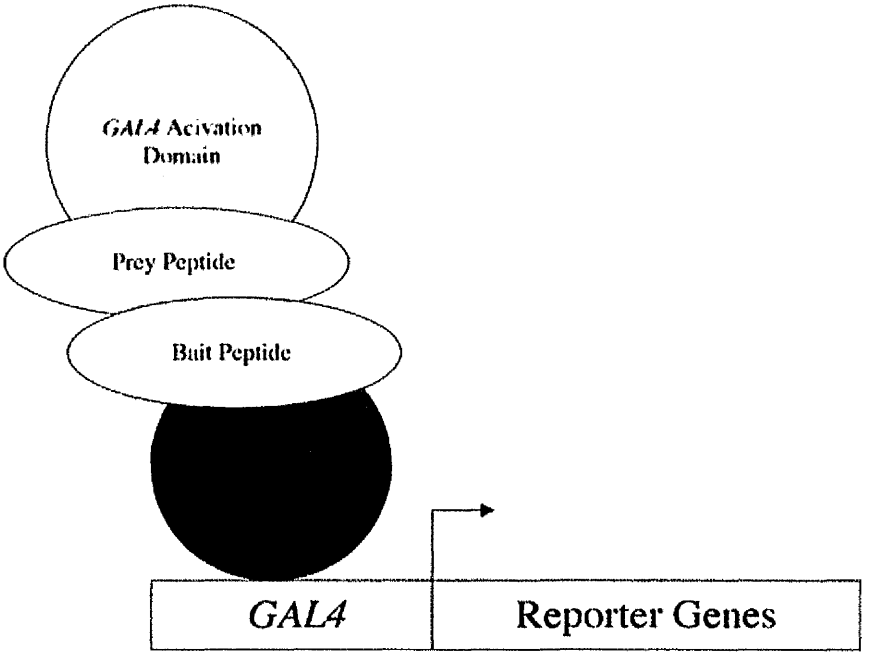


Figure 5.1. Schematic representation of how the 2-hybrid system works. Interaction between the bait and prey peptides result in localisation of the activation domain to the *GAL4* promoter, activating expression of the reporter genes.

domain plasmid was self-activating in the yeast 2-hybrid system (Data not shown). This finding has subsequently been reported by other labs (Tang *et al*, 2000). While an increasing body of data exists on the interactions of the C-terminus of Sla1p, little is known about the central region of Sla1p. This region of Sla1p contains two regions termed Homology Domains 1 and 2 (HD1 and HD2), which are the regions of highest homology between the *S.cerevisiae* and homologues of Sla1p identified in *S.pombe*, *N.orassa* and *C.albicans* (Figure 5.2; Ayscough *et al*, 1999). This could indicate that these regions of Sla1p have some evolutionary conserved function. The 2-hybrid system was used to identify interacting partners to the central region of Sla1p containing these conserved regions.

5.2 Results.

5.2.1 Yeast 2-hybrid screen using the central region of Sla1p as bait.

The central region of *SLA1* (nucleotides 1461-2190), containing both HD1 and HD2, was fused to the binding domain of *GAL4*. An outline of the yeast 2-hybrid system is shown in figure 5.3. The bait plasmid alone or with empty activation domain plasmid did not activate expression of the reporter genes when transformed into yeast strain PJ69-2A (Figure 5.4). The GAP1+SH3#3 region (see figure 1.6) of *SLA1* fused to the pGBDUC-3 plasmid and an fragment of *SLA2* fused to the pGAD plasmid that had previously been shown to interact in the yeast 2-hybrid system (Hilary Dewar, unpublished data) was used as a positive control (Figure 5.5).

Transformation of the activation domain library into the yeast strain containing the bait plasmid resulted in the isolation of 124 activation colonies. Plasmids were rescued and then retransformed into the yeast strain containing the bait plasmid to check the plasmids

(A).

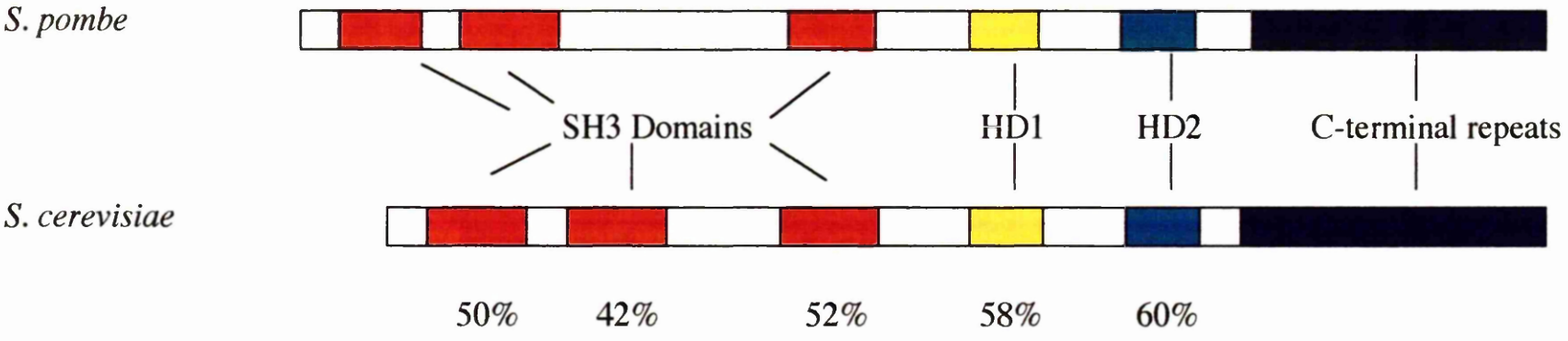
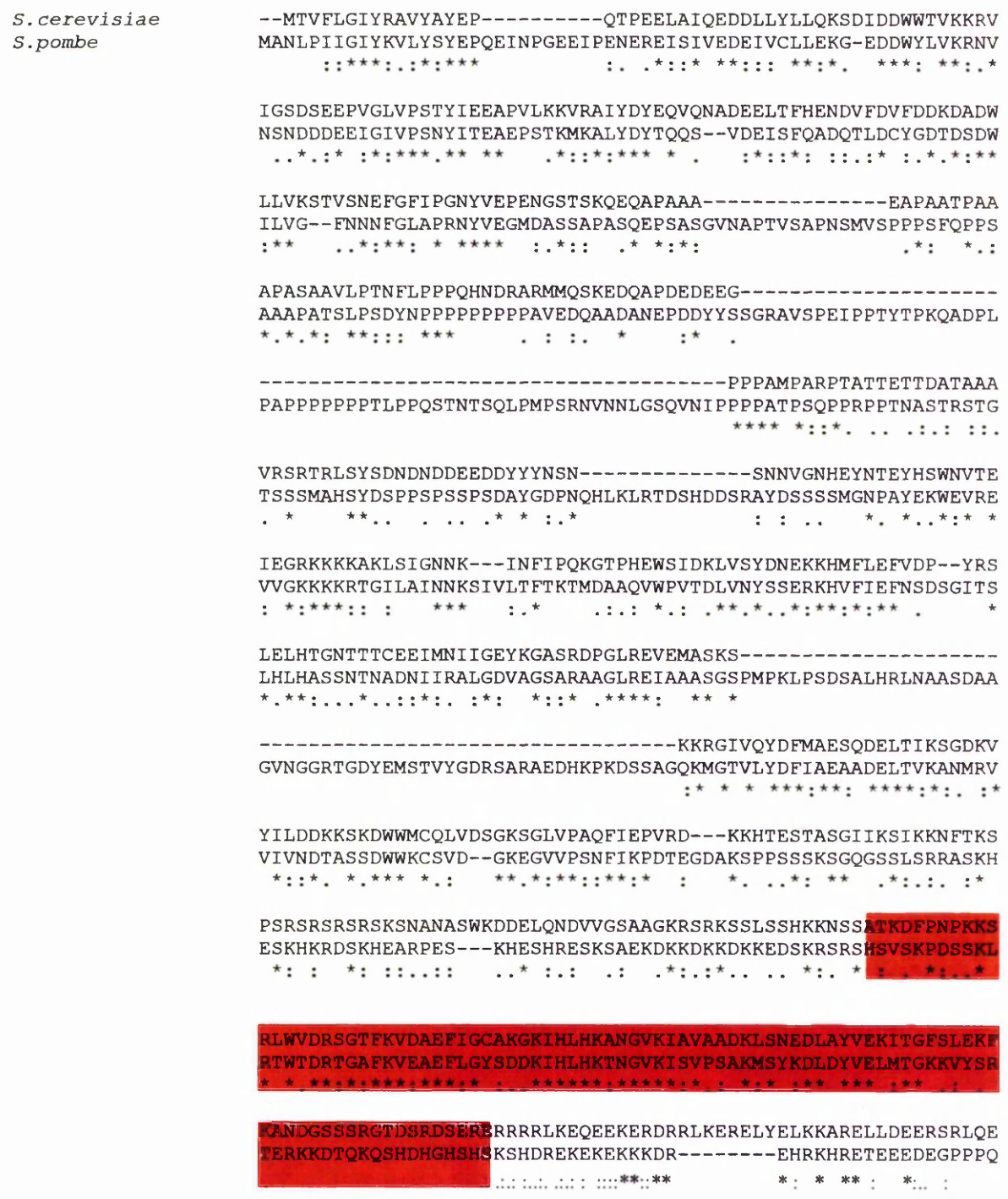


Figure 5.2. Sequence similarity between Sla1p homologues. (A) *S.pombe* homologue of Sla1p has a domain structure similar to *S.cerevisiae* Sla1p. The two domains with highest homology HD1 and HD2 (Homology Domain 1 and 2). The percentage homology of the SH3 domains and HD1 and 2 are noted below the respective motifs. (B) Sequence alignment of *S.pombe* and *S.cerevisiae* homologues of Sla1p. Adapted from Ayscough *et al*, 1999.

Figure 5.2.b. **Sequence alignment of *S. cerevisiae* and *S. pombe* Sla1p homologues.**




S.cerevisiae
S.pombe
KELPPIKPPRPTSTTSVPNTTSVPPAESSNNNNSSNKYDWFEFFLNCGVDVSNCQRYTIN
----PARPESTRPALAPPSSSHSNDKYDVIQERPKISYDWFDFFLRCGVDFTVCNRYTHN
FDREQLTEDMMPDINNSMLRTLGLREGDIVRVMKHLDKKFGR----------ENIASIPT
FNNEHLDEACIPSLNPDTLRTLGLKEGDIIRVMNHVNELNGVSTKPASAITPETKSTVNQ
NATGN-------MFSQPDGSLNVATSPETSLPQQLLPQTTSPAQTAPSTSAET---DDAW
IMSGGEALAAPVAVPAPIPAPVAEPAPPAAPAKEVVEKAPSPPATRPKSTTPQKFDDDAW
TVKPASKSESNLLSKKSEFTGSMQDLLDLQPLEPKKAAASTPEPNLKDLEPVKTGGTTVP
ANKPVTEPPVRASSVTVEPARVTESMNKMNISEEAKKPEAPSRPRTAPIPEPEEQ-KKAP
AAPVSSAPVSSAPAPLDPFKTGGNNILPLSTGFVMMPMITGGDMLP---MQRTGGFVVPQ
VEKKDAEKSVQAPIPAQPTGN-----ITIQNAYFTAPQPMAADPFQSPLYVQPTGFQPPP
TTFGMQSQVTGGILPVQKTGNGLIPISNTGGAMMPQTTFGAAATVLPLQKTGGGLIPIAT
SALPIQPTGYMQPIPVQATGYQPLMVQPTG---LQPHMTGVMPQVTGVMPQMTGVVPQMT
TGGAQFPQTSFNVQGQQQLPTGSILPVQKTANGLISANTGVSMPTVQRTGGTMIPQTSFG
G--VMPQMTGVQVQKTGAMPQQPVNYGYQVAGMQPQATGIISQPTGIRAQATGIMTQPTG
VSQQLTGGAMMTQPQNTGSAMMPQTS-----FNAVPQITGGAMMPQTSFNALPQVTGGAM
LHTQATGMMQPTGMQPQATGIMPQATGMMQPTGMQPQVTG--IMPQS-MPMQPQMTGVQV
MPLQRTGGALNTFNTGGAMIPQTSFSSQAQNTGGFRPQSQFGLTLQKTGGIAPLNQNQFT
QKTGMVAQPMLSQYTG-YQQNYTPTAMPAADGYGMQPSMNDTQAYYNMNAQTPVNYG-FA
GGAMNTLSTG-----GVLQQQQPQTMNTFNTGGVMQELQMMTTFNTGGAMQQPQ------
GGQDTSFGYEQQQMYSPMQQQQQQYYGTEMQPDMGYQQPMMSNYYDPMQMQQQTPYGYNQ
-MMNTFNTDGIMQQPQMMN---TFN--TGGAMQQPQQQALQNQPT-----GFGFGNGPQQ
TGMEGYSEYGYAQPAGNMANPMSYDPVSNASLYMPSDYNQQTQPANYYDSSFGGAQGANE
S-RQANIFNATASNPFGF
AGKKASIYQATPDNPFGF
Homology Domain-1
Homology Domain-2

Outline of the yeast 2-hybrid screen.

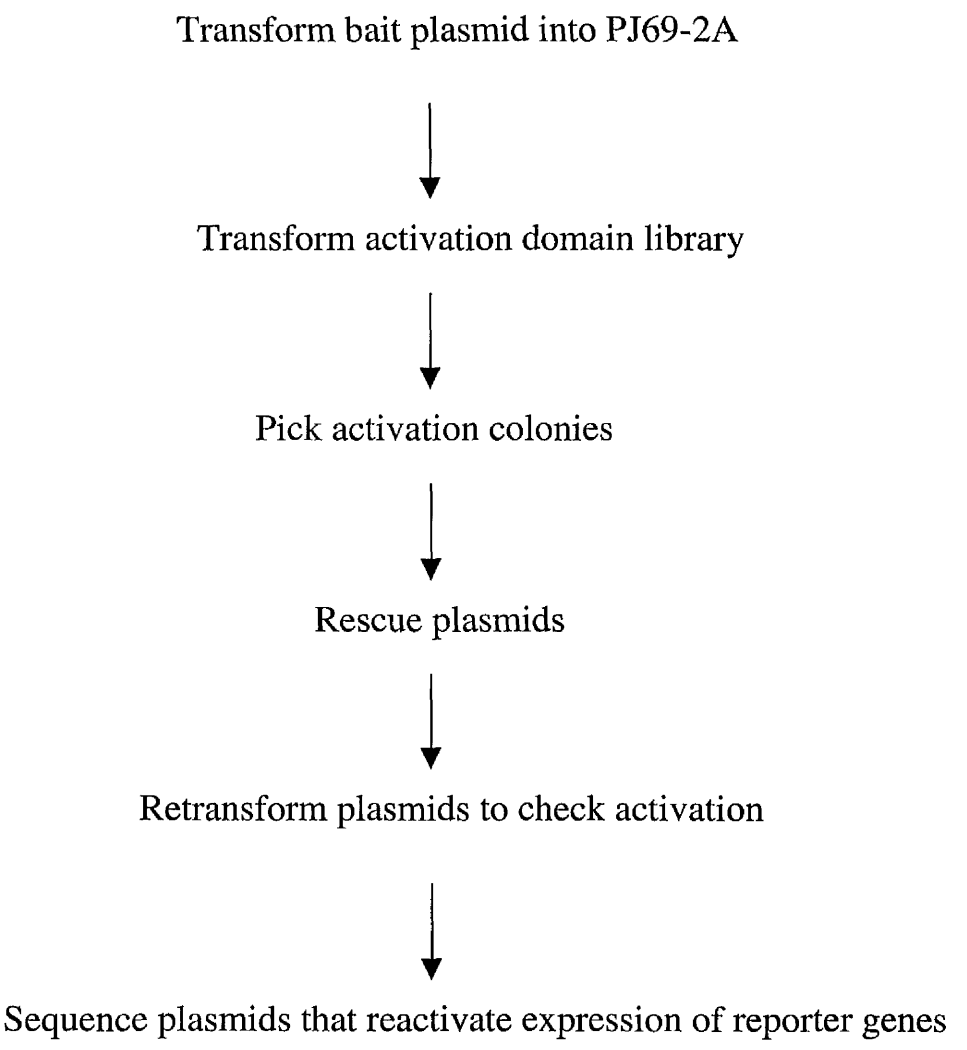


Figure 5.3 Flow chart of the yeast 2-hybrid screen

still activated the reporter genes. Of the 124 rescued plasmids, 20 reactivated expression (Figure 5.4) of the reporter genes (see Methods and Materials; James *et al*, 1996). All of the activation plasmids were then sequenced and the insert was identified using the WU-BLAST2 search on the *Saccharomyces* genome database (http://genome-www.stanford.edu/saccharomyces). The inserts contained on the prey plasmids are listed in Table 5.5.

