THE PCH FAMILY PROTEIN, CDC15P, INTERACTS DIRECTLY WITH TWO ACTIN NUCLEATION PATHWAYS TO CONTRIBUTE TO CYTOKINETIC ACTIN RING FORMATION IN *SCHIZOSACCHAROMYCES POMBE*

By

Robert Herschel Carnahan Jr.

Dissertation
Submitted to the Faculty of the
Graduate School of Vanderbilt University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

in

Cell and Developmental Biology

December, 2003

Nashville, Tennessee

Approved by

Professor Kathleen Gould

Professor Gary Olson

Professor David Greenstein

Professor Todd Graham

Professor Carl Johnson

ACKNOWLEDGEMENTS

I would like to thank Kathy Gould and the entire Gould lab, past and present, for all of the support and assistance they have provided throughout my time in graduate school. I am especially grateful to Kathy for the great training, the supportiveness, the freedom and independence to pursue ideas, and for her friendship. I have truly enjoyed my years in the lab and found it to be the most dedicated, intelligent, and fun group I have ever worked with (and considering I had over twenty jobs prior to grad school, that is saying something!). I'll never forget coffee time, Tuesdays at Sitar, and beer Fridays. Oh yeah, and the work was good too.

I also owe a debt of gratitude to all of my past science mentors and teachers. I would like to specifically thank Dr. James Holland for inspiration, Dr. George Malacinski and Dr. Pam Hanratty for the opportunity to grow through teaching, Dr. Flynn Picardal for introducing me to research, Dr. Yves Brun for helping me learn how to think, Dr. David White for getting my foot in the door at Eli Lilly, Dr. Richard Jaskunas for his faith in me and his supportiveness and mentoring during my time at LRL, Claude Thirion and Guy Roehrig for their support and friendship at Lilly France, Moses Vijaykumar for taking me under his wing at the Pasteur Institute and helping me to get moving in the right direction, Dr. Roger Chalkley and Dr. Mark Denison for their support and enthusiasm during my time in IGP, and finally to my entire committee, Dr. Kathy Gould, Dr. Gary Olson, Dr. David Greenstein, Dr, Todd Graham, and Dr. Carl Johnson, who proved that a room full of scientists can in fact work well together. I would also like to express my deepest appreciation to William Alborn, who has shown me that science is more than Ph.D.'s and papers. Bill is not only one of the brightest and most enthusiastic scientist I have ever had the great fortune to learn from, but also one of the most caring (despite

cultivating a crusty and codgerly reputation!). I wouldn't be writing this today if not for his mentoring, dogged support, encouragement, and true concern for me.

I would like to thank all of those outside science who have contributed in so many ways to helping me succeed at Vanderbilt. Thanks to my parents and family for the unquestioning belief in me, to the entire Pearson family for all of their support and help, to my friends in Nashville, to the guys and girls in the basketball group for all of the fun and injuries, and to my friends elsewhere, especially Doug and Lar for giving me something to look forward to every spring. Finally, thanks to Zucchi, Chani, and Baby and especially my wife, Nikki, for their unconditional support and love, and to Nikki for not only helping me to persevere in school, but for making sure that I didn't forget to live while doing it.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

aa	amino acid
APC	anaphase promoting complex
bp	base pair
BSA	bovine serum albumin
C	carboxy
CAR	cytokinetic actin ring
cdc	cell division cycle
Cdk	cyclin-dependent kinase
cDNA	complementary DNA
CFP	cyan fluorescent protein
coIP	coimmunoprecipitation
DAPI	4',6-diamidino-2-phenylindole
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
ECL	enhanced chemilluminescence
F-actin	filamentous actin
G	gap phase
GFP	green fluorescent protein
GST	glutathione-s-transferase
HA	influenza hemagglutinin epitope
HCl	hydrogen chloride
H_20	water
IgG	immunoglobulin-G
IPTG	isopropyl-□-D-thiogalactopyranoside
Kan^R	kanamycin resistant
KGY	Kathy Gould yeast
l	liter
□l	microliter (10^{-6} liter)
M	molar, mitosis

MBC	methyl-benzimidazol-2-yl carbamate
MBP	maltose binding protein
MEN	mitotic exit network
$MgCl_2$	magnesium chloride
ml	milliliter
mM	millimolar
$MnCl_2$	manganese chloride
Myc	myc epitope
N	amino
NaCl	sodium chloride
NETO	new end take-off
nmt	no message in thiamine
NP-40	Nonidet® P-40
ORF	open reading frame
^{32}P	phosphorus-32
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
pKG	plasmid Kathy Gould
RLU	relative light unit
RNA	ribonucleic acid
rpm	revolutions per minute
^{35}S	sulfur-35
SB	sample buffer
SDS	sodium dodecyl sulfate
ser	serine
SH3	Src homology 3
SIN	Septum Initiation Network
SPB	Spindle Pole Body
TBZ	thiabendazole
Thr	threonine

TnT	transcription and translation
ts	temperature sensitive
Ura	uracil
YE	yeast extract
YFP	yellow fluorescent protein
U	unit
Ub	ubiquitin
UV	ultraviolet
Δ	null
α	anti

CHAPTER I

INTRODUCTION

The eukaryotic cell cycle

Regulation of growth and division is one of the most fundamental aspects of the biology of cells. Understanding the pathways that regulate these processes not only further our knowledge of basic biology, but also move us closer to understanding diseases, such as cancer, which stem from a disruption of these control mechanisms.

In eukaryotic systems, cell division is accomplished by an ordered progression through a regulated series of intracellular events called the cell division cycle or cell cycle. This cycle essentially consists of alternating rounds of DNA synthesis (S-phase) and DNA segregation to daughter cells (Mitosis or M-phase) (Murray and Hunt, 1993). S-phase and M-phase each consist of a number of stepwise processes, which must not only be accomplished in the correct fashion, but also with the correct timing. For example, initiation of chromosome segregation prior to the completion of DNA synthesis could lead to incomplete genomes being inherited to daughter cells and thereby significantly lower the fitness of the resultant cells. This cell cycle is coordinated and monitored by a diverse set of surveillance mechanisms called checkpoints (Hartwell and Weinert, 1989). These checkpoints serve as biochemical road barriers to further cell cycle progression until prerequisite events have been successfully completed. S-phase and M-phase are separated by intervening "GAP" phases, G1 between M- and S-phases and G2 between S- and M-phases (Fig. 1). The GAP phases are critical times for monitoring of numerous checkpoint contingencies to ensure that cells are fully prepared to enter the highly complex and orchestrated events of S- or M-phases. The GAP phases, referred to as interphase when grouped together with S-phase, are also a time during which the majority of cell growth occurs.

The core machinery for control of cell cycle progression has principally been elucidated by genetic screens in budding yeast (*S. cerevisiae*) and fission yeast (*S. pombe*). One of the most important observations stemming from these Cell Division Cycle (cdc) screens was the identification of the Cyclin-dependent kinases (CDK) as the master regulators of the cell cycle. A complex and finely tuned system of governing the activities of this family of kinases is a

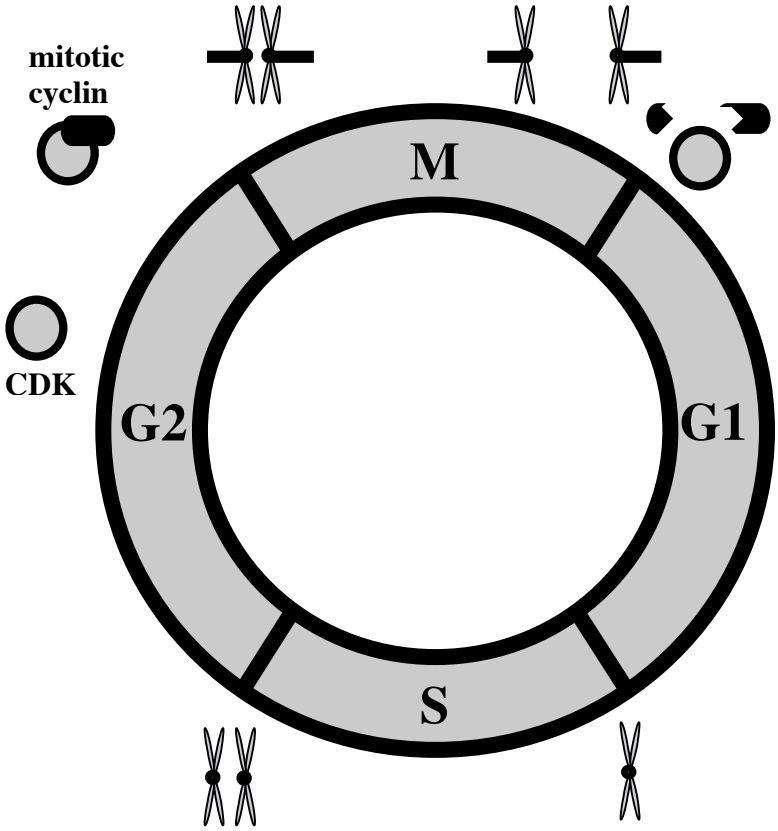


Figure 1. The eukaryotic cell cycle. Schematic diagram depicting the phases of the eukaryotic cell cycle and the regulation of mitotic entry by cyclin-dependent kinase.

cornerstone of cell cycle control in all eukaryotic systems. One key means of CDK regulation is through its association with its cyclin subunit. As implied by their name, cyclins are only expressed at specific times within the cell cycle. Complexing of the cyclin subunit with a CDK generally leads to activation of the kinase activity and phosphorylation of downstream targets. CDKs are also regulated by several other means including phosphorylation/ dephosphorylation, CDK inhibitors, and ubiquitin-mediated proteolysis of cyclin subunits (reviewed in Nigg, 2001). Active CDK is universally required for G2/M progression, as well as, other eukaryotic cell cycle transitions.

The actin cytoskeleton

Remodeling of the internal cytoskeletal structures by cells is involved at numerous points during the transit through the cell cycle. For example, chromosome segregation requires the microtubule-based spindle apparatus (Wang et al., 2003). Rearrangements of the actin cytoskeleton also occur at discrete phases of the cell cycle and are required for its successful completion. The actin cytoskeleton is made up of a complex network of actin polymers, called

actin filaments. Actin filaments grow and shrink by virtue of addition or subtraction of monomers onto filaments ends. These filaments display an intrinsic polarity having a faster growing barbed end and the slow growing pointed end (Cooper and Schafer, 2000). Actin filament formation from monomers is a dynamic process that occurs in two steps: nucleation of the new filament, followed by elongation (Higgs and Pollard, 2001). Formation of new actin filaments from monomer pools is highly unfavorable compared with existing polymer elongation (Pollard and Cooper, 1986). Thus, nucleation of new filaments is the rate limiting factor in control of actin networks.

The fission yeast, *Schizosacchromyces pombe*, as a model organism

Model organisms have proven to be invaluable tools in furthering our understanding of the mechanisms that govern biological processes. Among these model systems, simple unicellular eukaryotes, such as the fission yeast, *Schizosaccharomyces pombe*, have been extensively utilized for the study of processes conserved across the eukaryotic lineage. As with higher eukaryotes, the *S. pombe* cell cycle consists of repeated rounds of G1-, S-, G2-, and M-phases. Furthermore, all eukaryotic systems have maintained a considerable conservation of the biochemical mechanisms driving the cell cycle. A particularly useful aspect of *S. pombe* morphology is that the rod-shaped cells grow by tip elongation and divide by medial fission at a constant size. Thus, cell cycle stage can typically be determined visually (Nurse et al., 1976). Similarly, there is a conservation of the key molecules and mechanisms of cytoskeletal regulation, including those proteins critical to the functioning of the cleavage apparatus (Guertin et al., 2002). In addition, *S. pombe* is readily amenable to genetic, biochemical and cytological analyses, making it both a powerful and flexible system for studying basic cell biology.

Cytokinesis in *S. pombe*

After cells have successfully accomplished both replication and segregation of the genetic material, there remains the physical division of the mother cell into two daughter cells. This process, called cytokinesis, must not only be tightly temporally regulated with respect to DNA synthesis and segregation, but also spatially regulated to ensure proper partitioning between the resultant daughter cells. In animal and fungal cells, cytokinesis requires major reorganization of the actin cytoskeleton to form an actomyosin based cleavage apparatus

(Balasubramanian et al., 2000; Marks et al., 1986). This apparatus, also called the cleavage furrow or cytokinetic actin ring (CAR), generally assembles as an equatorial ring structure at the presumptive site of cell division. After mitosis is completed, the constriction of this ring, coupled with new membrane addition, leads to the creation of two separate daughter cell compartments. In contrast to higher eukaryotes, *S. pombe* cells synthesize a septum in the wake of the constricting ring. This "primary" septum creates a barrier of cell wall material between the daughter compartments. Subsequently, two secondary septa are synthesized flanking the primary septum. Finally, cell separation is accomplished by centripetal degradation of the primary septum (Johnson et al., 1973). The events of ring constriction and septation require the function of a conserved signaling cascade termed the Septation Initiation Network (SIN) (Fig. 2, reviewed in McCollum and Gould, 2001). The SIN consists of three kinases (Cdc7p, Sid1p, and Sid2p), the small GTPase Spg1p, and a two-component GAP (Cdc16p and Byr4p). The kinases Sid1p and Sid2p also require an additional subunit, Cdc14p and Mob1p, respectively, for *in vivo*

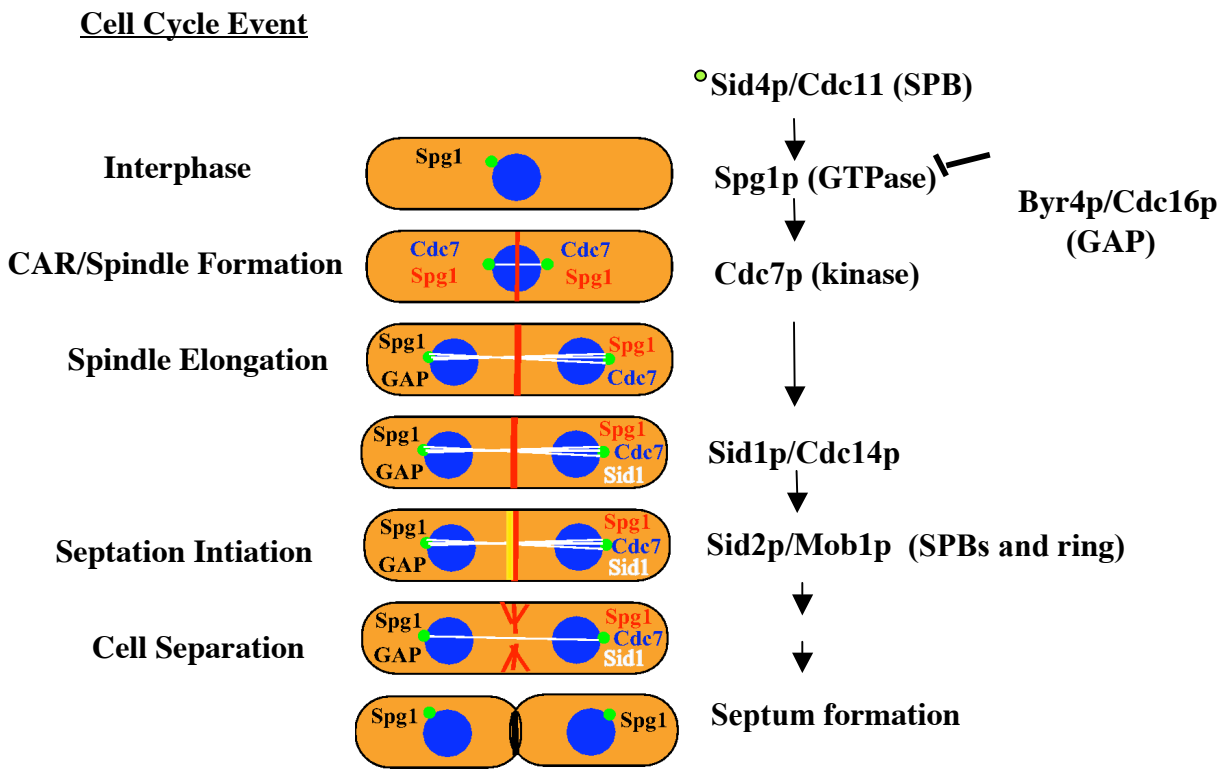


Figure 2. The Septation Initiation Network (SIN). A schematic diagram of the control of septation in *S. pombe* by the SIN.

function. Finally, there are two proteins, Cdc11p and Sid4p, which function to anchor various members of the pathway to the spindle pole bodies (SPBs). While all other members of the network are required for septation, loss of Cdc16p or Byr4p function leads to repeated rounds of septation in the absence of nuclear division, indicating a negative regulatory role for these proteins (Fankhauser et al., 1993; Song et al., 1996). An analogous pathway exists in *S. cerevisiae* (MEN pathway), and many SIN- and MEN-related proteins have homologues in higher eukaryotes (Guertin et al., 2002).

Cytokinetic actin ring assembly in *S. pombe*

The timing of CAR assembly in *S. pombe* appears tightly associated with entry into mitosis (Balasubramanian et al., 2000). *S. pombe* cells arrested, even for long periods, at the G2/M boundary fail to form a CAR. However, after the transition into M-phase CAR assembly rapidly begins in the medial region (Fig. 3). As the site of CAR assembly determines the site of eventual division, it is crucial that its formation also be spatially regulated such that cytokinesis occurs only between the segregated chromosomes. In animal cells, the mitotic spindle is first established and subsequent signals from the spindle midzone determine the site of CAR placement. However, in yeast and plant cells it appears that the CAR position is established independently of the mitotic spindle, which is subsequently oriented with respect to the CAR (Guertin et al., 2002) . In *S. pombe*, spatial cues for placement of the CAR have been linked to nuclear position. Thus, mutants which display misplaced nuclei lead to misplaced division sites correlating with nuclear location. Two proteins, Mid1p, an anillin family protein, and Plo1p kinase, have clearly been shown to play a role in the establishment of CAR position (Wang et al., 2003). During interphase Mid1p resides principally in the nucleus. Upon entry into mitosis Mid1p becomes hyper-phosphorylated and exits the nucleus in a Plo1p-dependent manner. This cytoplasmic Mid1p localizes to a broad band at the cell cortex overlying the nucleus and is thought to thereby mark the site of recruitment for actomyosin ring proteins. Several other proteins, such as the protein kinase Pom1p, have also been shown to be involved in signaling for ring placement. However, their roles are currently less well understood. After the site of ring placement is established actin, myosin, and numerous other proteins are recruited and assembled into the ring structure. Considerable conservation across all eukaryotes has been observed in both the proteins found within the CAR (Table 1) and its assembly process (Guertin et al., 2002).

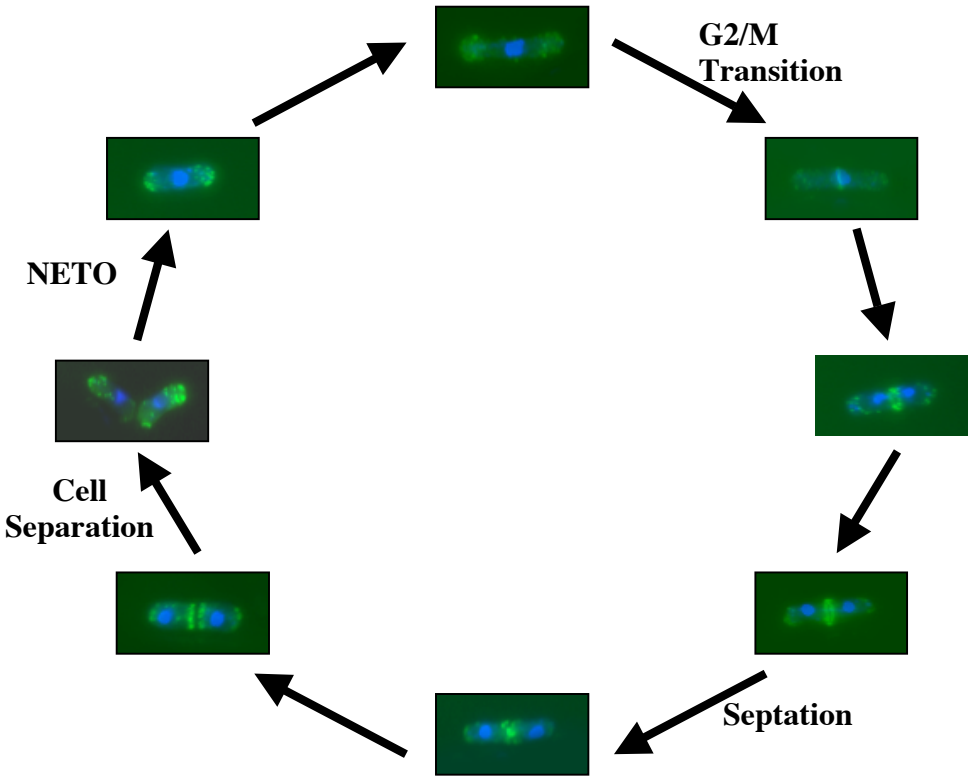


Figure 3. F-Actin localization across the *S. pombe* cell cycle. Representative images of *S. pombe* cells at various stages across the cell cycle, fixed and stained with AlexaFluor 488-phalloidin to visualize actin (green) and DAPI to visualize DNA (blue).

