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Identification of Actin Cytoskeletal Protein Interactions with the Fission Yeast Scaffold, Mid1

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Abstract

Cancer is a disease of uncontrolled cell division. Cell division in eukaryotic cells occurs by the formation of a contractile ring, predominantly composed of filamentous actin (F-actin). During mitosis, actin filaments polymerize to form rings at the medial plane of the cell that constrict causing the cell membrane to pinch and divide. Proteins that regulate this process are essential because dysregulation can lead to uninhibited cell growth and division. In fission yeast, the protein Mid1 functions as a scaffold to recruit regulatory proteins required for actin filament formation and simultaneously anchors the contractile ring at the cell division site. The Mid1 Pleckstrin Homology (PH) domain can bind lipids in the membrane, but direct binding of the PH domain to other contractile ring proteins has not been described. The GTPase Rho1 is an important activator of proteins that regulate actin filaments, and has been shown to localize to the contractile ring during cell division. Since F-actin, Mid1, and Rho1 are important components for contractile ring formation and proper cytokinesis, it is important to clarify their interactions. Co-sedimentation experiments indicated that the Mid1 PH domain specifically binds F-actin. The PH domain also inhibits F-actin filaments from elongating by actin polymerization assays. Low speed actin co-sedimentation showed that the PH domain also bundles actin filaments. Creation of a Rho1-GFP yeast strain was attempted to perform co-immunoprecipitation of Rho1 and Mid1 *in vivo*. The confirmation and importance of the interaction of Rho1 and Mid1 will be explored in future studies. The interaction of Mid1 with F-actin and Rho1GTPase may reveal important regulatory events for contractile ring assembly during cell division.

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Chapter I: Introduction:

The cell cycle

All new cells are progeny from previously existing cells. Cells reproduce by duplication and division to generate genetically identical daughter cells. This process is controlled by a complex cell cycle that includes phases and checkpoints required for successful division. The cell cycle proteins involved in its regulation are highly conserved throughout eukaryotic organisms (Alberts et al., 2008). Passage through each stage in the cell cycle provides assurance that each daughter cell holds a genetic blueprint that is free of damage, aberration, and mutation. The first phase in the cell cycle is called interphase, which includes three stages that prepare the cell for division (Figure 1). In the first gap phase, G1, the cell grows and develops while being sensitive to extracellular conditions. The cell will commit to division during G1 phase based on whether it resides within favorable conditions including nutrient availability, physical space, and presence of damaging agents. Recognition of tolerable conditions permits the passage through the first checkpoint in the cell cycle, committing the cell to duplicate its DNA. Synthesis (S) phase initiates DNA replication and the entire genome is duplicated within the nucleus of the cell. Many proteins are involved in this process including synthesizing enzymes, DNA damage detecting proteins, and DNA repair proteins. The centrosome, which contains two centrioles and nucleates the microtubules in the cell, also begins to duplicate during S phase. The second gap phase, G2, follows S phase. The second cell cycle checkpoint is during G2 phase when the cell verifies that the DNA was replicated without damage and elects to move into cell division or mitosis (M) phase. The centrosomes are completely duplicated at the G2/M transition. M phase is the shortest phase of the cell cycle and includes the steps necessary for precise division of the genetic material and abscission of the cell membrane to create independent daughter cells.

Prophase is the first step of mitosis in which the newly replicated chromosomes condense. Also, centrosomes migrate along the nuclear envelope during prophase. The centrosomes move to the poles of the nucleus by the opposing forces of the microtubule plus ends extending from each centrosome. For cells with an open mitosis, the break down of the nuclear envelope initiates during prometaphase. Mitotic spindle microtubules can then extend from the centrosomes and attach to the chromosomes. These microtubules attach at the centromere of the chromosomes, which contains multiple heterochromatic proteins that make up the kinetochore. During metaphase, the chromosomes are aligned along the metaphase plate at the equator of the cell. Kinetochore microtubules from opposite poles attach to sister chromatids along the metaphase plate. The final checkpoint in the cell cycle is at the transition of metaphase to the next stage, anaphase. At this point, the cell verifies that all the chromosomes are attached to the spindles by microtubules. Once confirmed, anaphase is initiated and spindle poles begin to move outward and kinetochore microtubules begin to shorten towards the poles. This movement causes sister chromatids to be pulled apart and segregated to opposite ends of the cell. Nuclear envelopes are produced in telophase, which incase the daughter chromosomes creating two new nuclei. During telophase, an actin-myosin contractile ring is formed at the equator of the cell. Cytokinesis is the final stage of mitosis when the contractile ring constricts allowing for the pinching and abscission of the plasma membrane. The final stage results in two identical daughter cells. The cell cycle restarts as the daughter cells enter interphase.

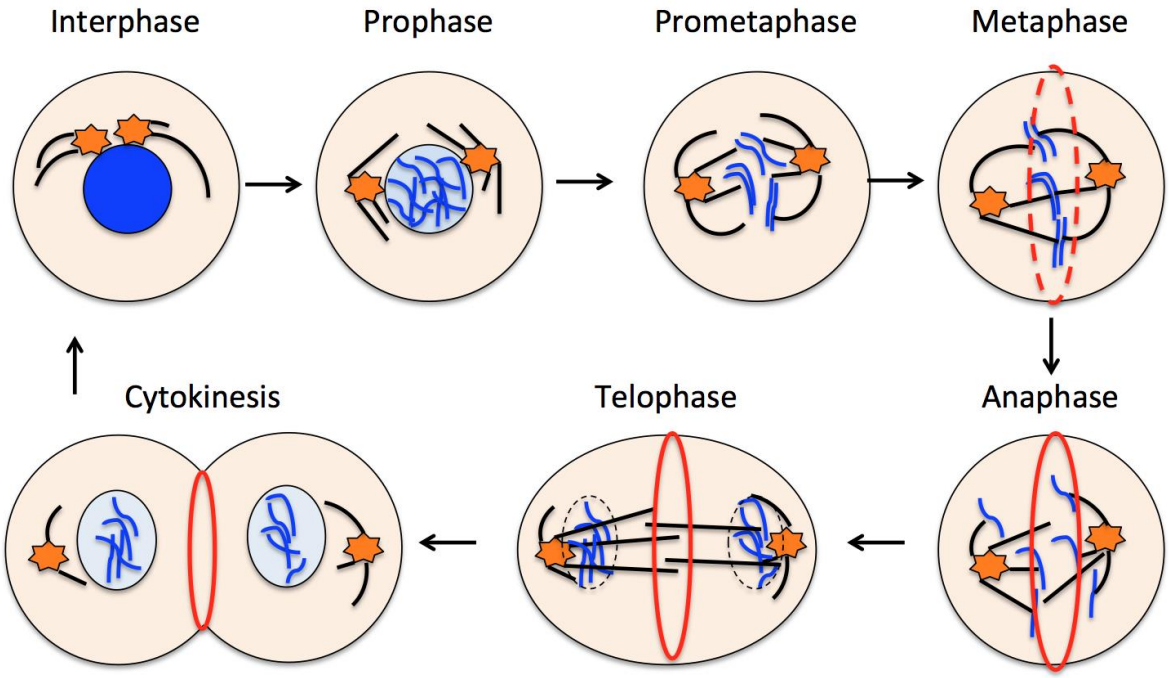


Figure 1: **Cell cycle phases:** In interphase, the cell duplicates its DNA and centrosomes. In prophase, the DNA begins to condense and centromeres separate to poles of the cell. Mitotic spindles extending from the centrosomes attach to chromosomes during prometaphase. The chromosomes are aligned at the metaphase plate during metaphase, and the contractile ring begins to form. In anaphase the mitotic spindles contract towards the centromere poles resulting in separation of sister chromatids. In anaphase, the contractile ring is fully formed. In telophase, the nuclear envelope begins to reform around the separated chromosomes. The contractile ring constricts during cytokinesis to result in two genetically identical daughter cells. Blue lines represent chromosomes, orange stars represent centrosomes, black lines represent spindle pole microtubules, and red lines represent the contractile ring.

Actin cytoskeleton

The cell cycle and cell division results in major modifications to the shape of the cell, which requires coordination and rearrangement of the cytoskeleton. The cytoskeleton of the cell regulates cell shape, growth, polarity, mobility, intracellular transport, adhesion, and division (Pollard and Cooper, 2009). One of the main components of the cytoskeleton is actin. Actin filaments are formed from globular actin monomers. Filament formation occurs

in three phases: nucleation, elongation, and steady state. The lag phase consists of nucleating subunits that must come together to form an oligomer. This oligomer nucleus provides the starting point for extension of the filament during the elongation phase, which occurs at the highest rate (Figure 2). This phase will continue until the filament reaches critical concentration in which the addition of subunits is equal to the loss of subunits. This is when the filament reaches steady state or equilibrium. Actin filaments form in a helical structure of two protofilaments constructed from multiple actin monomers. Actin monomers are added in a specific direction, making actin filaments distinctly polarized with a plus (barbed) end and a minus (pointed) end (Figure 2). The association of the actin subunit with ATP or ADP regulates nucleation, elongation, and dissociation from the filament. When actin monomers are bound to ATP, they can be added to the filament, which occurs more rapidly to the plus end. Once incorporated, the nucleotide is hydrolyzed to ADP. Most monomers in the filament are ADP bound, but this form causes the monomers to have less affinity for adjacent monomers in the filament. The decrease in affinity allows for depolymerization of the filament and release of ADP bound actin monomers. Therefore, the ratio of ATP actin addition and hydrolysis to ADP determines the growth rate and stability of the actin filament. Although the basic mechanism of actin monomer addition is important for filament growth, there are multiple other mechanisms for regulating actin dynamics and formation of higher actin structures. These mechanisms involve actin accessory proteins. Formins bind actin and help initiate nucleation of filaments and enhance elongation (Figure 2). Profilin binds actin subunits to facilitate their addition to elongating filaments (Figure 2). Tropomyosin binds alongside actin filaments to stabilize them. Capping proteins bind the plus end of filaments and halts association or dissociation of actin monomers. There are also other proteins that can

sever actin filaments, accelerate the dissociation of actin monomers, or bind subunits and prevent their addition. More proteins are involved in forming higher actin structures such as crosslinking and bundling of filaments to form mesh works, protrusions and contractile structures (Figure 2). Regulation of actin nucleation, elongation, and dissociation is fundamental for modifying the actin cytoskeleton for different purposes for the cell. Thus, understanding the functions of actin binding proteins that regulate and reorganize actin will provide insight into the necessary cytoskeletal manipulations during cell division.