5.2.2 Summary of interacting partners of the central region of Sla1p identified in the yeast 2-hybrid screen.

This work has identified 6 interacting partners of the central region of Sla1p. The most abundant insert identified in this 2-hybrid screen was a fragment of *Ycl034w*, with 6 hits. All of these inserts contained different but overlapping regions of *YCL034w*, the smallest insert contained amino acids 169-289 of a 354 amino acid protein. Ycl034w has an unknown biological function but has previously been shown to interact with Las17p in the yeast 2-hybrid system. The function of this Sla1p binding partner is investigated in more detail later in this chapter.

Two Fragments of *ZRT3* were also identified as interacting partners of Sla1p in this screen, however, these *ZRT3* fragments are to different regions of the protein that do not overlap. The Zrt3p protein has been shown to encode a zinc ion transporter that is found on the vacuolar membrane (MacDiarmid *et al*, 2000). Cells lacking *ZRT3* have been demonstrated to be viable (MacDiarmid *et al*, 2000).

Another interacting partner identified was Ynr065cp. Two overlapping fragments of the central region of the protein were identified in this screen. Ynr065cp has an unknown biological function but is very similar in sequence to Pep1p and Vth1p, sharing 87%

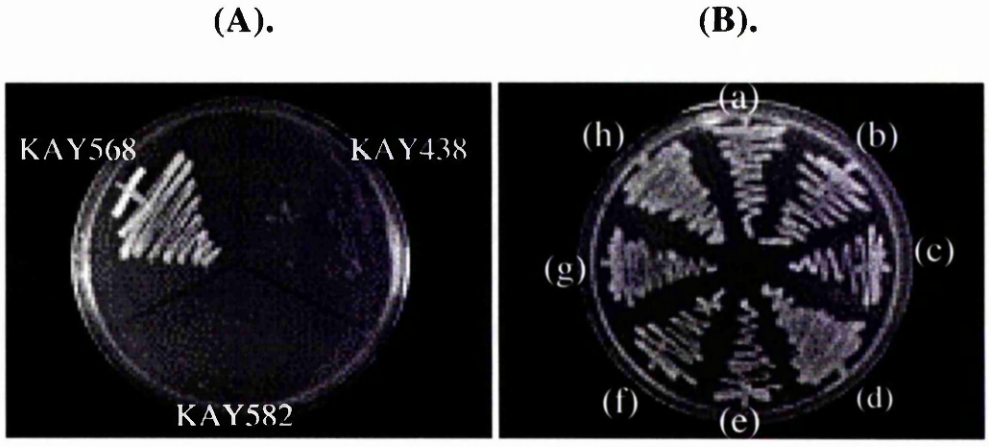


Figure 5.4 Two-hybrid control and activation colonies. (A) Cells containing either pDW1 (KAY438) alone or pDW1 + empty pGADC-1 (KAY582) failed to activate expression of the reporter genes. Cells containing plasmids previously shown to interact in the yeast 2-hybrid (KAY568) activated the expression of the reporter genes. (B) The pGAD-fusion library was transformed into KAY cells expressing pGBDU-C3-HD1/2 region of Sla1p (pDW1). Colonies growing on activation plates were struck onto fresh activation plates. (a) yDW105 (*YCL034w*), (b) yDW109 (*YSC84*), (c) yDW101 (*YDR128w*), (d) yDW88 (*YNR065c*), (e) yDW97 (*ZRT3*), (f) yDW111 (*YPR171w*), (g) yDW59 (*GAL4*) and (h) yDW102 (*ADH*).

INSERT	HITS	FUNCTION	FUSION SITES
YCL034w	7	Unknown biological function. Interacts with Las17p in the yeast 2-hybrid system. Deletion is viable.	Chromosome III co-ordinates 62375-62670 , 62342-62917, 62271-62839, 62317-62787, 62330-62839, 62385-62646 and 62306-62844.
YNR065c	2	Unknown biological function. 87% similarity to Pep1p and Vth1p. Deletion is viable.	Chromosome XIV co-ordinates 752049-752578 and 752053-752642.
ZRT3	2	Zinc ion transporter located on the vacuolar membrane. Deletion is viable.	Chromosome XI co-ordinates 119876-120479 and 119408-110817.
YSC84	1	Unknown biological function. Interacts with Las17p, Ste2p and the GAP1+SH3#3 region of Sla1p in the 2-hybrid system. Deletion is viable.	Chromosome VIII co-ordinates 137073-137599.
YDR128w	1	Unknown biological function. Contains WD repeats. Deletion is viable.	Chromosome IV co-ordinates 709419-709962.
YPR171w	1	Unknown biological function. Interacts with Cap1p, Rvs167p and Sla1p in the 2-hybrid system. Displays cytoskeletal localisation pattern	Chromosome XVI co-ordinates 884943-885445.
GAL4	2		
ADH	4		

Table 5.5 Inserts contained on activation plasmids.

homology with these two proteins. It also shares homology with two human proteins, known as Sortilin-1 and Sorl-1. Sortilin-1 has been proposed to function in localising proteins to endosomal vesicles and Golgi (Petersen *et al*, 1997; 2000). Sorl-1 is a sortilin-related receptor that may be involved in the uptake of lipoproteins and proteases (Jacobsen *et al*, 1996). This may be relevant as Sla1p is required for efficient endocytosis and therefore may be part of the endocytic machinery.

The other inserts contained fragments of *YSC84*, *YPR171w* and *YDR128w*. Ysc84p has been shown to interact with a number of proteins including Las17p (Madania *et al*, 1999), the GAP1+SH3#3 region (amino acids 118-511) of Sla1p (Hilary Dewar, unpublished data) and Sla2p (Drees *et al*, 2001). Ypr171w also has an unknown biological function, but has been reported to display a cytoskeletal localisation (Drees *et al*, 2001). It has also been reported to interact with a number of proteins including Cap1p, Rvs167 and Sla1p in the yeast 2-hybrid system. This work has narrowed down the Sla1p/Ypr171wp interaction to involve the central region of Sla1p. Ydr128wp is predicted to contain WD repeats but has an unknown biological function. Yeast cells lacking either *YSC84*, *YNR171w* or *YDR128w* are viable.

This screen has identified a number of proteins that were reported by previous studies to interact with Sla1p. The data presented here refines the regions involved in these interactions and identifies new interacting partners of Sla1p. This validates the interactions identified in this study. This 2-hybrid screen identified two Las17p interacting proteins, Ycl034wp and Ysc84p, that interact with the central region of Sla1p. Ysc84p has also been demonstrated to interact with an adjacent region of Sla1p, the GAP1+SH3#3 region. Sla1p has also been shown to interact with Las17p by both yeast 2-hybrid and GST pull-down approaches (Rong Li, 1997; Warren *et al*, in press). This could imply that Las17 is

interacting with Sla1p via these two proteins that interact with adjacent regions of Sla1p. For the rest of this work we decided to focus on Ycl034wp and Ysc84p.

5.2.3 *YSC84.*

The *YSC84* gene is located on chromosome VIII and encodes a protein containing 468 amino acids with a predicted molecular weight of 51kDa. Ysc84p has been shown to interact with Las17p (Madania *et al*, 1999), Ste2p (Ito *et al*, 2001), the yeast alpha factor receptor, and also the GAP1+SH3#3 region of Sla1p (Hilary Dewar, unpublished data) in the yeast 2-hybrid system. Database searches revealed that Ysc84p contains a SH3 domain in its C-terminal region and the central region consists of arginine rich and aspartic acid rich tracts.

The region of Ysc84p demonstrated previously to interact with the GAP1+SH3#3 region of Sla1p is different from the region identified in this screen. Work carried out by Hilary Dewar demonstrated that Ysc84p was interacting with the GAP1+SH3#3 region of Sla1p via its C-terminal SH3 domain. However the region identified in this screen does not contain the SH3 domain of Ysc84p, suggesting multiple interactions exist between Sla1p and Ysc84p.

5.2.4 Sequence analysis of *YCL034w.*

The *YCL034w* gene is located on chromosome III and encodes a 354 amino acid protein with a predicted molecular weight of 40kDa that has been shown to interact with Las17p in the yeast 2-hybrid system (Madania *et al*, 1999). Database searches revealed the presence of three predicted sequence homology domains within the sequence of Ycl034wp (figure 5.6).

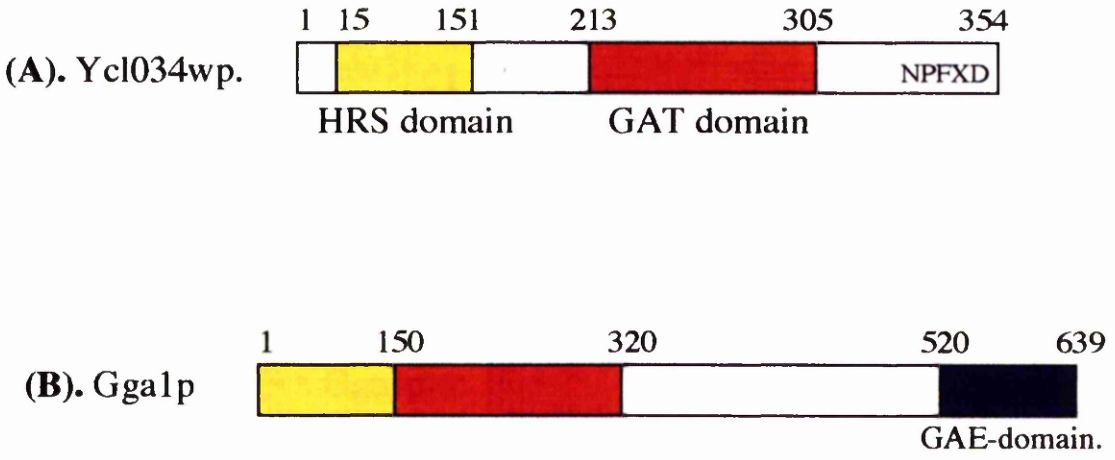


Figure 5.6 Schematic representation of predicted domains contained within Ycl034wp and Gga1p. (A) Ycl034wp contains an N-terminal HRS domain. Downstream of this a potential GAT domain is present. Also present in its extreme C-terminus exists an NPFXD motif. (B) GGA proteins Gga1p and Gga2p contain a similar domain structure to Ycl034wp. Gga1p contains an N-terminal VHS domain, downstream of this lies a GAT domain. Located in its C-terminus is a gamma adaptin ear (GAE) homology domain.

Located at the N-terminal end of the protein is a predicted VHS domain, a domain that has been identified in a growing number of proteins involved in endocytosis and vesicle trafficking. This domain is about 150 residues long and contains an unknown biological function but is always located in the N-terminus of proteins. The VHS domain of the *Drosophila* HRS protein has been shown to interact with FYVE domains and it has been proposed that these domains may interact with other domains (Mao *et al*, 2000). The crystal structure of the VHS domain of *Drosophila* HRS and human Tom1 revealed that it consists of eight helices arranged in a superhelix (Mao *et al*, 2000; Misra *et al*, 2000). Comparison of the structure of the VHS domain of human Tom1 with the ENTH domain of epsin-1 (Hyman *et al*, 2000) revealed a conserved surface containing a net positive charge and other studies have proposed that this surface plays a role in membrane binding (Misra *et al*, 2000).

Other yeast proteins that are VHS-domain containing proteins include GGA proteins and Vps27p (Figure 5.6). The GGA proteins have been demonstrated to be involved in the trafficking of proteins between the trans-Golgi network and the vacuole of yeast cells (Hirst *et al*, 2000). Vps27p has also been reported to be involved in vesicle trafficking and is thought to control membrane trafficking through the prevacuolar/endosomal compartment (Raymond *et al*, 1992; Piper *et al*, 1995). Alignment of the sequence of the VHS domains from Gga1p, Vps27p and Ycl034wp revealed that low sequence similarity existed between these VHS domains (Figure 5.7). However, prediction of the secondary structure of these VHS domains from the GGA proteins and Ycl034wp identified the existence of multiple potential α-helical regions (Figure 5.8), suggesting the possibility that these VHS domains also form superhelical structures similar to that of the VHS domains of human Tom1 and *Drosophilia* HRS.

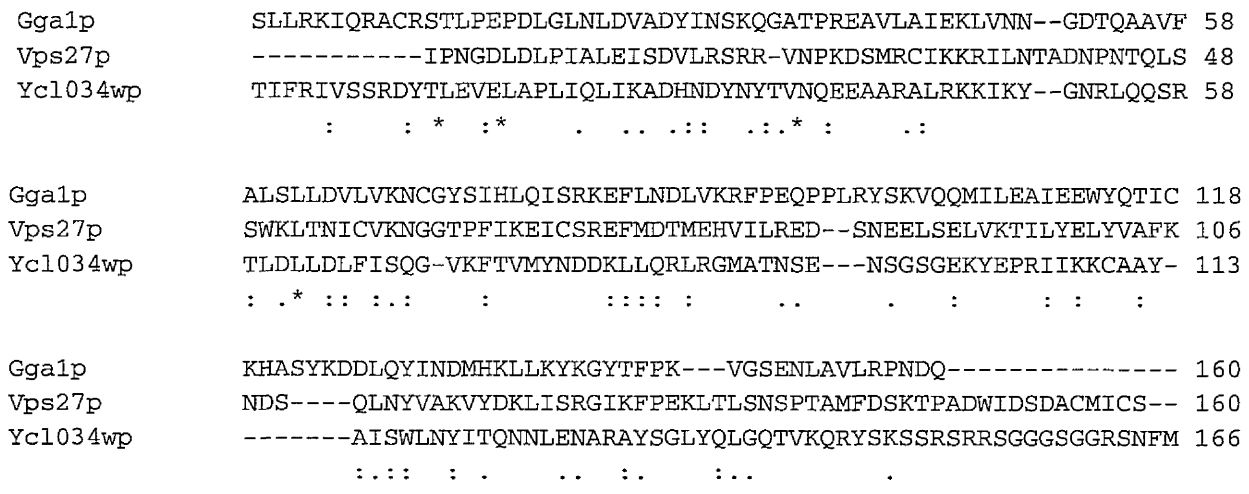


Figure 5.7 Alignment of the VHS domains from Gga1p, Vps27p and Ycl034wp. Alignment was carried out using the clustal-l software and revealed a low sequence similarity between the VHS domains of these proteins. There is about a 18% sequence identity between the VHS domains of Gga1p and Ycl034wp and a 15% sequence homology between the VHS domains of Vps27p and Ycl034wp.

(A).

Gga1p SKQGATPREA VLAIEKLVNN GDTQAAVFAL SLLDVLVKNC GYSIHLQISR
CCCCCCCHHH HHHHHHHHCC CCCCHHHHHH HHHHSSSCCC CCCSSSSHHH

Gga1p KEFLNDLVKR FPEQPPLRYS KVQQMILEAI EEWYQTICKH ASYKDDLQYI
HHHHHHHHHC CCCCCCCCHH HHHHHHHHHH HHHHHHHHHC CCCCCCHHHH

Gga1p NDMHKLLKYK GYTFPKVGSE NLAVLRPNDQ LRTPSELQEE QERAQAAKLE
HHHHHHHCCC CCCCCCCCCC CSSSSCCCCC CCCCHHHHHH HHHHHHHHHH

(B).

Gga2p MSHPHSHSIY LSELPVRKPQ ALGNPLLRKI QRACRMSLAE PDLALNLDIA
CCCCCCCSSS SSCCCCCCCC CCCCHHHHHH HHHHHHHCCC CHHHHHCCHH

Gga2p DYINEKQGAA PRDAAIALAK LINNRESHVA IFALSLLDVL VKNCGYPFHL
HHHHHHCCCC HHHHHHHHHH HHCCCCCCCC SSHHHHHHHS SCCCCCCCCC

Gga2p QISRKEFLNE LVKRFPGHPP LRYSKIQRLI LTAIEEWYQT ICKHSSYKND
HHHHHHHHHH HHHCCCCCCC CCHHHHHHHH HHHHHHHHHH HCCCCCCCCC

Gga2p MGYIRDMHRL LKYKGYAFPK ISESDLAVLK
CCHHHHHHHH HHCCCCCCCC CCCCCSSSSC

(C).