Other F-actin structures in *S. pombe*

In addition to the CAR, two other F-actin structures are detected in *S. pombe*, cables and patches (Fig. 3), and both are involved in the cell division process. During interphase, F-actin cables run longitudinally through cells and have been associated with both protein and organelle transport (Schott et al., 2002). Cables accumulate in the medial region during the initial stages of ring assembly (Arai and Mabuchi, 2002). These pre-existing cables are linked to the newly forming CAR and appear to be packed into this structure as it transitions from a thin primary ring to a thicker mature structure (Arai and Mabuchi, 2002).

F-actin patch formation during interphase is favored at growing ends of cells (Marks et al., 1986). Thus, in G2 when cells are actively growing from both ends, patches are observed in both locations (Fig. 3). At the onset of mitosis, patches cease to assemble at cell tips and instead form in the medial region of the cell (Marks and Hyams, 1985; Pelham and Chang, 2001), where

the components of these patches presumably contribute to CAR formation (Balasubramanian et al., 2000). After cytokinesis, patches are observed only at the actively growing "old" cell end, until cells undergo New-End Take-Off (NETO), when growth again redistributes to both ends (Marks and Hyams, 1985). Patches are believed to play a role in the deposition of new cell

Table 1. Cytokinetic actin ring components (adapted from Guertin et al., 2002)

Protein Family	Gene	Organism
Actin	*act1*	*S. pombe*
	ACT1	*S. cerevisiae*
Myosin II heavy chain	*myo2*	*S. pombe*
	myo3/myp2	*S. pombe*
	MYO1	*S. cerevisiae*
Myosin regulatory light chain	*rlc1*	*S. pombe*
	RMLC	*D. discoideum*
	spaghetti squash	*D. melanogaster*
Myosin essential light chain	*cdc4*	*S. pombe*
	MLC1	*S. cerevisiae*
	ELC	*D. discoideum*
Septin	*spn1, -2, -3, -4, -5, -6*	*S. pombe*
	CDC3, -10, -11, -12	*S. cerevisiae*
	peanut, Sep1, Sep2	*D. melanogaster*
	unc-59, unc-61	*C. elegans*
	Nedd5, H5, Diff6	Mouse
PCH	*cdc15, imp2*	*S. pombe*
	HOF1	*S. cerevisiae*
	PSTPIP, PSTPIP2	Mouse
Formin	*cdc12*	*S. pombe*
	BNI1, BNR1	*S. cerevisiae*
	diaphanous	*D. melanogaster*
	cyk-1	*C. elegans*
IQGAP	*rng2*	*S. pombe*
	CYK1/IQG1	*S. cerevisiae*
	GAPA	*D. discoideum*
	IQGAP1	Human
Tropomyosin	*cdc8*	*S. pombe*
Polo kinase	*plo1*	*S. pombe*
	CDC5	*S. cerevisiae*
SIN components	*sid2* (kinase)	*S. pombe*
	mob1	*S. pombe*
	DBF2 (kinase)	*S. cerevisiae*
	MOB1	*S. cerevisiae*

wall material and in maintenance of cell polarity (Ishiguro and Kobayashi, 1996; Kobori et al., 1989). F-actin patches are highly dynamic, undergoing rapid movements and cycles of assembly and disassembly (Pelham and Chang, 2001). Formation and motility of actin patches depends on Arp2/3 complex driven F-actin polymerization (Li et al., 1995; Pelham and Chang, 2001; Winter et al., 1997).

Regulation of the actin cytoskeleton through the Arp2/3 complex

The Arp2/3 complex is an abundant, highly conserved, seven-member protein complex, which has been shown to catalyze the formation of new actin filaments (Mullins et al., 1998) . Two subunits, Arp2 and Arp3 are actin-related proteins, while the other five subunits are novel (Higgs and Pollard, 2001). The complex is capable of binding the side of an existing filament. Arp2 and Arp3 are believed to then serve as the first two subunits for the formation of a new daughter filament with a free barbed end (Fig. 4). By this mechanism Arp2/3 contributes to the branched actin network observed in cells. In *S. pombe*, actin nucleation by the Arp2/3 complex is not only required for F-actin patch formation, but also for CAR formation (Pelham and Chang, 2002).

Regulators of the Arp2/3 complex

The Arp2/3 complex alone possesses a low intrinsic activity. Thus, the complex requires interaction with one of several activator proteins to achieve a high level of nucleation activity

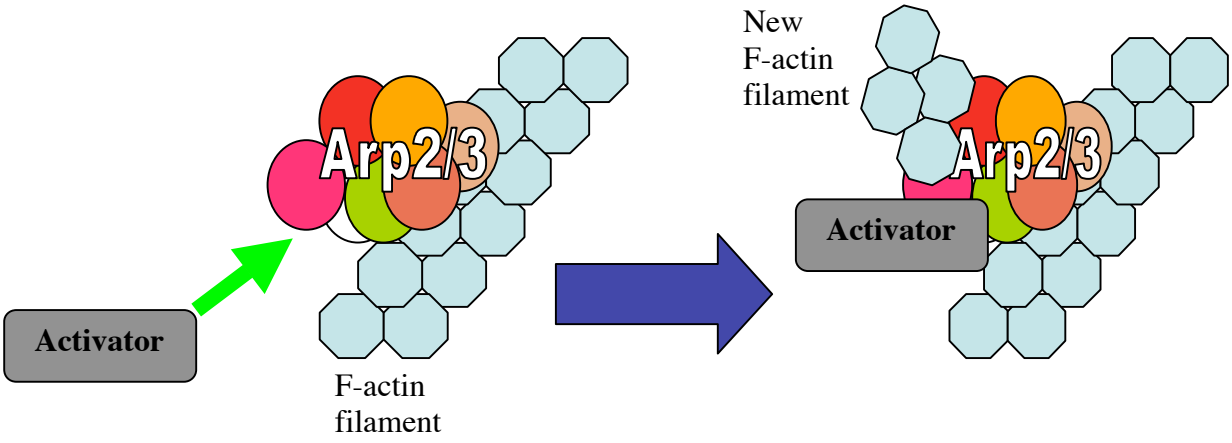


Figure 4. A cartoon depicting Arp2/3 mediated nucleation of branched actin filament.

(Fig. 4). Arp2/3 activators, such as the type I myosins and WASp/Scar protein family, bind directly to Arp2/3 complex and stimulate its ability to promote actin filament assembly (Bear et al., 2001; Cooper et al., 2001; Higgs and Pollard, 2001). These activators possess C-terminal A(acidic)-domains by which they interact with Arp2/3 complex (Lee et al., 2000). The Arp2/3 activators in *S. pombe*, Wsp1p and Myo1p, are functionally redundant (Lee et al., 2000; Toya et al., 2001). Similar redundancy is seen in *S. cerevisiae* where the WASp homolog (Las17p/Bee1p) and type I myosins exist in an Arp2/3 regulatory complex that includes verprolin (Vrp1p), a homologue of the Human WASp Interacting Protein (WIP) (Evangelista et al., 2000; Lechler et al., 2000).

Regulation of the actin cytoskeleton by formins

Recently, formins have been shown to represent a second F-actin nucleator family (Evangelista et al., 2002; Pelham and Chang, 2002; Sagot et al., 2002). The *S. cerevisiae* formin Bni1p nucleates linear, unbranched actin filaments, while remaining bound to their barbed ends (Evangelista et al., 2002; Pruyne et al., 2002; Sagot et al., 2002). This mechanism is distinct from that of Arp2/3 complex, which nucleates *de novo* filaments by binding to pointed ends and creates branched actin meshworks by binding to the sides of existing filaments (Higgs and Pollard, 2001).

Formins are large multidomain proteins (Fig. 5). The FH1 region interacts with the actin monomer binding protein profilin. The FH2 domain is unique to this family of proteins and has been shown to be the actin nucleation control region (Evangelista et al., 2003). Constructs encompassing solely the FH1 and FH2 domains of *S. cerevisiae* Bni1 possess profilin-dependent *in vitro* actin nucleation activity (Evangelista et al., 2002; Sagot et al., 2002). The FH3 domain has been less well characterized, but evidence indicates that it is involved in subcellular targeting of the proteins (Petersen et al., 1998). Formins also typically possess one of a variety of N-terminal regulatory domains (e.g. Rho-binding domain), as well as, multiple coiled-coil regions.

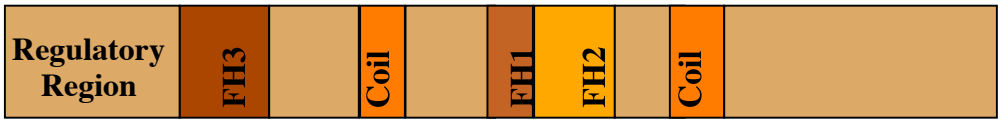


Figure 5. A cartoon of Formin protein structure.

The *S. pombe* formin, Cdc12p

The *S. pombe* formin, Cdc12p, is a component of the CAR and is required for the earliest steps of ring assembly (Arai and Mabuchi, 2002; Pelham and Chang, 2002). In interphase cells, Cdc12p is present in a single motile cytoplasmic spot (Chang et al., 1997). This motile particle is targeted to the medial region prior to CAR formation and serves as a nucleating structure for the CAR into which it is simultaneously incorporated (Chang, 2001). Intracellular movement of Cdc12p appears to involve both the actin and microtubule cytoskeletons (Chang, 2000). Movement of the Cdc12p spot to the medial region is also dependent on *cdc3* (profilin homologue), *cdc8* (tropomyosin homologue), and the ring component *cdc15*. Furthermore, though *cdc12* is expressed normally in *cdc15* mutant cells, the Cdc12p spot fails to form (Chang et al., 1997).

The Cdc15p family of proteins

Though recent progress has been made towards elucidating formin function, the roles of other proteins important for CAR formation are less well established. Furthermore, an important question still remains as to how these molecules are organized and coordinated in order to build the CAR. One protein linked to ring establishment, but whose function is poorly understood is *S. pombe* Cdc15p. *cdc15* is an essential gene, mutations of which were first isolated in the original *S. pombe* cdc screen (Nurse et al., 1976). Subsequent cloning and analysis revealed it to be a unique multidomain protein involved in mitotic actin rearrangement and required for septum formation (Fankhauser et al., 1995). Furthermore, the importance of Cdc15p in CAR initiation was reinforced by the observation that it was one of a very small class of proteins whose overproduction leads to medially directed actin rearrangement in interphase cells (Fankhauser et al., 1995). Work in other systems has led to the discovery of numerous homologous proteins, which have now been classified as the PCH (Pombe Cdc15 Homology) family of proteins (Fig. 6, Fankhauser et al., 1995; Lippincott and Li, 2000). Members of this family localize to actin-rich regions, such as the CAR, and are important for actin cytoskeletal functions. These proteins characteristically have an N-terminal FER-CIP4 homology (FCH) domain, which is unique to this family, a central coiled-coil region, a PEST sequence, and one or more carboxy-terminal Src homology 3 (SH3) domains (Fig. 6, Fankhauser et al., 1995; Lippincott and Li, 2000).

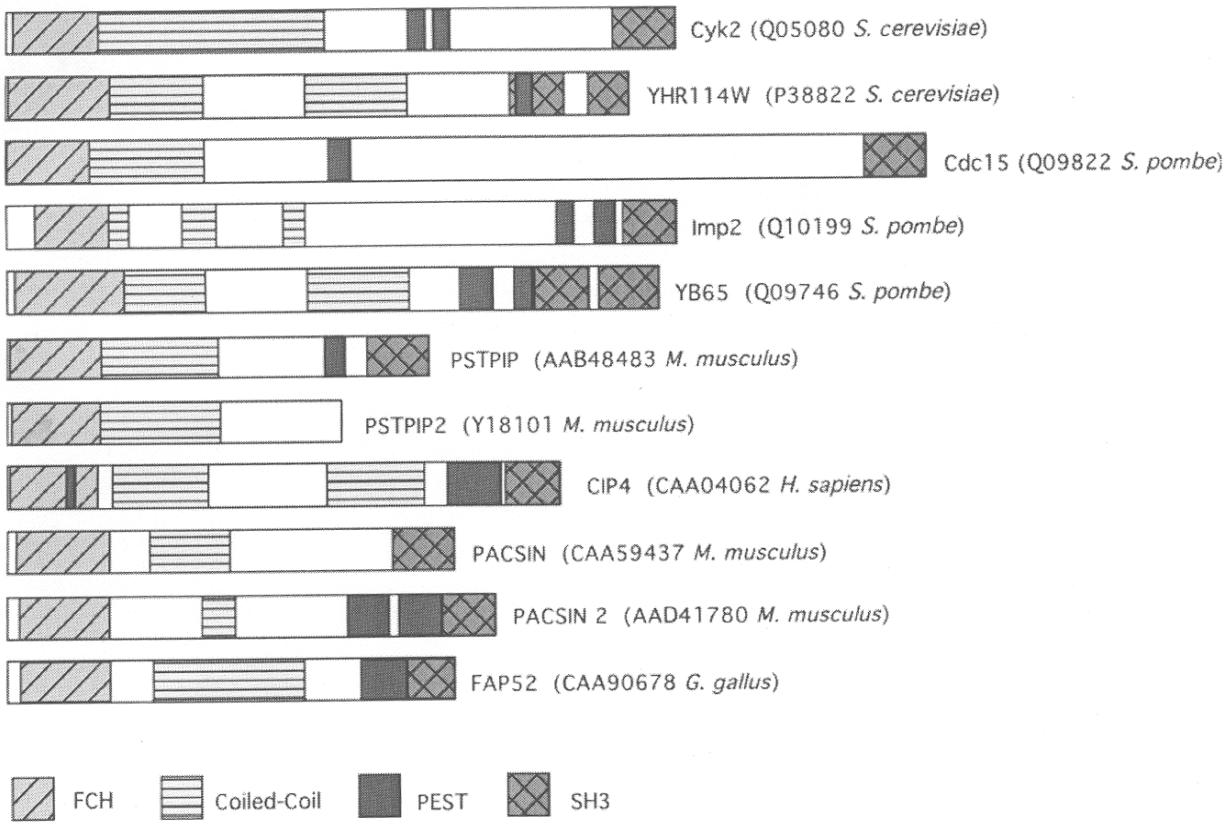


Figure 6. Schematic representation of several known PCH family proteins (adapted from Lippincott and Li, 2000).

Summary

Recent work has suggested an exciting and somewhat unexpected role for the formin Cdc12p in actin polymerization at the cytokinetic ring. While numerous reports have been issued examining various aspects of Cdc12p, very little work has appeared on Cdc15p, a protein linked to proper Cdc12p functioning. Cdc15p has also been suggested to be important for other aspects of mitotic actin reorganization (Balasubramanian et al., 1998). Here we investigate the role of Cdc15p in CAR formation (Chapter III). We find that Cdc15p interacts directly with both the Arp2/3-dependent and the formin-dependent actin nucleation pathways and is required for their medial recruitment during mitosis. As such, Cdc15p plays an integral role in, and is strictly required for CAR formation during cytokinesis. We also go on to more fully characterize the Cdc15p interaction with F-actin (Chapter IV) and establish several other previously unreported protein-protein interactions (Chapter V).

CHAPTER II

MATERIALS AND METHODS

Strains and media

S. pombe strains (Table 2) were grown in YE medium or EMM minimal medium with the appropriate supplements (Moreno et al., 1991). Expression of constructs under control of the thiamine-repressible *nmt* promoter system was carried out as previously described (Maundrell, 1993). Cells were synchronized using arrest-release of a *cdc25-22* mutant, as previously described (Fankhauser et al., 1995), lactose gradients (Barbet and Carr, 1993) or centrifugal elutriation (Chang et al., 2001). Yeast transformations were performed using either a lithium acetate method (Keeney and Boeke, 1994) or electroporation (Prentice, 1992).

Molecular biology methods

Standard genetic and recombinant DNA methods were used except where noted. Gene fragments were obtained by PCR amplification from either *S. pombe* genomic DNA or a cDNA library, as appropriate utilizing either TaqPlus Precision polymerase (Stratagene) or Vent polymerase (New England Biolabs). Oligonucleotides were obtained from Integrated DNA Technologies, Inc. Sequencing was performed using Thermosequenase (USB) and Redivue ^{33}P terminator kit (Amersham Biosciences). . pK294, a plasmid expressing GFP fused to Cdc15p(1-425), under control of the native promoter, was obtained from Y. Hiraoka (Ding et al., 2000). All plasmids used in this study are listed in Table 3.

Table 2. Strains

Strain	Genotype	Source
KGY188	*cdc15-140 h-*	P. Nurse
KGY246	*ade6-M210 ura4-D18 leu1-32 h-*	Lab Stock
KGY3958	*myo1-GFP ade6-M210 ura4-D18 leu1-32 h-*	This Study
KGY3963	*myo1-GFP cdc15-140 leu1-32 h-*	This Study
KGY3966	*vrp1-GFP ade6-M210 ura4-D18 leu1-32 h-*	This Study
KGY108	*vrp1-GFP cdc15-140 leu1-32 h-*	This Study
KGY358	*wsp1-GFP cdc15-140 ura4-D18 leu1-32 h+*	This Study
KGY3019	*cdc15-GFP ade6-M210 ura4-D18 leu1-32 h-*	This Study
KGY3020	*cdc15-HA ade6-M210 ura4-D18 leu1-32 h-*	This Study
KGY3960	*myo1-HA ade6-M210 ura4-D18 leu1-32 h-*	This Study
KGY3359	*cdc15-GFP cdc12-112 ade6-M210 ura4-D18 leu1-32 h-*	This Study
KGY3066	*cdc12-GFP ade6-M210 ura4-D18 leu1-32 h-*	This Study
KGY3362	*cdc15-GFP sid4-GFP ade6-M210 ura4-D18 leu1-32 h-*	This Study
KGY746	*vrp1::ura4 ade6-M210 ura4-D18 leu1-32 h+*	This Study
KGY586	*myo1::kanR ade6-M210 leu1-32 h-*	M. Yamamoto
KGY318	*cdc12-112 ura4-D18 h-*	P. Nurse
KGY748	*cdc12-299 h-*	P. Nurse
KGY1463	*cut9-HA ura4-D18 h-*	Lab Stock
KGY3042	*cdc15-GFP cdc25-22 ade6-M210 ura4-D18 leu1-32 h-*	This Study
KGY4224	*cdc15/cdc15::ura4 ade6-M210/ade6-M216 ura4-D18/ura4-D18 leu1-32/leu1-32 h+/h-*	This Study
KGY3352	*cdc15-CFP ade6-M210 ura4-D18 leu1-32 h-*	This Study
KGY751	*cdc15-140 cdc12-112 ura4-D18 leu1-32 h+*	This Study
KGY752	*cdc15-140 cdc12-299 h+*	This Study
KGY753	*vrp1::ura4 wsp1::ura4*	This Study
KGY753	*cdc15-CFP, arc15-GFP*	This Study
KGY4409	*wsp1::ura4 cdc15-GFP*	This Study
KGY662	*myo1::kanR cdc15-GFP*	This Study

Table 3. Plasmids

Plasmid Number	Vector	Insert
pKG1743	pGAD424	none
pKG1744	pGBT9	none
pKG1699	pGEX4T-1	none
pKG1826	pMAL-2C	none
pKG2312	pGAD424	Cdc15p 1-405
pKG2997	pGAD424	Cdc15p 1-282
pKG2629	pGAD424	Cdc15p 440-927
pKG2630	pGAD424	Cdc15p 843-927
pKG2995	pGEX4T-1	Cdc15p 1-405
pKG2321	pEG3	Cdc15p 1-425
pKG2996	pMAL-2C	Cdc15p 1-282
pKG2210	pREP81-GFP	Cdc15p 1-927
pKG2998	pREP81	Cdc15p 1-927
pKG2316	pREP81-GFP	cdc15-140p 1-927
pKG2999	pSK	Cdc15p 1-282
pKG2226	pGBT9	Cdc12p 1-999
pKG2397	pGBT9	Cdc12p 1-764
pKG3000	pGBT9	Cdc12p 1-524
pKG3001	pGBT9	Cdc12p 1-314
pKG3002	pGBT9	Cdc12p 302-524
pKG3003	pMAL-2C	Cdc12p 1-764
pKG3004	pGEX4T-1	Cdc12p 1-764
pKG2317	pREP81	Cdc12p 1-1841
pKG3005	pREP81-GFP	Cdc12p 1-1841
pKG3022	pREP81-GFP	Cdc12p 1-999
pKG3023	pREP81-GFP	Cdc12p 1-764
pKG3024	pREP81-GFP	Cdc12p 1-524
pKG3025	pREP81-GFP	Cdc12p 1-314
pKG3026	pREP81-GFP	Cdc12p 302-524
pKG3006	pGBT9	Myo1p 1077-1218
pKG2790	pGBT9	Myo1p 727-1218
pKG2798	pGBT9	Myo1p 727-1104
pKG3007	pGBT9	Myo1p 727-1041
pKG3008	pGBT9	Myo1p 957-1041
pKG3009	pGBT9	Myo1p 727-981
pKG3010	pGEX4T-1	Myo1p 1077-1218
pKG3011	pMAL-2C	Myo1p 727-1041
pKG3012	pSK	Myo1p 727-1041
pKG3013	pGAD424	Wsp1p 1-283
pKG3014	pGAD424	Wsp1p 1-497
pKG3015	pGAD424	Wsp1p 215-497
pKG2747	pGAD424	Wsp1p 1-574
pKG3016	pSK	Wsp1p 1-497
pKG3017	pSK	Wsp1p 1-283
pKG2794	pGBT9	Vrp1p 1-309
pKG3018	pGBT9	Vrp1p 206-309
pKG3019	pMAL-2C	Vrp1p 1-309
pKG3020	pGEX4T-1	Sid4p
pKG3021	pMAL-2C	Cdc11p

In vivo tagging and gene deletions

Strains expressing epitope tagged versions of proteins were constructed using a PCR-based approach as previously described (Bahler et al., 1998). Each open reading frame (ORF) was tagged at its endogenous locus at its 3' end with a variety of tag-Kan^r cassettes. Appropriate tagging was confirmed by PCR and either microscopic or immunoblot inspection, as appropriate. All tagged strains were viable at temperatures ranging from 25 to 36ºC. Unless otherwise noted, proteins are detected using endogenous tags throughout this study.