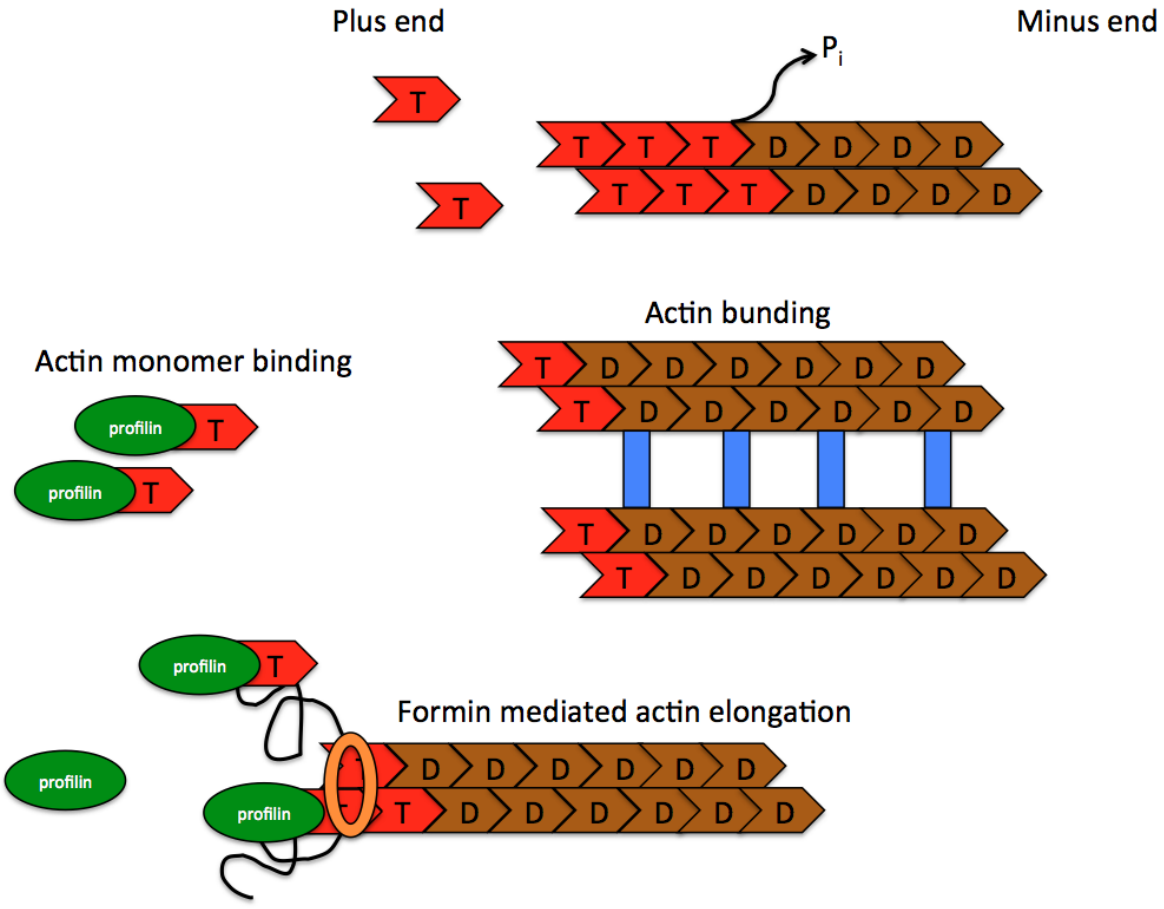


Figure 2: Actin polymerization. T represents actin monomers bound to ATP and D represents actin monomers bound by ADP. ATP actin is added more rapidly to the plus end of the filament. Actin bundling proteins can crosslink actin filaments. Profilin binds actin monomers and prevents them from spontaneously assembling. Formin binds to the plus end of actin filaments and adds profilin bound actin to elongate the actin filament.

Actin and cell division

The final stage in the cell cycle includes the formation of a contractile ring, which constricts to physically separate the daughter cells (Alberts et al., 2008). The use of a contractile ring during cytokinesis is conserved in eukaryotes (Guertin et al., 2002). Although there are differences in regulation of cytokinesis in different eukaryotic species, there are three conserved steps that are necessary for successful cell division. In the first step, the cell chooses the division site/cleavage furrow, which typically is the equator of the cell (Guertin et al., 2002). The midzone of the mitotic spindle seems to be the determining factor for division plane determination in animal cells while position of the nucleus seems to determine the division site in yeast (Guertin et al., 2002). Proteins involved in contractile ring assembly are located at the furrow including the primary components of the contractile ring, actin and myosin (Guertin et al., 2002). In the second step, the actin filaments are polymerized to form the contractile ring and myosin motor proteins attach and move along filaments (Alberts et al., 2008). The kinetic activity of myosin allows for the actin filaments in the contractile ring to constrict. The final step is the abscission of the cell membrane, which is facilitated by the eventual collapse of the contractile ring to a single point, called the midbody (Guertin et al., 2002). This results in physical separation of the cell into independent daughter cells. Although the broad mechanism of contractile ring assembly and constriction is conserved throughout eukaryotes, many details remain unknown. Because of its similarity to animal cells, the fission yeast *Schizosaccharomyces pombe* has become a widespread model for the study of contractile ring assembly and cytokinesis (Goyal et al., 2011).

Fission Yeast Cytokinesis

The fission yeast *Schizosaccharomyces pombe* is a common model for studying the cell cycle and cytokinesis for many practical reasons (Goyal et al., 2011). *S. pombe* has an oblong shape and large size, which allows clear microscopic imaging. Fission yeast lives most of their lives as haploid organisms making them easy to manipulate genetically. They also have a fast generation time, completing the cell cycle between two and four hours (Pollard et al., 2010; Wu et al., 2003). Similar to mammalian cells, the site of cell division in fission yeast occurs in a medial plane and constricts by an actin/myosin ring (Goyal et al., 2011). Fission yeast also contain proteins that are analogous to essential mammalian cytokinesis proteins (Pollard & Wu, 2010). These proteins include the motor myosin proteins, septins, SH3 domain proteins, small GTPases, IQGAP proteins, and formins (Guertin et al., 2002). These proteins contribute to the many molecular steps to position, assemble, and constrict the contractile ring properly.

The cell division process requires multiple proteins that allow for the accurate placement of the division site, formation of the actin-myosin contractile ring, and timely constriction and separation of daughter cells. Mid1 is an essential protein for medial placement of the division plane and correct cytokinesis of fission yeast cells (Sohrmann et al., 1996, Chang et al., 1996). During interphase, Mid1 shuttles from the nucleus to the cell cortex, but is mostly concentrated in the nucleus (Sohrmann et al., 1996, Wu et al., 2003, Paoletti and Chang, 2000). Mid1 shuttling between the nucleus and the cell cortex couples the location of the nucleus to the location of the contractile ring (Almonacid et al., 2009). Secured to the cell membrane, Mid1 provides a scaffold for other proteins to form cytoplasmic regions called cytokinesis nodes (Celton-Morizur et al., 2004). Mid1 establishes

the location of the cytokinesis nodes during the G2/M transition (Wu et al., 2003). These nodes are the precursors to the actin-myosin contractile ring, and the recruitment of many of the node proteins to the medial plane is dependent on Mid1 (Huang et al., 2008).

Pom1 and Cdr2 proteins are also involved in division site placement. Pom1 is a polarity protein that creates a gradient from the cell tips toward the equator of the cell (Celton-Morizur et al., 2006, Moseley et al., 2009). Pom1 phosphorylates Cdr2, which prevents its localization to the cell equator during interphase (Moseley et al., 2009). Regulation of Cdr2 localization and Pom1 gradient blocks the cell entry into mitosis (Moseley et al., 2009). Cdr2 dephosphorylation triggers it to interphase nodes at the medial plane. Cdr2 binds the N-terminus of Mid1 and helps anchor it to the cell membrane (Figure 4, Almonacid et al., 2009). However, this anchoring mechanism seems to be redundant with the amphipathic helix of Mid1, since deletion of Cdr2 does not affect Mid1 localization to cytokinesis nodes (Almonacid et al., 2009). Moreover, when nuclei were physically displaced in the live cells, Cdr2 localizes to the cell center irrespective of the nucleus while Mid1 localizes to the equator of the displaced nucleus (Almonacid et al., 2009). A Mid1 nuclear shuttling mutant caused Mid1 to localize in the center of the cell regardless of nuclei position indicating that Mid1 nuclear shuttling is the most significant mechanism for Mid1 mediated division site positioning (Almonacid et al., 2009).

At the G2/M transition, the Polo-like kinase Plo1 facilitates the complete translocation of Mid1 from the nucleus to the cell cortex where it anchors to the plasma membrane at the medial plane of the cell (Paoletti & Chang, 2000, Almonacid et al., 2011). The Mid1 C-terminus includes an amphipathic helix that helps anchor the protein to the lipids in the cell membrane (Figure 4, Celton-Morizur et al., 2004). Proteins are recruited in a

specific order. Mid1 first recruits the actin bundling protein, Rng2 and the myosin light chain, Cdc4 to the nodes (Refer to figure 3, Laporte et al., 2011, Almonacid et al., 2011, Padmanabhan et al., 2011). Shorty after, myosin II (Myo2) and the myosin regulatory protein Rlc1 localize to the Mid1-Rng2-Cdc4 complex (Wu et al., 2003, Laporte et al., 2011). The F-BAR protein Cdc15 recruits the actin nucleating formin Cdc12 and the profilin Cdc3 to join the nodes and initiate actin filament polymerization (Wu et al., 2003, Roberts-Galbraith et al., 2010, Carnahan and Gould, 2003). Cdc12 is essential for actin nucleation and Cdc3 is required for rapid elongation of actin filaments in the mature cytokinesis nodes (Kovar et al., 2003). Nucleation of actin filaments from each cytokinesis node is followed by the search, capture, pull, and release (SCPR) mechanism, which allows for the mature contractile ring to form (Vavylonis et al., 2008). The filaments extending from nodes begin the SCPR process by searching for Myosin II (Myo2) associated with an adjacent node (Vavylonis et al., 2008). Once the actin filament has been captured by the Myo2 at another node, the motor activity of Myo2 links and pulls the nodes closer together. Actin modifying proteins Ain1 and Fim1 bundle and condense actin filaments into a closed ring structure. The tension between nodes is then released by one of three mechanisms: the dissociation of Myo2, the severing of actin by cofilin, or the displacement of Cdc12 from the actin filaments (Lee et al., 2012). Once the ring is assembled, Mid1 dissociates and translocates to the daughter cell nuclei before cytokinesis (Sohrmann et al., 1996, Wu et al., 2003). A group of proteins within the Septation Initiation Network (SIN) are recruited to the medial plane where they regulate the constriction of the contractile ring and the deposition of the septum to complete cytokinesis (Gould and Simanis, 1997).

In the absence of Mid1, the proteins within the cytokinesis nodes localize incorrectly, which leads to inaccurate location of the subsequent contractile ring (Sohrmann et al., 1996, Wu et al., 2003, Saha et al., 2012). When Mid1 is deleted, actin localization is disorganized and contractile rings take more than two times longer to form compared to wild type (Huang et al., 2008). These rings are not medially located and most result in cytokinesis failure (Sohrmann et al., 1996, Chang et al., 1996). An alternative mechanism that is mediated by the SIN signaling pathway allows for the contractile ring to be constructed in the absence of Mid1 (Hachet and Simanis, 2008). When Mid1 is deleted, the misplacement of contractile rings can be attributed in part to the mislocalization and/or absence of proteins within the cytokinesis nodes (Saha et al., 2012). Although the formin Cdc12 can localize to the cell cortex in these cells, Rng2, Myo2, and Cdc15 do not accumulate into nodes (Saha et al., 2012). Cdc12 is able to nucleate and elongate actin filaments and eventually they are able to merge into a ring structure (Saha et al., 2012). The formation of the actin filaments by Cdc12 allows for the association of Rng2, Myo2, and Cdc15 so that the ring can mature and condense. Without Mid1 anchoring and localizing the cytokinesis node proteins to the medial plane, contractile rings do not form in the equator of the cell. Because the node proteins are more dispersed in the cell without Mid1 present, the contractile ring may not materialize or the ring takes longer to form (Saha et al., 2012). Therefore, understanding how Mid1 organizes the contractile ring will reveal important mechanisms for accurate cytokinesis.