Ycl034wp MGFLSDHPHT AITETIFRIV SSRDYTLEVE LAPLIQLIKA DHNDYNYTVN
CCCCCCCCCCCCCCCCHHSSSCCCCCCSSSSCCHHHHHHHHCCCCCCCCCCC

Ycl034wp QEEAARALRK KIKYGNRLQQ SRTLDLLDLF ISQGVKFTVM YNDDKLLQRL
HHHHHHHHHHHHHHHCCCHHHHHHHHHHHHHHHHCCCCSSSSSCCCHHHHHHH

Ycl034wp RGMATNSENS GSGEKYEPRI IKKCAAYAIS WLNYITQNNL ENARAYSGLY
HCCCCCCCCCCCCCCCCCCHHHHHHHHHHHHHHHHCCCCCCHHHHHHHHHH

Ycl034wp QLGQTVKQRYSKSSRSRRS
HHCCCSSSSCCCCCSSSSCC

Figure 5.8 Comparison of the predicted secondary structure of the VHS domains of (A) Gga1p, (B) Gga2p and (C) Ycl034wp. All of these VHS domains contain predicted regions of alpha-helices (H), coils or turns (C) and β-sheet (S).

Downstream of this VHS domain, a potential GAT domain was identified, however, no functional studies have been carried out and the sequence similarity with other identified GAT domains is very low. Functional studies have been carried out on the GAT domains belonging to the yeast GGA proteins. The GGA proteins (Gga1p and Gga2p) have been demonstrated to interact with several members of the ARF family and this interaction occurs via their GAT domain (Zhdankina *et al*, 2001). The GAT domain is thought to stabilise the membrane bound ARF1 in its GTP bound state by disrupting the interaction with GAP proteins (Zhdankina *et al*, 2001). Further analysis of the sequence of the GGA proteins and Ycl034wp revealed that these proteins were divergent in their C-terminus. The GGA proteins contain a gamma-adaptin ear (GAE) homology domain, a proposed protein-protein interaction domain (Hirst *et al*, 2000), in their C-terminal regions, whereas an NPFxD motif was identified at the C-terminus of Ycl034wp. NPFxD is a motif that has been demonstrated to interact with EH domain-containing proteins involved in endocytosis (Tan *et al*, 1996; Salcini *et al*, 1997; Wendland *et al*, 1998). This may imply that Ycl034wp is involved in trafficking of proteins through yeast cells, but may be involved in an early step of the trafficking pathway that the GGA proteins.

5.2.5 Ycl034wp-myc localises to the cell cortex via an actin cytoskeleton independent process.

Sla1p has been demonstrated to localise to the cell cortex in small, punctate patches that become polarised to the bud (Ayscough *et al*, 1999). In order to determine if Ycl034wp was in a position to interact with Sla1p in cells, a myc tag was integrated at the C-terminus of *YCL034w*. Indirect immunofluorescence microscopy was used to investigate the localisation of myc tagged Ycl034wp (see Methods and Materials; Ayscough and Drubin, 1998).

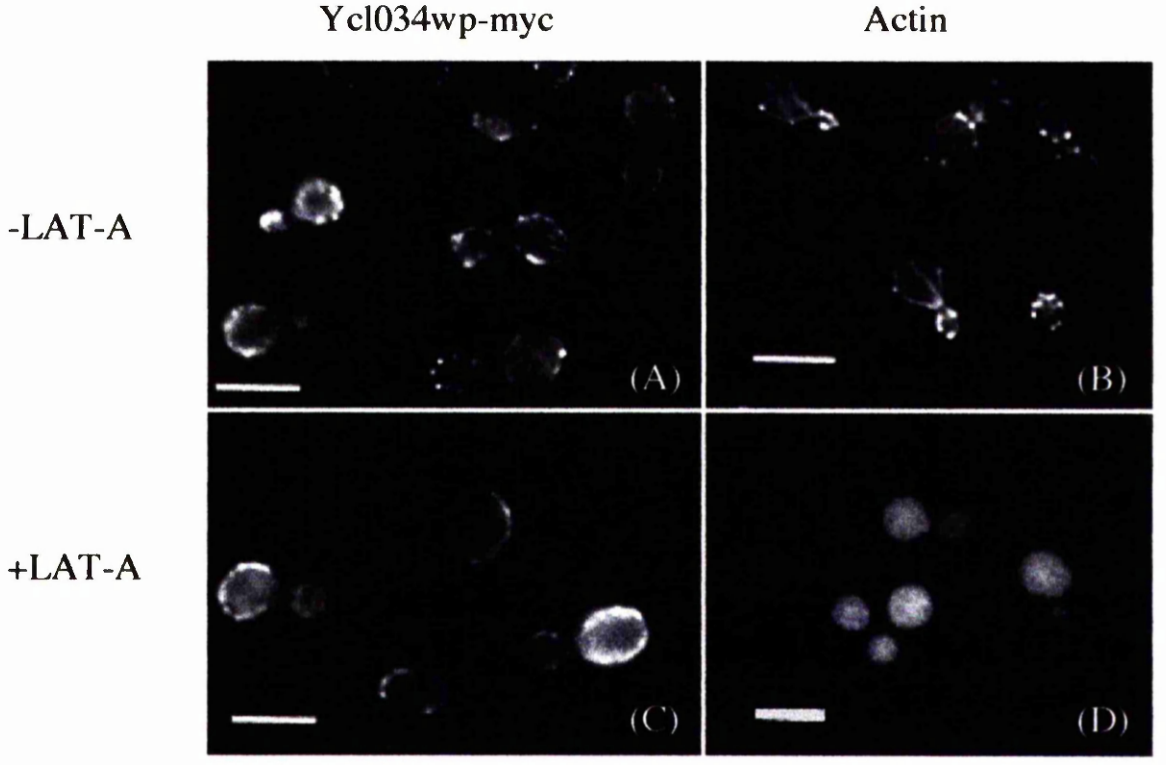


Figure 5.9 Localisation of Ycl034wp-myc. Actively growing Ycl034wp-myc (KAY514) cells were fixed and myc-tagged proteins were localised indirect fluorescence microscopy using rabbit A14 (1:100) and anti-rabbit FITC (1:200) antibodies (A) before and (C) after treatment with 200 μM LAT-A for 5 minutes. Half the cells were stained with rhodamine phalloidin and observed by fluorescence microscopy (B) before and (D) after treatment with LAT-A to check that the actin cytoskeleton was disrupted. Bar 10 μM

Ycl034wp-myc localised in small, punctate cortical patches, mainly in the mother cell although there was also a small amount present in the buds. Association with actin can be tested by incubating cells with LAT-A (Ayscough *et al*, 1997; Morton *et al*, 2000). Treatment with LAT-A results in actin and proteins directly associated with actin become disassembled and dispersed. After the actin cytoskeleton was disrupted by LAT-A, the localisation pattern of Ycl034wp-myc was slightly altered. Ycl034wp-myc was still present at the cell cortex but rather than being organised into small punctate patches it appeared to be more uniform through the cell cortex. This suggests that localisation of Ycl034wp-myc to the cell cortex does not require an intact actin cytoskeleton, however it requires the actin cytoskeleton to organise into discrete structures at the cell cortex (Figures 5.9).

5.2.6 Characterisation of cells lacking *YCL034w* and *YSC84*.

Both Ycl034wp and Ysc84 were identified as Las17p interacting proteins in the yeast 2-hybrid system (Madania *et al*, 1999) and have also been demonstrated to interact with adjacent regions of Sla1p (Hilary Dewar, unpublished data). This may indicate that these proteins function to link Sla1p to Las17p. To investigate this possibility strains were constructed lacking *YCL034w*, *YSC84* or both *YCL034w* and *YSC84*.

5.2.7 Growth of cells lacking *YCL034w* and *YSC84*.

Cells lacking either *YCL034w* or *YSC84* displayed no noticeable growth defect at 30°C or 37°C on YPAD plates (Figure 5.10). However cells lacking both of these genes display a slow growth defect at 30°C and are not viable at 37°C. This synthetic lethality at elevated temperatures suggests that these proteins may have overlapping functions in yeast cells.

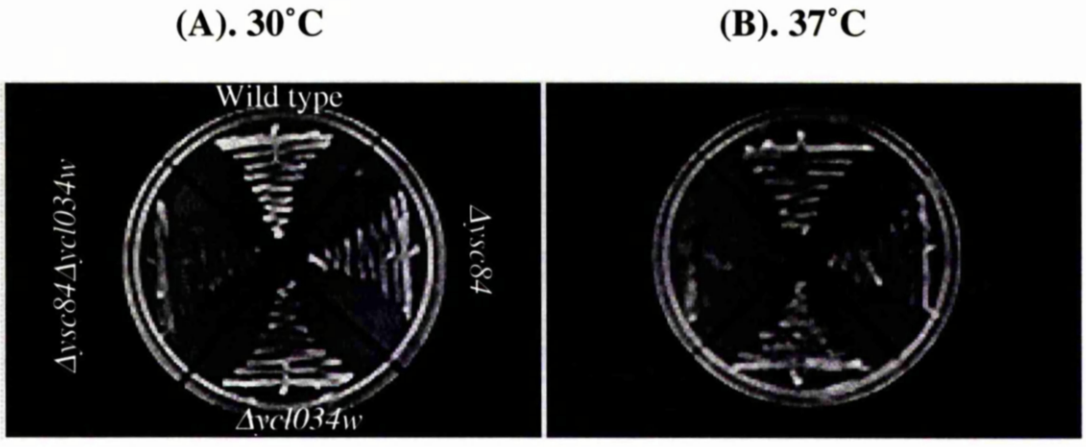


Figure 5.10 Growth of mutant strains on YPAD plates. (A) Wild type (KAY302), *Δysc84* (KAY510), *Δycl034w* (KAY515) and *Δysc84Δycl034w* (KAY516) cells were struck out on YPAD plates and incubated at 30°C for 2 days. (B) Wild type (KAY302), *Δysc84* (KAY510), *Δycl034w* (KAY515) and *Δysc84Δycl034w* (KAY516) cells were struck out on YPAD plates and incubated at 37°C for 2 days.

5.2.8 Cells lacking *YCL034w* and *YSC84* contain an aberrant cortical actin cytoskeleton organisation.

Ysc84p and Ycl034wp have been shown to interact with Las17p in the yeast 2-hybrid system (Madania *et al*, 1999) and Las17p is the yeast WASP homology that has been shown to stimulate the actin nucleation activity of the Arp2/3 complex. Ycl034wp and Ysc84p could therefore be in a position to link Sla1p to actin dynamics. Rhodamine phalloidin was used to observe the effects of deleting these genes from the cells on the cortical actin cytoskeleton organisation (see Methods and Materials and Pringle *et al*, 1989).

Wild-type cells contained small, punctate cortical actin patches that are highly polarised and are found throughout the cortex of the bud in small-medium budded cells (Figure 5.11a). Cells lacking either *YSC84* or *YCL034w* contained a normal cortical actin cytoskeleton organisation. However, cells lacking both *YSC84* and *YCL034w* contained an aberrant cortical actin organisation. In about 70 % of these cells the cortical actin patches were depolarised and found throughout the cortex of mother cells of small-medium budded cells (Figure 5.11b). These cells appeared to contain fewer cortical actin patches than wild type or cells lacking either *YCL034w* or *YSC84*.

5.2.9 Sensitivity to the actin cytoskeleton disrupting drug LAT-A.

LAT-A is a drug that has been previously shown to disrupt the yeast actin cytoskeleton by binding to actin monomers and preventing the assembly step in a rapid cycle of assembly and disassembly (Ayscough *et al*, 1997, Morton *et al*, 2000). Comparing different mutants sensitivity to LAT-A suggests roles for proteins in stabilising or destabilising actin filaments.

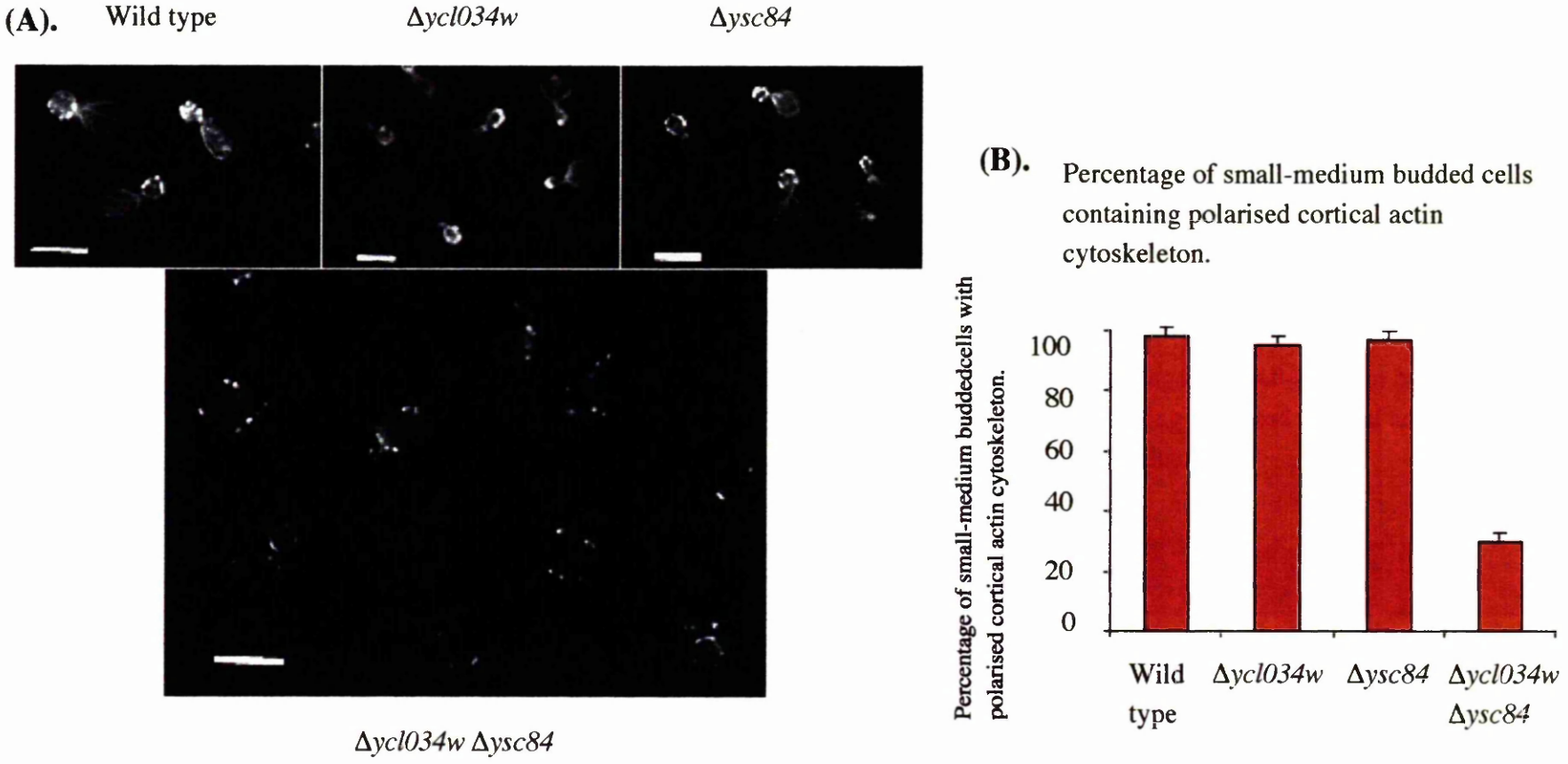


Figure 5.11 Organisation of the cortical actin cytoskeleton. (A) Actively growing wild type (KAY302), Δ*ycl034w* (KAY515), Δ*ysc84* (KAY510) and Δ*ycl034w* Δ*ysc84* (KAY516) cells were fixed and stained with rhodamine phalloidin. Fluorescence microscopy was used to visualise the actin cytoskeleton. (B) Counts of small-medium budded cells containing a polarised cortical actin cytoskeleton. Bar 10 μm.