Gene replacements were accomplished by replacing the coding sequences with the *ura4* $^+$ gene using a one-step PCR-based approach as previously described (Bahler et al., 1998). Ura $^+$ transformants were confirmed by PCR amplification using primers inside the *ura4*$^+$ gene and primers outside the disruption cassette. In the case of *myo1*Δ (KGY586, a generous gift of M. Yamamoto), a similar strategy was used to replace the entire coding region with the Kan^r cassette. For phenotypic analysis of *cdc15::ura4* cells, the heterozygous *cdc15*$^+$/*cdc15::ura4*$^+$ diploid was induced to sporulate on glutamate plates, spores were isolated following glusulase-treatment and were inoculated into YE media for 3h to assist spore germination. Spores were washed extensively and then incubated in media without uracil to allow growth of *cdc15::ura4*$^+$ cells, but not of the Ura$^-$ *cdc15*$^+$ cells. Spores were grown 10-12h in selection media prior to being harvested and processed for actin staining.

Microscopy

All microscopy, except germination of *cdc15::ura4* cells, was carried out on a Zeis Aviovert II inverted microscope equipped with a Plan Apo 100/1.40 lens, piezo-electric Z-axis stepper objective motor (Physik Instumente, Germany), an UltraView LCI real-time scanning head confocal (PerkinElmer Life Sciences), a 488 nm argon-ion laser (for GFP and YFP excitation), a 442 nm Helium Cadmium laser (for CFP excitation) and images were captured on an Orca-ER charge-coupled device (CCD) camera (Hamamatsu, Japan). Images were captured using Ultra-View software (Perkin Elmer Life Sciences) and subsequently processed using Volocity 2.0 software (Improvision, Lexington, MA). For time-lapse experiments, cells were placed on a YE agar pad and a coverslip was then sealed over the sample using VALAP (vaseline:lanolin:parafin). For spore germination experiments, fluorescence microscopy was performed on a Zeiss Axioskop II equipped with a z-focus motor drive, and images

were captured with an Orca II charge-coupled device camera (Hamamatsu, Japan). Images were obtained, processed, and analyzed with OpenLab 2.1.3 software (Improvision, Lexington, MA). In all microscopy experiments on temperature sensitive strains, the temperature of the sample was maintained during examination using an objective heater (Bioptechs). For visualization of actin structures, cells were fixed and processed as previously described (Pelham and Chang, 2001). Latrunculin-A (Molecular Probes) was used at a concentration of 100 □M.

Yeast two-hybrid analysis

The yeast two-hybrid system used in this study was described previously (James et al., 1996). Various portions of the *cdc15*$^+$, *myo1*$^+$, *wsp1*$^+$, *vrp1*$^+$, and *cdc12*$^+$ cDNAs were cloned into the bait plasmid pGBT9 and/or the prey plasmid pGAD424 (Clontech, Palo Alto, CA) and sequenced to ensure the absence of PCR-induced mutations and that the correct reading frame had been retained. To test for protein interactions, both bait and prey plasmids were co-transformed into *S. cerevisiae* strain PJ69-4A. ß-galactosidase reporter enzyme activity in the two-hybrid strains was measured using Galacto-Star™ chemiluminescent reporter assay system according to the manufacturer's instructions (Tropix Inc., Bedford, MA), with the exception that cells were lysed by glass bead disruption. Each sample was measured in triplicate. Reporter assays were recorded on the Mediators PhL luminometer (Aureon Biosystems, Vienna, Austria).

***In vitro* binding assays of recombinant proteins**

Glutathione-S-transferase (GST)- or Maltose Binding Protein (MBP)-fusion proteins were produced in *Escherichia coli* from pGEX-2T or pMAL-2C, respectively, and purified on either glutathione-Sepharose beads (for GST) or amylose-Sepharose beads (for MBP). To elute fusion protein from beads they were incubated in the presence of excess amounts of either glutathione or maltose, as appropriate. The supernatant, containing the eluted proteins, was then separated from the beads. For binding reactions, bead-bound recombinant proteins were incubated for 1h at 4ºC with the eluted fusion protein indicated in binding buffer (20 mM Tris-HCL, pH 7.0, 150 mM NaCl, 2 mM EDTA, 0.1% NP-40). The beads were washed extensively in binding buffer, and the proteins were resolved by SDS-PAGE followed by Coomasie blue staining to visualize the proteins.

In vitro binding assays in reticulocyte lysates

The indicated fragments were cloned into pSK(+) and translated *in vitro* in the presence of [^{35}S]-Trans label (ICN Pharmaceuticals, Irvine, CA) with the use of the T_NT-coupled reticulocyte lysate system (Promega, Madison, WI). Recombinant fusion proteins and binding reactions were carried out as described above except that SDS-PAGE gels were treated with Amplify (Amersham Pharmacia Biotech, Piscataway, NJ), and bound ^{35}S-Wsp1p was visualized by a Storm 860 phosphoimager (Amersham Pharmacia Biotech).

Protein lysates, immunoprecipitations, lysate bindings, and immunoblotting.

Protein lysates were prepared in either NP-40 (Gould et al., 1991) or KCl (Morrell et al., 1999) buffer . For immunoprecipitations, cells were lysed natively, incubated with anti-HA or anti-Myc monoclonal antibodies and washed as previously described (Gould et al., 1991; McDonald et al., 1999). Denatured lysates were obtained as detailed (Burns et al., 2002). For lysate binding experiments, the indicated bead-bound recombinant proteins were added to lysates, incubated for 1h at 4ºC, and extensively washed with NP-40 buffer. Proteins were resolved by SDS-PAGE and transferred by electroblotting to a PVDF membrane (Immobilon P, Millipore Corp., Bedford, MA). Anti-HA (12CA5) and Anti-Myc (9E10) antibodies were detected using horseradish-peroxidase-conjugated goat anti-mouse secondary antibodies (0.8 mg/ml; Jackson ImmunoResearch Laboratories, West Grove, PA) at a dilution of 1:50,000, Immunoblots were visualized using ECL reagents (Amersham Pharmacia Biotech).

Immunoprecipitation/phosphatase assay

Following cell lysis, Cdc15-HA was immunoprecipitated with 12CA5-sepharose beads and washed two times in NP-40 buffer then four times in phosphatase buffer (25 mM HEPES-NaOH pH 7.4, 150 mM NaCl, 0.1 mg/ml BSA). Beads were then split into two samples, pelleted and resuspended in 9 μl of 1X phosphatase buffer + 2mM $MnCl_2$. One microliter of either lambda phosphatase (New England Biolabs) or H_2O (mock) was added to each tube, followed by incubation at 30ºC for 30-45 minutes with frequent mixing. Beads were then washed extensively with NP-40 buffer, resuspended in 30 μl 2X SB, and immunoblotted.

In vivo labeling cells with ^{32}P orthophosphate and phospho-amino acid analysis

Cells were inoculated from a pre-culture into phosphate free media plus supplements and 50 m NaH_2PO_4, grown 16 hours, collected, and resuspended in phosphate free media. ^{32}P orthophosphate label (NEN, New Jersey) was added and cells were incubated 4 hours prior to harvesting. Cells were lysed and the proteins resolved by SDS-PAGE. Proteins were transferred by electroblotting to a PVDF membrane (Immobilon P, Millipore Corp., Bedford, MA) and detected by auotradiography. ^{32}P -Labeled Cdc15p was subjected to partial acid hydrolysis while bound to the PVDF membrane, and the phosphoamino acids were separated in two dimensions by thin-layer electrophoresis at pH 1.9 and 3.5. Phosphoamino acids were visualized by autoradiography or with the use of a Molecular Dynamics PhosphorImager.

CHAPTER III

CDC15P INTERACTS WITH TWO ACTIN NUCLEATION PATHWAYS

Introduction

Our lab showed previously that neither actin patches nor the Arp2/3 complex are recruited to the medial region of *cdc15-140* cells grown at its restrictive temperature (Balasubramanian et al., 1998). Furthermore, previous work has also demonstrated that the only other identified actin nucleator in *S. pombe*, Cdc12p, is also incapable of medial localization in *cdc15* mutants (Chang et al., 1997). Therefore, we wished to determine the nature of the interaction between Cdc15p and these two actin nucleation pathways. Using genetic, yeast two-hybrid, and biochemical approaches we observed that Cdc15p interacted directly with components of both nucleation pathways. Cdc15p bound directly to the tail homology 1 and 2 (TH1/TH2) regions of the Arp2/3 activator Myo1p. Cdc15p also interacted directly with Cdc12p in a manner dependent on the Formin Homology 3 (FH3) region of the protein. We have also characterized the dependence of Cdc15p localization on actin nucleators and thereby discovered a previously undetected localization pattern of Cdc15p in live cells.

Results

Cdc15p is required for medial localization of Arp2/3 activators during mitosis.

Given the lack of medial recruitment of Arp2/3, we first asked whether activators of this complex are also not properly localized in a *cdc15* mutant. We examined in live cells the localization of endogenously GFP-tagged Myo1p and Wsp1p, as well as, verprolin (Vrp1p), a potential regulator of these activators. Consistent with known roles in actin patch regulation (Lee et al., 2000; Naqvi et al., 2001; Toya et al., 2001), Myo1p-GFP, Wsp1p-GFP, and Vrp1p-GFP were all localized as patches at growing cell ends during interphase, while in mitosis they were detected in the medial region of cells (Fig. 7 A-C). In *cdc15-140* cells at 36°C, however, all three of these proteins remained primarily in patches at the cell cortex (Fig. 7 A-C). Examination of *cdc15-140 myo1-gfp* cells, synchronized in G2 and released to either permissive or restrictive temperature, revealed that Myo1p was never detected in the medial region in the

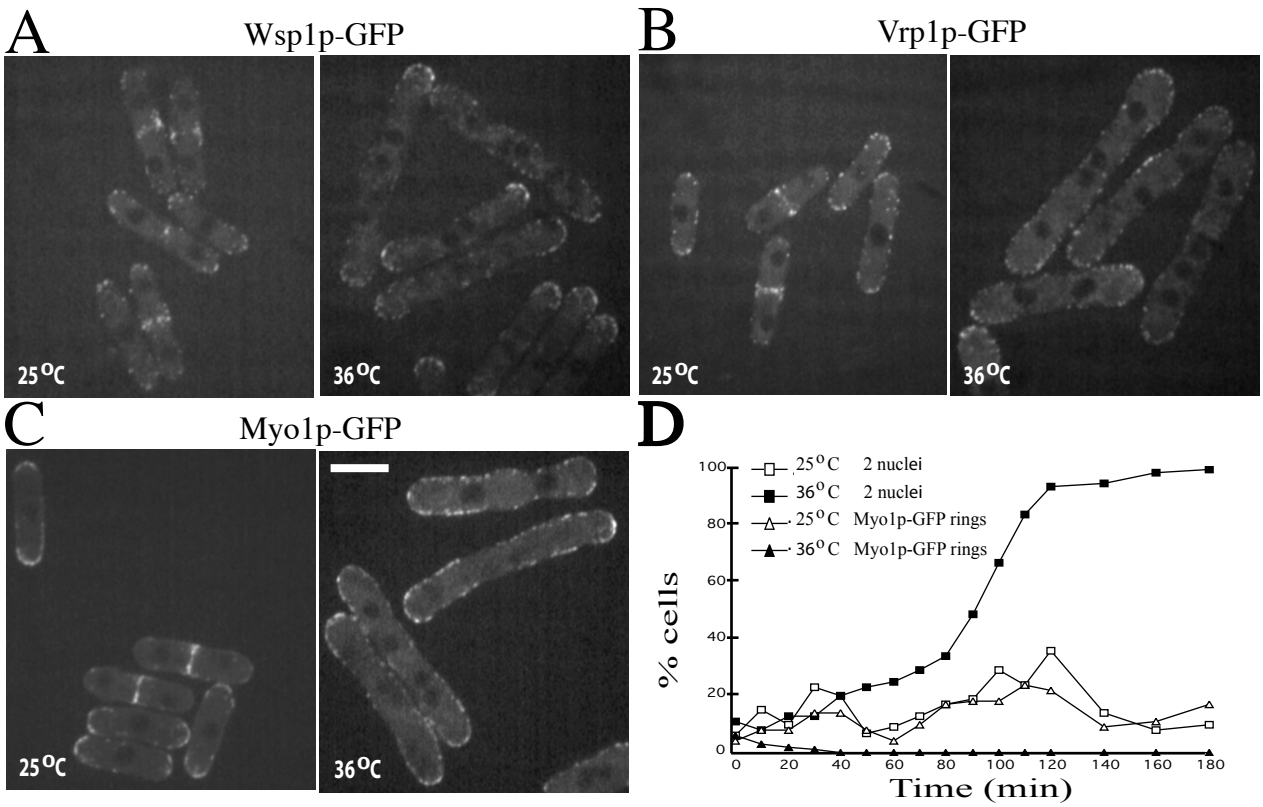


Figure 7. GFP-tagged Arp2/3 activators fail to localize to the medial region in a cdc15 mutant at the restrictive temperature. (A-C) *cdc15-140* cells with either *wsp1-GFP* (KGY358), *vrp1-GFP* (KGY108), or *myo1-GFP* (KGY3963) alleles, were grown to log phase at 25ºC and shifted to 36ºC for 4h before live cells were imaged. Scale bars on this and all subsequent figures represent 5 μm. (D) *myo1-GFP cdc15-140* cells (KGY3963) were synchronized by lactose gradients and released to 25˚C or 36ºC. Live cells were monitored for Myo1p-GFP and parallel samples were fixed and stained with DAPI to visualize nuclei. Localization of Myo1p to the medial region is indicated by triangles and cells with two or more nuclei are indicated by squares.

absence of Cdc15p function (Fig. 7 D). Similar results were obtained for Wsp1p-GFP and Vrp1p-GFP (data not shown).

Interactions of Arp2/3 complex regulators with one another.

In order to understand possible interactions between Cdc15p and regulators of the Arp2/3 complex, we first needed to determine how these factors interacted with one another. The *S. pombe* verprolin homolog has not previously been characterized. Examination of the *S. pombe* genome sequence data in the Sanger Centre database revealed a protein (SPBC13E7.09) related to *S. cerevisiae* Vrp1p and human WASp-interacting protein (WIP), that we have called *S. pombe* Vrp1p (Fig. 8 A). We determined that *vrp1* is not an essential gene, but similar to the case in *S. cerevisiae* (Naqvi et al., 2001), *S. pombe vrp1*Δ cells display morphological defects at high temperatures and are cold-sensitive (Fig. 8 B-C).

In *S. cerevisiae*, Vrp1p, Las17p/Bee1p, and type I myosins form an Arp2/3 regulatory complex, with direct protein-protein interactions observed between Vrp1p and Las17p/Bee1p and between both of these proteins and the type I myosins (Evangelista et al., 2000; Lechler et al., 2000). Therefore, we asked whether the *S. pombe* homologs of these proteins also interact with one another. Similar to observations in *S. cerevisiae*, Wsp1p interacted strongly with the SH3 domain of Myo1p (residues 1077-1218), as well as with the C-terminus of Vrp1p (residues 206-309), by two hybrid analysis (Fig. 9 A). Further, GST-Myo1p(1077-1218) and MBP-Vrp1p(206-309) fusion proteins bound directly to Wsp1p fragments produced in a coupled transcription/ translation *in vitro* system (Fig. 9 B-C). These binding regions corresponded to those found to interact in the *S. cerevisiae* homologs (Evangelista et al., 2000; Lechler et al., 2000). Contrary to expectations, however, we found no evidence for an interaction between any regions of Myo1p and Vrp1p by two-hybrid and direct binding analyses. Additionally, while *vrp1*Δ *wsp1*Δ cells were viable (Fig. 8 C), deletion of both *vrp1* and *myo1* was synthetically lethal.

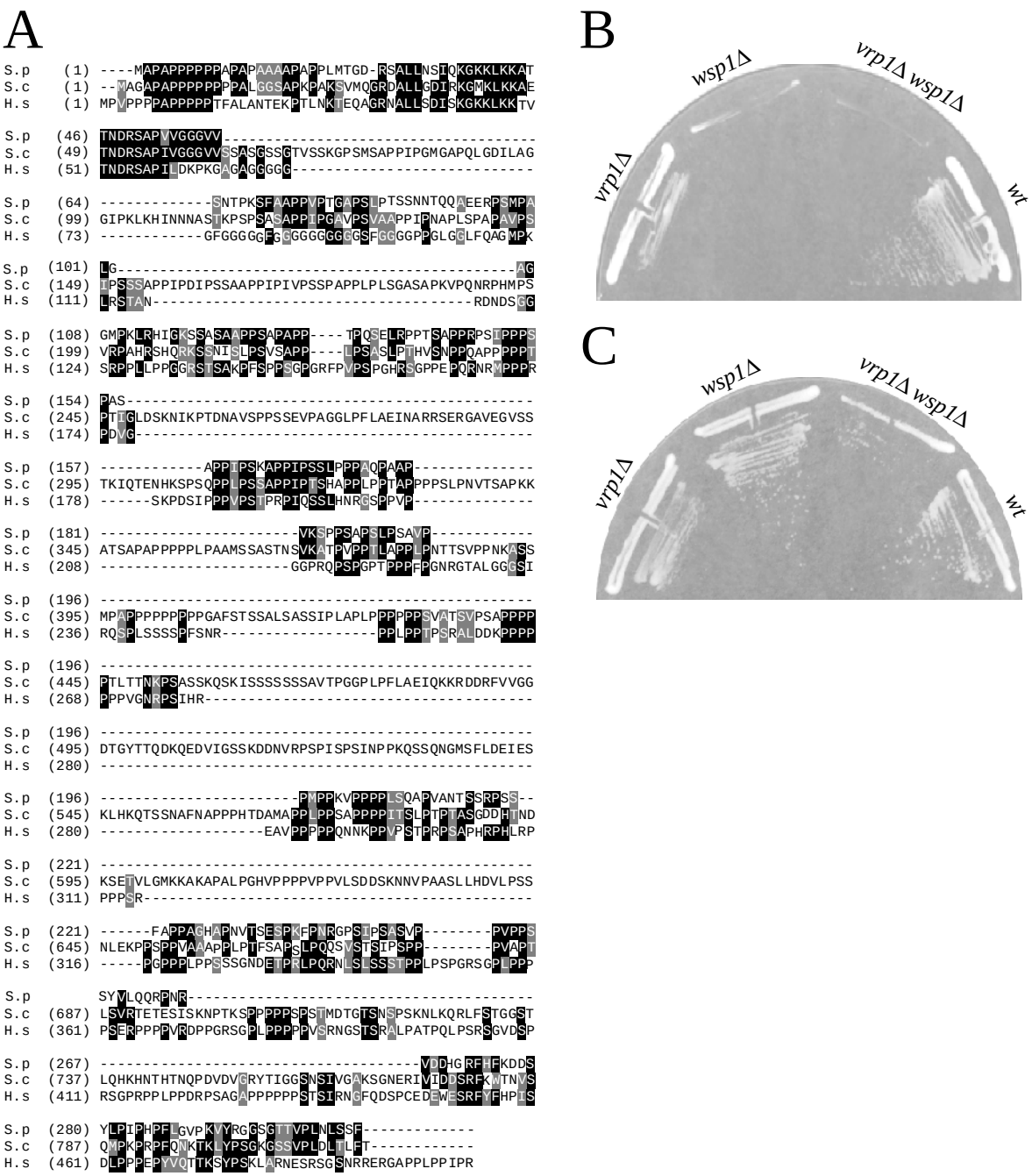


Figure 8. Characterization of *S. pombe* Vrp1p. (A) Alignment of *S. pombe* Vrp1p (S.p) with *S. cerevisiae* Vrp1p (S.c) and human WIP (H.s). Identical amino acids are in solid boxes and similar residues are shaded. (B-C) The entire coding regions of *vrp1* and *wsp1* were replaced with the *ura4+* cassette to create deletion strains. *vrp1*Δ, *wsp1*Δ, *vrp*1Δ *wsp1*Δ, and wild-type cells (KGY246) were struck to YE medium at 18˚C (B) or 36˚C (C).