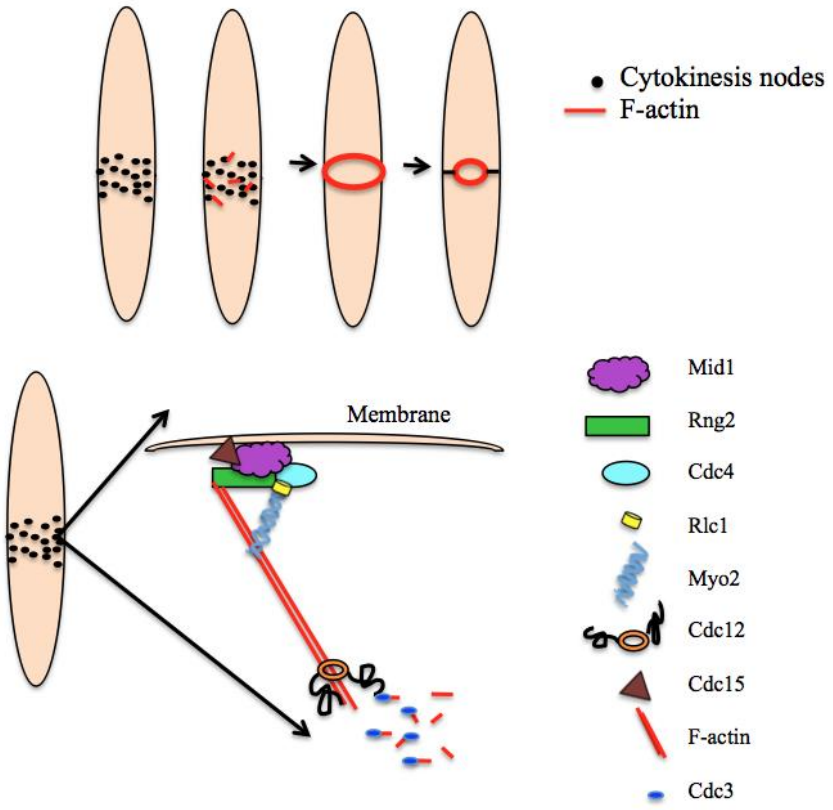


Figure 3: The formation of cytokinesis nodes during contractile ring assembly in fission yeast. Proteins are sequentially recruited to the cytokinesis nodes (bottom) where they assemble the contractile ring, which eventually matures and constricts (top).

Mid1 domains

Because Mid1 functions to recruit and anchor multiple proteins important for contractile ring assembly, it has many domains that are important for theses interactions (Figure 4). Mid1 contains direct binding domains to Rng2 (actin bundling), Gef2 (Rho GTPase activator), Cdr2 (kinase), Clp1 (phosphatase), and Plo1 (kinase) (Padmanabhan et al., 2011, Ye et al., 2012, Almonacid et al., 2009, Clifford et al., 2008, Almonacid et al., 2011). Mid1 also includes nuclear export sequences (NES) and a nuclear localization sequence (NLS) that aids in its shuttling from the nucleus and cytoplasm (Celton-Morizur et

al., 2004, Paoletti et al., 2003). The C-terminus of Mid1 contains an amphipathic helix that is known to interact with lipids in the membrane to anchor it to the cell cortex (Celton-Morizur et al., 2004, Paoletti et al., 2003). The final 122 amino acids of the Mid1 C-terminus contains a Pleckstrin Homology (PH) domain. PH domains are typically involved in protein-protein interactions and are known to bind phosphoinositol lipids (Lemmon et al., 2000). Localization studies of fluorescently labeled Mid1 fragments revealed that Mid1 1-800 (missing the PH domain) retained 20% lower fluorescence intensity in cytokinesis nodes than full length Mid1 (Lee and Wu, 2012). This suggests that the PH domain is important for Mid1 localization to the cytokinesis nodes at the cell cortex (Lee and Wu, 2012). In the same study, fluorescence recovery after photobleaching (FRAP) experiments indicated that Mid1 1-800 had a significantly reduced (2 fold) half-life at the cytokinesis nodes compared to full length Mid1 (Lee and Wu, 2012). Since the Mid1 1-800 protein was more dynamic at cytokinesis nodes than full length, the PH domain may serve to stabilize Mid1 at the cell cortex (Lee and Wu, 2012). Based on *in vitro* binding assays, the Mid1 PH domain alone can also bind phospholipids that are found in the cell membrane (Lee and Wu, 2012). Taken together, the PH domain may help localize and stabilize Mid1 in cytokinesis nodes by interacting with lipids in the membrane (Lee and Wu, 2012). However, the PH domain does not localize to the cell membrane alone, possibly because its interaction with lipids is fairly weak, indicating that another PH domain related mechanism might cooperate to stabilize Mid1 at cytokinesis nodes.

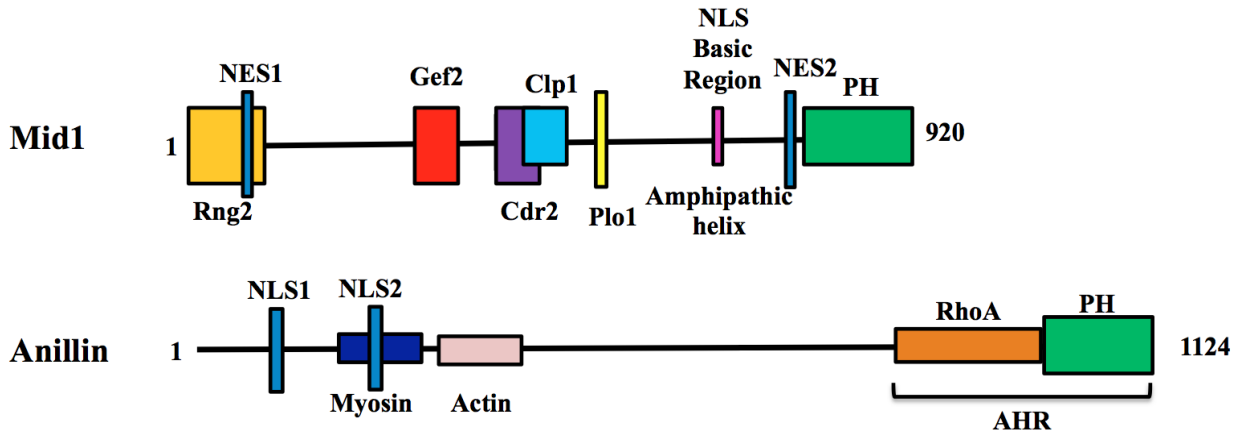


Figure 4: Mid1 and human anillin protein binding domains and localization sequences. Both Mid1 and anillin are scaffolding proteins that contain multiple protein binding domains (colored boxes). They also contain signaling sequences that aid in nuclear shuttling (NES=nuclear export sequence, NLS=nuclear localization sequence). Mid1 also contains an amphipathic helix that aids in its insertion into the cell membrane. Both Mid1 and anillin have a C-terminal Pleckstrin Homology domain (PH). In addition, Anillin contains an anillin homology region (AHR) that is shared with other homologous anillins.

Anillin related Mid1 functions

The Mid1 analogue in humans is called anillin (Pollard et al., 2007). Anillin-related proteins are found in all eukaryotic organisms and are necessary for recruitment of contractile ring components to the cleavage furrow to promote ring assembly and constriction (D'Avino et al 2009). Anillins in *Drosophila*, vertebrates, and *S.pombe* have remarkably similar localizations during the cell cycle (D'Avino et al., 2009). Anillins are nuclear during interphase then transport to the cell cortex during mitosis (D'Avino et al., 2009). The N-terminus of human anillin has three nuclear localization sequences (NLS) and a SH3 binding domain (Figure 4, Oegema et al., 2000). The human anillin N-terminus also contains a binding domain for myosin II and an actin domain that both binds and bundles filamentous actin (F-actin) (Straight et al., 2005). Its C-terminus contains an anillin homology region

(AHR) that includes a RhoA GTPase binding domain and a PH domain (Oegema et al., 2000). The PH domain of human anillin is essential but not sufficient for its accumulation to the cell cortex during cytokinesis (Oegema et al, 2000). The sequences of the PH domain of anillin and Mid1 are not highly conserved (23% homology by NCBI BLAST), however they have very strong structural homology, which is shared by other PH domains. Although the Mid1 PH domain is dispensable for Mid1 localization in nodes, it appears to be important for Mid1 stability at the cell cortex (Lee and Wu, 2012).

Actin and myosin binding domains of human anillin are also found in *Drosophila melanogaster* and *C. elegans*, but canonical binding domains for these proteins have not been identified in Mid1 (D'Avino et al., 2009). However, the absence of *S. pombe* Mid1 and vertebrate anillin result in similar phenotypes involving myosin II and F-actin defects during mitosis (D'Avino et al., 2009). Human anillin and Mid1 are important for recruitment of mysosin II to the equator of the cell and also co-localize with F-actin during contractile ring assembly (Sohrmann et al., 1996, Wu et al., 2003, Saha et al., 2012). Although Mid1 is known to bind and recruit myosin II to the cytokinesis nodes, a binding locus has not been identified (Wu et al., 2003, Motegi et al., 2004). When F-actin is depleted in cells using Latrunculin A (LatA), anillin localizes to the center of the cell, but in a broken band that is unorganized (D'Avino et al., 2008). In a similar experiment where *S. pombe* cells were treated with LatA, Mid1 localized to the cell equator in the absence of actin filaments but it did not form a tight ring as in untreated wild type cells (Wu et al., 2003). These parallel data suggest that although Mid1 and anillin do not have completely homologous sequences, many of their functions are conserved. Likewise, human anillin contains a RhoA GTPase binding site that is essential for actin filament formation in the contractile ring by activating formins

(Hickson et al., 2008). RhoA is also important for recruitment of anillin to the cell equator (Hickson et al., 2008). In *S. pombe* cells, the RhoA homologue Rho1 also localizes the contractile ring and has similar functions as RhoA but has not yet been shown to bind Mid1 (Arellano et al., 1997, Nakano et al., 1997). A GTPase activator for the contractile ring assembly formin in fission yeast, Cdc12 also has not been found. There are many questions regarding Mid1 interactions with contractile ring components that are not yet clear. Therefore, it would be valuable to discover novel functional domains in Mid1 that are homologous to other anillins.

Rho1 and cytokinesis

Proteins that regulate cytoskeleton organization, cell movement, and polarity are ideal candidates for understanding cytokinesis since major cytoskeletal rearrangements and organization are required for cell division. RhoGTPases are GTP binding proteins that are molecular switches responding to cellular signals that result in modification of the actin cytoskeleton (Alberts et al., 2008). GTPases cycle between active and non-active forms coinciding with whether GTP (guanine triphosphate) or its hydrolyzed form, GDP (guanine diphosphate) is bound (Figure 5). GTPases are modified by GTPase activating proteins (GAPs) that induce hydrolysis of the GTP molecule to GDP. The inorganic phosphate is released and GDP remains tightly bound causing the GTPase to be in an inactive conformation. Guanine nucleotide exchange factors (GEFs) bind GTPases causing it to release the GDP and the GTP is quickly replaced resulting in an active conformation. GTPases are involved in a wide range of cellular processes but Cdc42, Rac, and RhoA of the RhoGTPase family are specific to the regulation of the cytoskeleton. Activation of these proteins results in major reorganization of the cytoskeleton due to their many downstream

targets. Activation of the RhoA protein in particular causes bundling of actin filaments with myosin II into stress fibers by interactions with several kinases and phosphatases and by activation of formin proteins (Hall 1998). RhoA binds to the autoinhibited form of formins and activates them by changing their protein conformation (Alberts et al., 2008, Hall 1998). Formins build actin filaments by nucleation and are recruited for actin polymerization during contractile ring assembly in fission yeast (Kovar et al., 2003). The involvement of Rho proteins in cytokinesis has not been completely described, but research in recent years has begun to uncover its role in this complicated process.