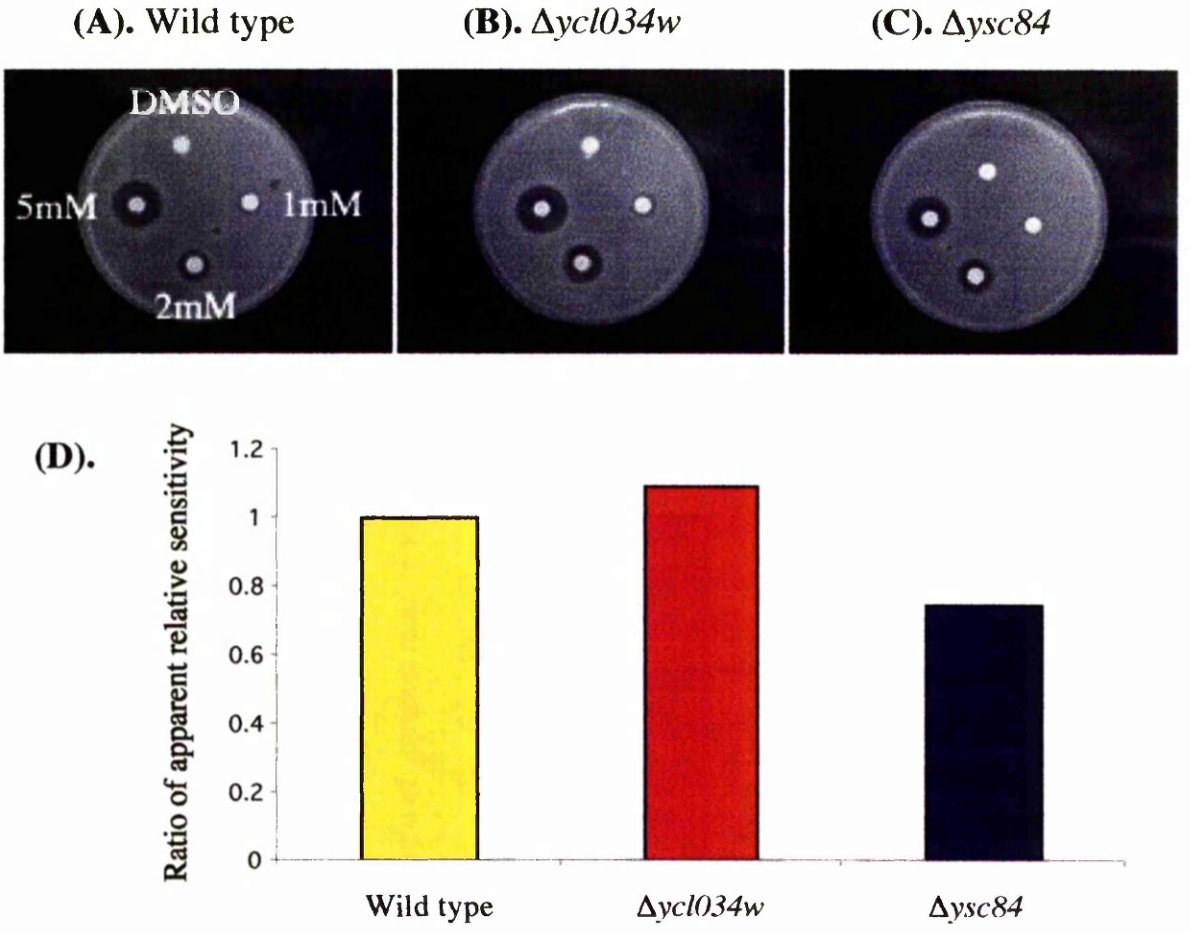


Figure 5.12. Sensitivity of Δ*ycl034w* and Δ*ysc84* cells to LAT-A. Halo assays were used to assess the sensitivity of cells lacking either *YCL034w* or *YSC84*. Concentrations measured 1 mM, 2 mM and 5 mM. (A) Wild type (KAY447), (B) Δ*ycl034w* (KAY479) and (C) Δ*ysc84* (KAY510.1). (D) Comparison of the apparent sensitivity of wild type (KAY302), Δ*ysc84* (KAY510.1) and Δ*ycl034w* (KAY479) as described in Methods and Materials section and Reneke *et al*, 1988).

To assess the effect of Ycl034wp and Ysc84p on actin cytoskeleton dynamics, halo assays were performed on strains lacking genes encoding these proteins (Figure 5.12). Cells lacking *YSC84* are less sensitive to the effects of LAT-A than wild-type cells indicating that the protein encoded by this gene acts to decrease actin filament stability. Cells containing the *YCL034w* deletion displayed a similar sensitivity to the effects of LAT-A as wild-type cells suggesting that Ycl034wp functions do not effect the stability of actin filaments. The sensitivity of the double knock out cells was unable to be determined as these cells grow very slowly at 30°C. After incubating plates for a week at this temperature, a lawn of cells was visible however no halos were observed, probably due to degradation of the LAT-A

5.2.10 Cells lacking both *YCL034w* and *YSC84* display defects in fluid phase endocytosis.

Ycl034wp and Ysc84p have been demonstrated to interact with proteins involved in endocytosis. In this study we have shown that cells lacking *YCL034w* and *YSC84* display an aberrant cortical actin organisation. Previous studies have demonstrated that defects in the cortical actin cytoskeleton often lead to defects in endocytosis.

To examine if cells lacking *YCL034w* and *YSC84* had defects in fluid phase endocytosis, the small fluorescent molecule Lucifer yellow was used (see Methods and Materials; Dulic *et al*, 1991). Lucifer yellow is rapidly endocytosed to the vacuole in wild-type cells (Figure 5.13a). Cells lacking either *YCL034w* or *YSC84* rapidly accumulated Lucifer yellow in their vacuoles. Cells lacking both *YCL034w* and *YSC84* were severely defective in fluid phase endocytosis. In these cells there is an almost complete block in the accumulation of Lucifer yellow, only 6 % of these cells contained Lucifer yellow in the vacuoles, indicating fluid phase endocytosis is severely impaired (Figure 5.13b).

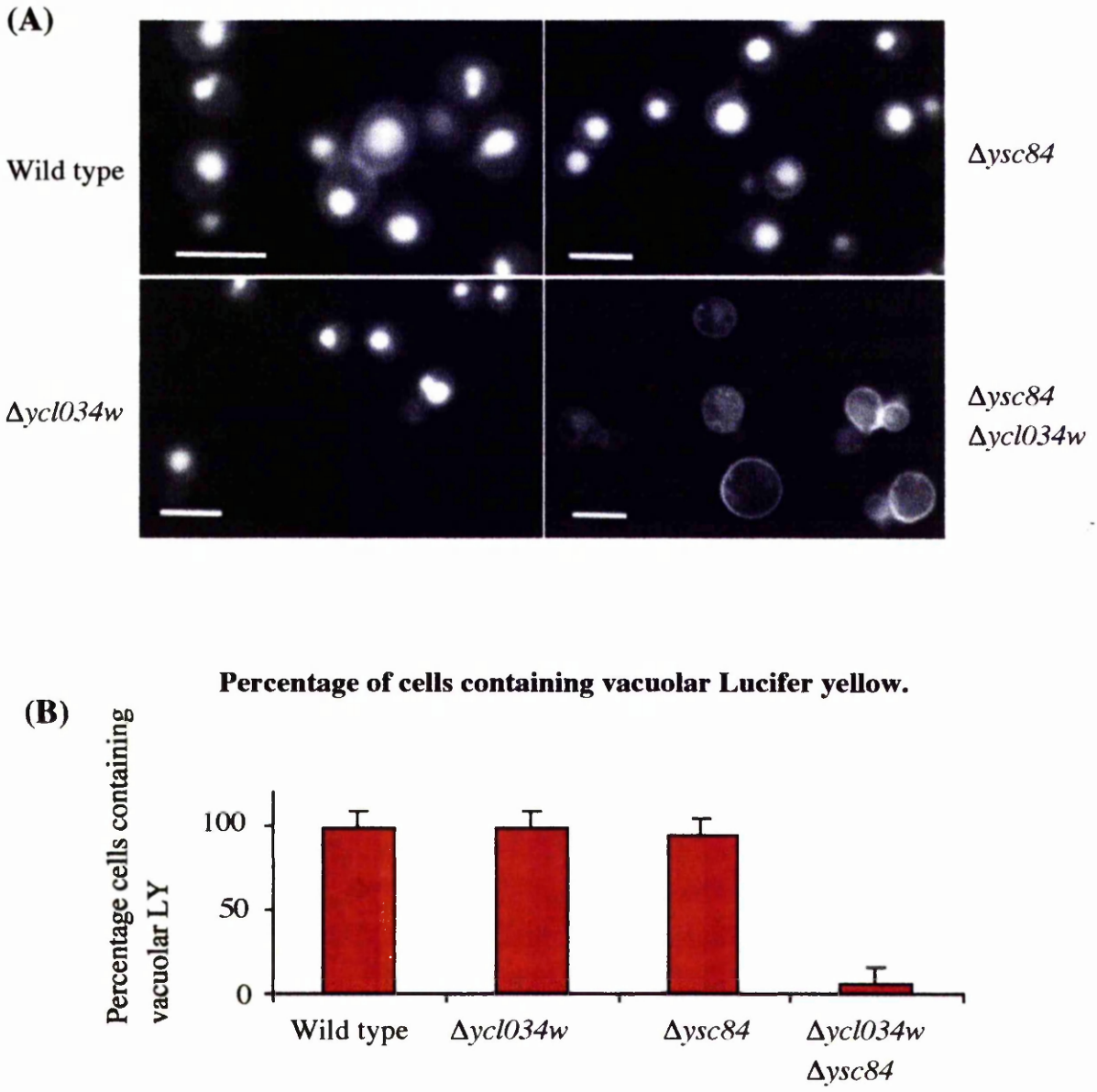


Figure 5.13 Fluid phase endocytosis. (A) Lucifer yellow was used to assess fluid phase endocytosis in wild type (KAY302), Δ*ycl034w* (KAY515), Δ*ysc84* (KAY510) and Δ*ycl034w*Δ*ysc84* (KAY516) cells. Actively growing cells were incubated at room temperature for one hour in the presence of Lucifer yellow before visualisation by fluorescence microscopy. (B) Percentage of cells containing vacuolar Lucifer yellow staining. Bar 10 μm

5.2.11 Characterisation of cells lacking *YCL034w* and either *SLA1* or *ABP1*.

Loss of both *YCL034w* and *YSC84* results in a depolarised cortical actin cytoskeleton organisation and a severe block in endocytosis observed in these cells. To study functions of *YCL034w* further, we decided to study other possible genetic interactions of this gene with genes encoding other proteins involved in actin dynamics. To investigate the possible genetic interactions of *YCL034w*, cells lacking *YCL034w* in combination with either *SLA1* or *ABP1* were constructed. However, neither *Δycl034w* and *Δsla1* or *Δycl034w* and *Δabp1* showed any synthetic lethality. To investigate the effects of these mutations further the phenotypes were analysed.

5.2.12 Organisation of the actin cytoskeleton in cells lacking *YCL034w* in combination with *SLA1* or *ABP1*.

Sla1p is required for the proper organisation of the cortical actin cytoskeleton (Holtzman *et al*, 1993; Ayscough *et al*, 1999) and shares a functional redundancy with Abp1p, loss of both of these proteins from cells is lethal. To observe the effects on cells lacking *YCL034w* in combination with either deletion of *SLA1* or *ABP1* on the organisation of the cortical actin cytoskeleton, rhodamine phalloidin was used (See Materials and Methods; Pringle *et al*, 1989).

Wild type cells contain small punctate cortical actin patches that are highly polarised. As described previously, loss of *ABP1* had little effect on the cortical actin cytoskeleton, whereas loss of *SLA1* results in an aberrant cortical actin cytoskeleton morphology, these cells contain fewer cortical chunks of actin (Holtzman *et al*, 1993; Ayscough *et al*, 1999). Cells lacking both *YCL034w* and *ABP1* displayed a relatively normal cortical actin (Figure

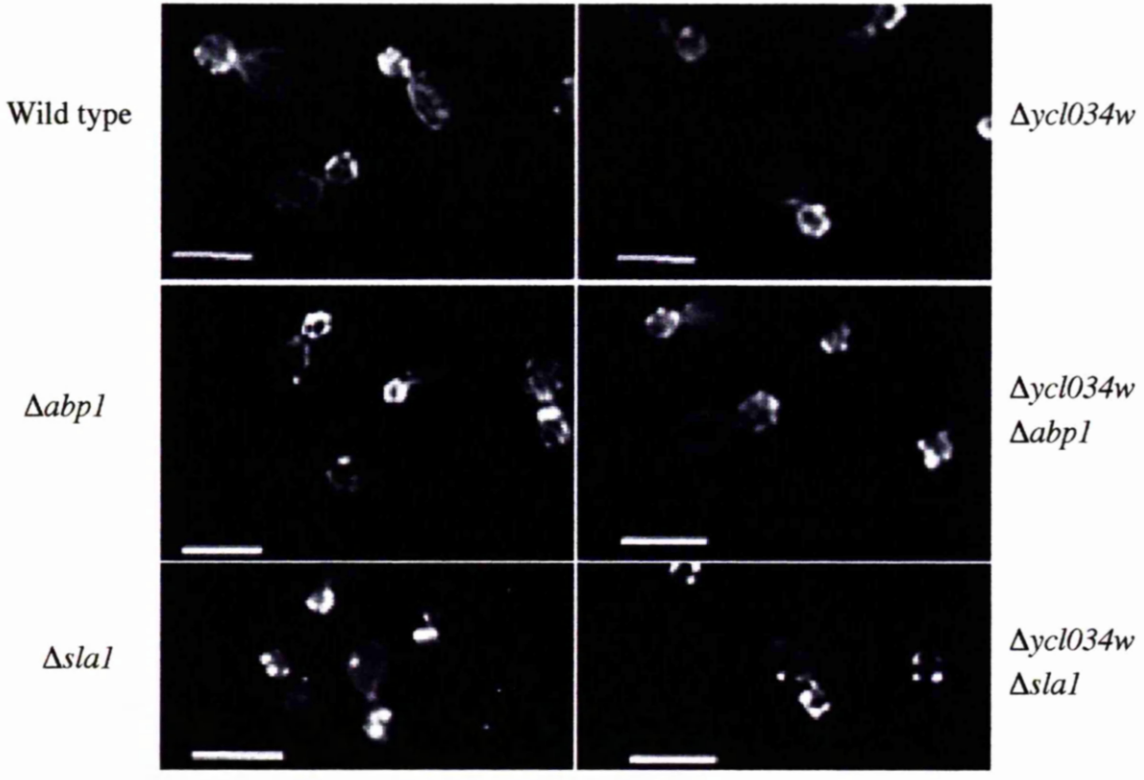


Figure 5.14 Organisation of the cortical actin cytoskeleton in double knock out cells. Actively growing wild type (KAY302), Δ*ycl034w* (KAY465), Δ*abp1* (KAY126), Δ*sla1* (KAY300), Δ*ycl034w*Δ*abp1* (KAY466) and Δ*ycl034w*Δ*sla1* (KAY467) cells were fixed and stained with Rhodamine phalloidin. Fluorescence microscopy was used to visualise the actin cytoskeleton. Bar 10 μm.

5.14) cytoskeleton. Loss of both *YCL034w* and *SLA1* from cells causes an aberrant cortical actin cytoskeleton morphology similar to that of cells lacking just *SLA1*.

5.2.13 Fluid phase endocytosis in cells lacking *YCL034w* in combination with *SLA1* or *ABP1*.

Sla1p contains a multidomain structure and has been shown to interact with the Pan1p/End3p complex to play roles in endocytosis (Tang *et al*, 2000). Cells either lacking *SLA1* or expressing *sla1ΔCt* are defective in fluid phase endocytosis, although there is not a complete block of the process (See Figure , Chapter 1). Abp1p has also been shown to interact with components of the endocytic machinery, such as the yeast homologue of Amphiphysin Rvs167p, in the yeast 2-hybrid system (Lila and Drubin, 1997; Colwill *et al*, 1999; Drees *et al*, 2001).

To investigate the effects of deletion of *YCL034w* and *SLA1* or *YCL034w* and *ABP1* on fluid phase endocytosis, the small fluorescent molecule Lucifer yellow was used (see Materials and Methods; Dulic *et al*, 1991). As reported previously, cells lacking *YSC84* or *ABP1* rapidly accumulated Lucifer yellow in their vacuoles (Hilary Dewar, unpublished data; Wesp *et al*, 1997), whereas cells lacking *SLA1* displayed a partial block in the accumulation of Lucifer yellow in their vacuoles (Chapter 1, Figure1.2.). Cells lacking *YCL034w* and *ABP1* displayed no obvious defect in fluid phase endocytosis (Figure 5.15). Deletion of *YCL034w* and *SLA1* resulted in a partial block in fluid phase endocytosis that appeared very similar to that displayed by *SLA1* null cells. This suggests that Sla1p function lies upstream of Ycl034wp function and that Abp1p plays no role in the functions of Ycl034wp.