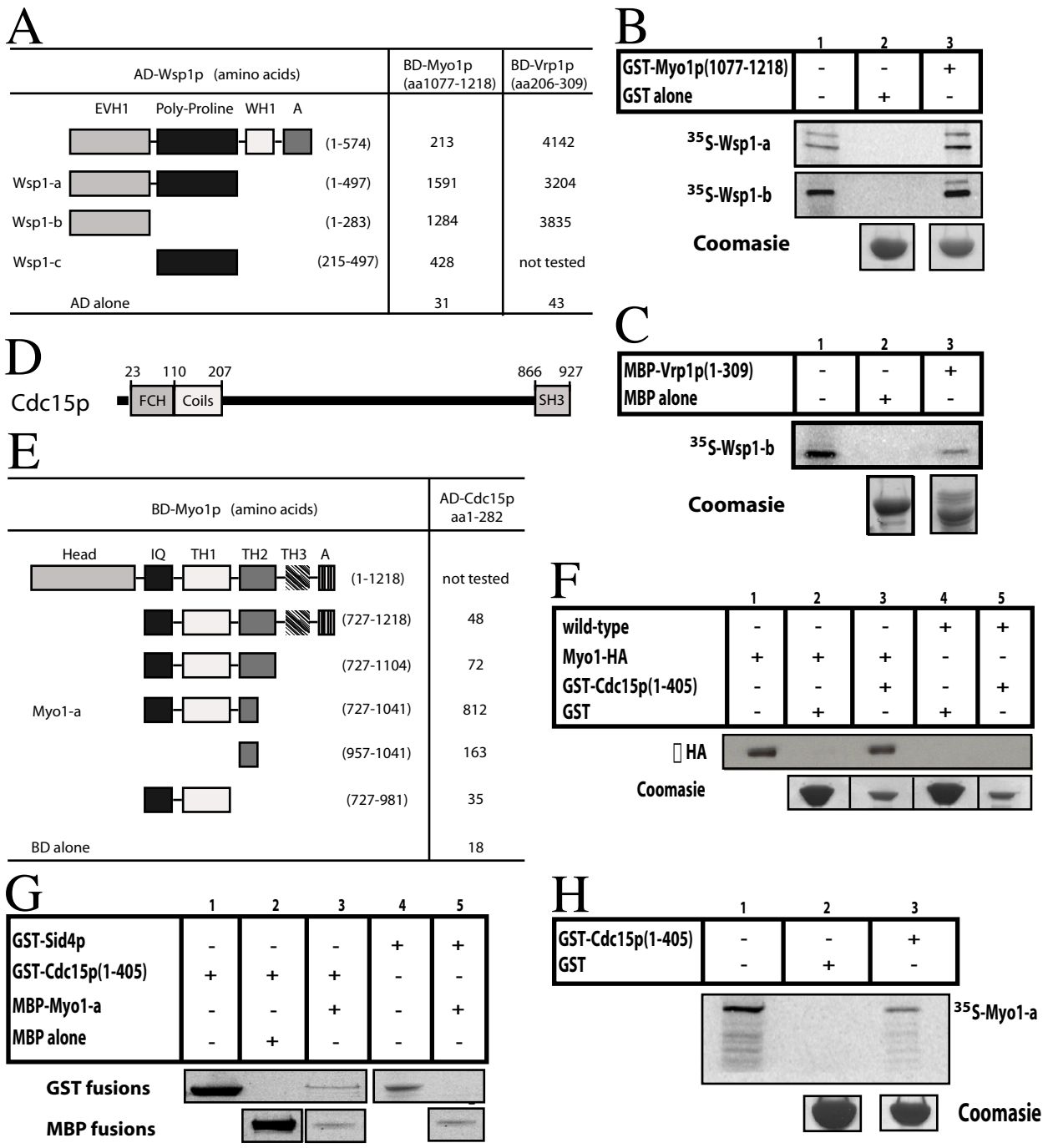


Figure 9. Protein-protein Interactions of Arp2/3 complex regulators. (A) The indicated regions of Wsp1p were tested for interaction with Myo1p and Vrp1p by two-hybrid analysis. LEU+ TRP+ transformants were tested for growth on selective media (data not shown) and assayed for β-galactosidase activity measured in relative light units. (B) Approximately equal amounts of GST (lane 2, bottom panel) and GST- Myo1p(1077-1218) (lane 3, bottom panel) bound to glutathione beads were mixed with *in vitro* translated Wsp1p-a (amino acids 1-497, top panel) or Wsp1p-b (amino acids 1-283, middle panel). Beads were collected, washed and eluted as described in materials and methods. Proteins were resolved by SDS-PAGE and detected by fluorography (top 2 panels) or Coomasie staining (bottom panel). Only relevant portions of the Coomasie-stained gel are shown to indicate loading, however all proteins ran at the predicted sizes. Lane 1 contains 10% of the input into the reactions. (C) Approximately equal amounts of MBP (lane 2, bottom panel) and MBP-Vrp1p(1-309) (lane 3, bottom panel)

Figure 9 – continued. bound to amylose beads were mixed with *in vitro* translated Wsp1p-b (top panel). Proteins were further analyzed as in B. (D) A graphic representation of Cdc15p. Amino acid residues at the borders of known domains are shown. (E) The indicated regions of Myo1p were tested for interaction with Cdc15p (1-282) by two-hybrid analysis as in A. (F) GST (lanes 2 and 4) or GST-Cdc15p(1-405) (lanes 3 and 5) bound to glutathione beads were incubated with protein lysates from *myo1-HA* cells (KGY3960) and subsequently extensively washed in binding buffer. Bound proteins were then divided and analyzed by immunoblotting with □-HA antibodies (top panel) and Coomasie staining (bottom panel). Lane 1 contained lysate from *myo1-HA* cells as an input control. (G) Approximately equal amounts of MBP (lane 2, bottom panel) or MBP-Myo1-a (amino acids 727-1041) (lanes 3 and 5, bottom panel) bound to amylose beads were mixed with either soluble GST-Cdc15p(1-405) (lanes 2-3) or GST-Sid4p (lane 5). Beads were collected, washed and eluted as described in Materials and methods. Proteins were resolved by SDS-PAGE and detected by Coomasie staining. Only relevant portions of the Coomasie gel are shown to indicate loading, however all proteins ran at the predicted sizes. Lanes 1 and 4 contain samples of the GST fusion proteins before the binding reactions. (H) Approximately equal amounts of GST (lane 2, bottom panel) and GST-Cdc15p(1-405) (lane 3, bottom panel) bound to glutathione beads were mixed with in vitro translated Myo1-a (top panel). Proteins were further analyzed as in B.

Cdc15p and Myo1p interact directly.

Having observed that Arp2/3 complex regulators are not medially recruited in a *cdc15* mutant, we asked whether any of these proteins physically interacted with Cdc15p. Extensive two-hybrid analysis revealed no detectable interactions between any regions of Cdc15p and either Wsp1p or Vrp1p (data not shown). However, a strong interaction was observed between the N-terminus of Cdc15p and the C-terminus of Myo1p (Fig. 9 D-E). The Myo1p-interacting region of Cdc15p contained the conserved FCH domain and the two predicted coiled-coils of the protein. In Myo1p, residues 727-1041, which contains the IQ repeats as well as the tail homology 1 (TH1) and 60% of the tail homology 2 (TH2) motif, interacted strongly with Cdc15p. Furthermore, the partial TH2 region from this construct also supported a significant interaction with Cdc15p.

We next tested whether Cdc15p and Myo1p could interact in *S. pombe* cell lysates. GST-Cdc15p(1-405) associated with Myo1-HA from lysates (Fig. 9 F, lane 3), while no interaction was seen with GST alone (lane 2). Furthermore, GST-Cdc15p(1-405) also interacted directly with both MBP-Myo1p(727-1041) (Fig. 9 G), and ^{35}S-Myo1p(727-1041), produced in a coupled

transcription/translation *in vitro* system (Fig. 9 H), while GST alone was unable to bind Myo1p in either case.

Localization of Cdc15p.

Given that medial mitotic recruitment of Arp2/3 regulators is dependent upon Cdc15p function, we wished to determine whether loss of these proteins affected Cdc15p localization. Consistent with the non-essential nature of *wsp1*, *vrp1*, or *myo1* for cytokinesis, Cdc15p-GFP localization to the CAR appeared normal in the individual deletion strains (Fig. 11). Live cell imaging analysis, however, revealed a previously unappreciated interphase localization of Cdc15p to numerous spots at cell tips in wild-type and *wsp1*, *vrp1*, or *myo1* deletion cells (Fig. 10 A-C, Fig. 11, see also Video 1). Previous work on Cdc15p has been limited to examination of the protein localization by indirect immuno-fluorescence (IF) in fixed cells (Fankhauser et al., 1995). However, by live cell imaging of Cdc15p-GFP, we typically observed a small number (2-5/cell) of large bright non-motile spots, as well as numerous smaller fast-moving spots (Fig. 10 C, see also Video 2). Small motile spots could occasionally be observed emerging from the larger static spots (Fig 10 C). Upon entry into mitosis, Cdc15p-GFP spots moved to the medial region of the cell where they were incorporated into the newly forming Cdc15p ring structure (Fig. 12 A, see also Video 3). The Cdc15p ring initially appeared as a network of interconnected fibers encircling the medial region (Fig. 10 E, see also Video 4), which quickly coalesced into a thicker ring structure. The appearance of the Cdc15p ring is subsequent to SPB duplication (Fig. 10 D). The Cdc15p-GFP ring then constricted as cells divided and Cdc15p-GFP reformed as spots and re-localized to the old cell ends. Cdc15p-GFP spots were then detected at both cell ends when cells resumed bipolar growth (Fig. 10 A-B).

As cells entered mitosis, a prominent Cdc15p-GFP spot could be detected associated with the nucleus prior to ring assembly (Fig. 12 A, see also Video 3). This medial spot of Cdc15p-GFP was reminiscent of a similar spot structure containing the formin Cdc12p that associates with the nuclear periphery just prior to ring assembly (Chang, 1999). The motile Cdc12p spot localizes to the medial cortex region as cells enter mitosis, presumably serving as a nucleating structure for the formation of the CAR (Chang, 1999; Chang et al., 1997). The nuclear associated spot, and all other localizations of Cdc15p, were easily visible when expressing only the N-terminal region of Cdc15p (residues 1-425) from a plasmid under control of the native

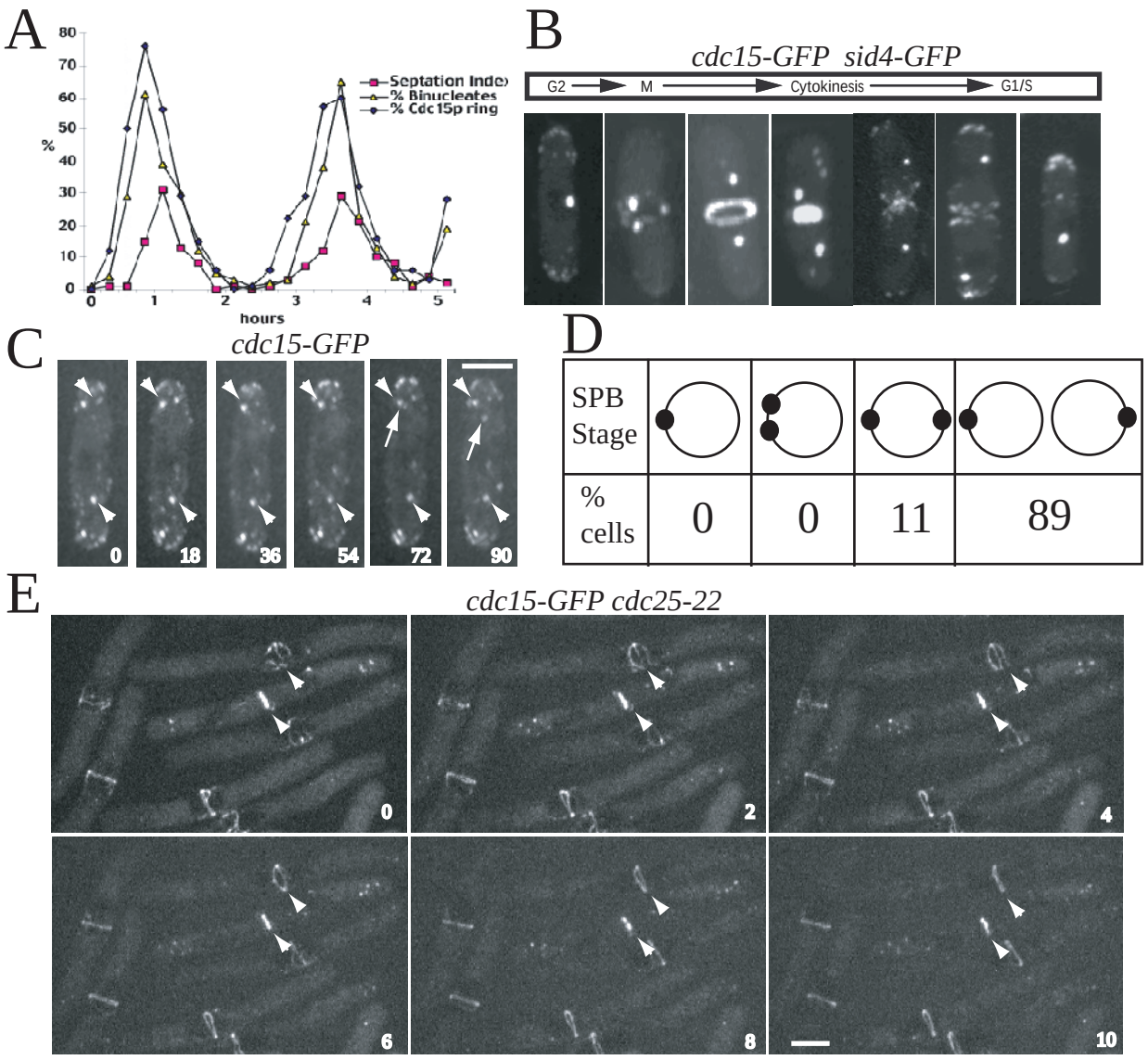


Figure 10. Localization of Cdc15p-GFP. (A-B) *cdc15-GFP sid4-GFP* Cells (KGY3362)were grown to mid-log at 25˚C and synchronized in early G2-phase by centrifugal elutriation. Sid4p-GFP localization was used to monitor spindle pole bodies and to indicate cell-cycle stage of cells. (A) Cells were collected at 15 min intervals and examined for septation index, percentage of binucleate cells, and percentage of cells with Cdc15p-GFP medial rings. (B) Representative live cell images of *cdc15-GFP sid4-GFP* cells from A at various cell-cycle stages. (C) Time-lapse images of an interphase *cdc15-GFP* cell (KGY3019) with two non-motile spots indicated by arrowheads. The arrow indicates the formation of a smaller patch from a larger one. Time is indicated in seconds. (D) *cdc15-GFP sid4-GFP* cells from A were examined for the presence of Cdc15p-GFP rings with respect to SPB duplication, as determined by Sid4p-GFP localization. (E) Time-lapse images of synchronized *cdc15-GFP cdc25-22* cells (KGY3042) that had been arrested for 4h at 36˚C and released to 25˚C for 10 minutes prior to the beginning of the time course at the early stages of ring formation. The arrowhead indicates a branched network of fibers encircling the medial region that is the initial Cdc15p ring structure. This network then coalesced to form a thick bright ring structure. Time is indicated in minutes.

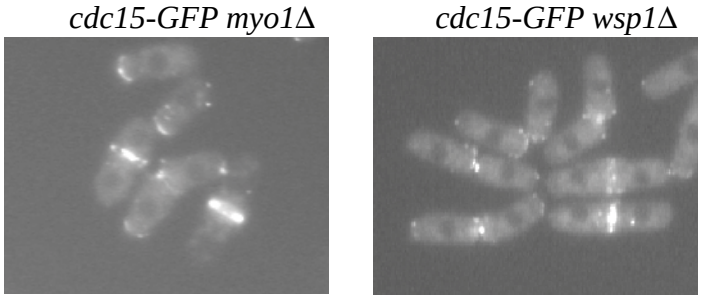


Figure 11. Cdc15p-GFP localizes to both a medial ring and spots at cell ends in the absence of Myo1p (left panel), Wsp1p (middle panel) at 25˚C, or in arp3 mutant cells at the restricitve temperature of 18˚C (right panel).

See attached file Video1.mov

Video 1. Cdc15p-GFP localizes to both cells ends and the medial ring. Partial rotation of a three-dimensional reconstruction of cells expressing endogenously tagged Cdc15p-GFP and Sid4p-GFP.

See attached file Video2.mov

Video 2. Cdc15p-GFP spots exhibit both motile and non-motile behavior. Time-lapse images of live *cdc15-GFP* cells (KGY3019). Z-series optical sections (spacing of 0.5 mm) were collected at 17 s intervals.

See attached file Video3.mov

Video 3. A medial spot of Cdc15p-GFP arrives prior to ring formation and appears to nucleate Cdc15p ring assembly. Time-lapse images of *cdc15-GFP* live cells. Z-series optical sections (spacing of 0.5 μm) were collected at 25 s intervals. The cell shown in Fig. 12 A can be seen in the center of the frame. In this cells a Cdc15p medial spot can be seen arriving with the subsequent formation of a ring.

See attached file Video4.mov

Video 4. Cdc15p-GFP ring first appears as a network of interconnected fibers, which are subsequently bundled to form the mature ring. Time-lapse images of live *cdc15-GFP* cells. Z-series optical sections (spacing of 0.2 μm) were collected at 30 s intervals.

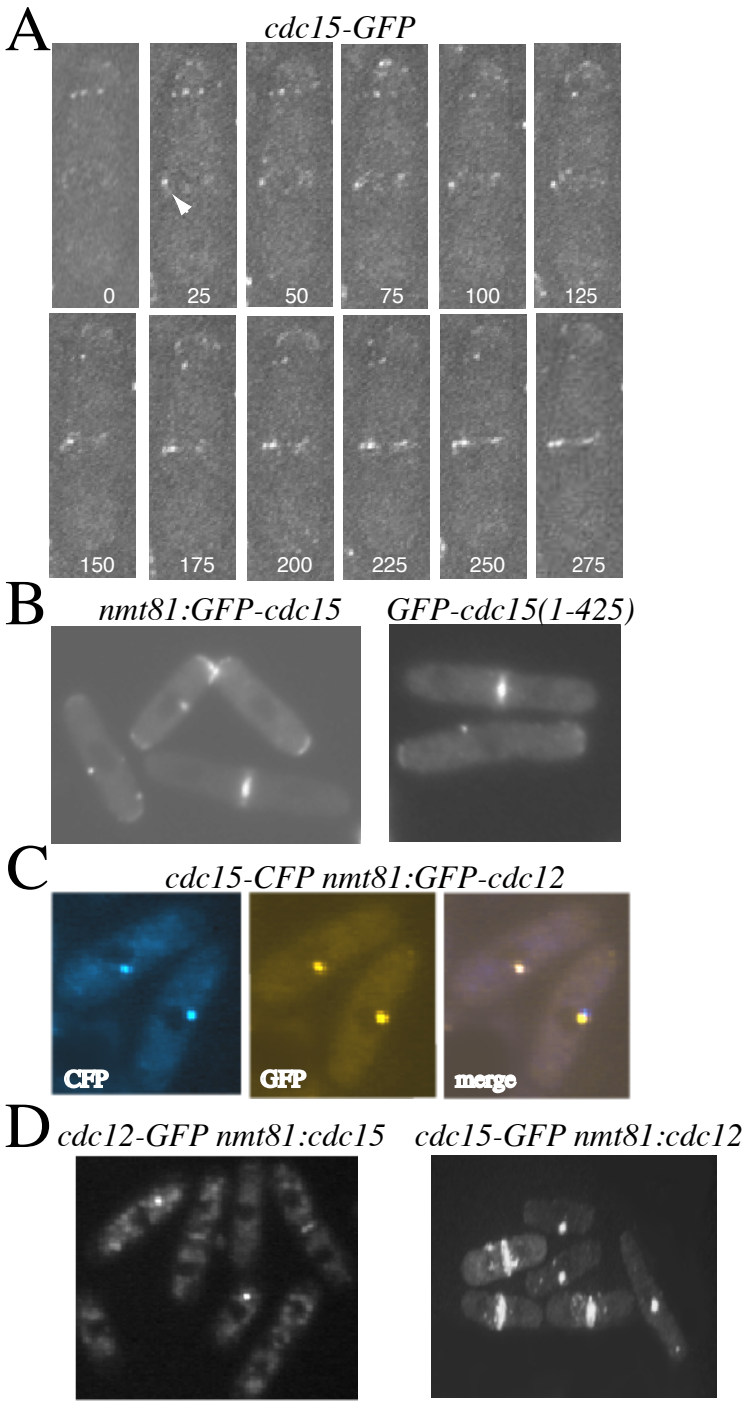


Figure 12. Cdc15p colocalizes with the formin, Cdc12p, in a medial spot. (A) Time-lapse images of a *cdc15-GFP* cell (KGY3019). The arrowhead indicates the initial localization of a Cdc15p-GFP spot to the medial region with subsequent incorporation of other Cdc15p patches into this structure. (B) GFP-Cdc15p, expressed under control of *nmt81* promoter, and GFP-Cdc15p(1-425), under control of the *cdc15+* promoter were visualized in live wild-type cells. (C) GFP-Cdc12p was expressed under control of the *nmt81* promoter and visualized in live *cdc15-CFP* cells (KGY3352). (D) Cdc15p was produced under control of the *nmt81* promoter in cdc12p-GFP cells (KGY3066) and live cells were imaged (left panel). Cdc12p was produced under control of the *nmt81* promoter in *cdc15-GFP* cells (KGY3019) and live cells were imaged (right panel).

promoter (Fig. 12 B). To ask whether the nuclear associated spots of Cdc12p and Cdc15p were in fact the same structure, we expressed GFP-Cdc12p under control of the low strength *nmt81* promoter in a strain where the chromosomal locus of *cdc15* had been modified to produce a C-terminal fusion to CFP (Cyan-fluorescent protein). In these cells, a spot containing both GFP-Cdc12p and Cdc15p-CFP was observed in interphase cells (Fig. 12 C). Furthermore, we observed that, while these spots were somewhat difficult to detect with endogenous levels of these proteins, mild overexpression from the *nmt81* promoter of either untagged Cdc12p or Cdc15p promoted higher levels of either endogenously-tagged Cdc15p-GFP or endogenously-tagged Cdc12p-GFP, respectively, into a spot structure (Fig. 12 D).