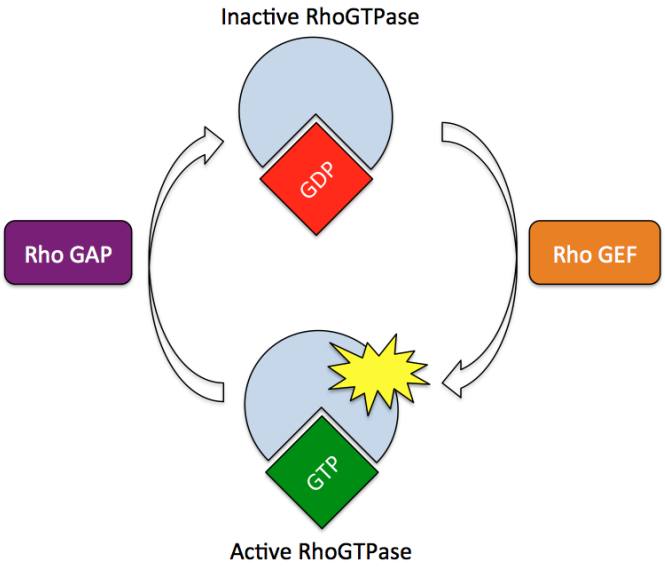


Figure 5: RhoGTPase activation cycle. RhoGTPases act as molecular switches. RhoGEF proteins activate RhoGTPase by stimulating GTP to bind to the RhoGTPase, while RhoGAP proteins deactivate RhoGTPases by enhancing hydrolysis of the GTP molecule to GDP.

The RhoA protein in fission yeast is called Rho1. Normally, Rho1 localizes to the growing ends of yeast and the division plane during mitosis (Arellano et al., 1997, Nakano et al., 1997). Overexpression of Rho1 causes cell swelling, loss of cell polarity, actin mislocalization, and multiple septation sites in fission yeast and budding yeast (Arellano et al., 1997, Nakano et al., 1997). Deletion of Rho1 is lethal and suppression of its levels by gene disruption causes the disappearance of actin patches and lysis of cells before cytokinesis (Arellano et al., 1997).

Since the actin contractile ring is not made from previously existing actin bundles, new actin filaments must be nucleated at the bud neck of dividing budding yeast (Tolliday et al., 2002). Therefore recruitment of formins to the bud neck is critical for contractile ring assembly. When Rho1 expression is suppressed, cells are deficient in contractile ring formation and do not complete cytokinesis. This in part, is due to inadequate recruitment of formins to the division plane since overexpression of formin can reverse this Rho1 deficient phenotype (Tolliday et al., 2002). Many lines of evidence indicate that the polo-like kinase is necessary for contractile ring assembly (Barr et al., 2004). Among its other substrates, the budding yeast Polo-like kinase Cdc5 phosphorylates the GEF Tus1, which regulates the activation of Rho1 and recruits it to the division site (Yoshida et al., 2006, Yoshida et al., 2009). This process is necessary for Rho1 to activate formins at the division plane, which are needed to assemble the actin filaments of the contractile ring (Yoshida et al., 2006). These studies indicate that Rho1 activation of formins at the division site is necessary for proper contractile ring assembly through a polo-kinase/GEF recruitment mechanism in budding yeast.

Studies in *S. pombe* have also indicated the importance of Rho1 in cytokinesis. Deletion of one of the Rho1 regulatory GEFs, Rgf3, is lethal in fission yeast (Mutoh et al., 2005). Rgf3 and Rho1 both localize to the medial plane before cytokinesis in structures reminiscent of interphase nodes. Depletion of Rgf3 expression causes defective contractile ring assembly including disorganized actin filament formation and disappearance of septa. This phenotype can be reversed by overexpression of Rho1 indicating an important role of Rho1 activity during contractile ring assembly (Mutoh et al., 2005).

Mid1 localization has recently been connected to GEF recruitment to the contractile ring in fission yeast. Gef2 is the activator of another RhoGTPase, Cdc42 which has downstream targets that regulate actin nucleation (Ye et al., 2012). Gef2 was found to physically interact with Mid1 and affect its localization to the contractile ring during early mitosis (Ye et al., 2012). This interaction is important for contractile ring assembly and division site positioning (Ye et al., 2012). Though this study does not elucidate whether the relationship between Mid1 and Gef2 is dependent on RhoGTPase, other research suggests the importance of RhoGTPase for cytokinesis (Mutoh et al., 2005).

Our lab previously performed Tandem Affinity Purification (TAP) of Mid1 complexes during fission yeast mitosis and potential binding partners were identified using 2D liquid chromatography tandem mass spectrometry. In this analysis, Rho1 was indicated as a putative binding partner for Mid1 with 31.7% peptide sequence coverage. Given the evidence that RhoGTPases play an essential role in contractile ring assembly and cytokinesis, and human anillin directy binds to the Rho1 homologue RhoA, the possible interaction of Mid1 and Rho1 is of great interest.

Given the central role of Mid1 in cytokinesis, the research objectives of this thesis aim to identify binding partners of Mid1 important for contractile ring assembly during cell division.

1.) Characterize the interaction of Mid1 and filamentous actin

2.) Determine whether Mid1 and Rho1 GTPase physically bind

Chapter II: Materials and Methods:

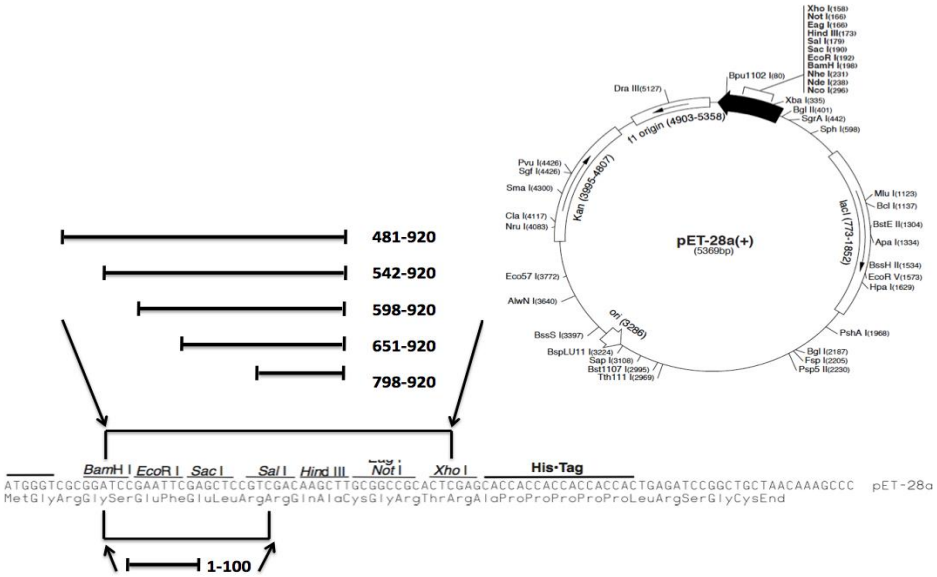
Plasmids

Mid1 fragments were cloned into different vectors as follows. All primers are listed in table 1. Mid1 fragments (481-920, 542-920, 598-920, 651-920, 798-920) were each amplified from the full-length gene and cloned into the pET28a vector (Novagen, Madison, WI). Each fragment was cloned with primers that included BamH1 and Xho1 cut sites. Mid1 1-100 fragment was also cloned into the pET28a vector using BamH1 and Sal1 cut sites. Restriction digest (enzymes from Promega, Madison, WI) and ligation of the inserts into the pET28a vector resulted in a N-terminal 6x Histidine tag (Figure 6A, Epicentre Fast Link Ligation Kit Madison, WI). Mid1 fragment 798-920 encoding the PH domain was also cloned into the pET21a vector (Figure 6B, Novagen, Madison, WI) using primers with BamH1 and Sal1 cut sites, which resulted in a C-terminal 6x Histidine tag.

The sequence of *rho1* gene was cloned into the pGEX vector as follows. Total RNA was extracted from about 1 x 10^7 wild type *S. pombe* cells (Strain 246) through glass bead lysis (Acid washed Glass bead 710-1180, G8772 Sigma-Aldrich, St. Louis, MO) and extraction using an RNeasy Minikit (Qiagen Gaithersburg, MD) according to the manufacturer's protocol. Total mRNA was reverse transcribed into cDNA using an oligo dT primer (Omniscript Reverse Transcription Kit, Qiagen, Gaithersburg, MD). Rho1 cDNA was amplified from total cDNA using primers located in the 3'UTR and 5'UTR of the *rho1* gene using Taq polymerase that leaves 3' overhangs on the PCR product (Syzygy Biotech, Grand Rapids, MI). Rho1 cDNA was ligated into the pGEMT vector (Promega Easy Vector Systems, Madison, WI) and transformed into DH5α competent cells (New England Bioloabs,

C2987, Beverley, MA). The *rho1* gene was subcloned from the pGEMT vector by amplifying the *rho1* gene with BamH1 and Xho1 cut site primers. Insert was ligated into the pGEX vector (Figure 6C) and resulted in a N-terminal gluthathione S-transferase (GST) tag. All plasmids were sequenced to verify correct insertions within the plasmids.

A.)



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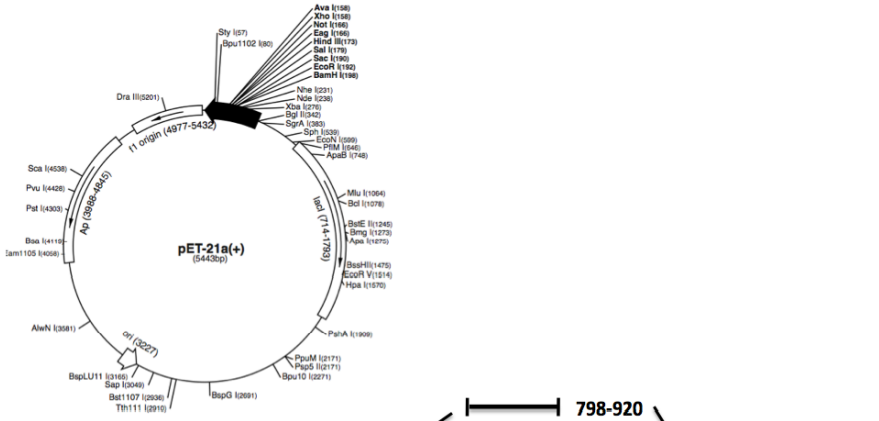


C.)

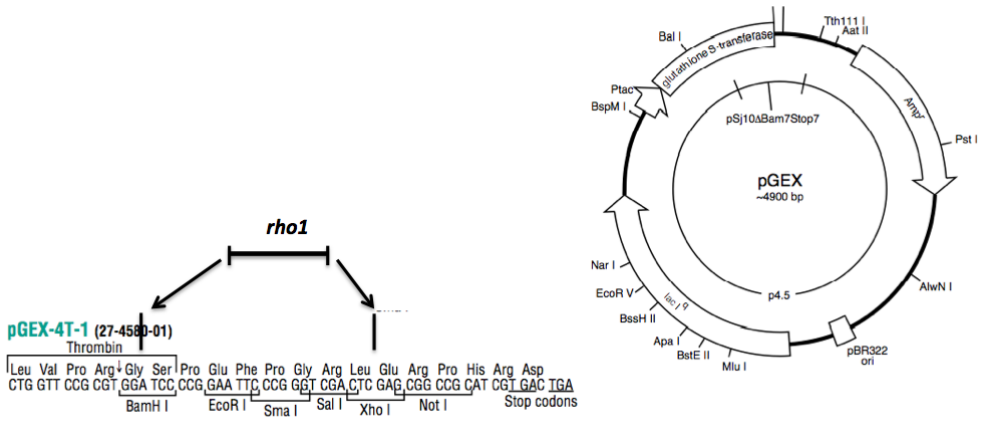


Figure 6: Vector maps for cloning *mid1* fragments (A and B) and *rho1* (C) gene for protein expression.