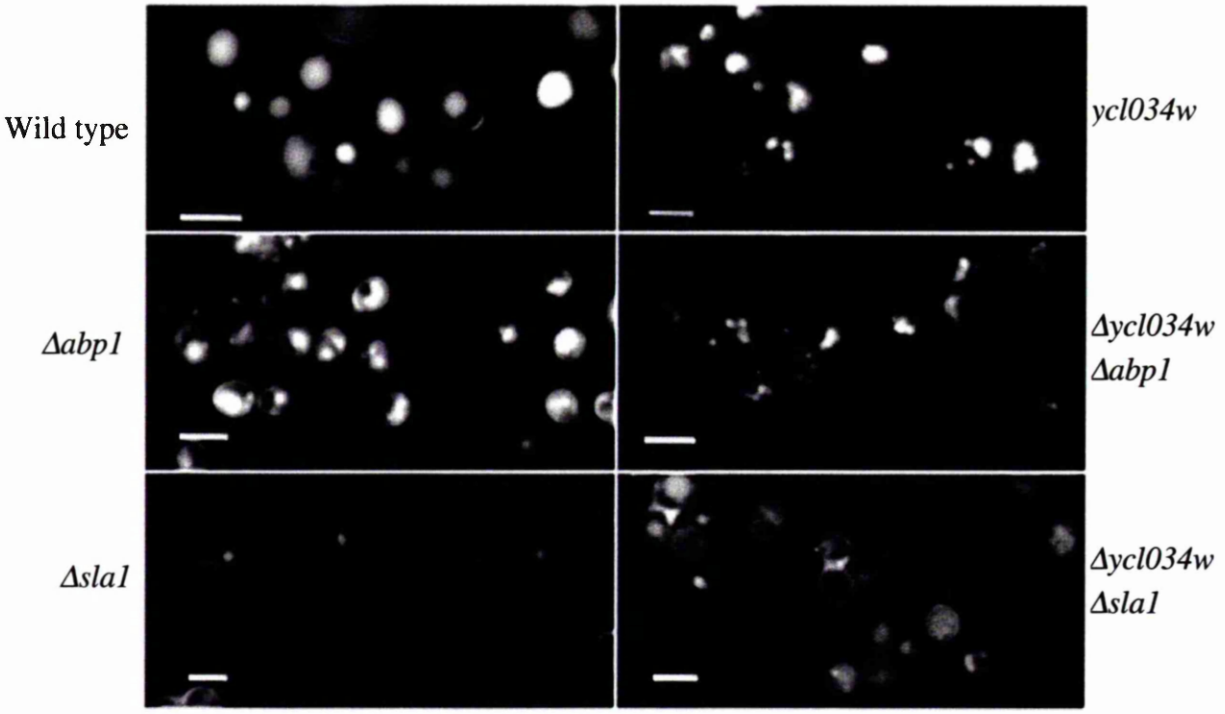


Figure 5.15 Fluid-phase endocytosis in cells lacking *YCL034w* and either *ABP1* or *SLA1*. Actively growing wild type (KAY302), *Δycl034w* (KAY465), *Δabp1* (KAY126), *Δsla1* (KAY300), *Δycl034wΔabp1* (KAY466) and *Δycl034wΔsla1* (KAY467) cells were incubated with Lucifer yellow for one hour at room temperature. Fluorescence microscopy was used to visualise the vacuolar staining. Bar 10 μm

5.3 Discussions.

Sla1p has been shown to interact with a number of different proteins. The GAP1+SH3#3 region has been demonstrated to interact with both Sla2p and Ysc84p in the yeast 2-hybrid system (Hilary Dewar, unpublished data). Studies on the C-terminal repeat region of Sla1p using both yeast 2-hybrid and immunoprecipitation approaches have demonstrated interactions with the Pan1p/End3p complex (Tang *et al*, 2000).

While a lot of data exists on the interactions of the C-terminus of Sla1p, little is known about the central region of Sla1p. This part of the protein contains two regions that show the highest sequence identity to the *S. pombe* Sla1p homologue which may suggest that these regions may have some evolutionary conserved function (Ayscough *et al*, 1999). To shed light on the function of the central region of Sla1p the yeast 2-hybrid system was utilised to identify interacting partners of this region.

The most frequent interacting partner identified was Ycl034wp, a protein that has also been demonstrated to interact with Las17p in the yeast 2-hybrid system (Madania *et al*, 1999). Little is known about the functions of Ycl034wp but database searches identified three distinct domains within the protein, an N-terminal VHS domain, a central GAT domain and a C-terminal NPFxD motif. Another Las17p interacting protein, Ysc84p (Madania *et al*, 1999), was also shown to interact with the central region of Sla1p. This protein has also been demonstrated to interact with the GAP1+SH3#3 region of Sla1p via its C-terminal SH3 domain (Hilary Dewar, unpublished data)..

Sla1p has been shown to interact with Las17p by both GST pull down and immunoprecipitation approaches (Li, 1997; Warren *et al*, in press). Two Las17p

interacting proteins have been demonstrated to interact with adjacent regions of Sla1p, suggesting they are linking Las17p to Sla1p. Sla1p could be acting as an adaptor protein linking the endocytic machinery, via interactions with the Pan1p/End3p complex, to actin dynamics, via interaction with Ycl034wp and Ysc84p. Deletion of either *YCL034w* or *YSC84* had no observable effect on yeast cells. However cells lacking both *YCL034w* and *YSC84* had a depolarised cortical actin cytoskeleton and were defective in fluid phase endocytosis.

Ysc84p has been demonstrated to colocalise with cortical actin patches (Hilary Dewar and Kathryn Ayscough, unpublished data). Charaterisation of cells lacking *YSC84* revealed that these cells are less sensitive to the effects of LAT-A than wild type cells suggesting that Ysc84p decreases actin filament stability. This implies that Ysc84p is involved in processes that require cortical actin patches.

The localisation pattern of Ycl034wp does not resemble that of the cortical actin patches. Deletion of *YCL034w* had no noticable effect upon the yeast cells sensitivity to LAT-A. This suggests that Ycl034wp has no direct role in the functions of cortical actin patches. However, this protein contains three predicted domains that indicate a role for this protein in endocytosis and vesicle trafficking. So far no functional studies have been carried out on these domains but the GAT domain may potentially allow Ycl034wp to interact with vesicular membranes via interactions with the ARF family proteins.

Arfs are members of the Ras-GTPase superfamily that have been implicated in the regulation of vesicle trafficking and the actin cytoskeleton in mammalian cells. Studies carried out using Arf6 have demonstrated that this protein is found at the plasma membrane and its overexpression leads to alterations in the actin cytoskeleton (Radharkrishna *et al*,

1996; D'souza-Schorey *et al*, 1998). More recently an activated form of Arf6 has been shown to induce actin assembly that resulted in the movement of vesicle-like particles (Schafer *et al*, 2000). This particle motility induced by Arf6 was also demonstrated to involve the Arp2/3 complex (Schafer *et al*, 2000). These data indicate a role for Ycl034wp in linking vesicular structures to actin dynamics via interactions with the Arf family proteins and Las17p.

Deletion of *YCL034w* or *YSC84* from cells lead to no obvious phenotype, however, cells lacking both *YCL034w* and *YSC84* display an aberrant cortical actin cytoskeleton morphology and a severe block in fluid phase endocytosis. As these two proteins may potentially have different cellular functions this suggests that multiple stages of the endocytic pathway may be defective in cells lacking both these proteins. These defects could be due to multiple stages of the endocytic pathway becoming uncoupled from proteins involved in actin dynamics.

We also provide evidence that Sla1p function lies upstream of Ycl034wp function as cells lacking both *YCL034w* and *SLA1* display a cortical actin cytoskeleton organisation and endocytic defects that resemble those of *SLA1* null cells. However, Abp1p appears to contain no overlapping functions with Ycl034wp as cells lacking both *YCL034w* and *ABP1* display no obvious phenotypes.

Future work to elucidate further the cellular role of Ycl034wp would include determining whether it is associated with vesicular membranes by cell fractionation experiments. Identifying interacting partners for this protein may also determine the cellular functions of this protein. One candidate interacting protein is the yeast homologue of Arf6 (Arf3p) and may potentially be a candidate for allowing yeast cells to control endocytosis.

Chapter 6.

Final Discussion.

6.1 Aims of this project.

Previous studies have suggested that the C-terminal repeat region of Sla1p is required to rescue the Abp1p-dependent phenotype associated with *SLA1* knockout cells (Ayscough *et al*, 1999). To further our understanding of the roles and interactions of this region, a strain lacking the entire C-terminal repeat encoding sequence was constructed.

It has previously been reported that the C-terminal repeat region of Sla1p contains motifs that are a potential target for the Actin Regulating Kinases (Zeng and Cai, 1999). These kinases may regulate the functions of Slap through phosphorylation of residues within these motifs. This study aimed to determine whether the C-terminal repeat region is phosphorylated and to expand our knowledge of how the functions of Sla1p are regulated *in vivo*.

The final aim of this study was to identify interacting partners of Sla1p, using the yeast 2-hybrid system. Elucidation of Sla1p interacting proteins may allow a better understanding of how Sla1p fulfils its functions at the cell cortex.

6.2 Findings of this study.

Characterisation of the integrated *sla1ΔCt* mutant demonstrated that these cells were viable at high temperatures, displayed a relatively normal actin cytoskeleton organisation and

required expression of *ABP1* for viability. The data presented in Chapter 3 demonstrates that the C-terminal repeat region of Sla1p is required to localise the protein to the cell cortex. This study has also demonstrated, using a temperature sensitive allele of *END3*, that End3p is also required to localise Sla1p to the cells cortex. This is in line with a previous study which demonstrated that Sla1p is able to interact with the Pan1p/End3p complex (Tang *et al*, 2000). Data presented in Chapter 3 also suggests a role of Sla1p in endocytosis. Cells either lacking *SLA1* or expressing the *sla1ΔCt* mutation were partially defective in fluid phase endocytosis. Further analysis of *SLA1* null cells, using radio-labelled alpha factor peptide indicates that these cells are partially defective in the internalisation step of endocytosis.

Chapter 4 is concerned with the possible phosphorylation and regulation of Sla1p. The data presented here demonstrates that Sla1p is phosphorylated *in vivo*. It suggests there are multiple phosphorylation sites both within the C-terminal region and out with this domain. This work also provides evidence suggesting that Sla1p is phosphorylated by the Actin Regulating Kinases and that phosphorylation of Sla1p by these kinases is required for its proper localisation. In combination the *Δark1*, *Δprk1*, and *sla1ΔCt* mutations are synthetically lethal, further indicating the importance of these proteins. Data presented in the final part of this chapter also indicates that Sla1p may potentially be dephosphorylated by the yeast Protein Phosphatase-1 Glc7p. This indicates that the functions of Sla1p may be regulated by cycles of phosphorylation and dephosphorylation.

In this study, the yeast 2-hybrid system has also been utilised to determine novel interacting partners of Sla1p. Originally, this study attempted to identify interacting partners for the C-terminal repeat region of Sla1p. Unfortunately, this proved impossible as this region fused to the *GAL4* binding domain is self-activating in the yeast 2-hybrid system.

The central region of Sla1p contains two regions that share the highest homology between the Sla1p homologues, suggesting that this region may contain some evolutionary function. In Chapter 5, the interacting partners identified in this study for the central region of Sla1p are presented. This screen identified novel interacting partners including Ycl034wp and Ynr065cp as well as previously reported interacting partners such as Ysc84p and Ypr171wp (Drees *et al*).

The remaining part of chapter 5 concentrates upon the cellular roles of Ycl034wp. The data presented here demonstrates that Ycl034wp is localised in small, punctate cortical patches that were found in both the mother cell and the bud. This work also indicates that localisation of Ycl034wp is an actin independent process. This study also demonstrates that cells lacking either Ycl034wp or Ysc84p display no obvious phenotypes. However, cells lacking both Ycl034wp and Ysc84p are temperature sensitive, display an aberrant cortical actin cytoskeleton organisation and have a severe block in fluid phase endocytosis. To further analyse the role of Ycl034wp, mutants lacking both *YCL034w* and *SLA1* or *YCL034w* and *ABP1* were charaterised. Data presented here shows that cells lacking *YCL034w* and *SLA1* display phenotypes similar to *SLA1* null cells whereas cells lacking both *YCL034w* and *ABP1* display no obvious phenotype. This suggests that the function of Sla1p lies upstream to the function of Ycl034wp in yeast cells.

6.3 Sla1p interacts with components of the endocytic machinery.

In these studies we have demonstrated that the C-terminal repeat region of Sla1p is required for its localisation to the cell cortex. Data presented in this work also demonstrates that functional End3p is required for the cortical localisation of Sla1p, suggesting that the

interaction between the C-terminal repeat region of Sla1p and End3p is required to mediate localisation of Sla1p to the cell cortex. Data presented here also shows cells either lacking *SLA1* or expressing *sla1ΔCt* displayed a partial block in fluid phase endocytosis. Further analysis of *Δsla1* cells demonstrated that these cells have a partial block in the internalisation step of endocytosis (Warren *et al*, in press). This supports previous studies suggesting that the Sla1p/End3p/Pan1p complex plays roles in endocytosis (Tang *et al*, 2000).

6.4 Sla1p interacts with proteins that may play roles in vesicle trafficking.

Using the central region of Sla1p as bait, this study identified Ycl034wp and Ynr065cp as proteins that interact with Sla1p. Sequence analysis revealed that Ycl034wp contains a predicted VHS domain at its N-terminus. VHS domains have been identified in a growing number of proteins involved in vesicle trafficking. This domain has an unknown function, however, a number of potential functions have been proposed. The VHS domain of the *Drosophila* HRS protein has been shown to interact with FYVE domains and it has been proposed to be protein-protein interaction module (Mao *et al*, 2000). Another possibility is that VHS domains are involved in membrane binding. Comparison of the structures of the VHS domain from human Tom1 with the ENTH domain of epsin revealed a conserved surface containing a net positive charge that may be able to interact with membranes (Hyman *et al*, 2000; Misra *et al*, 2000). Also present in Ycl034wp is a predicted central GAT domain. Although no functional studies have been carried out on the predicted domains identified in Ycl034wp and the homology shared with other GAT domains is very low, this region may potentially link Ycl034wp to the ARF family proteins. This would implicate Ycl034wp in the process of vesicle trafficking.

Ycl034wp has been reported to interact with Las17p in the yeast 2-hybrid system (Madania *et al*, 1999). This may suggest that Ycl034wp is functioning to link proteins involved in actin dynamics to vesicle transport. Ycl034wp has the potential to interact with GTP-bound ARF family members via the predicted GAT domain. This may allow yeast cells to regulate Las17p and allow actin dynamics to move a vesicle. Studies in mammalian cells support a role for the Arf family proteins in regulating Arp2/3 dependent actin polymerisation (Schafer *et al*, 2000).

This study also identified Ynr065cp as an interacting partner of Sla1p. Although very little is known about this yeast protein, its mammalian homologues (Sortilin-1 and Sorl-1) have been implicated in vesicle trafficking (Jacobsen *et al*, 1996; Petersen *et al*, 1997; 2000). This implicates the central region of Sla1p in the vesicle trafficking. Sla1p may be a potential docking site at the cell cortex for proteins involved in vesicle transport. In agreement with this, vesicles accumulate in *Δsla1* cells (Kathryn Ayscough, unpublished data).

6.5 Sla1p interacts with proteins involved in actin dynamics.

The screen carried out in this study identified Ysc84p as an interacting partner of the central region of Sla1p. Other studies have demonstrated that Ysc84p localises to cortical actin patches and that proper localisation of Ysc84p requires an intact cortical actin cytoskeleton (Hilary Dewar and Kathryn Ayscough, unpublished data). Data presented in Chapter 5 suggests that Ysc84 plays roles in actin dynamics, cells lacking *YSC84* are less sensitivity to the effects of LAT-A, suggesting that Ysc84p decreases actin filament stability. These data indicate that Ysc84p is an actin patch associated protein that may play roles in actin dynamics.

Other work has demonstrated that Sla1p interacts with both Las17p and Abp1p (Li, 1997; Warren *et al*, in press), proteins that have been shown to interact with and stimulate the actin nucleating activity of the Arp2/3p complex (Winter *et al*, 1999; Goode *et al*, 2001). The domains within Sla1p responsible for this interaction have not been elucidated, however, given that cells expressing *SLA1* lacking the GAP1+SH3#3 region display an aberrant actin phenotype (Ayscough *et al*, 1999), it would imply that these proteins interact with Sla1p via this domain.

6.6 A model for Sla1p function in linking actin dynamics to the endocytic machinery and vesicle trafficking.

Data from this study and from other studies have demonstrated that Sla1p interacts with proteins involved in actin dynamics, components of the endocytic machinery and also proteins that may be involved in vesicle transport. The simplest model is that Sla1p is functioning as an adaptor protein that assembles with the endocytic machinery and recruits proteins that are activators of actin dynamic turnover to the proper sites promoting endocytosis and allowing efficient vesicle trafficking to occur (figure 6.1).