Cdc15p interacts directly with the formin Cdc12p.

Given the co-localization of Cdc15p and Cdc12p to both the CAR and the medial interphase spot, we asked if these proteins could interact. By two-hybrid analysis a strong interaction was detected between the N-terminus of Cdc15p (residues 1-282) and N-terminal constructs of Cdc12p that included its FH3 domain (Fig. 13 A). Previous work has suggested that FH3 domains of formins are involved in targeting them to discrete locations within *S. pombe* cells (Petersen et al., 1998). The N-terminus and FH3 domain of Cdc12p suffice not only for its localizations to the CAR and the motile spot, but also for its interaction with Cdc15p (Fig. 13 A). In contrast to the interaction observed between the *S. cerevisiae* proteins Bni1p and Hof1p, the C-terminal SH3 domain of Cdc15p is not involved in its interaction with Cdc12p. Indeed, a construct of Cdc15p lacking its SH3 domain is able to rescue *cdc15* null cells indicating it does not play an essential role in Cdc15p function during cytokinesis.

We next tested whether Cdc15p and Cdc12p could interact in *S. pombe* cell lysates. We found that MBP-Cdc12p(1-764) but not MBP, incubated with protein lysates from a *cdc15-HA* strain, strongly interacted with Cdc15p-HA (Fig. 13 B, lanes 1 and 3), while it did not interact with the unrelated protein Cut9p-HA (lanes 4 and 5). Furthermore, we observed that GST-Cdc12p(1-764), bound to glutathione beads, was able to bind the soluble recombinant Cdc15p N-terminal region (Fig. 13 C, lane 3), while no binding was seen with an unrelated control protein (Fig. 13 C, lane 5). Thus, Cdc15p and Cdc12p are able to bind directly to one another.

Evidence supporting the biological relevance of this interaction is two-fold. First, while Cdc15p-GFP could be observed both in spots and in the medial cortex region, no Cdc15-GFP

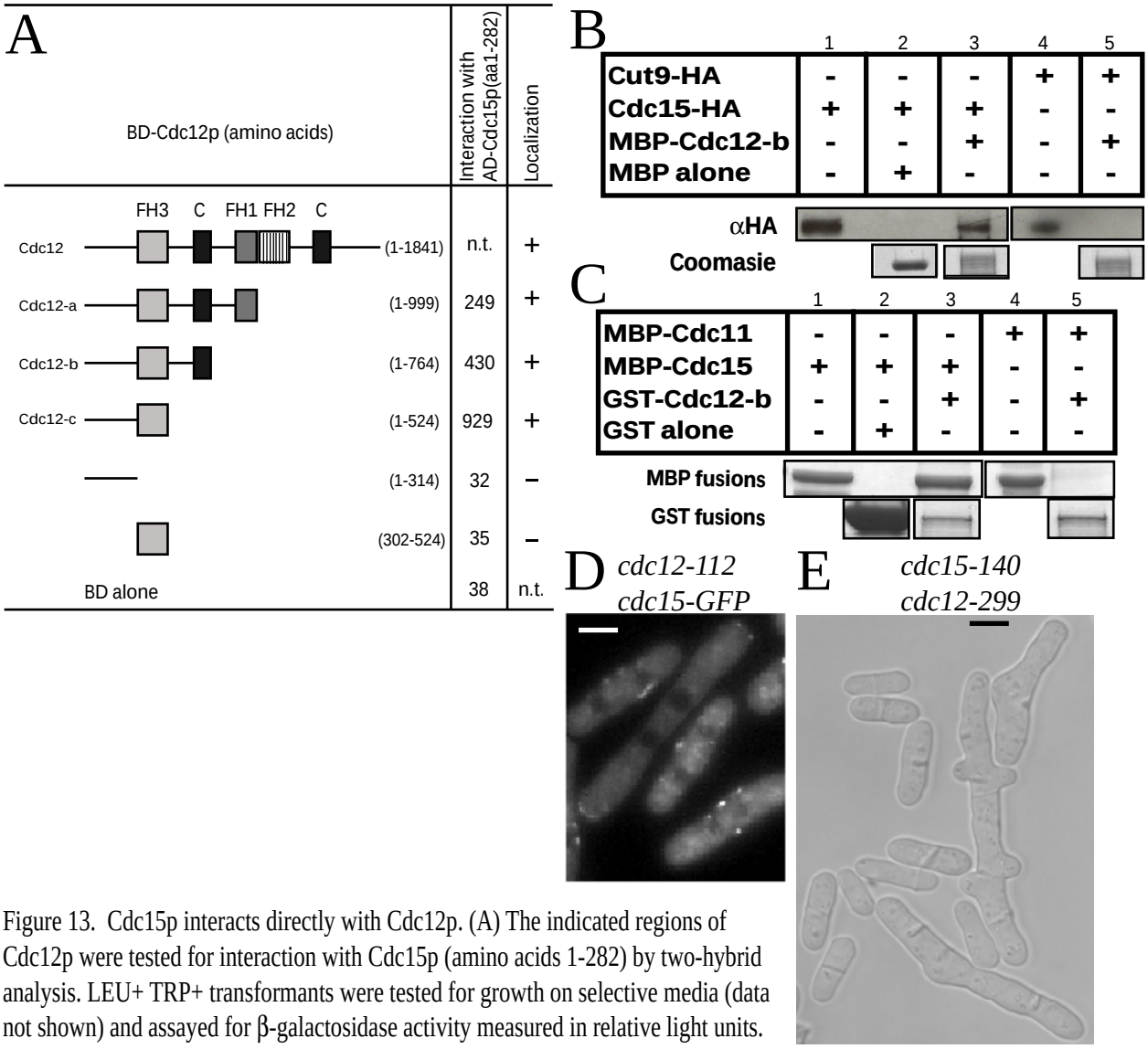


Figure 13. Cdc15p interacts directly with Cdc12p. (A) The indicated regions of Cdc12p were tested for interaction with Cdc15p (amino acids 1-282) by two-hybrid analysis. LEU+ TRP+ transformants were tested for growth on selective media (data not shown) and assayed for β-galactosidase activity measured in relative light units. GFP fusions of the *cdc12* constructs were also expressed under control of the *nmt81* promoter and visually examined for their ability (+) or lack of ability (-) to localize to an interphase spot structure and also to the CAR (column 3). (B) MBP (lane 2) or MBP-Cdc12p-b (amino acids 1-764) (lanes 3 and 5) both bound to amylose beads were incubated with protein lysates from either a *cdc15-HA* strain (KGY3020) or a *cut9-HA* strain (KGY1463) and subsequently extensively washed in binding buffer. Bound proteins were then divided and analyzed by immunoblottimg (top panel) and Coomasie staining (bottom panel). Only relevant portions of the gels are shown, however all proteins ran at the predicted sizes. (C) GST (lane 2, bottom panel) or GST-Cdc12-b (lanes 3 and 5, bottom panel) both bound to glutathione beads were mixed with either soluble MBP-Cdc15p(1-282) (lanes1-3, top panel) or soluble MBP-Cdc11p(1-660) (lanes 4-5, top panel). Proteins were resolved by SDS-PAGE and detected by Coomasie staining. Only relevant portions of the Coomasie gel are shown to indicate loading, however all proteins ran at the predicted sizes. Lanes 1 and 4 contain samples of the MBP fusion proteins before the binding reactions. (D) Localization of Cdc15p-GFP in *cdc15-gfp cdc12-112* cells after 4 hours at 36° C. (E) Phase contrast image of *cdc15-140 cdc12-299* cells grown at 25° C.

rings were detected in *cdc12-112* cells at the restrictive temperature (Fig. 13 D). Further, there is a strong negative genetic interaction between *cdc15* and *cdc12* mutant alleles (Table 4). Even at 25˚C, double mutant cells were barely able to form colonies with many cells in the population failing at cytokinesis (Fig. 13 E).

Table 4. Genetic Interactions of *cdc15* with *cdc12*

Genotype	25ºC	29ºC	32ºC	36ºC
cdc15-140	++	++	+/-	-
cdc12-299	++	++	-	-
cdc15-140 cdc12-299	+/-	-	-	-
cdc12-112	++	++	+/-	-
cdc12-112 cdc15-140	+	-	-	-

Conclusions

We conclude that Cdc15p function is required for medially directed mitotic recruitment of not only the Arp2/3 complex, but also of its known regulators in *S. pombe*. While no evidence was found to indicate an interaction between Cdc15p and either Wsp1p or Vrp1p, Cdc15p and Myo1p were shown to interact directly. We also examined the interaction of Arp2/3 regulators with one another and found that Wsp1p interacts directly with both Myo1p and Vrp1p. However, Myo1p and Vrp1p do not appear to interact with one another. Previous work has indicated that Wsp1p and Myo1p represent redundant pathways of Arp2/3 complex activation in *S. pombe* (Lee et al., 2000). Our data support this model and suggest that Vrp1p is most important for the Wsp1p pathway. We also conclude that Cdc12p and Cdc15p interact directly with one another and exist together in a potential ring nucleating structure at the onset of mitosis. This interaction between Cdc15p and Cdc12p is mutually important for their proper subcellular localization and *in vivo* functions.

CHAPTER IV

CDC15P INVOLVEMENT IN ACTIN DYNAMICS

Introduction

Recent work has established that formins represent an Arp2/3 complex independent pathway for the nucleation of actin filaments (Evangelista et al., 2002; Pelham and Chang, 2002; Sagot et al., 2002). Furthermore, formation of the CAR in *S. pombe* depends upon both the Arp2/3 complex and Cdc12p (Pelham and Chang, 2002). Since Cdc15p is involved in recruitment of both actin nucleation pathways required for CAR formation, we predicted that in the absence of Cdc15p function, actin rings should not be able to form. Contrary to this prediction, however, actin rings were reported to form in *cdc15-140* cells (Balasubramanian et al., 1998). Here by utilizing a Cdc15 heterozygous null strain we find that Cdc15p function is required for actin ring formation. We also show that Cdc15p is semi-dependent on F-actin for its localization and appears to interact with actin *in vivo*.

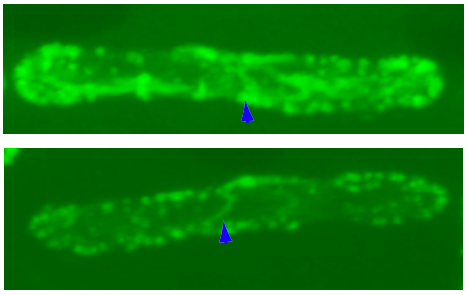
Results

Cdc15p is required for medial actin ring formation.

We initially re-examined actin ring dynamics in the *cdc15-140* mutant and found that while actin rings were detected after a 4h incubation at the restrictive temperature of 36ºC in *cdc15-140* cells, this occurred in fewer than 2% of cells (vs. 12% in a similarly treated wild-type culture). In accordance with another report on CAR formation in *S. pombe* (Arai and Mabuchi, 2002), these rings are often poorly organized and incomplete (Fig. 14 A). To further complicate the analysis of ring formation in this *cdc15* mutant, we also observed that GFP-*cdc15-140*p, expressed under the control of the low strength *nmt81* promoter in a *cdc15-140* background, localized to the medial region at the restrictive temperature in a subset of cells (Fig. 14 B). After 4 hours at the restrictive temperature, *Cdc15-140*p-GFP was still observed in rings and/or a medial spot in some cells. To clarify the ability of cells to form actin rings in the absence of Cdc15p function we examined cells lacking the protein. Spores from a *cdc15*$^{+}$/*cdc15::ura4*$^{+}$ Ura4+ growth. As the diploid was a homozygous *ura4* mutant at the endogenous locus, only

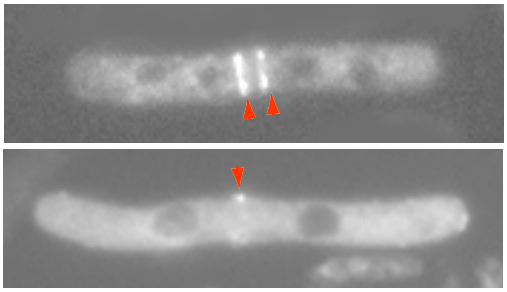
A

cdc15-140



B

cdc15-140 nmt81-GFP-cdc15-140



C

Ura Selection	nuclei	% rings	total cells
+	mononucleates	0	120
+	binucleates	0	111
-	mononucleates	19	108
-	binucleates	47	125

D

cdc15::ura^{+}4

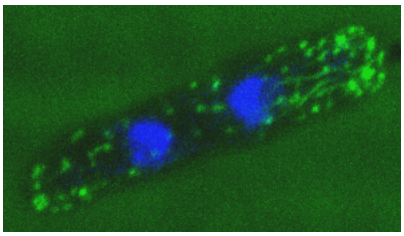


Figure 14. Cdc15p is required for formation of the medial actin ring. (A) Representative images of *cdc15-140* cells grown to mid-log phase at 25 C and shifted to 36 C for 4 hours. Cells were then fixed and stained with AlexaFluor 488-phalloidin to visualize actin (green) Medial actin structures are marked by blue arrowheads. (B) GFP-*Cdc15-140p* expressed from a plasmid under the control of the *nmt81* promoter in *cdc15-140* cells. Medial GFP-*Cdc15-140*p structures are marked by red arrowheads. (C) Spores from the heterozygous *cdc15+/cdc15:ura4+* diploid were released into selective (-uracil) indicated by (+) or non-selective media (YE) indicated by (-) and after 16 hours were fixed and stained with AlexaFluor 488-phalloidin. The presence of a CAR in germinated cells was determined in binucleates and mononucleates. (D) A representative image of a binucleate *cdc15::ura4* cell, fixed and stained with AlexaFluor 488-phalloidin to visualize actin (green) and DAPI to visualize DNA (blue).

spores possessing the *ura4*$^{+}$ knockout cassette at the *cdc15* locus, therefore lacking Cdc15p, would have been able to germinate in the uracil free media. Under these selective conditions, no CARs were detected as spores germinated and underwent their first mitosis (Fig. 14 C-D). Rather, actin remained principally polarized at cell tips. This is in contrast to spores released into non-selective medium (+uracil), where CARs were observed in approximately 50% of binucleate cells and a significant portion of germinated mononucleate cells (Fig. 14 C), as would be predicted.

As *cdc15* overexpression is sufficient for directing medial actin rearrangement in G2-arrested cells (Fankhauser et al., 1995), we tested whether it was also sufficient for accumulation of regulators of F-actin nucleation. Myo1p-GFP localized predominantly at cell ends in *cdc25-22* G2-arrested cells (Fig. 15 A). In contrast, induction of *cdc15* expression led to medial accumulation of Myo1p-GFP in *cdc25-22* arrested cells (Fig. 15 A). Similarly, *cdc15* overexpression was also sufficient for medial accumulation of a Cdc12p spot in G2-arrested cells (Fig. 15 B).

Cdc15p spots are distinct from actin patches

Because the localization and motility of Cdc15p-GFP interphase spots were reminiscent of actin patches, we examined whether these structures were identical. We found there was not a high degree of spatial overlap between Cdc15p and the known actin patch protein Arc15p during interphase (Fig. 16), suggesting that Cdc15p spots are distinct from actin patches.

Dependence of Cdc15p localization on the actin cytoskeleton

To determine the requirement of the F-actin cytoskeleton for proper Cdc15p localization, we examined Cdc15p localization in *cdc15-GFP cdc25-22* cells synchronized in G2 and subsequently released into mitosis in the presence of either DMSO or Latrunculin A (Lat A). Lat A is a rapid and potent F-actin de-polymerizing agent (Ayscough, 1998). In control (DMSO) cells, Cdc15p rings were observed within 10-15 minutes after release into mitosis (Fig. 17 A). However, in the presence of Lat A, while a band of Cdc15p spots appeared at the medial cortex, a tight Cdc15p ring failed to form even after 60 minutes of observation (Fig. 17 A). While formation of Cdc15p rings depended upon F-actin cytoskeletal integrity, previously established rings persisted after Lat A treatment. At the concentration used, Lat A typically leads to a loss of

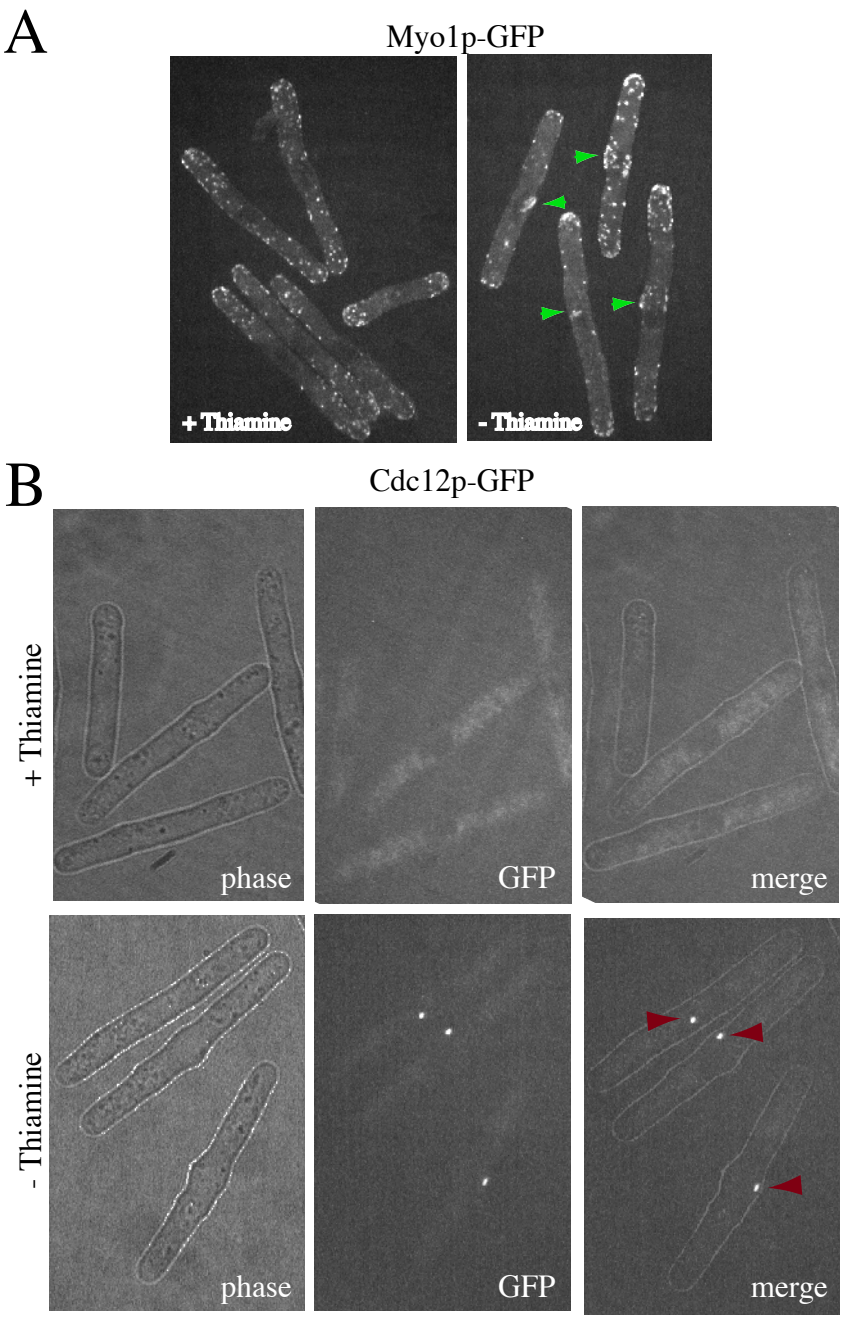


Figure 15. (A) Myo1p-GFP localization in G2-arrested *cdc25-22* cells either expressing (- Thiamine) or not (+ Thiamine) *nmt1-cdc15*. Medial Myo1p-GFP is marked by green arrowheads. (B) Cdc12p-GFP localization in G2-arrested *cdc25-22* cells either expressing (- Thiamine) or not (+ Thiamine) *nmt1-cdc15*. Phase contrast images (left panels) and GFP Images (middle panels) of cells were merged (right panels) to aid in identifying cell boundaries. Medial Cdc12p-GFP spots are marked by red arrowheads.