Table 1: List of primers used in this study

Primer Name	Primer sequence (5'-3')
Mid1 1 BamH1 Forward	TCCCGGATCCATGAAAGAGCAAGAGTTCTC
Mid1 443 BamH1 Forward	TCCCGGATCCATGAGCTCTGAAGATT
Mid1 481 BamH1 Forward	TCCCGGATCCATGGAGTACCATAACG
Mid1 542 BamH1 Forward	TCCCCGATCCATGGATAAGAGTACGT
Mid1 598 BamH1 Forward	TCCCGGATCCATGAGTGAAGTCCATT
Mid1 651 BamH1 Forward	TCCCGGATCCATGATTGAGGACAAGT
Mid1 798 BamH1 Forward	TCCCGGATCCATGAGAAGTAACTATCTATATAA
Mid1 920 Sal1 Reverse	ACGCGTCGACTTAAGCCATAAAATTCAC.
Mid1 920 Xho1 Reverse	GCAGCTCGAGAGCCATAAAATTCACG
Rho1 3'UTR Forward	GAAAGTAAACAAATCTAGGG (anneals to 5'UTR)
Rho1 5' UTR Reverse	AAAATTTGGAAACAAGCTGT (anneals to 3'UTR)
Rho1 BamH1 Forward	TCCCGGATCCATGGCGACAGAACTTC
Rho1 Xho1 Reverse	GCAGGGGCCCTTACAACAAGATACAA
Rho 1 Tag Forward	CTCGTGCTGCTATGCTCAAACACAAGCCCAAA GTGAAGCCCTCTAGTGGAACTAAGAAGAAGAA GCGTTGTATCTTGTTGCGGATCCCCGGGTTA ATTAA
Rho1 Tag Reverse	TTGAATGTGCTTCGACTGAAGAGATGTATAAA AACAGAAAAGATAAATTCTCTAAGTTAAAAAA TTTGGAAACAAGCTGT GAA TTCGAG CTC GTT TAAAC

Protein Purification

The plasmid was transformed into BL21 RIL *E.coli* cells and the protein was produced from an IPTG inducible promoter. The resulting N-terminal 6x histidine tagged PH domain protein was purified by incubating with Talon resin (Clontech, Mountain View, CA) in Extraction buffer (50 mM NaH_2PO_4, 500 mM NaCl, 10% glycerol, 10 mM Imidazole, 10 mM beta-mercaptoethanol (BME), 0.5mM PMSF, 1 tablet Roche EDTA Free Inhibitor tablets, pH 8.0). Histidine tagged PH domain was eluted from resin by collecting 1 mL fractions of Elution Buffer (50 mM NaH_2PO_4, 500 mM NaCl, 10 % glycerol, 250 mM imidazole, 10 mM BME, pH 8.0). Fractions containing purified protein were combined and concentrated using an Amicon Filter tube 10,000MW (Millipore Bedford, MA) in Storage Buffer (20 mM HEPES, 1 mM EDTA, 200 mM KCl, 0.01% NaN_3, 10% glycerol, 1 mM DTT, pH 7.4). Cdc12 FH1 and FH2 domains, and Cdc3 profilin proteins were obtained from the laboratory of Dr. David Kovar from the University of Chicago, Chicago, IL. Cdc12(FH1FH2)-HIS was purified from bacteria as described in Scott et al., 2008. Purified Cdc12(FH1FH2)-HIS protein was stored in formin buffer: 20 mM HEPES, pH 7.4, 1 mM EDTA, 200 mM KCl, 0.01% NaN_3, and 1 mM DTT. The profilin gene was inserted into the pMW172 vector and was purified from bacteria with poly-L proline sepharose as described in Lu and Pollard, 2001. Profilin was stored in 1 mM EDTA, 1 mM DTT, 20 mM Tris-Cl, pH 8.0.

Actin co-sedimentation

Aliquots of His-tagged PH domain in Storage buffer and bovine serum albumin (BSA, Thermo Scientific, Rockford, IL) were pre-cleared by centrifugation at 150,000 x g for 1 hour at 4°C. Actin monomers (Rabbit Muscle Actin AKL99-A, Human platelet Actin

APHL99, Cytoskeleton, Inc Denver, CO) were allowed to assemble by the manufacturer's protocol. Filaments were mixed with different concentrations of Mid1 PH domain, alpha actinin (AT01 Cytoskeleton, Inc Denver, CO), or BSA. High-speed cosedimentation samples were centrifuged at 150,000 x g at 24°C for 1.5 hours. Low speed cosediementation samples were centrifuged at 10,000 x g at room temperature for 20 minutes. Total supernatant was removed from each sample and placed into new tubes and SDS sample buffer was added. Pellets were resuspended in SDS sample buffer. Samples were resolved on a 15 % SDS PAGE gel and visualized by Coommassie Blue staining. Gels were imaged and protein band densities were determined with the Odyssey Fc Imager (LI-COR Biosciences, Lincoln NE). For high-speed actin binding assays, the fraction of PH domain bound was determined by dividing the density of the PH domain found in the supernatant by the density found in the pellet for each concentration of F-actin. These data were used to create a curve (y=fraction PH domain bound, x=concentration F-actin). Dissociation constants (K_d) were calculated from three independent experiments (y=x/Kd+x). The K_d is 1.7 μM with a standard deviation of 0.55 μM. The average of the three experiments is graphed on the curve. Error bars represent standard deviations for each data point. For low-speed actin bundling assays, the fraction of actin in low speed pellets was found by calculating the density pellet/density pellet+supernatant, for each the concentration of PH domain. The averages of three experiments were used to graph the fraction of actin in the pellet versus the concentration of PH domain in the reaction. Error bars represent standard deviations. Bundling efficiency was calculated as the amount of PH domain necessary to pellet half of the actin in the reaction. The bundling efficiency is 0.23 μM with a standard deviation of 0.11 μM calculated from the three independent experiments.

Pyrene-actin assembly

Spontaneous Assembly: Calcium associated actin monomers were put into magnesium buffier (500 mM KCl, 10 mM $MgCl_2$, 10 mM ethylene glycol tretraacetic acid (EGTA), and 100 mM imidazole PH 7.0) to produce Mg-ATP actin. Mg-ATP Actin monomers were mixed with pyrene labeled actin to produce a 20% labeled stock (Kuhn and Pollard 2005). 15 μM of the 20% pyrene labeled stock was aliquotted into 12 wells along a 96 well black plate (Corning, Corning NY). Proteins of interest in storage buffer (Mid1 PH domain, Cdc12 FH1FH2, or Cdc3) were diluted to different concentrations in 10x KMEI (500 mM KCl, 10 mM $MgCl_2$, 10 mM ethylene glycol tretraacetic acid (EGTA), and 100 mM imidazole PH 7.0) into new wells. Using a 12-well multipipetter, the protein/ buffer solution in each well was simultaneously transferred to the wells containing pyrene labeled actin monomers. Fluorescence was measured immediately with a Safire2 fluorescence plate reader (Tecan, Durham, NC) reading at 364 nm excitation and 407 nm emission.

Seeded Assembly: 5.0 μM Mg-ATP actin monomers were allowed to assemble in a 96 well black plate for 2.5 hours. Proteins of interest were added to new wells along with 2.5 μM 20% pyrene labeled actin monomers in 10X KMEI. Protein/monomer/buffer solution was simultaneously transferred to the pre-assembled actin seeds. Fluorescence was measured immediately with the Safire2 fluorescence plate reader (Tecan, Durham, NC) reading at 364 nm excitation and 407 nm emission.

For both assembly assays, fluorescence measurements were taken every 10 seconds for 2000 seconds. Each data point was normalized to the actin only control so that starting fluorescence was the same. Fluorescence measurements for every 100 seconds were used to

create the graphs of fluorescence intensity of the actin polymer compared to the time in seconds.

Phalloidin staining of Actin Filaments

2.5 μM Mg-ATP actin was allowed to assemble in 1X KMEI for 2.5 hours. 3.5 μM, 2.5 μM, 1.5 μM and 0.5 μM of 6x histidine tagged Mid1 PH domain in storage buffer (described in protein purification) was added to each reaction. Actin filaments in each reaction were incubated with 1 μM TRITC-Phalloidin (Fluka Biochemika, Switzerland) for 5 minutes. Reactions were diluted 1:250 into fluorescence buffer (50 mM KCl, 1 mM $MgCl_2$, 100 mM DTT, 20 μg/mL catalase, 100 μg/mL glucose oxisase, 10 mM imidazole PH 7.0, 3 mg/mL glucose, and 0.5% methylcellulose). Diluted samples were spotted onto coverslips coated with 0.05 μg/uL poly-L-lysine. Filaments were viewed and imaged with a CCD camera (Orca-ER, Hamamatsu) on an Olympus IX-81 microscope. Two separate slides were prepared from each of the 5 treatment reactions and 10 frames per slide were imaged. Number of filaments and filament lengths were measured using Image Pro Premier software. Means and standard deviations were calculated from all frames taken. Student's Ttest was used to compare the number of filaments found for each treatment. Due to mild outliers in the data set, actin filament lengths were compared using the Jonckheere-Terpstra nonparametric test for order alternatives.

Yeast strains

S. pombe yeast strains were grown in Yeast Extract (YE) at 25°C or 32°C. Table 3 includes yeast strains used in this study. The *rho1-gfp* (strain 0052 *rho1-GFP:KanR ade6-M210 ura4-D18 leu1-32, h-*) was created by amplifying a GFP-Kan insert from the pFA6

SUPERENCHANCED GFP kan cassette plasmid (Wach et al., 1997, Bahler et al., 1998). The forward primer included sequence flanking the GFP-Kan insert and the C-terminus of the *rho1* gene (Table 2, Rho1 tag Forward). The reverse primer also included GFP-Kan flanking sequence with the sequence downstream of *rho1* (Table 2, Rho1 tag Reverse). These primers were used to create an insert with homology to the C-terminus of endogenous *rho1* gene site. This insert was transformed into wild type fission yeast strain 246 (Table 3) by the standard lithium acetate procedure (Keeney and Boeke, 1994). Briefly, cells were washed with 0.1 M lithium acetate/ 10 mM Tris HCl/1 mM EDTA. PCR product and 40 ug boiled herring sperm carrier DNA were added to cells and they were incubated at 32°C for 45 minutes, heat shocked at 42°C for 5 minutes, washed and plated on Yeast Extract plates for 16-18 hours, then replica plated onto G418 and YE to select for recombinants. Incorporation of *rho1-GFP-Kan* was confirmed by fluorescence microscopy to view GFP signal.

The *rho1-GFP* strain (0052) was crossed with *mid1-myc* (strain 3136) and also with *mid1*Δ (strain 2711) by random spore analysis. The two strains were grown together on low nutrient plates. Cells that had undergone meiotic recombination were identified by presence of tetrad spores encased in asci. Cells were incubated with glusalase overnight to dissolve asci and release spores. 30% ethanol wash was used to remove vegetative cells and spores were plated on selective media to identify *rho1-GFP mid1-myc* or *rho1-GFP mid1Δ* recombinants.