In support of this model, Sla1p has been reported to interact with both Las17p (Li, 1997; Warren *et al*, in press) and Abp1p (Warren *et al*, in press). Both of which have been demonstrated to stimulate the activity of the Arp2/3 complex (Winter *et al*, 1999; Goode *et al*, 2001). Pan1p has also been reported to interact with the Arp2/3 complex via an acidic region within its sequence (Duncan *et al*, 2001). Although the authors suggest that Pan1p is also able to stimulate the nucleating activity of the Arp2/3 complex, their data did not show a reduction in the lag phase of actin polymerisation by adding increasing amounts of Pan1p to purified Arp2/3 complex *in vitro*. An alternative model to that suggested by authors is that the interaction between Pan1p and the Arp2/3 complex serves to stabilise the

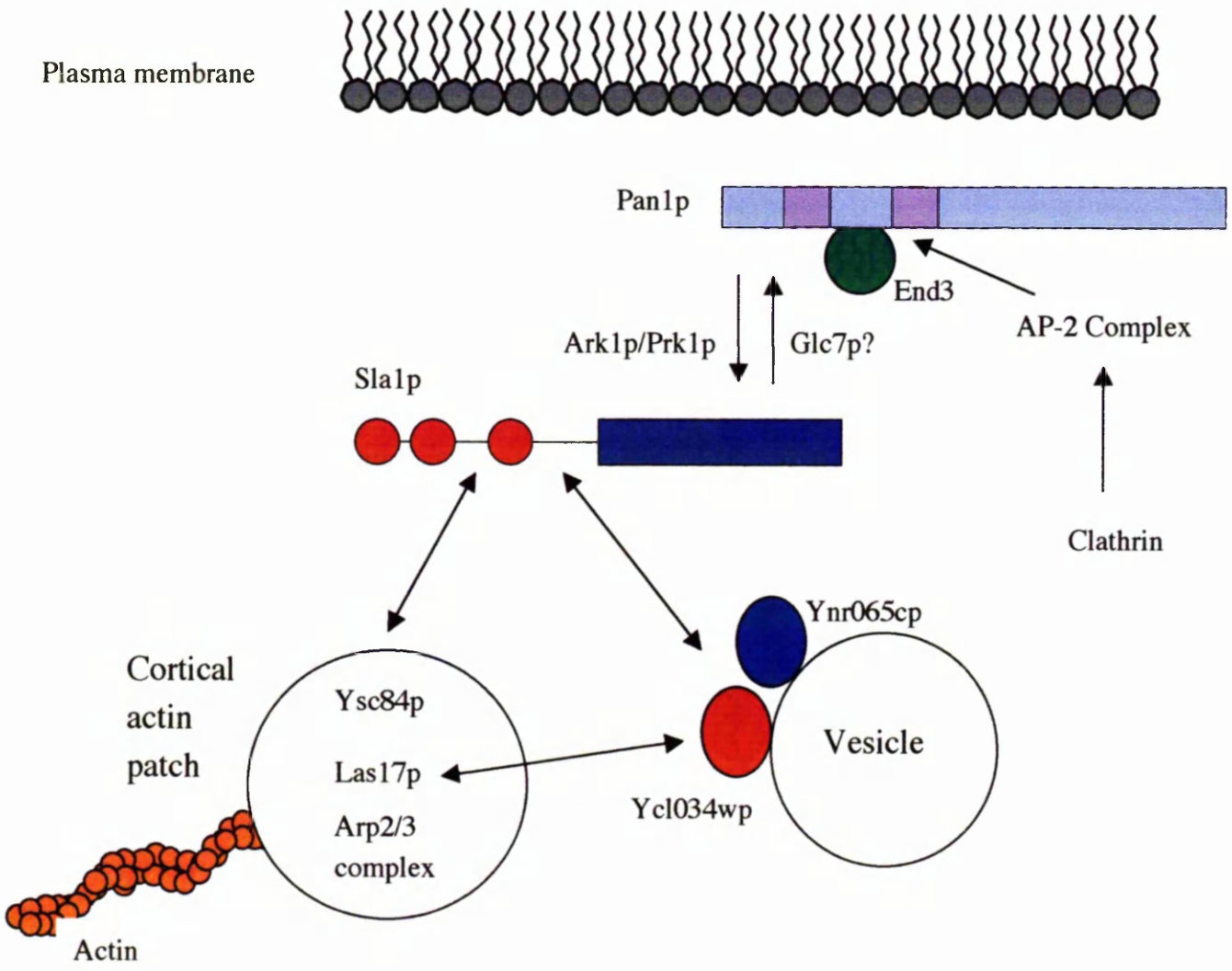


Figure 6.1 Working model of Sla1p linking actin dynamics to endocytic events. Sla1p interacts with the endocytic machinery through its C-terminal repeat region. This interaction is regulated by cycles of phosphorylation and dephosphorylation. Sla1p interacts with Las17p via Ycl034wp and Ysc84p. The Arp2/3 complex has been demonstrated to interact with an acidic region in Pan1p, this may allow this cortical complex to be further stabilised and allow actin dynamics to promote the internalisation step of endocytosis. Our data also suggests that Sla1p interacts with proteins that play roles in vesicle-transport. This suggests that Sla1p is an adaptor protein that allows factors involved in multiple steps of the endocytic pathway to assemble.

whole assembled complex. This may allow Las17p and Abp1p to stimulate the nucleating activity of the Arp2/3 complex at sites that are in close proximity to the endocytic machinery, promoting endocytosis.

In these studies we have demonstrated that central region of Sla1p interacts with Ycl034wp and Ysc84p. Other work has shown that Ysc84p also interacts with the GAP1+SH3#3 region of Sla1p via a C-terminal SH3 domain (Hilary Dewar and Kathryn Ayscough, unpublished data). Both Ycl034wp and Ysc84p have been demonstrated to interact with Las17p in the yeast 2-hybrid system (Madania *et al*, 1999), suggesting that both of these proteins may link Sla1p to actin dynamics via Las17p. However, the data presented here suggests that Ycl034wp and Ysc84p may play roles during different stages of the endocytic process. Ycl034wp may potentially contain domains that allow it to function in vesicle trafficking, whereas Ysc84p may be playing roles in actin dynamics at the cell cortex.

Although Las17p has been demonstrated to interact with other proteins involved in endocytosis, such as Rvs167p, it is not known if these interactions are direct. If Ysc84p and Ycl034wp play roles in different stages endocytosis, they may allow Las17p to be linked to different stages of endocytic pathway. Identifying functions and interacting partners of Ysc84p and Ycl034wp may be useful in furthering our understanding of how these proteins are involved in actin regulation and endocytosis.

6.7 Regulation of the assembly and disassembly of protein complexes at the cell cortex.

Other studies have demonstrated that Sla1p associates with a subset of cortical actin containing patches (Warren *et al*, in press). This is evidence that the distribution of cortical patch associated proteins do not always overlap. Data presented in this work demonstrates

that the cell fractionation patterns of the cortical actin patch proteins Sla1p, Sla2p. Abp1p and End3p were different but overlapping. This is further evidence that cortical actin patch proteins do not exclusively colocalise. These data imply that the assembly/disassembly of complexes at the cell cortex is regulated.

The Actin Regulating Kinase Prk1p has been shown to phosphorylate a region of Pan1p comprised of multiple copies of the LxxQxTG motif (Zeng and Cai, 1999). The C-terminal repeat region of Sla1p contains 5 exact copies of this repeat, but also contains a further 9 copies of the QxTG motif suggesting that Sla1p is a likely target for the Prk1p kinase. Using wild type cells and cells lacking both *ARK1* and *PRK1* we were able to show that Sla1p may be phosphorylated by these Actin Regulating Kinases.

In cells lacking both Ark1p and Prk1p we were able to demonstrate that Sla1p is localised exclusively too the aberrant chunk of actin present in these cells. However, a recent study carried out during the course of this work demonstrated that Sla1p does not exclusively localise to actin patches in wild type cells (Warren *et al*, in press). This suggests that phosphorylation of cortical patch associated proteins leads to the disassembly of cortical Sla1p containing structures. We have also demonstrated the requirement for phosphorylation in the disassembly of Sla1p containing structures. Expression of functional Prk1p, but not kinase dead form of the Prk1p, in cells lacking both *ARK1* and *PRK1* resulted in the disassembly of the large, aberrant actin chunk (Cope *et al*, 2000) and uncoupled the Sla1p patches from actin patches.

There is increasing evidence that phosphorylation is a common regulatory mechanism of the cortical actin patch associated proteins. A recent study has demonstrated that the yeast Epsin homologues, Ent1p and Ent2p, are also targets of the Actin Regulatory Kinases

Ark1p/Prk1p (Watson *et al*, 2001). Both Ent1p and Ent2p are cortical actin patch associated proteins that contain 2 copies of the L/IxxQxTG motif (Watson *et al*, 2001). Sequence analysis has also revealed that both Las17p and Arp2p contain 1 copy of the LxxQxTG motif, suggesting that these proteins are also potentially regulated by the actin regulating kinase Ark1p/Prk1p.

More recently the Actin Regulating Kinases have been shown to interact with the SH3 domain of Abp1p and this interaction is required to localise these kinases to the actin patches (Fazi *et al*, 2001). Abp1p completely colocalises to the cortical actin patches whereas Sla1p localisation only partially overlaps with the actin patches. This led to the hypothesis that Abp1p localises Ark1p/Prk1p to the cortical actin patch, where they can function to disassembly structures via phosphorylation of QxTG motifs.

As phosphorylation appears to be important for the disassembly of cortical complexes then it is likely that these proteins also are dephosphorylated, allowing them to assemble into new complexes. In support of this, both Sla1p and Pan1p have been shown to interact with the protein phosphatase-1 Glc7p in the yeast 2-hybrid system (Tu *et al*, 1996; Venturi *et al*, 2000; Uetz *et al*, 2000). Using wild type and cells expressing the *glc7-13* temperature sensitive allele we were able to demonstrate that Glc7p may dephosphorylate Sla1p.

The data presented in these studies suggest that the cortical patch protein Sla1p undergoes cycles of phosphorylation and dephosphorylation allowing the assembly/disassembly of cortical structures. The identification of different Sla1p and Abp1p containing patch populations at the cell cortex (Warren *et al*, in press) implies that different protein complexes exist at the cells cortex. In support of this, in this study we present data showing

that cortical patch associated proteins display different but overlapping fractionation patterns.

6.8 Linking actin dynamics to the endocytic machinery in higher eukaryotes.

To date no known mammalian homologue of Sla1p has been identified, however, there is increasing evidence for links between actin dynamics and endocytosis in mammalian cells. There is now evidence that the mammalian proteins Intersectin and mAbp1 function to link actin dynamics to endocytosis in a similar manner that we propose for Sla1p. Intersectin-s functions as a scaffold protein in the formation of endocytic vesicles. It contains an EH domain that has been demonstrated to target the protein to clathrin coated pits (Hussain *et al*, 1999) and multiple SH3 domains that recruit dynamin to these endocytic structures (Sengar *et al*, 1999). The neural variant Intersectin-I, an alternatively spliced form of intersectin-s, includes a C-terminus Dbl homology (DH) domain in tandem with a pleckstrin homology (PH) domain. The DH domain has been shown to function as a guanine nucleotide exchange factor for Cdc42p (Hussain *et al*, 2001), a small GTP-binding protein that has been shown to be involved in regulating actin cytoskeleton rearrangements (for review see Nobes and Hall, 1995).

A recent study has demonstrated that stimulation of Cdc42p with Intersectin-I accelerates actin assembly via N-WASP and the Arp2/3 complex. N-WASP binds directly to Intersectin-I and this has been shown to stimulate the GEF activity of Intersectin-I, promoting the formation of GTP bound Cdc42p (Hussain *et al*, 2001). Cdc42p has been demonstrated to be a critical activator of N-WASP. These data suggest that Intersectin-I stimulates actin assembly in an N-WASP dependent manner. Intersectin-I has also been shown to interact with Eps15 (Sengar *et al*, 1999), the mammalian homologue of Pan1p, so

Intersectin is able to interact with both components of the endocytic machinery and proteins involved in actin dynamics.

There are also examples of actin-binding proteins that are able to interact with components of the endocytic machinery, forming a direct link between the actin cytoskeleton and the endocytic machinery. The mammalian homologue of Abp1p (mAbp1) has been shown to interact with and stimulate the nucleating activity of the Arp2/3p complex (Winter *et al*, 1999; Goode *et al*, 2001). mAbp1 has also recently been shown to bind dynamin (Kessels *et al*, 2001), a key GTPase involved in the formation of endocytic vesicles (Sever *et al*, 2000). Work by Kessels and others has also demonstrated that mAbp1 is enriched at the sites of endocytosis (Kessels *et al*, 2001).

This model proposed for the function of Intersectin-I is similar to the model we propose for the function of Sla1p. Both proteins are proposed to interact with the endocytic machinery via Pan1p/Eps15p (Sengar *et al*, 1999; Tang *et al*, 2000). In mammalian cells Eps15 has been shown to interact with the AP-2 complex and is necessary for the formation of clathrin coated pits (Benmerah *et al*, 1995; 1998; 1999; Delft *et al*, 1997). Both Sla1p and Intersectin have been shown to interact with proteins involved in actin dynamics through Las17p/N-WASP, which have in turn been shown to interact with and stimulate the nucleating activity of the Arp2/3 complex (Madania *et al*, 1999; Winter *et al*, 1999; Machesky and Insall, 1998; Yarar *et al*, 1999).

The models differ in the requirement for GTP-bound Cdc42p, the critical activator of N-WASP. Las17p does not possess a GTPase-binding domain (Symons *et al*, 1996) indicating that is a fundamental difference between yeast and higher eukaryotes. This could reflect differences in how the formation of these complexes is controlled between yeast and

higher eukaryotes. We propose that in yeast, Sla1p undergoes cycles of phosphorylation and dephosphorylation allowing cells to spatially and temporally regulate these processes. In mammalian cells, these processes may be regulated by the availability of N-WASP or its ability to interact with Intersectin-I and stimulate its GEF activity.

6.9 Summary and future directions.

In summary, the data presented here indicates that in wild type cells the interaction between Sla1p and End3p is required to localise Sla1p to the cells cortex. Sla1p is required for efficient endocytosis and may function to bring proteins involved in actin dynamics into close proximity to the endocytic machinery and to proteins involved in vesicle trafficking. The assembly of these proteins into a complex that promotes endocytosis is regulated by cycles of phosphorylation and dephosphorylation mediated by the actin regulating kinases and protein phosphatase-1 (Figure 6.1). We propose that Sla1p functions to localise actin dynamics to the sites of endocytosis but its function is regulated within cells allowing both spatial and temporal control of its function (Figure 6.1).

Future directions would include further analysis of how these complexes are controlled. For example, determining whether Ycl034wp is membrane associated when GTP-Arf family proteins are available. This study also provides evidence for the phosphorylation of Sla1p at sites outside the C-terminal repeat region. Sequence analysis has identified potential phosphorylation sites for both protein kinase C and cAMP dependent protein kinase within Sla1p. These sites may regulate the links between Sla1p and proteins involved in actin dynamics and vesicle trafficking. Elucidation of how Sla1p and its interacting partners are regulated will be of value in furthering our understanding of the relationship between these essential cellular functions.

Chapter 7.

References Used

1. Adams,A.E., D.Botstein, and D.G.Drubin. 1991. Requirement of yeast fimbrin for actin organization and morphogenesis in vivo. *Nature* 354:404-408.

2. Adams AE, Johnson DI, Longnecker RM, Sloat BF, Pringle JR. 1990. CDC42 and CDC43, two additional genes involved in budding and the establishment of cell polarity in the yeast *Saccharomyces cerevisiae. J. Cell Biol.* 111: 131-142.

3. Adams AE, Pringle JR. 1991. Staining of actin with fluorochrome-conjugated phalloidin. *Methods Enzymol.* 194: 729-731.

4. Amatruda JF, Cooper JA. 1992. Nucleotide, Protein Purification, characterization, and immunofluorescence localization of *Saccharomyces cerevisiae* capping protein. *J. Cell Biol.* 117:1067-76.

5. Ayscough,K.R. 1998. In vivo functions of actin-binding proteins. *Curr. Opin. Cell Biol.* 10:102-111.

6. Ayscough,K.R., J.J.Eby, T.Lila, H.Dewar, K.G.Kozminski, and D.G.Drubin. 1999. Sla1p is a functionally modular component of the yeast cortical actin cytoskeleton required for correct localization of both Rho1p-GTPase and Sla2p, a protein with talin homology. *Mol. Biol. Cell* 10:1061-1075.