arc15-GFP cdc15-CFP

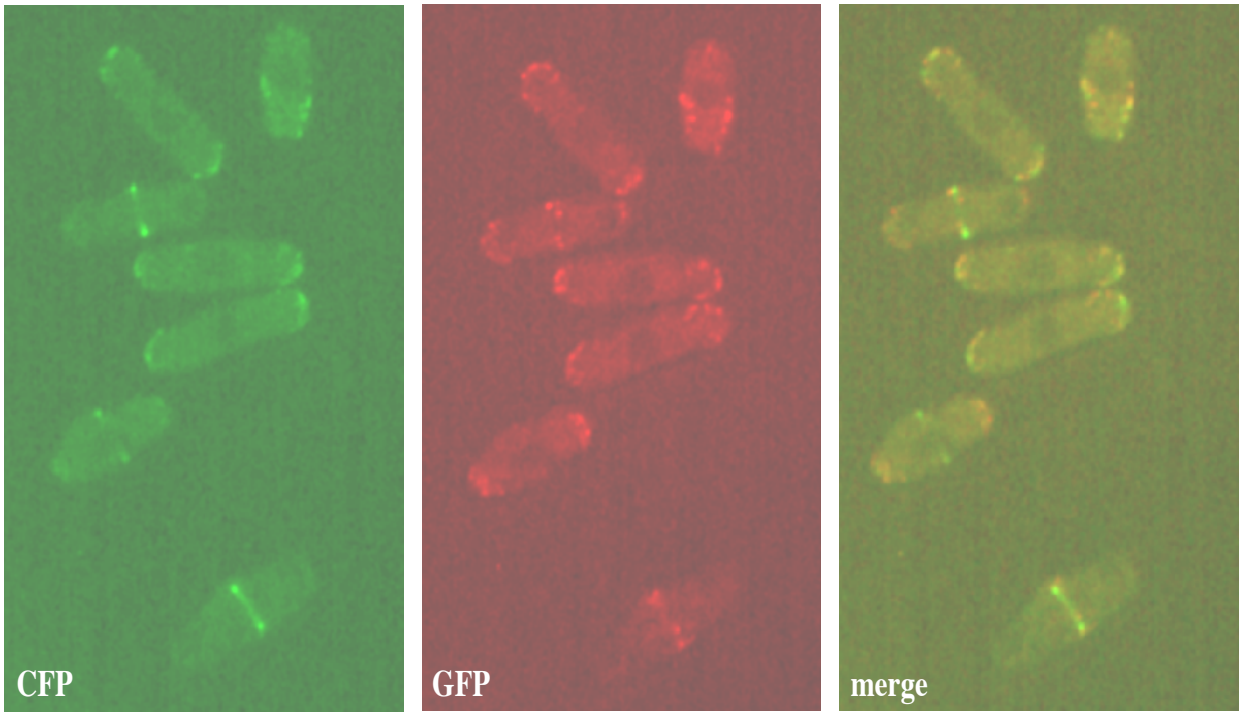


Figure 16. Three-dimensional reconstructions of live cells expressing endogenously tagged Cdc15p-CFP (left panel) and Arc15p-GFP (middle panel) (KGY754) imaged for protein colocalization (right panel).

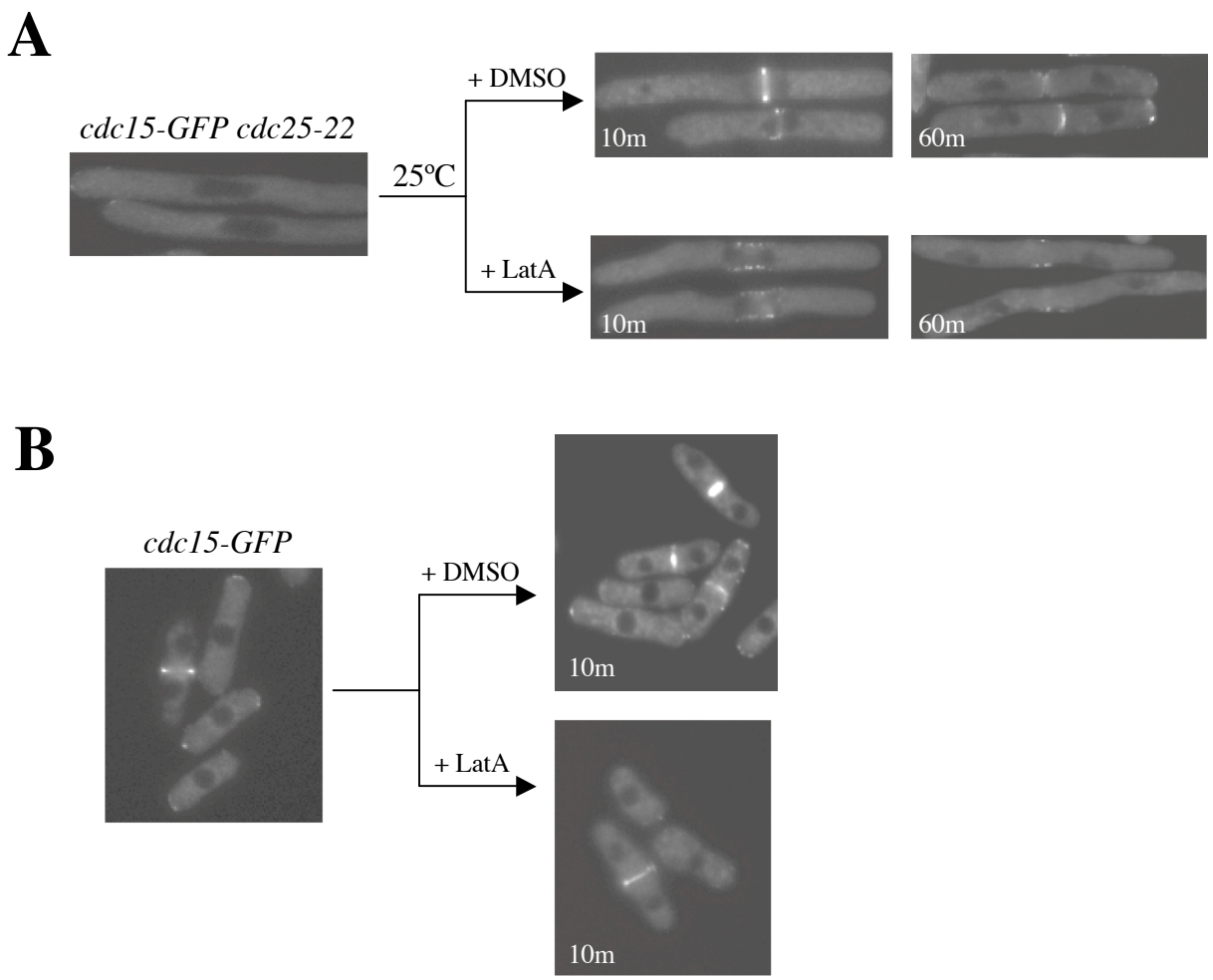


Figure 17. (A) Representative images of *cdc15p-GFP cdc25-22* cells (KGY3042) synchronized by incubation at 36ºC for 4h and released to 25 ºC in the presence of either DMSO or Lat A. Time is indicated in minutes. (B) DMSO or Lat A was added to log phase *cdc15-GFP* cells (KGY 3019) growing at 25 ºC. Time indicated is in minutes after addition of DMSO or Lat A (C) Either -actin or - Arp3p antibodies were added to *cdc15-HA* (KGY3020) native cell. Antibodies and bound proteins were then isolated by incubation of samples with IgG-sepharose. Beads were collected, washed, and eluted as described in materials and methods. Proteins were then resolved by SDS-PAGE and detected by immunoblotting with monoclonal -HA antibodies (12CA5).

F-actin at the ring within one minute of application (Pelham and Chang, 2002). However, Cdc15p-GFP rings were still observed in an asynchronous cell population 10 minutes after treatment with Lat A (Fig. 17 B).

Interaction of Cdc15p with actin

Based on the close association we have observed between Cdc15p and F-actin regulation, we tested whether Cdc15p itself interacted with actin. In order to test this possibility, native cell lysates were prepared from *Cdc15-HA* (KGY3020) cells, then immunoprecipitated utilizing polyclonal antibodies to either actin or Arp3p. Probing of western blots from these immunoprecipitations (IP's) with monoclonal anti-HA antibodies revealed a specific band of the approximate size of Cdc15p-HA (~120 kDa) in the anti-actin IP sample, but not the anti-Arp3p IP sample (Fig. 17 C). As both actin and Arp3p co-migrate with the IgG heavy chain, demonstrating complementary IP's utilizing anti-HA antibodies were not possible. Though Cdc15p appears to interact with actin *in vivo*, this may not be direct as we failed to co-sediment fragments of bacterially-produced Cdc15p with actin filaments (data not shown). One caveat is that constructs of the Cdc15p C-terminus alone appeared to be unstable in the assays.

Conclusion

We conclude that Cdc15p function is strictly required for assembly of the CAR, and is able to direct medial recruitment of key factors involved in this process. Cdc15p appears to have a semi-dependent relationship with the actin cytoskeleton. The actin cytoskeleton is required for Cdc15p ring formation, but not for its relocalization to the medial region during mitosis. Furthermore, once Cdc15p rings assemble they can persist even in the absence of organized F-actin structures. As might be expected Cdc15p associates with actin *in vivo*, but this interaction may not be direct as *in vitro* co-sedimentation with F-actin was not detected. Given the direct association of Cdc15p with numerous other actin regulators, the interaction with actin may take place through Cdc15p binding to these molecules.

CHAPTER V

OTHER MODES OF CDC15P REGULATION

Introduction

Cdc15p has been reported to be a phosphoprotein, which appears to vary in phosphorylation state across the cell cycle. Here we examine Cdc15p phosphorylation and find that it is phosphorylated at serine residues as cells enter mitosis. Furthermore, it returns to a hyperphosphorylated state in a manner that may involve the SIN kinase Sid2p. We also examined a number of other possible means of Cdc15p regulation, including interactions with itself and dependence of its localization on the microtubule cytoskeleton.

Results

Cdc15p phosphorylation across the cell cycle

Cdc15p is a phosphoprotein (Fig. 18 A and E) with an SDS-PAGE protein migration pattern suggesting that the phosphorylation state of the protein varies across the cell cycle (Fankhauser et al., 1995). To more carefully examine this variation, we analyzed Cdc15p-HA in denatured cell lysates from *cdc15-HA* cells (KGY3020), which had been synchronized in early G2-phase by centrifugal elutriation (Fig. 18 B). We observed that the protein appeared maximally phosphorylated in late-G2 cells and rapidly underwent a downward mobility shift as cells entered mitosis. The peak of hypophosphorylation preceded binucleate formation, a time which correlates with CAR formation. The maximal downward mobility shift observed in cells appeared to run slightly higher than lambda-phosphatase treated Cdc15p-HA. As septation began, an upward mobility shift of Cdc15p was observed. The occurrence of septation initiation marks a time when the CAR is constricting. In order to further examine the state of Cdc15p while at the CAR we utilized an *nda3-km311* genetic background. These cells possess a mutation in the alpha-tubulin gene, which after 8 hours at the restrictive temperature of 18°C, leads to an arrest in metaphase with mature actin rings (Chang et al., 1996). As expected, Cdc15p-GFP was also present in medial rings in *nda3-km311* arrested cells (Fig. 18C). Examination of protein lysates from *cdc15p-HA nda3-km311* arrested cells by western blotting for HA revealed that the protein appeared to be hypophosphorylated at the arrest point (Fig. 18D)

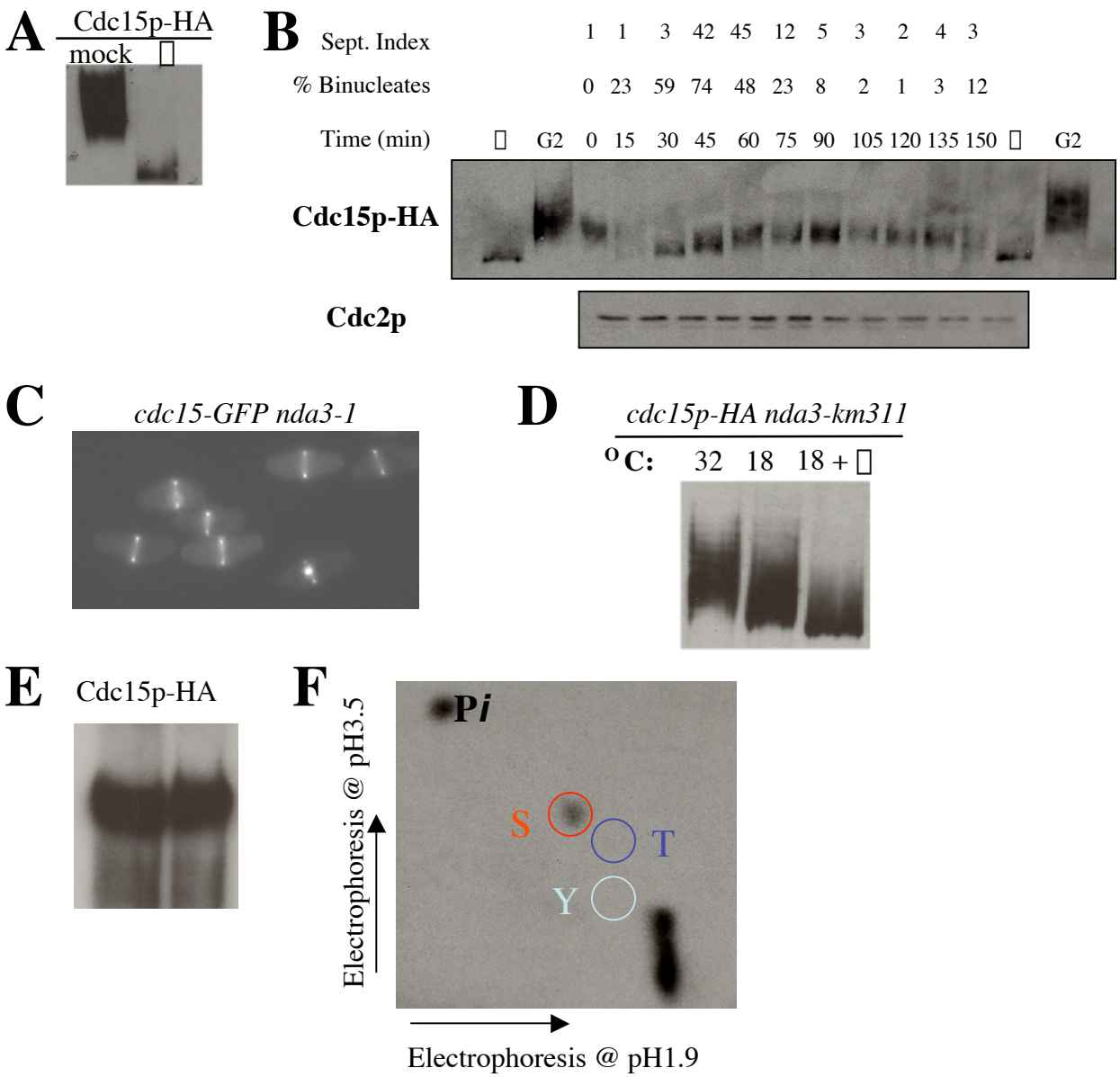


Figure 18. (A) Denatured cell lysates from G2-arrested *cdc15-HA cdc25-22* (KGY3350)cells were immunoprecipitated with anti-HA antibodies bound to sepharose beads. Beads were then either mock treated or lambda-phosphatase treated, collected and washed as described in materials and methods. Proteins were eluted and resolved by SDS-PAGE, then immunoblotted with anti-HA antibodies. (B) *cdc15-HA* cells (KGY3020) were synchronized in early G2 by centrifugal elutriation. Cells were collected at 15 minute intervals and examined for septation index, percentage of binucleate cells, and by SDS-PAGE/immunoblotting for λ-HA. (C) Image of *cdc15-GFP nda3-km311* (KGY3207) cells arrested at metaphase by incubation at 18°C for 8 hours. (D) *cdc15-HA nda3-km311* (KGY4410) cells were lysed and processed as in A. (E) Auto-radiogram of denatured cell lysate from ^{32}P metabolically-labelled *cdc15-HA* cells resolved by SDS-PAGE and transferred to PVDF-membrane. (F) ^{32}P-Labelled Cdc15p-HA from E was extracted from the membrane and subjected to phospho-amino acid analysis to separate phospho-serine (S), phospho-threonine (T), phospho-tyrosine (Y).

Further substantiating Cdc15p as a phosphoprotein was that we were able to readily radio-label endogenously HA-tagged Cdc15p via ^{32}P metabolic labeling (Fig. 18E). The ^{32}P labeled full-length Cdc15p-HA was subsequently isolated and subjected to phospho-amino acid analysis (PAA) and revealed that Cdc15p is phosphorylated at serine residues, and not at threonine or tyrosine residues (Fig. 18 F). This is in contrast to the Cdc15p-related murine protein, PSTPIP, which is phosphorylated primarily at tyrosine residues (Wu et al., 1998).

Dependence of Cdc15p ring formation on the SIN pathway

To determine the involvement of SIN signaling for proper Cdc15p localization, Cdc15p-GFP localization was examined in numerous mutants within this pathway (Fig. 19 A). As expected, considering actin rings are still observed in these mutants (Guertin et al., 2002), one or more Cdc15p-GFP rings were observed in most of these mutants at the restrictive temperature (Fig. 19 A). However, we were surprised to find no detectable Cdc15p-GFP rings in the *sid2* mutant under similar conditions. To further examine this potential connection between Cdc15p and Sid2p, we examined the SDS-PAGE protein migration of Cdc15p-HA in *sid2-250* at permissive and restrictive temperatures (Fig. 19 B). We observed that there was an apparent downward mobility shift of the protein in the *sid2* mutant, indicating that in the absence of Sid2p, Cdc15p is hypophosphorylated.

Dependence of Cdc15p ring formation on the microtubule cytoskeleton

Given that Cdc15p was able to localize independently of the actin cytoskeleton, we also examined the dependence of Cdc15p ring formation on the microtubule cytoskeleton. Synchronized *cdc15p-GFP cdc25-22* cells released to enter into mitosis in the presence of either of two microtubule depolymerizing drugs, TBZ or MBC, failed to form medial Cdc15p-GFP rings (Fig. 20). This was somewhat surprising given that Cdc15p-GFP rings are present in virtually 100% of *nda3-km311* arrested cells (Fig. 18 C), a mutant which reportedly destabilizes the microtubule cytoskeleton (Chang et al., 1996). This indicated that either these drugs have secondary effects, which prevent Cdc15p ring formation, or that the microtubule cytoskeleton is required for Cdc15p ring formation, and the *nda3-km311* is not completely penetrant for microtubule destabilization.