Table 2: *S. pombe* strains used in this study

Strain	Genotype
0052	*rho1-GFP:KanR ade6-M210 ura4-D18 leu1-32, h-*
3136	*mid1-myc:ura4 ade6-M210 ura4-D18 leu1-32 h+*
0053	*rho1-GFP:KanR mid1-myc:ura4 ade6-M210, ura4-D18, leu1-32, h-*
2415	*mid1-GFP:KanR ade6-M210 ura4-D18 leu1-32 h+*
0054	*rho1-GFP:KanR mid1::ura4 ade6-M210 ura4-D18 h-*
0246	*ade6-M210 ura4-D18 leu1-32 h-*
2711	*mid1::ura4 ade6-M210 ura4-D18 leu1-32 h+*

Staining and Microscopy

rho1-GFP, *mid1-GFP*, and *rho1-GFP mid1Δ* strains were grown in 50 mL cultures of YE overnight at 25°C. Cells were methanol fixed and stained with 4',6-diamidino-2-phenylindole (1 μg/μL DAPI, 1 mg/mL p-phenylenediamine, 90% glycerol). Another sample of cells were fixed with paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA) and stained with Alexa Fluor 546 Phalloidin (Invitrogen, Carlsbad, CA) and DAPI. Cells were viewed with an Olympus BX51 Upright Microscope with an UPlan apo 100x/1.35 oil iris objective.

Co-immunoprecipitation

400 µL Protein G Dynabeads (Invitrogen, Carlsbad, CA) were coated with c-myc antibody (4.5 µg/reaction, 9E10, Roche, Indianapolis, IN). *rho-GFP*, *mid1-GFP*, and *rho-GFP mid1Δ* yeast cells (2x 50 OD_{595} cultures) were lysed with glass beads (710-1180 um Sigma-Aldrich, St. Louis, MO) in NP40 buffer (6 mM Na_2HPO_4, 4 mM NaH_2PO_4, 1% NONIDET-P-40, 150 mM NaCl, 2 mM EDTA, 50 mM, NaF, 0.1 mM Na_3VO_4) with Protease Arrest (GBiosciences, Maryland Heights, MO), 1 mM phenylmethylsulfonyl fluoride (PMSF, Sigma-Aldrich, St. Louis, MO), and 12 µM benzamidine (Sigma-Aldrich, St Louis, MO). 0.5 M Diisopropyl fluorophosphate (DiFP) was added immediately prior to lysis. Mid1-myc protein was immunoprecipitated by incubating the cell lysates with the coated beads for 1 hour at 4°C. Beads were washed with phosphate citrate buffer (100 mM Na_2HPO_4, 50 mM $Na_3C_6H_5O_7$) and protein was eluted from the beads by boiling in SDS sample buffer. Western blots were performed by resolving Mid1-myc immunoprecipitate on a 3-8% Tris Acetate Gel (NuPAGE Invitrogen, Carlsbad, CA), probing with the c-myc antibody (1:1000 dilution in Odyssey Blocking Buffer, LICOR Biosciences, Lincoln, NE) and detecting Mid1-myc by a goat-anti mouse IRDye-800W secondary antibody (1:1000 dilution in Odyssey Blocking Buffer, LICOR Biosciences, Lincoln, NE). Rho1-GFP was detected by resolving the same immunoprecipitates on a 4-20% Tris glycine gel (BioRad, Des Plaines, IL) probing with anti-GFP (1:1000 dilution, Roche, Indianapolis IN), and detecting with the goat anti-mouse IRDye-800W secondary antibody (1:1000 dilution in Odyssey Blocking Buffer, LICOR Biosciences, Lincoln, NE). Membranes were imaged on an Odyssey Fc Imager (LI-COR Biosciences, Lincoln NE)

Chapter III: Results

Preliminary Results:

Given that Mid1 is an essential component for correct division site placement and recruitment of contractile ring proteins to the medial plane of the cell, exploring the proteins with which it interacts is important for understanding the mechanism of cell division. Mid1 has many functional similarities to anillin analogues, although the two proteins do not contain complete sequence conservation. Since anillin analogues in metazoans are known to bind F-actin, and Mid1 and F-actin have similar functions during contractile ring assembly in yeast cells, the interaction of Mid1 and F-actin was explored in the following studies. The aim of these studies was to determine whether Mid1 physically binds F-actin and whether the interaction has consequence for actin assembly. The Mid1 sequence was divided into two regions, the N-terminus (amino acids 1-424) and C-terminus (amino acids 443-920) (Refer to Figure 8). High-speed actin co-sedimentation assays were performed with purified Mid1 protein regions to determine whether either could bind F-actin. The result clearly indicated that the C-terminus of Mid1 could bind F-actin while the N-terminus did not (Jakubowski and Clifford-Hart, unpublished data).

The Mid1 C-terminal sequence was aligned to the Mid1 homologues in closely related species. Comparison of the Mid1 homologues of *S. japonicas, S. cryophilus* and *S. octosporus* revealed the most conserved amino acids between species. Under the assumption that sequence similarity is closely associated with functional importance, Mid1 C-terminal fragments were constructed without disruption of these conserved sites. These included fragments with amino acids 481-920, 542-920, 598-920, 651-920, and the sequence of the

PH domain, 798-920. Because the function of the PH domain of Mid1 has not yet been determined, it was the first fragment analyzed for F-actin binding. The PH domain was initially cloned into the pGEX vector, (Figure 6) which resulted in a purified protein with a N-terminal GST tag. High-speed co-sedimentation assays were performed by mixing filamentous actin, and centrifuging reactions. The supernatant fraction was removed from each reaction and the pellets were re-suspended. Proteins that do not bind to F-actin will remain soluble in the supernatant while proteins that bind will be found in the pellet with F-actin. Results indicate that the GST-PH domain binds F-actin (Figure 7, Lanes 1 and 2). Bovine serum albumin (BSA) was used as a negative control, which does not bind F-actin and was found in the supernatant. The positive control, α-actinin binds F-actin and was found in the pellet with F-actin (Figure 7, Lanes 3 and 4). The PH domain does not pellet by itself (Figure 7, Lanes 5 and 6). However, when combined with F-actin the PH domain was found in pellet (Figure 7, Lanes 7 and 8). Because GST is a large tag (~30 kDa) and can dimerize, the PH domain was cloned into the pET21a vector and purified with a C-terminal 6x Histidine tag. This His-tagged PH domain did not bind F-actin with similar affinity to the GST-Mid1 PH domain fragment previously tested (data not shown). We speculate that the change in the location of the tag on the protein (N-terminus to C-terminus) or the kind of tag (GST to His) could change the binding affinity of the purified PH domain to F-actin by disrupting its folding. Therefore, the PH domain was cloned into the pET28a vector so that the purified protein contained a N-terminal 6x His tag (Figure 6). This purified protein was used in further actin association studies.

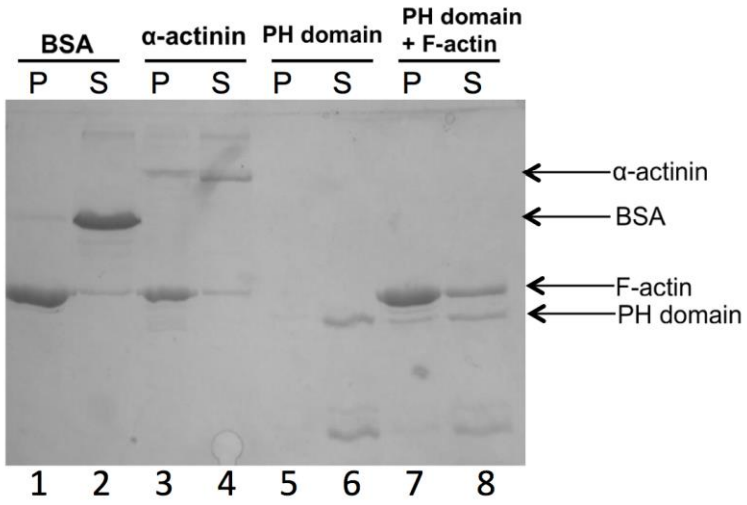


Figure 7: GST-Mid1 PH domain binds F-actin. 3 µM actin monomers were allowed to polymerize for 1.5 hours. 3 µM PH domain, 1µg/µL BSA and 1µg/µL α -actinin were added to different reactions and samples were centrifuged at 150,000 x g for 1.5 hrs. Supernatants from the reactions were removed, the pellets were resuspended, and SDS sample buffer was added to each sample. Samples were resolved on a 15% SDS PAGE gel and stained with Coommassie Blue.

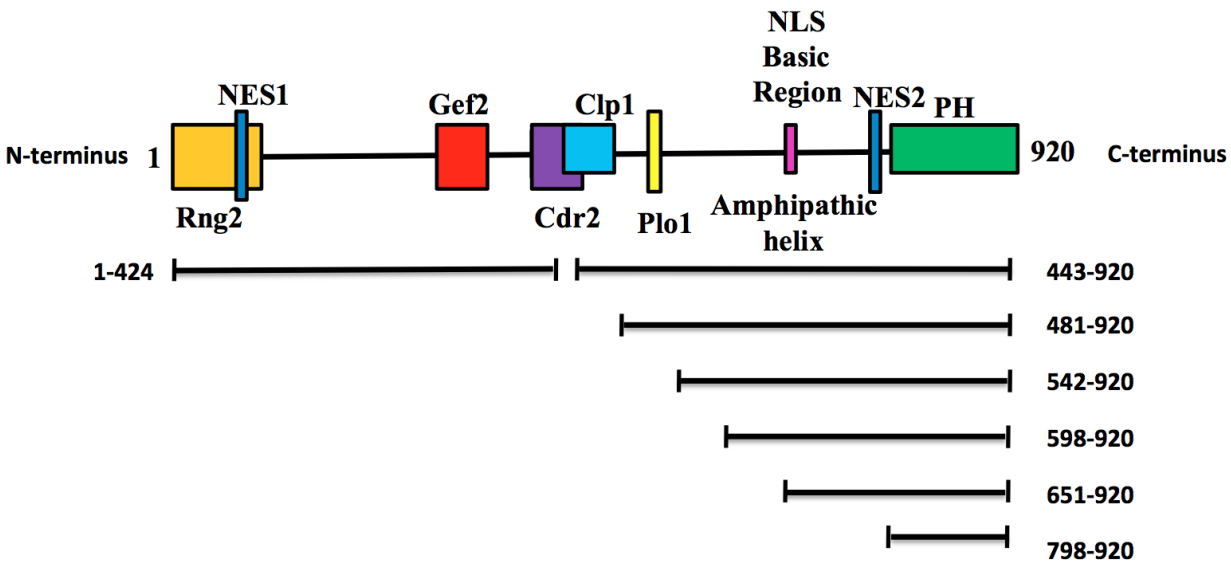
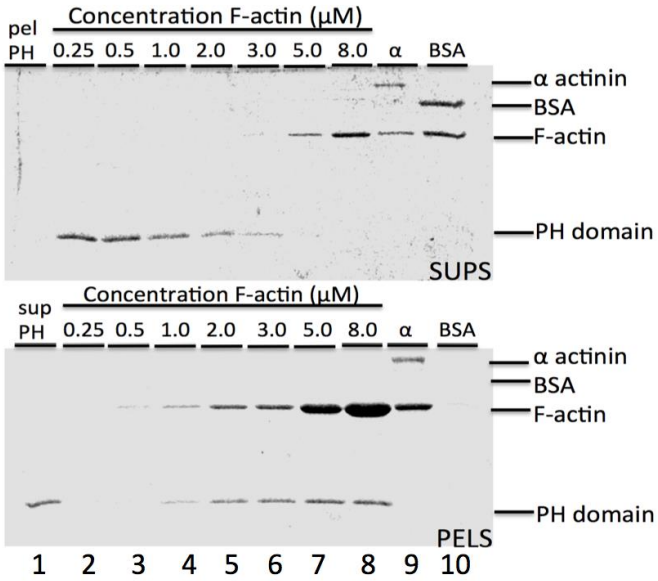


Figure 8: Schematic of Mid1 with cloned fragments. N-terminus (1-424) and C-terminus (443-920) were produced with N-terminal GST tags. 481-920, 542-920, 598-920, and 651-920 were produced with N-terminal 6x His tags. 798-920 (PH domain) fragment was produced with a C-terminal 6x His tag, N-terminal GST tag, and a N-terminal 6x His tag.