7. Ayscough,K.R., J.Stryker, N.Pokala, M.Sanders, P.Crews, and D.G.Drubin. 1997. High rates of actin filament turnover in budding yeast and roles for actin in establishment and maintenance of cell polarity revealed using the actin inhibitor latrunculin-A. *J. Cell Biol.* 137:399-416.

8. Belmont,L.D. and D.G.Drubin. 1998. The yeast V159N actin mutant reveals roles for actin dynamics in vivo. *J. Cell Biol.* 142:1289-1299.

9. Belmont,L.D., G.M.Patterson, and D.G.Drubin. 1999. New actin mutants allow further characterization of the nucleotide binding cleft and drug binding sites. *J. Cell Sci.* 112: 1325-1336.

10. Benedetti,H., S.Raths, F.Crausaz, and H.Riezman. 1994. The END3 gene encodes a protein that is required for the internalization step of endocytosis and for actin cytoskeleton organization in yeast. *Mol. Biol. Cell* 5:1023-1037.

11. Benmerah,A., M.Bayrou, N.Cerf-Bensussan, and A.Dautry-Varsat. 1999. Inhibition of clathrin-coated pit assembly by an Eps15 mutant. *J. Cell Sci.* 112 (Pt 9):1303 1311.

12. Benmerah,A., J.Gagnon, B.Begue, B.Megarbane, A.Dautry-Varsat, and N.Cerf Bensussan. 1995. The tyrosine kinase substrate eps15 is constitutively associated with the plasma membrane adaptor AP-2. *J. Cell Biol.* 131:1831-1838.

13. Benmerah,A., C.Lamaze, B.Begue, S.L.Schmid, A.Dautry-Varsat, and N.Cerf Bensussan. 1998. AP-2/Eps15 interaction is required for receptor-mediated endocytosis. *J. Cell Biol.* 140:1055-1062.

14. Bloecher,A. and K.Tatchell. 2000. Dynamic localization of protein phosphatase type 1 in the mitotic cell cycle of Saccharomyces cerevisiae. *J. Cell Biol.* 149:125 140.

15. Brenner,S.L. and E.D.Korn. 1980. The effects of cytochalasins on actin polymerization and actin ATPase provide insights into the mechanism of polymerization. *J. Biol. Chem.* 255:841-844.

16. Brown SS. Myosins in yeast.1997. *Curr. Opin. Cell Biol.* 9: 44-48.

17. Brown,S.S. and J.A.Spudich. 1979. Cytochalasin inhibits the rate of elongation of actin filament fragments. *J. Cell Biol.* 83:657-662.

18. Carlier,M.F., V.Laurent, J.Santolini, R.Melki, D.Didry, G.X.Xia, Y.Hong, N.H.Chua, and D.Pantaloni. 1997. Actin depolymerizing factor (ADF/cofilin) enhances the rate of filament turnover: implication in actin-based motility. *J. Cell Biol.* 136:1307-1322.

19. Carlsson,L., L.E.Nystrom, I.Sundkvist, F.Markey, and U.Lindberg. 1977. Actin polymerizability is influenced by profilin, a low molecular weight protein in non-muscle cells. *J. Mol. Biol.* 115:465-483.

20. Christensen HE, Ramachandran S, Tan CT, Surana U, Dong CH, Chua NH. 1996. Arabidopsis profilins are functionally similar to yeast profilins: identification of a vascular bundle-specific profilin and a pollen-specific profilin. *Plant J.* 10: 269-279.

21. Cooper JA. 1987. Effects of cytochalasin and phalloidin on actin. *J. Cell Biol.* 105: 1473-1478.

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

23. Davison,M.D. and D.R.Critchley. 1988. alpha-Actinins and the DMD protein contain spectrin-like repeats. *Cell* 52:159-160.

24. Drees B, Brown C, Barrell BG, Bretscher A. 1995. Tropomyosin is essential in yeast, yet the TPM1 and TPM2 products perform distinct functions. *J. Cell Biol.* 128: 383-392.

25. Drees,B.L., B.Sundin, E.Brazeau, J.P.Caviston, G.C.Chen, W.Guo, K.G.Kozminski, M.W.Lau, J.J.Moskow, A.Tong, L.R.Schenkman, A.McKenzie, III, P.Brennwald, M.Longtine, E.Bi, C.Chan, P.Novick, C.Boone, J.R.Pringle, T.N.Davis, S.Fields, and D.G.Drubin. 2001. A protein interaction map for cell polarity development. *J. Cell Biol.* 154:549-571.

26. Drgonova,J., T.Drgon, K.Tanaka, R.Kollar, G.C.Chen, R.A.Ford, C.S.Chan, Y.Takai, and E.Cabib. 1996. Rho1p, a yeast protein at the interface between cell polarization and morphogenesis. *Science* 272:277-279.

27. D'Souza-Schorey C, van Donselaar E, Hsu VW, Yang C, Stahl PD, Peters PJ. 1998. ARF6 targets recycling vesicles to the plasma membrane: insights from an ultrastructural investigation. *J. Cell Biol.* 140: 603-616.

28. Dulic,V., M.Egerton, I.Elguindi, S.Raths, B.Singer, and H.Riezman. 1991. Yeast endocytosis assays. *Methods Enzymol.* 194:697-710.

29. Duncan,M.C., M.J.Cope, B.L.Goode, B.Wendland, and D.G.Drubin.2001. Yeast Eps15-like endocytic protein, Pan1p, activates the Arp2/3 complex. *Nat. Cell Biol.* 3:687-690.

30. Drubin,D.G. 1988. Yeast actin-binding proteins: evidence for a role in morphogenesis. *J. Cell Biol.* 107:2551-2561

31. Engqvist-Goldstein,A.E., M.M.Kessels, V.S.Chopra, M.R.Hayden, and D.G.Drubin. 1999. An actin-binding protein of the Sla2/Huntingtin interacting protein 1 family is a novel component of clathrin-coated pits and vesicles. *J. Cell Biol.* 147:1503-1518.

32. Estes,J.E., L.A.Selden, and L.C.Gershman. 1981. Mechanism of action of phalloidin on the polymerization of muscle actin. *Biochemistry* 20:708-712.

33. Evangelista M, Klebl BM, Tong AH, Webb BA, Leeuw T, Leberer E, Whiteway M, Thomas DY, Boone C. 2000. A role for myosin-I in actin assembly through interactions with Vrp1p, Bee1p, and the Arp2/3 complex. *J. Cell Biol.* 148:353-362.

34. Faulstich,H., A.J.Schafer, and M.Weckauf. 1977. The dissociation of the phalloidin actin complex. *Hoppe Seylers. Z. Physiol Chem.* 358:181-184.

35. Fazi B, Cope MJ, Douangamath A, Ferracuti S, Schirwitz K, Zucconi A, Drubin D, Willmanns M, Cesareni G, Castagnoli L. 2001. Unusual binding properties of the SH3 domain of the yeast actin binding protein Abp1: Structural and functional analysis. *J. Biol. Chem.* epub ahead of print

36. Flanagan,M.D. and S.Lin. 1980. Cytochalasins block actin filament elongation by binding to high affinity sites associated with F-actin. *J. Biol. Chem.* 255:835-838.

37. Gaidarov,I. and J.H.Keen. 1999. Phosphoinositide-AP-2 interactions required for targeting to plasma membrane clathrin-coated pits. *J. Cell Biol.* 146:755-764.

38. Goldschmidt-Clermont,P.J., M.I.Furman, D.Wachsstock, D.Safer, V.T.Nachmias, and T.D.Pollard. 1992. The control of actin nucleotide exchange by thymosin beta 4 and profilin. A potential regulatory mechanism for actin polymerization in cells. *Mol. Biol. Cell* 3:1015-1024.

39. Goode,B.L., A.A.Rodal, G.Barnes, and D.G.Drubin. 2001. Activation of the Arp2/3 complex by the actin filament binding protein Abp1p. *J. Cell Biol.* 153:627-634.

40. Goodson HV, Anderson BL, Warrick HM, Pon LA, Spudich JA. 1996. Protein Synthetic lethality screen identifies a novel yeast myosin I gene (MYO5): myosin I proteins are required for polarization of the actin cytoskeleton. *J. Cell Biol.* 133:1277-1291.

41. Gourlay CW, Dewar H, Warren DT, and Ayscough KA. Actin dynamics and endocytosis in budding yeast are regulated by an interaction between Sla1p and Sla2p. *J. Cell Biol.* Submitted

42. Haarer BK, Lillie SH, Adams AE, Magdolen V, Bandlow W, Brown SS 1990. Purification of profilin from *Saccharomyces cerevisiae* and analysis of profilin deficient cells. *J. Cell Biol.* 110:105-114.

43. Haarer BK, Petzold AS, Brown SS. 1993. Mutational analysis of yeast profilin. *Mol. Cell Biol.* 13: 7864-7873.

44. Hirst, J., Lui, W. W. Y., Bright, N. A., Totty, N., Seaman, M. N. J., and Robinson, M. S. 2000. A family of proteins with gamma-adaptin and VHS domains that facilitate trafficking between the trans-Golgi network and the vacuole/lysosome. *J. Cell Biol.* 149: 67-79.

45. Holmes,K.C., Popp,D., Gebhard,W. and Kabsch,W. 1990. Atomic model of the actin filament. *Nature* 347:44-49.

46. Holtzman,D.A., S.Yang, and D.G.Drubin. 1993. Synthetic-lethal interactions identify two novel genes, SLA1 and SLA2, that control membrane cytoskeleton assembly in Saccharomyces cerevisiae. *J. Cell Biol.* 122:635-644.

47. Honts JE, Sandrock TS, Brower SM, O'Dell JL, Adams AE.1994. Actin mutations that show suppression with fimbrin mutations identify a likely fimbrin-binding site on actin. *J. Cell Biol.* 126: 413-422.

48. Hussain,N.K., S.Jenna, M.Glogauer, C.C.Quinn, S.Wasiak, M.Guipponi, S.E.Antonarakis, B.K.Kay, T.P.Stossel, N.Lamarche-Vane, and P.S.McPherson. 2001. Endocytic protein intersectin-l regulates actin assembly via Cdc42 and N WASP. *Nat. Cell Biol.* 3:927-932.

49. Hussain,N.K., M.Yamabhai, A.R.Ramjaun, A.M.Guy, D.Baranes, J.P.O'Bryan, C.J.Der, B.K.Kay, and P.S.McPherson. 1999. Splice variants of intersectin are components of the endocytic machinery in neurons and nonneuronal cells. *J. Biol. Chem.* 274:15671-15677.

50. Hyman,J., H.Chen, P.P.Di Fiore, P.De Camilli, and A.T.Brunger. 2000. Epsin 1 undergoes nucleocytosolic shuttling and its eps15 interactor NH(2)-terminal homology (ENTH) domain, structurally similar to Armadillo and HEAT repeats, interacts with the transcription factor promyelocytic leukemia Zn(2)+ finger protein (PLZF). *J. Cell Biol.* 149:537-546.

51. Iida K, Moriyama K, Matsumoto S, Kawasaki H, Nishida E, Yahara I. 1993. Isolation of a yeast essential gene, COF1, that encodes a homologue of mammalian cofilin, a low-M(r) actin-binding and depolymerizing protein. *Gene*. 124: 115-120.

52. Ito,T., T.Chiba, R.Ozawa, M.Yoshida, M.Hattori, and Y.Sakaki. 2001. A comprehensive two-hybrid analysis to explore the yeast protein interactome *Proc. Natl. Acad. Sci. U. S. A* 98:4569-4574.

53. Jacobsen,L., P.Madsen, C.Jacobsen, M.S.Nielsen, J.Gliemann, and C.M.Petersen. 2001. Activation and functional characterization of the mosaic receptor SorLA/LR11. *J. Biol. Chem.* 276:22788-22796.

54. James,P., J.Halladay, and E.A.Craig. 1996. Genomic libraries and a host strain designed for highly efficient two- hybrid selection in yeast. *Genetics* 144:1425 1436.

55. Kabsch,W., Mannherz,H.G., Suck,D., Pai,E.F. and Holmes,K.C. 1990. Atomic structure of the actin: DNase I complex. *Nature* 347:37-44

56. Kang,F., D.L.Purich, and F.S.Southwick. 1999. Profilin promotes barbed-end actin filament assembly without lowering the critical concentration. *J. Biol. Chem.* 274:36963-36972.

57. Karpova,T.S., J.G.McNally, S.L.Moltz, and J.A.Cooper. 1998. Assembly and function of the actin cytoskeleton of yeast: relationships between cables and patches. *J. Cell Biol.* 142:1501-1517.

58. Karpova,T.S., K.Tatchell, and J.A.Cooper. 1995. Actin filaments in yeast are unstable in the absence of capping protein or fimbrin. *J. Cell Biol.* 131:1483-1493.

59. Kelleher,J.F., S.J.Atkinson, and T.D.Pollard. 1995. Sequences, structural models, and cellular localization of the actin- related proteins Arp2 and Arp3 from Acanthamoeba. *J. Cell Biol.* 131:385-397.

60. Kolluri,R., K.F.Tolias, C.L.Carpenter, F.S.Rosen, and T.Kirchhausen. 1996. Direct interaction of the Wiskott-Aldrich syndrome protein with the GTPase Cdc42. *Proc. Natl. Acad. Sci. U. S. A* 93:5615-5618.

61. Korn,E.D. 1982. Actin polymerization and its regulation by proteins from nonmuscle cells. *Physiol Rev.* 62:672-737.

62. Kubler,E. and Riezman,H. 1993. Actin and fimbrin are required for the internalisation step of endocytosis in yeast. *EMBO J.* 12:2855-2862.

63. Lamaze,C., L.M.Fujimoto, H.L.Yin, and S.L.Schmid. 1997b. The actin cytoskeleton is required for receptor-mediated endocytosis in mammalian cells. *J. Biol. Chem.* 272:20332-20335.

64. Lamaze,C., L.M.Fujimoto, H.L.Yin, and S.L.Schmid. 1997a. The actin cytoskeleton is required for receptor-mediated endocytosis in mammalian cells. *J. Biol. Chem.* 272:20332-20335.

65. Lappalainen,P. and D.G.Drubin. 1997. Cofilin promotes rapid actin filament turnover in vivo. *Nature* 388:78-82.

66. Lappalainen,P., E.V.Fedorov, A.A.Fedorov, S.C.Almo, and D.G.Drubin. 1997. Essential functions and actin-binding surfaces of yeast cofilin revealed by systematic mutagenesis. *EMBO J.* 16:5520-5530.

67. Lassing,L. and Lindberg,U. 1988. Specificity of the interaction between phosphatidylinositol 4,5-bisphosphate and the profilin: actin complex. *J. cell Biochem.* 37:255-267.

68. Lechler T, Shevchenko A, Li R. 2000. Direct involvement of yeast type I myosins in Cdc42-dependent actin polymerization. *J. Cell Biol.* 2000 148: 363-373.

69. Li,R. 1997. Bee1, a yeast protein with homology to Wiscott-Aldrich syndrome protein, is critical for the assembly of cortical actin cytoskeleton. *J. Cell Biol.* 136:649-658.

70. Lippincott J, Li R. 1998. Sequential assembly of myosin II, an IQGAP-like protein, and filamentous actin to a ring structure involved in budding yeast cytokinesis. *J. Cell Biol.* 140: 355-366.

71. Long, R. M., Singer, R. H., Meng, X., Gonzalex, I., Nasmyth, K., and Jansen, R. P. 1997. Mating type switching in yeast controlled by asymmetric localization of ASH1 mRNA. *Science.* 277: 383-387.

72. Longtine MS, McKenzie A 3rd, Demarini DJ, Shah NG, Wach A, Brachat A, Philippsen P, Pringle JR. 1998. Additional modules for versatile and economical PCR-based gene deletion and modification in *Saccharomyces cerevisiae. Yeast.* 14: 953-961.

73. Lorenz,M., K.J.Poole, D.Popp, G.Rosenbaum, and K.C.Holmes. 1995. An atomic model of the unregulated thin filament obtained by X-ray fiber diffraction on oriented actin-tropomyosin gels. *J. Mol. Biol.* 246:108-119.

74. MacDiarmid,C.W., L.A.Gaither, and D.Eide. 2000. Zinc transporters that regulate vacuolar zinc storage in Saccharomyces cerevisiae. *EMBO J.* 19:2845-2855.

75. Machesky,L.M. and K.L.Gould. 1999. The Arp2/3 complex: a multifunctional actin organizer. *Curr. Opin. Cell Biol.* 11:117-121.