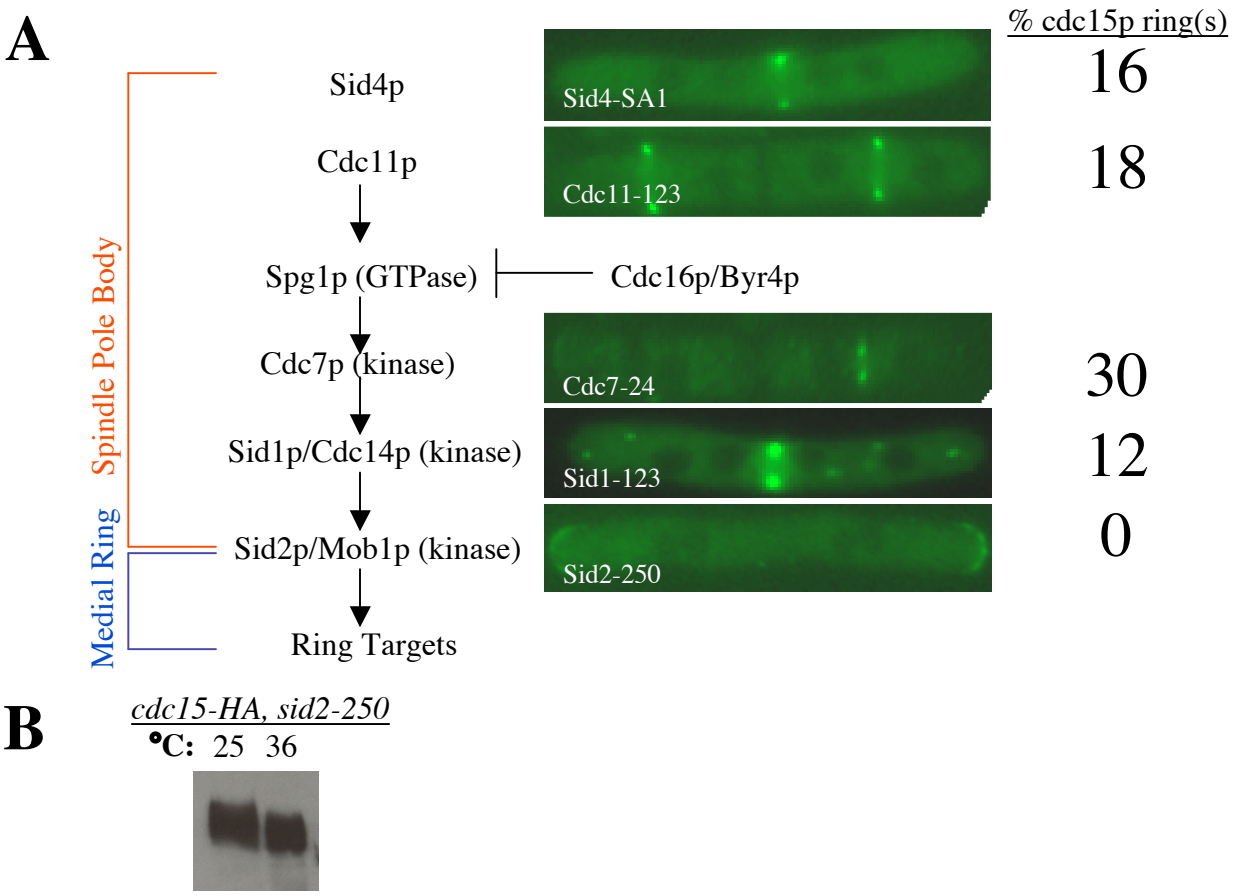


Figure 19. Cdc15p rings continue to form in all SIN mutants except *sid2*. (A) Representative images of cells from asynchronous cultures of the mutants indicated also carrying endogenously-tagged copy of *cdc15p-GFP* which had been shifted to 36°C for 4 hours prior to examination. Samples were also examined for the percentage of cells in each sample with one or more Cdc15p-GFP ring. (B) Denatured cell lysates from cdc15-HA sid2-250 cells which had been incubated at 36°C for 4 hours were lysed in NP-40 buffer. Proteins were resolved by SDS-PAGE and immunoblotted with -HA antibodies.

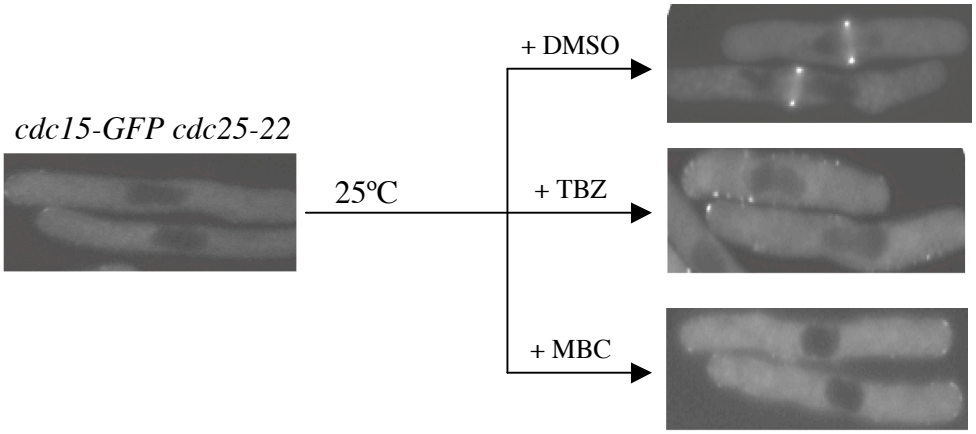


Figure 20. Cdc15p rings fail to form in the presence of microtubule destabilizing drugs. Figure 20 – continued. Representative images of *cdc15-GFP cdc25-22* (KGY 3042) cells synchronized in G2 and released to enter into mitosis in the presence of DMSO, TBZ or MBC. Cells were examined every 5 minutes for a total of 90 minutes and no rings were observed to form in TBZ or MBC treated cells. The images shown were taken after 30 minutes.

Cdc15p interacts with itself

Work with the PCH family protein FAP52 has indicated that this protein is likely to form trimers *in vivo* (Nikki et al., 2002). This self-interaction was dependent upon the N-terminally located coiled-coil region of the protein. We utilized yeast two-hybrid analyses to test the ability of Cdc15p to interact with itself. As expected, constructs of Cdc15p which contained the coiled-coil region robustly interacted with one another (Fig. 21 A). Furthermore, this interaction appeared to be direct as the same regions also bound, albeit somewhat weakly, to a Cdc15p coiled-coil containing fragment produced in reticulocyte lysate (Fig. 21 B). Interestingly, analyses of *cdc15-140* mutant allele DNA sequence revealed that there is a single base pair mutation within the predicted coiled-coil coding region. The indicated guanine to cytosine base pair alteration would result in a change from the wild-type alanine to a threonine at position 100 of the peptide sequence. This position represents the first highly scoring amino acid to participate in a coiled-coil as judged by the Coils prediction program (Fig. 21 C). However, altering the amino acid from an alanine to a threonine results in a drop in the Coils score from 0.365 to 0.050 (maximum score = 1.0) (Fig. 21 C). This might explain the thermostability of the *cdc15-140* gene product and suggest that a self-interaction mediated by the coiled-coil region is important for Cdc15p function

Conclusions

Cdc15p appears to be phosphorylated at serine residues. The peak of hyperphosphorylation is at the G2/M boundary. The protein then undergoes a rapid shift to hypophosphorylation at a time which correlates with its relocalization to the medial region and involvement in cytokinetic actin ring formation. Cdc15p then returns to a hyperphosphorylated state at the time of ring constriction and septation initiation. During this time the SIN kinase complex Sid2p/Mob1p kinase complex, at the bottom of the SIN cascade, translocates to the CAR and helps initiate these processes (Sparks et al, 1999; Salimova et al, 2000). Interestingly, unlike other SIN pathway mutants, no Cdc15p-GFP rings were observed in a *sid2* mutant at the restrictive temperature. Furthermore, Cdc15p-HA appears hypophosphorylated in a *sid2* mutant. We also found that Cdc15p interacts with itself through the predicted coiled-coils region in a manner which is likely to be important for protein function.

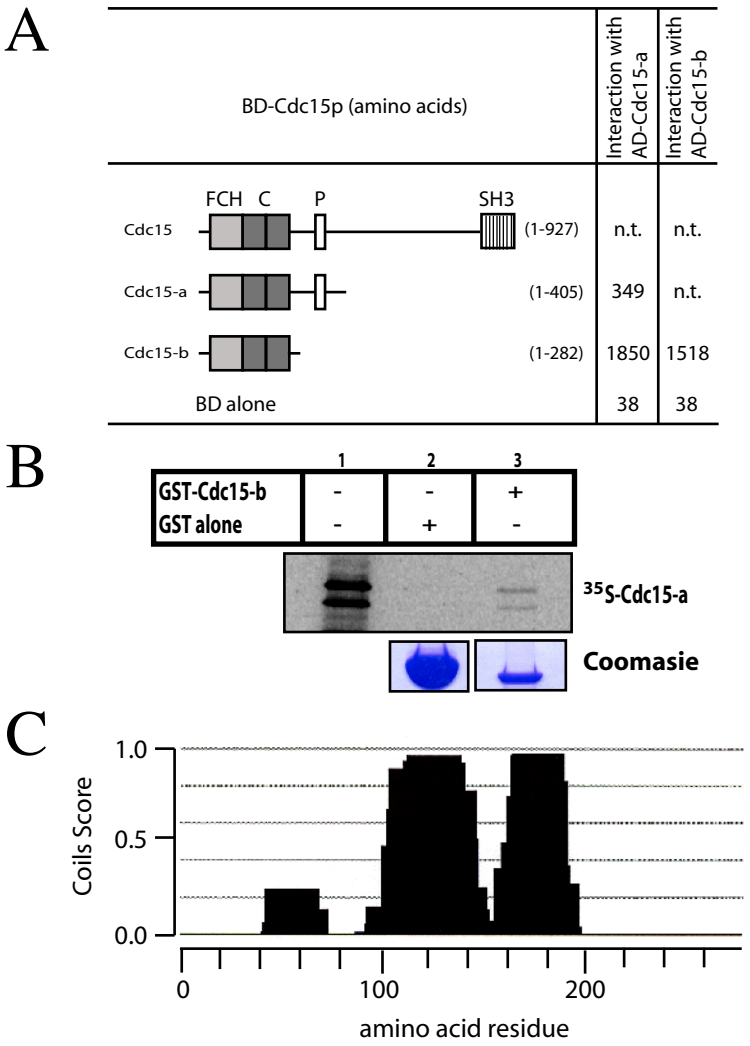


Figure 21. Cdc15p interacts with itself. (A) The indicated regions of Cdc15p were tested for interaction by two-hybrid analysis. LEU+ TRP+ transformants were tested for growth on selective media (data not shown) and assayed for □-galactosidase activity measured in relative light units. (B) GST (lane 2) and GST-Cdc15p-b (lane 3) bound to glutathione beads were mixed with in vitro translated Cdc15p-a. Beads were collected, washed, and eluted as described in materials and methods. Proteins were resolved by SDS-PAGE and detected by fluorography (top panel) or Coomasie staining (bottom panel).

CHAPTER VI

DISCUSSION

Cdc15p interacts directly with components of two actin nucleation pathways

The critical role of Cdc15p in CAR formation was suggested by the observation that overexpression of Cdc15p was sufficient to drive medial recruitment of actin during interphase (Fankhauser et al., 1995). We have extended this observation to show that Cdc15p recruits Myo1p and other F-actin nucleators (this study and our unpublished observations) that presumably allows this to occur. Plo1p overproduction has similarly been shown to lead to actin ring assembly in G2-arrested cells(Ohkura et al., 1995). However, other ring proteins, such as Cdc12p, do not appear to drive CAR formation in interphase (Balasubramanian et al., 2000; Chang, 1999). Therefore, our results solidify the role of Cdc15p as a critical factor in ring nucleation.

Formins have recently emerged as key factors in a novel actin nucleation pathway. The conserved N-terminal region of Cdc15p interacts directly with the *S. pombe* formin, Cdc12p, which is required for CAR assembly (Pelham and Chang, 2002). Further, these two proteins exist together in a medially located structure in cells prior to CAR formation and they show a mutual dependence for localization to both this structure and the CAR (Fig 5d, Chang et al., 1997). This Cdc12p containing structure has previously been reported to be the site of nucleation for the CAR (Chang, 1999). Furthermore, the initial actin filaments of the ring have also been shown to originate from a similar structure termed a medial aster (Arai and Mabuchi, 2002). It seems likely that the Cdc15p/Cdc12p spot and the actin aster are identical structures.

Cdc15p also interacts directly with the Arp2/3 complex regulatory factor Myo1p and is required for medial localization of all known Arp2/3 regulators in *S. pombe*. This is likely the explanation for the previously observed failure of the Arp2/3 complex itself to localize to the CAR in *cdc15-140* cells (Balasubramanian et al., 1998) and a major reason for CAR formation failure. Cdc15p appears to be the first reported factor required for proper localization of both Arp2/3 complex and its activator proteins. Other Cdc15-related proteins have previously been shown to interact with regulators of actin nucleation although the functional consequences of these interactions are not clear. For example, the mammalian PCH proteins PSTPIP and CIP4

bind to WASP (Tian et al., 2000; Wu et al., 1998), WRP binds to WAVE-1 (Soderling et al., 2002) and in *S cerevisiae*, Bzz1p and Hof1p have also been observed to interact with Las17/Bee1p, Vrp1p, and Myo1p (Kamei et al., 1998; Naqvi et al., 2001; Soulard et al., 2002; Vallen et al., 2000). However, all of these reported interactions occur via the SH3 domains of the PCH proteins, while we find that the SH3 domain of Cdc15p is not essential for the observed interactions. Nevertheless, it is worth noting that a similar network of physical interactions as we describe here may well take place in *S. cerevisiae* since two-hybrid interactions link Bni1p/Bnr1p-Myo3/5p-Bzz1p-Las17p (Tong et al., 2002). It was surprising that we were unable to detect association between Cdc15p and Wsp1p or Vrp1. However, this is consistent with our observation that the SH3 domain of Cdc15p is not essential for its function (data not shown).

S. cerevisiae Hof1p, has been previously suggested to be a functional homolog of Cdc15p (Lippincott and Li, 2000). However, unlike Cdc15p, Hof1p plays no known role in CAR formation but rather seems to be involved in coordinating ring contraction and septation (Lippincott and Li, 1998; Vallen et al., 2000). Indeed, Hof1p appears to be more functionally related to a second *S. pombe* PCH protein, Imp2p, which is involved in CAR dynamics during contraction (Demeter and Sazer, 1998).

Cdc15p localization across the cell cycle

Surprisingly, we found that Cdc15p also localizes to cell ends during interphase in a pattern very similar to that of actin patches. Though we found very little colocalization of these structures with actin patches, this localization pattern suggests that Cdc15p regulation of the actin cytoskeleton might not be limited to mitosis. Previous reports had characterized Cdc15p localization as at the CAR in mitosis and diffusely cytoplasmic during interphase (Fankhauser et al., 1995). However, by taking advantage of live cell microscopy utilizing endogenous GFP-tagging we were able to greatly enhance the visualization of this protein, as well as, examine the dynamic behavior of Cdc15p in cells. The localization of Cdc15p to the medial region is independent of Cdc12p and both the actin and microtubule cytoskeletons. However, formation of a ring structure by the medial localized Cdc15p does requires Cdc12p and intact F-actin structures, and possibly microtubules as well, although this is less clear.

Cdc15p regulation by phosphorylation

Cdc15p is heavily phosphorylated in interphase (Fankhauser et al., 1995 and our unpublished results). Immediately prior to CAR formation, Cdc15p becomes hypophosphorylated. Cdc15p at the mature CAR structure appears to be in a hypophosphorylated state. Then, as septation begins, Cdc15p returns to its hyperphosphorylated state. These observations lead one to speculate that the changes in Cdc15p phosphorylation might affect its interactions with other proteins. Consistent with this possibility, bacterially produced and presumably unphosphorylated Cdc15p binds to both Cdc12p and Myo1p. It will be interesting to determine if this interaction can be modulated by the phosphorylation state of Cdc15p, and if Cdc15p dephosphorylation might serve as a trigger for CAR formation. Interestingly, Cdc15p appeared hypophosphorylated in a *sid2* mutant. Furthermore, unlike other SIN mutants, Cdc15p-GFP rings are not readily observed in this mutant. It is unlikely that Cdc15p rings never form in the absence of Sid2p as this protein is predicted to be activated after the ring has already formed. However given that we observed cells 4 hours after shifting to the restrictive temperature, it is more likely that Cdc15p rings fail to persist and/or cycle in a *sid2* mutant. It is tempting to speculate that Cdc15p is one of the targets of Sid2p/Mob1p upon its arrival to the CAR and that this phosphorylation is involved in maintenance of Cdc15p at the ring. This phosphorylation may also be required for recycling Cdc15p back to a phosphorylated state in order to prepare for the following mitosis.

A model for Cdc15p involvement in CAR formation

Placing our results in the context of previous studies, particularly the careful analysis of CAR formation in Arai and Mabuchi (2001), leads us to propose a model for ring formation that includes Cdc15p (Fig. 22). In interphase, hyperphosphorylated Cdc15p is prevented from associating with Cdc12p. Upon commitment to mitosis Cdc15p is dephosphorylated and associates with Cdc12p and actin to form the pre-ring medial aster/spot. In metaphase the primary F-actin ring is formed. This step has been suggested to be dependent on Cdc12p (Chang et al., 1997), a hypothesis consistent with the role of formins in nucleating linear unbranched filaments (Evangelista et al., 2002; Pruyne et al., 2002; Sagot et al., 2002). The presence of Cdc15p in this primary ring leads to medial recruitment of the Arp2/3 complex and the subsequent creation of an actin network encircling the equator of the cell. Unlike Cdc12p

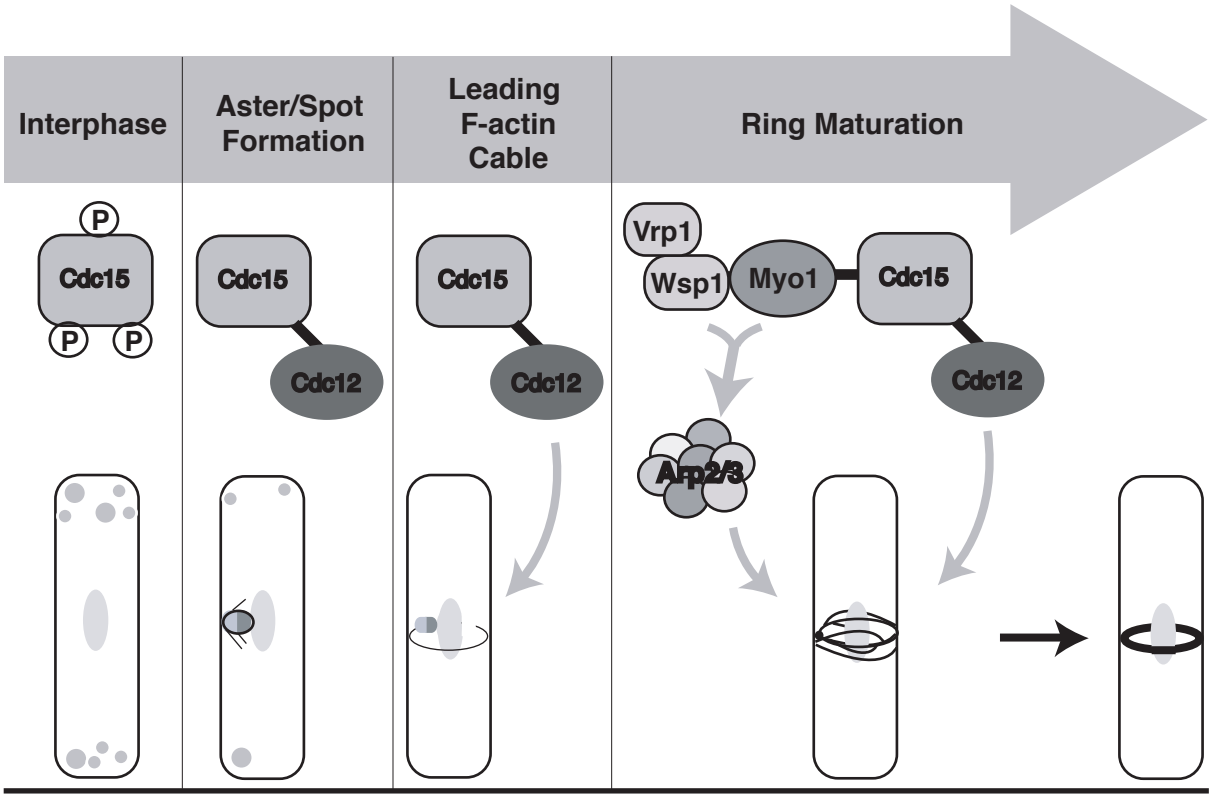


Figure 22. Model of Cdc15p involvement in ring formation. Hyperphosphorylated Cdc15p resides in patches at cell ends during interphase (indicated in grey spots). At the G2/M transition Cdc15p becomes hypophosphorylated and appears in a medial spot structure (grey spot) with Cdc12p (darker grey spot) near the nucleus (oblong grey structure). This complex recruits actin (black spot and lines) and through nucleation by Cdc12p initiates the assembly of the primary F-actin ring. Cdc15p also recruits the Arp2/3 actin nucleation machinery through its direct association with Myo1p. The Arp2/3 complex promotes the formation of the medial actin cable meshwork seen subsequent to primary ring formation. Finally, these actin fibers are bundled to create the mature CAR.

(Chang, 1999), Cdc15p seems to be a part of this actin meshwork. In late anaphase these thinner filaments coalesce into what is recognized as the mature CAR structure. Immediately prior to CAR constriction and septation, Cdc15p returns to a hyperphosphorylated state. This phosphorylation is potentially mediated by the SIN kinase Sid2p, which is believed to trigger ring constriction upon its arrival to the CAR. This places Cdc15p at a major convergence point for coordination of events required for initiation and formation of the CAR in *S. pombe*. In the future it will be interesting to determine if Cdc15p's function is limited to that of recruitment, or whether it might also have a catalytic role in regulating the activity of the proteins it recruits. It also suggests a potential role for Cdc15p in later events at the CAR such as initiation of ring constriction.

Future Directions

Our studies of Cdc15p have laid a foundation for many interesting avenues of research. Cdc15p appears to be a key player in establishment of the medial ring. As discussed in the introduction the protein Mid1p is believed to be critical for localizing the medial ring. As both Cdc12p and Cdc15p rely on Mid1p for proper medial localization, it would be interesting to understand the nature of the interaction of the medial Cdc15/Cdc12 spot with Mid1p. Are either of these proteins interacting directly with Mid1p? Another interesting possibility is the possibility that Cdc15p localizes through lipid binding. A recent report shows that the plasma membrane region at the site of CAR formation is a sterol-rich membrane domain (Wachtler, et al., 2002). Compromising the integrity of these domains results in actomyosin ring positioning defects, defects in ring stability, and failed septal material deposition. Furthermore, these sterol-rich domains appear to be perturbed in some *cdc15* mutants (F.Chang, personal communication). An attractive possibility is that Cdc15p interacts directly with sterols within these domains to direct it to the medial region and subsequently target ring nucleating factors.