Mid1 PH domain binds filamentous actin

Purified Mid1 PH domain with N-terminal 6x His tag was analyzed for actin binding by co-sedimentation with actin filaments at high speed as previously described (Figure 9A). BSA and alpha-actinin were again used as controls to ensure efficient separation of the supernatant from pellet and confirm actin binding ability. BSA does not bind F-actin and therefore was found in the supernatant while alpha-actinin is known to bind F-actin and was found in the pellet (Figure 9A, Lanes 9 and 10). As F-actin concentration increases, the amount of PH domain found in the pellet also increases until all of the PH domain protein is bound. As seen at F-actin concentrations from 3.0 μM to 6.0 μM, the PH domain protein moves from the supernatant to the pellet (Figure 9A, Lanes 6-8). The dissociation constant (K_d) for PH domain binding to F-actin was 1.7 μM (Figure 9B). The K_d was calculated from three independent experiments by finding the concentration of F-actin necessary to bind half of the total PH domain protein in the reaction. To verify the interaction of the PH domain with actin was not a function of the purification or the N-terminal 6x His tag of the PH domain, another Mid1 fragment that was purified in the same conditions was evaluated. Mid1 amino acids 1-100 contain a binding domain for Rng2, an actin bundling protein (Figure 4 and Figure 8, Almonacid et al., 2009). Also, the N-terminus of anillin contains the actin-binding region (Oegema et al., 2000). Because of these features, testing the actin binding ability of Mid1 1-100 provides a sufficient control to assess the specificity of PH domain binding to F-actin. High-speed co-sedimentation assays revealed that 6x His tagged Mid1 1-100 does not bind F-actin (Figure 9C, compare lanes 4 and 6) since it is found in the supernatant with or without F-actin. This data confirms that the PH domain of Mid1 can specifically bind F-actin *in vitro*.

A.)



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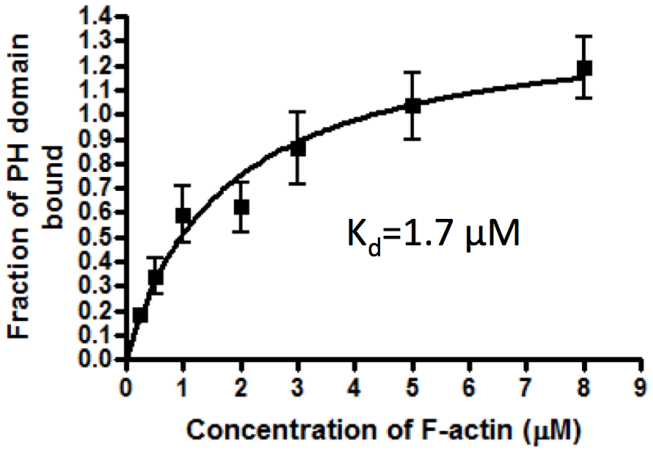


C.)

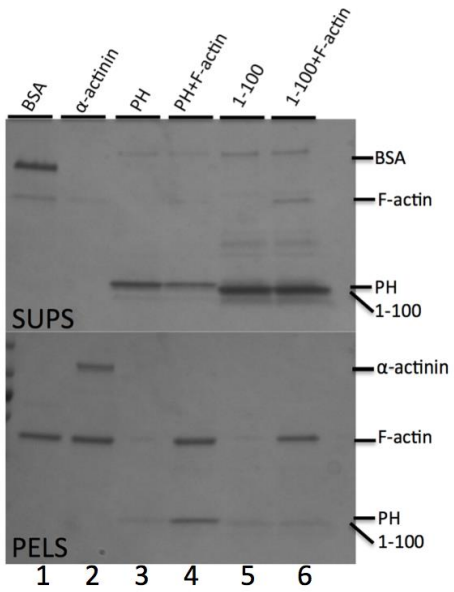


Figure 9: Mid1 PH domain binds filamentous actin. A.) Actin monomers were allowed to polymerize for 1.5 hours. Different concentrations of F-actin were added to 3 μM PH domain and samples were centrifuged at 150,000 x g for 1.5 hrs. Supernatants from the reactions were removed, the pellets were resuspended, and SDS sample buffer was added to each sample. Samples were resolved on a 15% SDS PAGE gel and stained with Coommassie Blue. B.)Dissociation constant (K_d) was found based on the fraction of PH domain bound as actin concentration increases (y=x/Kd + x) and was calculated from an average of three independent experiments. The curve was created from a the average of three independent experiments. Error bars represent standard deviation. C.) High speed co-sedimentation of Mid1 1-100 and PH domain proteins with 3 μM F-actin was performed as described in A.)

Mid1 PH domain inhibits spontaneous assembly of actin filaments

Mid1 recruits proteins to the cytokinetic nodes during mitosis, which is followed by actin nucleation and elongation to form the contractile ring (Padmanabhan et al. 2011, Pollard and Wu, 2010, Wu et al., 2006, Almonacid et al., 2011, Kovar et al., 2003, Lee and Wu 2012, Saha and Pollard, 2012). Since we found that the Mid1 PH domain specifically binds actin filaments, the effect of the PH domain on actin assembly was evaluated. In certain buffer conditions (refer to Materials and Methods), actin monomers will spontaneously assemble nuclei then elongate filaments. Actin monomers can be labeled with pyrene molecules that will fluoresce when incorporated into an actin filament (Kuhn and Pollard, 2005). Purified Mid1 PH domain was added to 2.5 uM actin monomers labeled with 20% pyrene. Actin monomers were spontaneously assembled in the presence of the PH domain. Actin monomers alone showed the most efficient filament assembly (Figure 10, filled in squares). Increasing concentrations of Mid1 PH domain inhibited actin assembly in a dose dependent manner as less fluorescence from actin polymers was detected (Figure 10). Therefore, the Mid1 PH domain is a novel inhibitor of *in vitro* actin monomer assembly into filaments.

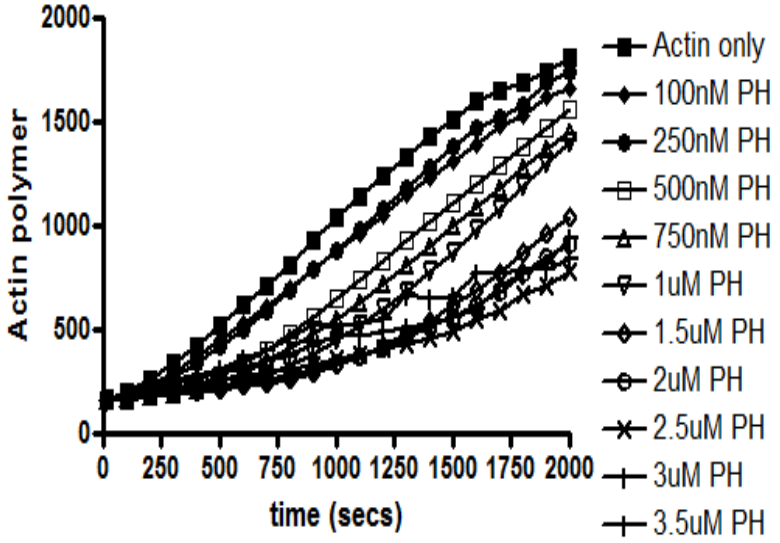


Figure 10: Mid1 PH domain inhibits spontaneous assembly of actin filaments. Mid1 PH domain was incubated with 2.5 uM 20% pyrene labeled actin and fluorescence from spontaneously assembled actin filaments was measured.

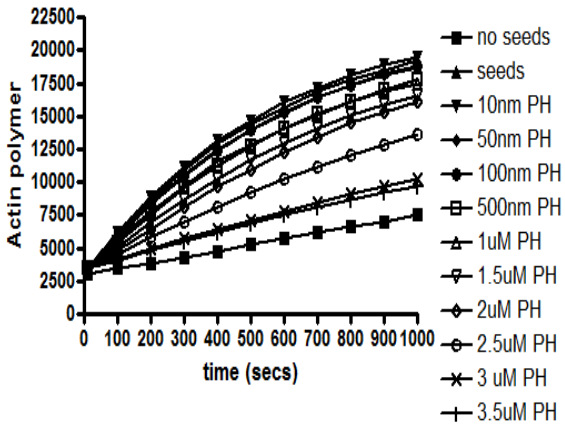
The Mid1 PH domain inhibits elongation of actin filaments in a dose dependent manner

Spontaneous actin assembly assays allow for the evaluation of the effect of the PH domain on nucleation of actin filaments. To determine whether the PH domain affects the elongation of actin filaments, seeded assembly assays were performed. 2.5 uM actin monomers were allowed to form filament seeds for 2.5 hours before addition of 20% labeled pyrene actin and increasing concentrations of PH domain. The reaction containing "no seeds" causes a lag in actin elongation and therefore shows the lowest amount of polymerization (Figure 11A, black squares) while the "seeds alone" reaction shows the highest amount of actin polymer detection (Figure 6A black triangles). As the concentration of PH domain increases, the amount of actin filament elongation decreases in the dose dependent manner (Figure 11A).

Pyrene-actin polymerization assays do not elucidate whether the inhibition caused by the PH domain is due to reduction of actin nuclei being formed or reduction in elongation of actin filaments. To determine whether the Mid1 PH domain is inhibiting actin elongation or actin nucleation, actin filaments were formed in the presence of increasing concentrations of Mid1 PH domain and stained with rhodamine-phalloidin. As the concentration of the PH domain increases, the length of actin filaments decreases (Figure 11B, 12A and 12C). PH domain concentrations of 1.5μM, 2.5μM and 3.5μM caused a noticeable decrease in actin filament length compared to actin alone. Average length of actin filaments without treatment was 11.8 μm ±10.5 μm. The average length of 0.5 μM PH domain treatment was 18.2 μm ±11 μm. Inhibition of actin filament length began with the 1.5 μM treatment with lengths averaging 7.6 μm ±7 μm. The 2.5 μM and 3.5 μM treatment further inhibited actin filament length at 4.1 μm ±3.8 μm and 2 μm ±1.5 μm respectively (Figure 12A). The Jonckheere-Terpstra test was performed to evaluate the differences in the lengths of PH domain treated actin filaments compared to control. Treatment with 0.5 μM PH domain did not significantly change the length of actin filaments ($p=0.99$). However, 1.5 μM, 2.5 μM, and 3.5 μM PH domain significantly decreased the lengths of actin filaments compared to control ($p<0.0001$ for each concentration). 90% of actin control filaments are longer than 3 μm while 3.5 μM PH domain treatment results in only 10% actin filaments longer than 3 μm (Figure 12C). However, a similar number of actin nuclei were detected (Figure 11B, and 12B). Average number of actin filaments without treatment was 40±29. Average number of filaments for the PH domain treated samples were 50±33 for 0.5 μM, 47±18 for 1.5 μM, 56±34 for 2.5 μM, and 27±12 for 3.5 μM. Student's T Tests determined that the number of filaments from all PH domain treated samples were not significantly different from actin control (0.5 μM

p=0.29, 1.5 µM p=0.37, 2.5 µM p=0.12, 3.5 µM p=0.10). This result indicates that the PH domain inhibits elongation of actin filaments but not nucleation of actin monomers. The ability of the PH domain to inhibit actin filament elongation indicates it may have an important role in regulating actin dynamics during filamentation in cytokinesis nodes.