76. Machesky,L.M. and R.H.Insall. 1999. Signaling to actin dynamics. *J. Cell Biol.* 146:267-272.

77. Machesky,L.M., R.D.Mullins, H.N.Higgs, D.A.Kaiser, L.Blanchoin, R.C.May, M.E.Hall, and T.D.Pollard. 1999. Scar, a WASp-related protein, activates nucleation of actin filaments by the Arp2/3 complex. *Proc. Natl. Acad. Sci. U. S. A* 96:3739-3744.

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

79. Mazzoni, C., Zarov, P., Rambourg, A., and Mann, C. 1993. The SLT2 (MPK1) MAP kinase homolog is involved in polarized cell growth in *Saccharomyces cerevisiae. J. Cell Biol.* 123: 1821-1833

80. Mao,Y., A.Nickitenko, X.Duan, T.E.Lloyd, M.N.Wu, H.Bellen, and F.A.Quiocho. 2000. Crystal structure of the VHS and FYVE tandem domains of Hrs, a protein involved in membrane trafficking and signal transduction. *Cell* 100:447-456.

81. McCann,R.O. and S.W.Craig. 1997. The I/LWEQ module: a conserved sequence that signifies F-actin binding in functionally diverse proteins from yeast to mammals. *Proc. Natl. Acad. Sci. U. S. A* 94:5679-5684.

82. McLaughlin,P.J., J.T.Gooch, H.G.Mannherz, and A.G.Weeds. 1993. Structure of gelsolin segment 1-actin complex and the mechanism of filament severing. *Nature* 364:685-692.

83. Merrifield,C.J., S.E.Moss, C.Ballestrem, B.A.Imhof, G.Giese, I.Wunderlich, and W.Almers. 1999. Endocytic vesicles move at the tips of actin tails in cultured mast cells. *Nat. Cell Biol.* 1:72-74.

84. Miki,H. 1997. Signal transduction through SH2, SH3 and PH domains. *Tanpakushitsu Kakusan Koso* 42:1484-1493.

85. Miki,H. and T.Takenawa. 1998. Direct binding of the verprolin-homology domain in N-WASP to actin is essential for cytoskeletal reorganization. *Biochem. Biophys. Res. Commun.* 243:73-78.

86. Misra,S., B.M.Beach, and J.H.Hurley. 2000. Structure of the VHS domain of human Tom1 (target of myb 1): insights into interactions with proteins and membranes. *Biochemistry* 39:11282-11290.

87. Mockrin,S.C. and E.D.Korn. 1980. Acanthamoeba profilin interacts with G-actin to increase the rate of exchange of actin-bound adenosine 5'-triphosphate. *Biochemistry* 19:5359-5362.

88. Moon,A.L., P.A.Janmey, K.A.Louie, and D.G.Drubin. 1993. Cofilin is an essential component of the yeast cortical cytoskeleton. *J. Cell Biol.* 120:421-435.

89. Morton,W.M., K.R.Ayscough, and P.J.McLaughlin. 2000. Latrunculin alters the actin-monomer subunit interface to prevent polymerization. *Nat. Cell Biol.* 2:376 378.

90. Mullins,R.D., J.A.Heuser, and T.D.Pollard. 1998. The interaction of Arp2/3 complex with actin: nucleation, high affinity pointed end capping, and formation of branching networks of filaments. *Proc. Natl. Acad. Sci. U. S. A* 95:6181-6186.

91. Mullins,R.D., J.F.Kelleher, J.Xu, and T.D.Pollard. 1998. Arp2/3 complex from Acanthamoeba binds profilin and cross-links actin filaments. *Mol. Biol. Cell* 9:841 852.

92. Nobes,C.D. and A.Hall. 1995. Rho, rac and cdc42 GTPases: regulators of actin structures, cell adhesion and motility. *Biochem. Soc. Trans.* 23:456-459.

93. Novick,P. and D.Botstein. 1985. Phenotypic analysis of temperature-sensitive yeast actin mutants. *Cell* 40:405-416.

94. Olah ME, Gallo-Rodriguez C, Jacobson KA, Stiles GL. 1994. ^{125}I-4-aminobenzyl-5' N-methylcarboxamidoadenosine, a high affinity radioligand for the rat A_3 adenosine receptor.*Mol. Pharmacol.* 45: 978-982

95. Orlova,A., Galkin,V.E., VanLoock,M.S., Kim,E., Shvetsov,A., Reisler,E. and Egelman,E.H. 2001. Probing the structure of F-actin: Cross links constrain atomic models and modify actin dynamics. *J. Mol. Biol.* 312:95-106.

96. Pantaloni,D. and M.F.Carlier. 1993. How profilin promotes actin filament assembly in the presence of thymosin beta 4. *Cell* 75:1007-1014.

97. Petersen,C.M., M.S.Nielsen, A.Nykjaer, L.Jacobsen, N.Tommerup, H.H.Rasmussen, H.Roigaard, J.Gliemann, P.Madsen, and S.K.Moestrup. 1997. Molecular identification of a novel candidate sorting receptor purified from human brain by receptor-associated protein affinity chromatography. *J. Biol. Chem.* 272:3599-3605.

98. Piper, R. C., Cooper, A. A., Yang, H., and Stevens, T. H. 1995. VPS27 controls vacuolar and endocytic traffic through a prevacuolar compartment in *Saccharomyces cerevisiae. J. Cell Biol.* 131: 603-617.

99. Pringle,J.R., R.A.Preston, A.E.Adams, T.Stearns, D.G.Drubin, B.K.Haarer, and E.W.Jones. 1989. Fluorescence microscopy methods for yeast. *Methods Cell Biol.* 31:357-435.

100. Pruyne,D. and A.Bretscher. 2000. Polarization of cell growth in yeast. *J. Cell Sci.* 113: 571-585.

101. Pucharcos,C., X.Estivill, and L.S.de la. 2000. Intersectin 2, a new multimodular protein involved in clathrin-mediated endocytosis. *FEBS Lett.* 478: 43-51.

102. Qadota,H., C.P.Python, S.B.Inoue, M.Arisawa, Y.Anraku, Y.Zheng, T.Watanabe, D.E.Levin, and Y.Ohya. 1996. Identification of yeast Rho1p GTPase as a regulatory subunit of 1,3- beta-glucan synthase. *Science* 272: 279-281.

103. Qualmann,B., M.M.Kessels, and R.B.Kelly. 2000. Molecular links between endocytosis and the actin cytoskeleton. *J. Cell Biol.* 150: F111-F116.

104. Radhakrishna H, Klausner RD, Donaldson JG. 1996. Aluminum fluoride stimulates surface protrusions in cells overexpressing the ARF6 GTPase. *J. Cell Biol.* 134: 935-947.

105. Raths,S., J.Rohrer, F.Crausaz, and H.Riezman. 1993. end3 and end4: two mutants defective in receptor-mediated and fluid- phase endocytosis in *Saccharomyces cerevisiae*. *J. Cell Biol.* 120:55-65.

106. Raymond, C. K., Howald-Stevenson, I., Vater, C. A., and Stevens, T. H. 1992. Morphological classification of the yeast vacuolar protein sorting mutants: evidence for a prevacuolar compartment in class E *vps* mutants. *Mol. Biol. Cell* 3: 1389-402.

107. Ren,R., B.J.Mayer, P.Cicchetti, and D.Baltimore. 1993. Identification of a ten amino acid proline-rich SH3 binding site. *Science* 259:1157-1161.

108. Reneke,J.E., K.J.Blumer, W.E.Courchesne, and J.Thorner. 1988. The carboxy terminal segment of the yeast alpha-factor receptor is a regulatory domain. *Cell* 55:221-234.

109. Rohatgi,R., L.Ma, H.Miki, M.Lopez, T.Kirchhausen, T.Takenawa, and M.W.Kirschner. 1999. The interaction between N-WASP and the Arp2/3 complex links Cdc42- dependent signals to actin assembly. *Cell* 97:221-231.

110. Rothkegel,M., O.Mayboroda, M.Rohde, C.Wucherpfennig, R.Valenta, and B.M.Jockusch. 1996. Plant and animal profilins are functionally equivalent and stabilize microfilaments in living animal cells. *J. Cell Sci.* 109 (Pt 1):83-90.

111. Salcini,A.E., S.Confalonieri, M.Doria, E.Santolini, E.Tassi, O.Minenkova, G.Cesareni, P.G.Pelicci, and P.P.Di Fiore. 1997. Binding specificity and in vivo targets of the EH domain, a novel protein-protein interaction module. *Genes Dev.* 11:2239-2249.

112. Santos, B., and Snyder, M. Targeting of chitin synthase 3 to polarized growth sites in yeast requires Chs5p and Myo2p. 1997. *J. Cell Biol.* 136: 95-110.

113. Schmid SL. 1997. Clathrin-coated vesicle formation and protein sorting: an integrated process. *Annu. Rev. Biochem.* 66: 511-548. Review.

114. Schutt,C.E., J.C.Myslik, M.D.Rozycki, N.C.Goonesekere, and U.Lindberg. 1993. The structure of crystalline profilin-beta-actin. *Nature* 365:810-816.

115. Schafer DA, Cooper JA. 1995. Control of actin assembly at filament ends. *Annu. Rev. Cell Dev. Biol.* 11: 497-518. Review.

116. Schafer DA, D'Souza-Schorey C, Cooper JA. 2000. Actin assembly at membranes controlled by ARF6. *Traffic.* 1: 892-903.

117. Sengar,A.S., W.Wang, J.Bishay, S.Cohen, and S.E.Egan. 1999. The EH and SH3 domain Ese proteins regulate endocytosis by linking to dynamin and Eps15. *EMBO J.* 18:1159-1171.

118. Sever S, Damke H, Schmid SL. 2000. Dynamin:GTP controls the formation of constricted coated pits, the rate limiting step in clathrin-mediated endocytosis. *J. Cell Biol.* 150: 1137-1148.

119. Sheterline,P., J.Clayton, and J.Sparrow. 1995. Actin. *Protein Profile.* 2: 1-103

120. Siegel EG, Gunther R, Schafer H, Folsch UR, Schmidt WE. 1999. Characterization of novel peptide agonists of the alpha mating factor of *Saccharomyces cerevisiae. Anal. Biochem.* 275 :109-115.

121. Singer-Kruger B, Ferro-Novick S. Use of a synthetic lethal screen to identify yeast mutants impaired in endocytosis, vacuolar protein sorting and the organization of the cytoskeleton. 1997. *Eur. J. Cell Biol.* 74: 365-375.

122. Sloat BF, Pringle JR. 1978. A mutant of yeast defective in cellular morphogenesis. *Science.* 200: 1171-1173.

123. Symons,M. 1996. Rho family GTPases: the cytoskeleton and beyond. *Trends Biochem. Sci.* 21:178-181.

124. Tan,P.K., J.P.Howard, and G.S.Payne. 1996. The sequence NPFXD defines a new class of endocytosis signal in Saccharomyces cerevisiae. *J. Cell Biol.* 135:1789 1800.

125. Tang,H.Y. and M.Cai. 1996. The EH-domain-containing protein Pan1 is required for normal organization of the actin cytoskeleton in Saccharomyces cerevisiae. *Mol. Cell Biol.* 16:4897-4914.

126. Tang,H.Y., A.Munn, and M.Cai. 1997. EH domain proteins Pan1p and End3p are components of a complex that plays a dual role in organization of the cortical actin cytoskeleton and endocytosis in Saccharomyces cerevisiae. *Mol. Cell Biol.* 17:4294 4304.

127. Tang,H.Y., J.Xu, and M.Cai. 2000. Pan1p, End3p, and S1a1p, three yeast proteins required for normal cortical actin cytoskeleton organization, associate with each other and play essential roles in cell wall morphogenesis. *Mol. Cell Biol.* 20:12-25.

128. Tellam,R. and C.Frieden. 1982. Cytochalasin D and platelet gelsolin accelerate actin polymer formation. A model for regulation of the extent of actin polymer formation in vivo. *Biochemistry* 21:3207-3214.

129. Tirion,M.M., D.ben Avraham, M.Lorenz, and K.C.Holmes. 1995. Normal modes as refinement parameters for the F-actin model. *Biophys. J.* 68:5-12.

130. Tu,J., W.Song, and M.Carlson. 1996. Protein phosphatase type 1 interacts with proteins required for meiosis and other cellular processes in Saccharomyces cerevisiae *Mol. Cell Biol.* 16:4199-4206.

131. Uetz,P., L.Giot, G.Cagney, T.A.Mansfield, R.S.Judson, J.R.Knight, D.Lockshon, V.Narayan, M.Srinivasan, P.Pochart, A.Qureshi-Emili, Y.Li, B.Godwin, D.Conover, T.Kalbfleisch, G.Vijayadamodar, M.Yang, M.Johnston, S.Fields, and J.M.Rothberg. 2000. A comprehensive analysis of protein-protein interactions in Saccharomyces cerevisiae. *Nature* 403:623-627.

132. van Delft,S., R.Govers, G.J.Strous, A.J.Verkleij, and van Bergen en Henegouwen PM. 1997. Epidermal growth factor induces ubiquitination of Eps15. *J. Biol. Chem.* 272:14013-14016.

133. Venturi,G.M., A.Bloecher, T.Williams-Hart, and K.Tatchell. 2000. Genetic interactions between GLC7, PPZ1 and PPZ2 in saccharomyces cerevisiae. *Genetics* 155:69-83.

134. Waelter,S., E.Scherzinger, R.Hasenbank, E.Nordhoff, R.Lurz, H.Goehler, C.Gauss, K.Sathasivam, G.P.Bates, H.Lehrach, and E.E.Wanker. 2001. The huntingtin interacting protein HIP1 is a clathrin and alpha-adaptin- binding protein involved in receptor-mediated endocytosis. *Hum. Mol. Genet.* 10:1807-1817.

135. Warren, DT., Andrews, PD., Gourlay, GW., Ayscough, KR. Sla1p couples the yeast endocytic machinery to proteins regulating actin dynamics. *J. Cell Sci.* Under revision.

136. Watson HA, Cope MJ, Groen AC, Drubin DG, Wendland B. 2001. In vivo role for actin-regulating kinases in endocytosis and yeast epsin phosphorylation. *Mol. Biol. Cell.* 12: 3668-3679.

137. Way,M., B.Pope, R.A.Cross, J.Kendrick-Jones, and A.G.Weeds. 1992. Expression of the N-terminal domain of dystrophin in E. coli and demonstration of binding to F-actin. *FEBS Lett.* 301:243-245.

138. Wendland,B., S.D.Emr, and H.Riezman. 1998. Protein traffic in the yeast endocytic and vacuolar protein sorting pathways. *Curr. Opin. Cell Biol.* 10:513-522.

139. Wendland,B., K.E.Steece, and S.D.Emr. 1999. Yeast epsins contain an essential N terminal ENTH domain, bind clathrin and are required for endocytosis. *EMBO J.* 18:4383-4393.

140. Wertman,K.F., D.G.Drubin, and D.Botstein. 1992. Systematic mutational analysis of the yeast ACT1 gene. *Genetics* 132:337-350.

141. Wesp,A., L.Hicke, J.Palecek, R.Lombardi, T.Aust, A.L.Munn, and H.Riezman. 1997. End4p/Sla2p interacts with actin-associated proteins for endocytosis in Saccharomyces cerevisiae. *Mol. Biol. Cell* 8:2291-2306.

142. Winter,D., A.V.Podtelejnikov, M.Mann, and R.Li. 1997. The complex containing actin-related proteins Arp2 and Arp3 is required for the motility and integrity of yeast actin patches. *Curr. Biol.* 7:519-529.

143. Yang,S., M.J.Cope, and D.G.Drubin. 1999. Sla2p is associated with the yeast cortical actin cytoskeleton via redundant localization signals. *Mol. Biol. Cell* 10:2265-2283.

144. Yonezawa,N., E.Nishida, K.Iida, I.Yahara, and H.Sakai. 1990. Inhibition of the interactions of cofilin, destrin, and deoxyribonuclease I with actin by phosphoinositides. *J. Biol. Chem.* 265:8382-8386.

145. Yonezawa,N., E.Nishida, and H.Sakai. 1985. pH control of actin polymerization by cofilin. *J. Biol. Chem.* 260:14410-14412.

146. Yu,H., J.K.Chen, S.Feng, D.C.Dalgarno, A.W.Brauer, and S.L.Schreiber. 1994. Structural basis for the binding of proline-rich peptides to SH3 domains. *Cell* 76:933-945.

147. Zeng,G. and M.Cai. 1999. Regulation of the actin cytoskeleton organization in yeast by a novel serine/threonine kinase Prk1p. *J. Cell Biol.* 144:71-82.

148. Zhdankina, O., Strand, N. L., Redmond, J. M., and Boman, A. L. 2001. Yeast GGA proteins interact with GTP-bound Arf and facilitate transport through the Golgi. *Yeast* 18: 1-18.