The Type II myosin Myo2 also localizes to a medial spot structure prior to ring formation (Motegi et al., 2000; Naqvi et al., 1999). Although actin and myosin localization to the cleavage furrow occurs partially independently (Motegi et al., 2000; Naqvi et al., 1999), there must be a mechanism for coordinating final CAR assembly and it will be interesting to determine whether the Cdc15p/Cdc12p spot structure also contain Myo2p. It would also be interesting to examine the ability of Cdc15p/Cdc12p and Myo2p spots to form in *cdc15* and *myo2* mutants, respectively.

Formin proteins are typically regulated via protein-protein interactions involving their N-terminus. For example, *S. cerevisiae* Bni1 and murine mDIA are both regulated by Rho, one of the most well studied formin regulators. The interaction is through a Rho-binding domain (RBD) within the formins (reviewed in Wasserman, 1997). The N-termini of formins also display an intramolecular interaction with the C-termini of the proteins. This interaction is believed to serve as a repression system of formin function. Binding of N-terminal regulators, such as Rho, is then proposed to free the C-terminally located FH1 and FH2 catalytic domains and relieve their repression (Wasserman, 1997). *S. pombe* Cdc12p, however, has no RBD and no other known N-terminal binding partners apart from Cdc15p. The timing of Cdc12p interaction with Cdc15p is also coincident with the requirement for Cdc12p activation in order to begin synthesis of the new actin ring. Is Cdc15p binding of Cdc12p involved in activating its nucleation activity? Furthermore, the hypophosphorylation of Cdc15p also coincides with its colocalization with Cdc12p and ring formation. It is possible that hypophosphorylated Cdc15p interacts preferentially with Cdc12p. Combining these ideas leads to the hypothesis that dephosphorylation of Cdc15p is a key means of initiation of CAR formation by its recruitment/activation of Cdc12p. Cdc15p function at the CAR also involves Arp2/3 recruitment and regulation. Therefore, It would also be interesting to examine the effect of phosphorylation on the Cdc15p/Myo1p interaction. Could the binding of Cdc15p also effect the ability of Myo1p to stimulate Arp2/3 nucleation activity?

We were unable to detect an interaction between Cdc15p and Wsp1p. However, further pursuing a possible interaction between these two proteins would be warranted. Our model predicts that medial recruitment of Myo1p by Cdc15p promotes the subsequent recruitment of the Arp2/3 complex. Myo1p and Wsp1p are both non-essential and are functionally redundant with respect to Arp2/3 activation. Therefore, *myo1Δ* cells must possess an alternate means of medial recruitment of Wsp1p and Arp2/3. Given the direct interaction of HOF1 to LAS17/BEE1 in *S. cerevisiae*, Cdc15p still remains a likely mediator of this recruitment. Based on *S. cerevisiae* data this interaction would presumably depend upon the SH3 domain of Cdc15p, a region we have observed to be non-essential for Cdc15p function. If this region is the Wsp1p binding region responsible for its medial recruitment, then it stands to reason it would display synthetic lethality with *myo1Δ*. In these cells there would presumably be no active Arp2/3 for effecting CAR formation.

In addition to the hypophosphorylation during CAR formation, another intriguing change occurs in Cdc15p phosphorylation state during ring constriction/septation initiation. This re-phosphorylation of Cdc15p correlates with Sid2p/Mob1p arrival at the CAR. We also observed that Cdc15p appears to be hypophosphorylated in a *sid2* mutant. Is Cdc15p one of the ring targets of Sid2p activity? Furthermore, could this proposed phosphorylation event be involved in signaling ring constriction, an event required to allow septal deposition?

CHAPTER VII

CONCLUDING REMARKS

The last few years have seen numerous advances in our understanding of the regulation of actin dynamics and more specifically in the contribution of actin regulatory molecules to cleavage furrow formation and function. Recent work in *S. pombe* and *S. cerevisiae* has identified formins as a new family of actin nucleators. Furthermore, both formin and Arp2/3 mediated nucleation has been shown to be required for CAR formation. Our work has now identified Cdc15p as a potential convergence point for coordination of these separate actin nucleation pathways.

cdc15 encodes a member of the PCH protein family, conserved from yeasts to mammals, which has been linked to actin cytoskeletal functions (Lippincott and Li, 2000). Though Cdc15p is the founding member of this protein family, little progress has been made towards understanding its precise function in cytokinesis. Here we have provided the first clues as to its critical role in this process. Cdc15p interacts directly with both the Arp2/3 complex activation machinery and the formin, Cdc12p, to orchestrate early events in CAR formation. As predicted by these interactions, the CAR does not form in the absence of Cdc15p function.

Preliminary evidence suggests that Cdc15p is regulated by phosphorylation/ dephosphorylation and that these modifications influence the ability of Cdc15p to participate in ring dynamics.

Domain architecture and localization to actin-rich regions are conserved across the PCH protein family. Given this structural conservation, it is likely that other family members are similarly involved in the organization of dynamic actin structures in higher eukaryotes

REFERENCES

Anderson, M., S.S. Ng, V. Marchesi, F.H. MacIver, F.E. Stevens, T. Riddell, D.M. Glover, I.M. Hagan, and C.J. McInerny. 2002. Plo1(+) regulates gene transcription at the M-G(1) interval during the fission yeast mitotic cell cycle. *Embo J.* 21:5745-55.

Arai, R., and I. Mabuchi. 2002. F-actin ring formation and the role of F-actin cables in the fission yeast Schizosaccharomyces pombe. *J Cell Sci.* 115:887-98.

Ayscough, K. 1998. Use of latrunculin-A, an actin monomer-binding drug. *Methods Enzymol.* 298:18-25.

Bahler, J., J.Q. Wu, M.S. Longtine, N.G. Shah, A. McKenzie, 3rd, A.B. Steever, A. Wach, P. Philippsen, and J.R. Pringle. 1998. Heterologous modules for efficient and versatile PCR-based gene targeting in Schizosaccharomyces pombe. *Yeast.* 14:943-51.

Balasubramanian, M.K., D. McCollum, L. Chang, K.C. Wong, N.I. Naqvi, X. He, S. Sazer, and K.L. Gould. 1998. Isolation and characterization of new fission yeast cytokinesis mutants. *Genetics.* 149:1265-75.

Balasubramanian, M.K., D. McCollum, and U. Surana. 2000. Tying the knot: linking cytokinesis to the nuclear cycle. *J Cell Sci.* 113:1503-13.

Barbet, N.C., and A.M. Carr. 1993. Fission yeast wee1 protein kinase is not required for DNA damage-dependent mitotic arrest. *Nature.* 364:824-7.

Bear, J.E., M. Krause, and F.B. Gertler. 2001. Regulating cellular actin assembly. *Curr Opin Cell Biol.* 13:158-66.

Burns, C.G., R. Ohi, S. Mehta, E.T. O'Toole, M. Winey, T.A. Clark, C.W. Sugnet, M. Ares, Jr., and K.L. Gould. 2002. Removal of a single alpha-tubulin gene intron suppresses cell cycle arrest phenotypes of splicing factor mutations in Saccharomyces cerevisiae. *Mol Cell Biol.* 22:801-15.

Chang, F. 1999. Movement of a cytokinesis factor cdc12p to the site of cell division. *Curr Biol.* 9:849-52.

Chang, F. 2000. Microtubule and actin-dependent movement of the formin cdc12p in fission yeast. *Microsc Res Tech.* 49:161-7.

Chang, F. 2001. Establishment of a cellular axis in fission yeast. *Trends Genet.* 17:273-8.

Chang, F., D. Drubin, and P. Nurse. 1997. cdc12p, a protein required for cytokinesis in fission yeast, is a component of the cell division ring and interacts with profilin. *J Cell Biol.* 137:169-82.

Chang, F., A. Woollard, and P. Nurse. 1996. Isolation and characterization of fission yeast mutants defective in the assembly and placement of the contractile actin ring. *J Cell Sci.* 109:131-42.

Chang, L., J.L. Morrell, A. Feoktistova, and K.L. Gould. 2001. Study of cyclin proteolysis in anaphase-promoting complex (APC) mutant cells reveals the requirement for APC function in the final steps of the fission yeast septation initiation network. *Mol Cell Biol.* 21:6681-94.

Cooper, J.A., and D.A. Schafer. 2000. Control of actin assembly and disassembly at filament ends. *Curr Opin Cell Biol.* 12:97-103.

Cooper, J.A., M.A. Wear, and A.M. Weaver. 2001. Arp2/3 complex: advances on the inner workings of a molecular machine. *Cell.* 107:703-5.

Demeter, J., and S. Sazer. 1998. imp2, a new component of the actin ring in the fission yeast Schizosaccharomyces pombe. *J Cell Biol.* 143:415-27.

Ding, D.Q., Y. Tomita, A. Yamamoto, Y. Chikashige, T. Haraguchi, and Y. Hiraoka. 2000. Large-scale screening of intracellular protein localization in living fission yeast cells by the use of a GFP-fusion genomic DNA library. *Genes Cells.* 5:169-90.

Evangelista, M., B.M. Klebl, A.H. Tong, B.A. Webb, T. Leeuw, E. Leberer, M. Whiteway, D.Y. Thomas, and C. Boone. 2000. A role for myosin-I in actin assembly through interactions with Vrp1p, Bee1p, and the Arp2/3 complex. *J Cell Biol.* 148:353-62.

Evangelista, M., D. Pruyne, D.C. Amberg, C. Boone, and A. Bretscher. 2002. Formins direct Arp2/3-independent actin filament assembly to polarize cell growth in yeast. *Nat Cell Biol.* 4:260-9.

Evangelista, M., S. Zigmond, and C. Boone. 2003. Formins: signaling effectors for assembly and polarization of actin filaments. *J Cell Sci.* 116:2603-11.

Fankhauser, C., J. Marks, A. Reymond, and V. Simanis. 1993. The S. pombe cdc16 gene is required both for maintenance of p34cdc2 kinase activity and regulation of septum formation: a link between mitosis and cytokinesis? *Embo J.* 12:2697-704.

Fankhauser, C., A. Reymond, L. Cerutti, S. Utzig, K. Hofmann, and V. Simanis. 1995. The S. pombe cdc15 gene is a key element in the reorganization of F-actin at mitosis. *Cell.* 82:435-44.

Gould, K.L., S. Moreno, D.J. Owen, S. Sazer, and P. Nurse. 1991. Phosphorylation at Thr167 is required for Schizosaccharomyces pombe p34cdc2 function. *Embo J.* 10:3297-309.

Guertin, D.A., S. Trautmann, and D. McCollum. 2002. Cytokinesis in eukaryotes. *Microbiol Mol Biol Rev.* 66:155-78.

Hartwell, L.H., and T.A. Weinert. 1989. Checkpoints: controls that ensure the order of cell cycle events. *Science*. 246:629-634.

Higgs, H.N., and T.D. Pollard. 2001. Regulation of actin filament network formation through ARP2/3 complex: activation by a diverse array of proteins. *Annu Rev Biochem.* 70:649-76.

Ishiguro, J., and W. Kobayashi. 1996. An actin point-mutation neighboring the 'hydrophobic plug' causes defects in the maintenance of cell polarity and septum organization in the fission yeast Schizosaccharomyces pombe. *FEBS Lett.* 392:237-41.

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.* 144:1425-36.

Johnson, B.F., B.Y. Yoo, and G.B. Calleja. 1973. Cell division in yeasts: movement of organelles associated with cell plate growth of Schizosaccharomyces pombe. *J Bacteriol.* 115:358-66.

Kamei, T., K. Tanaka, T. Hihara, M. Umikawa, H. Imamura, M. Kikyo, K. Ozaki, and Y. Takai. 1998. Interaction of Bnr1p with a novel Src homology 3 domain-containing Hof1p. Implication in cytokinesis in Saccharomyces cerevisiae. *J Biol Chem.* 273:28341-5.

Keeney, J.B., and J.D. Boeke. 1994. Efficient targeted integration at leu1-32 and ura4-294 in Schizosaccharomyces pombe. *Genetics.* 136:849-56.

Kobori, H., N. Yamada, A. Taki, and M. Osumi. 1989. Actin is associated with the formation of the cell wall in reverting protoplasts of the fission yeast Schizosaccharomyces pombe. *J Cell Sci.* 94:635-46.

Lechler, T., A. Shevchenko, and R. Li. 2000. Direct involvement of yeast type I myosins in Cdc42-dependent actin polymerization. *J Cell Biol.* 148:363-73.

Lee, W.L., M. Bezanilla, and T.D. Pollard. 2000. Fission yeast myosin-I, Myo1p, stimulates actin assembly by Arp2/3 complex and shares functions with WASp. *J Cell Biol.* 151:789-800.

Li, R., Y. Zheng, and D.G. Drubin. 1995. Regulation of cortical actin cytoskeleton assembly during polarized cell growth in budding yeast. *J Cell Biol.* 128:599-615.

Lippincott, J., and R. Li. 1998. Dual function of Cyk2, a cdc15/PSTPIP family protein, in regulating actomyosin ring dynamics and septin distribution. *J Cell Biol*. 143:1947-60.

Lippincott, J., and R. Li. 2000. Involvement of PCH family proteins in cytokinesis and actin distribution. *Microsc Res Tech*. 49:168-72.

Marks, J., I.M. Hagan, and J.S. Hyams. 1986. Growth polarity and cytokinesis in fission yeast: the role of the cytoskeleton. *J Cell Sci Suppl*. 5:229-41.

Marks, J., and J.S. Hyams. 1985. Localization of F-actin throughout the cell division cycle of *Schizosaccharomyces pombe*. *Eur J Cell Biol*. 39:27-32.

Maundrell, K. 1993. Thiamine-repressible expression vectors pREP and pRIP for fission yeast. *Gene*. 123:127-30.

McCollum, D., and K.L. Gould. 2001. Timing is everything: regulation of mitotic exit and cytokinesis by the MEN and SIN. *Trends Cell Biol*. 11:89-95.

McDonald, W.H., R. Ohi, N. Smelkova, D. Frendewey, and K.L. Gould. 1999. Myb-related fission yeast cdc5p is a component of a 40S snRNP-containing complex and is essential for pre-mRNA splicing. *Mol Cell Biol*. 19:5352-62.

Moreno, S., A. Klar, and P. Nurse. 1991. Molecular genetic analysis of fission yeast Schizosaccharomyces pombe. *Methods Enzymol*. 194:795-823.

Morrell, J.L., M. Morphew, and K.L. Gould. 1999. A mutant of Arp2p causes partial disassembly of the Arp2/3 complex and loss of cortical actin function in fission yeast. *Mol Biol Cell*. 10:4201-15.

Motegi, F., K. Nakano, and I. Mabuchi. 2000. Molecular mechanism of myosin-II assembly at the division site in Schizosaccharomyces pombe. *J Cell Sci*. 113 (Pt 10):1813-25.

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*. 95:6181-6.

Murray, A., and T. Hunt. 1993. The Cell Cycle: an introduction. Oxford University Press, New York, NY. 251 pp.

Naqvi, N.I., K. Eng, K.L. Gould, and M.K. Balasubramanian. 1999. Evidence for F-actin-dependent and -independent mechanisms involved in assembly and stability of the medial actomyosin ring in fission yeast. *Embo J*. 18:854-62.

Naqvi, S.N., Q. Feng, V.J. Boulton, R. Zahn, and A.L. Munn. 2001. Vrp1p functions in both actomyosin ring-dependent and Hof1p-dependent pathways of cytokinesis. *Traffic*. 2:189-201.

Nigg, E.A. 2001. Mitotic kinases as regulators of cell division and its checkpoints. *Nat Rev Mol Cell Biol*. 2:21-32.

Nikki, M., J. Merilainen, and V.P. Lehto. 2002. Focal adhesion protein FAP52 self-associates through a sequence conserved among the members of the PCH family proteins. *Biochemistry*. 41:6320-9.

Nurse, P., P. Thuriaux, and K. Nasmyth. 1976. Genetic control of the cell division cycle in the fission yeast Schizosaccharomyces pombe. *Mol Gen Genet*. 146:167-78.

Ohkura, H., I.M. Hagan, and D.M. Glover. 1995. The conserved Schizosaccharomyces pombe kinase plo1, required to form a bipolar spindle, the actin ring, and septum, can drive septum formation in G1 and G2 cells. *Genes Dev*. 9:1059-73.

Pelham, R.J., and F. Chang. 2002. Actin dynamics in the contractile ring during cytokinesis in fission yeast. *Nature*. 419:82-6.

Pelham, R.J., Jr., and F. Chang. 2001. Role of actin polymerization and actin cables in actin-patch movement in Schizosaccharomyces pombe. *Nat Cell Biol*. 3:235-44.

Petersen, J., O. Nielsen, R. Egel, and I.M. Hagan. 1998. FH3, a domain found in formins, targets the fission yeast formin Fus1 to the projection tip during conjugation. *J Cell Biol*. 141:1217-28.

Pollard, T.D., and J.A. Cooper. 1986. Actin and actin-binding proteins. A critical evaluation of mechanisms and functions. *Annu Rev Biochem*. 55:987-1035.

Prentice, H.L. 1992. High efficiency transformation of Schizosaccharomyces pombe by electroporation. *Nucleic Acids Res*. 20:621.

Pruyne, D., M. Evangelista, C. Yang, E. Bi, S. Zigmond, A. Bretscher, and C. Boone. 2002. Role of formins in actin assembly: nucleation and barbed-end association. *Science*. 297:612-5.

Salimova, E., M. Sohrmann, N. Fournier, and V. Simanis. 2000. The S. pombe orthologue of the S cerevisiae mob1 gene is essential and functions in signalling the onset of setum formation. *J Cell Sci*. 113 (Pt 10):1695-704.

Sagot, I., A.A. Rodal, J. Moseley, B.L. Goode, and D. Pellman. 2002. An actin nucleation mechanism mediated by Bni1 and profilin. *Nat Cell Biol*. 4:626-31.

Schott, D., T. Huffaker, and A. Bretscher. 2002. Microfilaments and microtubules: the news from yeast. *Curr Opin Microbiol*. 5:564-74.

Soderling, S.H., K.L. Binns, G.A. Wayman, S.M. Davee, S.H. Ong, T. Pawson, and J.D. Scott. 2002. The WRP component of the WAVE-1 complex attenuates Rac-mediated signalling. *Nat Cell Biol*. 4:970-5.

Song, K., K.E. Mach, C.Y. Chen, T. Reynolds, and C.F. Albright. 1996. A novel suppressor of ras1 in fission yeast, byr4, is a dosage-dependent inhibitor of cytokinesis. *J Cell Biol.* 133:1307-19.

Sparks, C.A., M. Morphew, and D. McCollum. 1999. Sid2p, a spindle pole body kinase that regulates the onset of cytokinesis. *J Cell Biol.* 146:777-90

Soulard, A., T. Lechler, V. Spiridonov, A. Shevchenko, R. Li, and B. Winsor. 2002. Saccharomyces cerevisiae Bzz1p is implicated with type I myosins in actin patch polarization and is able to recruit actin-polymerizing machinery in vitro. *Mol Cell Biol.* 22:7889-906.

Tian, L., D.L. Nelson, and D.M. Stewart. 2000. Cdc42-interacting protein 4 mediates binding of the Wiskott-Aldrich syndrome protein to microtubules. *J Biol Chem.* 275:7854-61.

Tong, A.H., B. Drees, G. Nardelli, G.D. Bader, B. Brannetti, L. Castagnoli, M. Evangelista, S. Ferracuti, B. Nelson, S. Paoluzi, M. Quondam, A. Zucconi, C.W. Hogue, S. Fields, C. Boone, and G. Cesareni. 2002. A combined experimental and computational strategy to define protein interaction networks for peptide recognition modules. *Science*. 295:321-4.

Toya, M., F. Motegi, K. Nakano, I. Mabuchi, and M. Yamamoto. 2001. Identification and functional analysis of the gene for type I myosin in fission yeast. *Genes Cells*. 6:187-99.

Vallen, E.A., J. Caviston, and E. Bi. 2000. Roles of Hof1p, Bni1p, Bnr1p, and myo1p in cytokinesis in Saccharomyces cerevisiae. *Mol Biol Cell.* 11:593-611.

Wachtler, V., Rajagopalan, S., and M.K. Balasubramanian. 2002. Sterol-rich plasma membrane domains in the fission yeast Schizosaccharomyces pombe. *J Cell Sci.* 116:867-74.

Wang, H., S. Oliferenko, and M.K. Balasubramanian. 2003. Cytokinesis: relative alignment of the cell division apparatus and the mitotic spindle. *Curr Opin Cell Biol.* 15:82-7.

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.* 7:519-29.

Wu, Y., S.D. Spencer, and L.A. Lasky. 1998. Tyrosine phosphorylation regulates the SH3-mediated binding of the Wiskott-Aldrich syndrome protein to PSTPIP, a cytoskeletal-associated protein. *J Biol Chem.* 273:5765-70.