A.)



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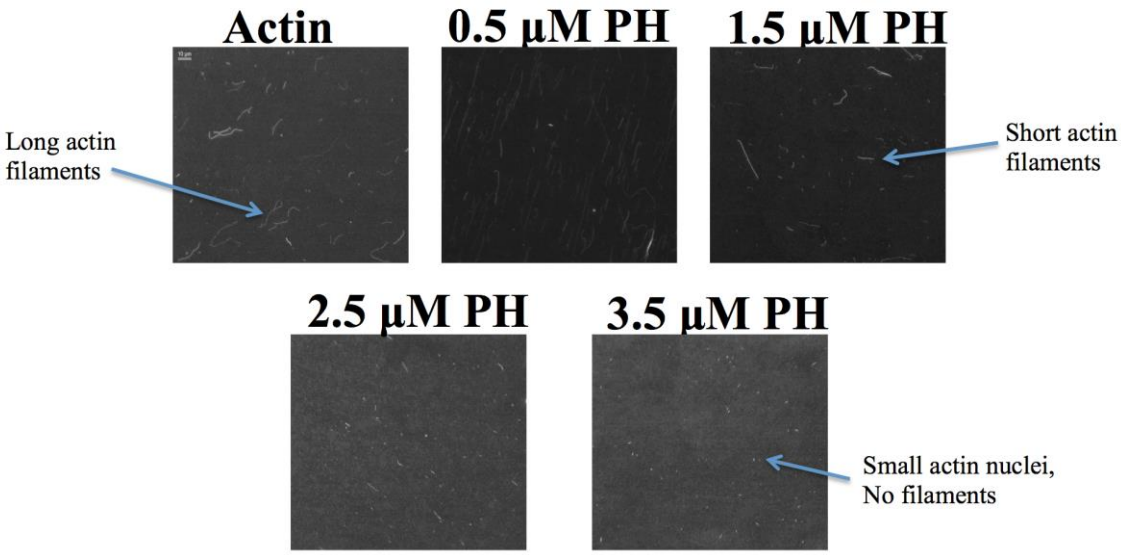


Figure 11: The Mid1 PH domain inhibits elongation of actin filaments in a dose dependent manner. A.) 0.5 µM Mg-ATP actin was preassembled for 2.5 hours followed by addition of different concentrations of Mid1 PH domain and 0.2 µM 20% pyrene actin. Elongation of actin filaments was measured by detection of fluorescence. B.) Different concentrations of Mid1 PH domain were incubated with 2.5 µM of actin for 2.5 hours. Representative image examples from were stained with rhodamine-phalloidin and viewed by fluorescence microscopy. Experiment was repeated 2 times.

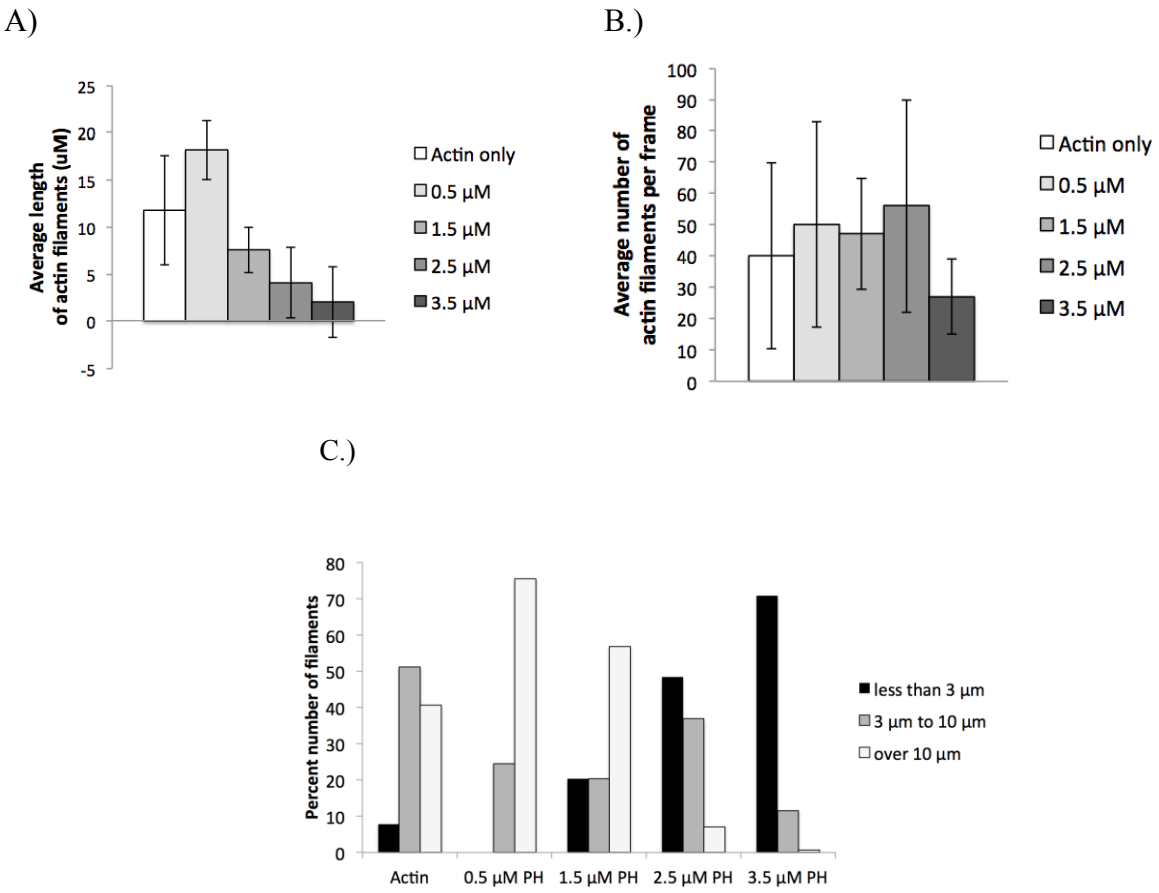


Figure 12: The Mid1 PH domain inhibits elongation but not nucleation of actin filaments. Bar graphs include means of filament lengths (A) and filament numbers (B) per frame. Error bars represent standard deviations (Actin only n=712, 0.5 µM n=958, 1.5 µM n=936, 2.5 µM n=1072, 3.5 µM n=620). The Jonckheere-Terpstra test was performed to determine significant differences between lengths of PH treated actin filaments compared to control. The addition of 0.5 µM PH was not significantly different than actin alone (p=0.99). However, 1.5 µM, 2.5 µM, and 3.5 µM PH domain treatments were significantly different than the actin control ($p<0.0001$ for all three concentrations). Student's T Test was used to calculate the p value for average number of filaments compared to actin control (0.5 µM p=0.29, 1.5 µM p=0.37, 2.5 µM p=0.12, 3.5 µM p=0.10). C.) Bar graph of the number of filaments (percent of total) with filament lengths less than 3 µm, 3 µm to 10 µm, and over 10 µm. About 90% of actin filaments are larger than 3 µm. A decrease in filament length is seen at 2.5 µM PH treatment with about 40% of filaments larger than 3 µm. The 3.5 µM PH domain treatment results in only about 10% of filaments longer than 3 µm.

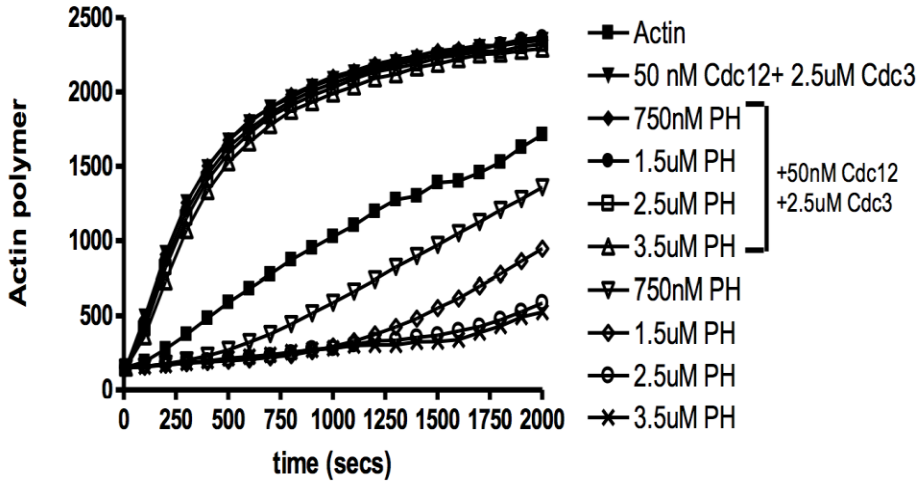
Inhibition of spontaneous actin assembly by the PH domain can be alleviated by Cdc12-Cdc3 (formin-profilin).

The formin Cdc12 is essential for the formation of the actin contractile ring during cell division in fission yeast (Chang et al., 1997). Mid1 arrives at the medial plane of the cell 60 seconds prior to recruitment of other contractile ring proteins including Cdc12 and the profilin, Cdc3 (Wu et al., 2003) Cdc12 efficiently nucleates and elongates actin filaments that are eventually bundled into the contractile ring. Cdc3 binds and inhibits actin monomers from forming nuclei in the absence of Cdc12 (Kovar et al., 2003). However, Cdc12 elongates actin filaments most efficiently when actin monomers are bound by Cdc3 (Kovar et al 2003). Since the Mid1 PH domain binds and inhibits actin, it may prevent spontaneous actin elongation at the medial plane before Cdc12 arrives. To determine whether Cdc12 can overcome inhibition of actin filamentation by the PH domain, increasing concentrations of Mid1 PH domain were added to spontaneous actin assembly reactions with and without Cdc12 and Cdc3. Consistent with previous experiments, as the concentration of the PH domain increased, detection of assembled actin filaments decreased (Figure 13A, All PH domain concentration curves are below actin alone represented by black squares). However, when Cdc12 and Cdc3 were added to reactions including the PH domain, filamentation was restored to levels equivalent to Cdc12 and Cdc3 without the PH domain (Figure 13A all PH domain concentrations in combination with Cdc12 and Cdc3 have same actin polymerization dynamics as Cdc12 and Cdc3 alone).

The spontaneous assembly of actin filaments was also evaluated with increasing concentrations of Cdc12 in the presence of 2.5 μM PH domain and 2.5 μM Cdc3. As expected, the addition of Cdc3 alone inhibits actin filament formation as does addition of the

PH domain alone. When Cdc3 and PH domain are added in combination, the inhibition of actin filamentation is additive and there is virtually no actin filament formation (Figure 13B, bottom three curves). But, as Cdc12 concentration increases in the presence of the PH domain and Cdc3, the efficiency of actin filament formation also increases in a dose dependent manner (Figure 13B). The additive effect of Cdc3 and PH domain inhibition may indicate that the two proteins are acting to inhibit actin filaments by different mechanisms and they may collaborate to simultaneously inhibit actin filaments from forming until the formin is available for rapid filament elongation. Although the PH domain and Cdc3 can completely abolish actin filamentation, this result can be reversed by the addition of the formin Cdc12. This allows proficient filament formation and eventually the inhibitory effect of the PH domain and Cdc3 is completely overcome. The inhibitory activity of the PH domain may indicate its participation in regulating actin contractile ring formation. Cooperation and competition of the PH domain with other proteins such as Cdc12 and Cdc3 may reveal important mechanisms for proper timing of actin organization in the contractile ring.

A.)



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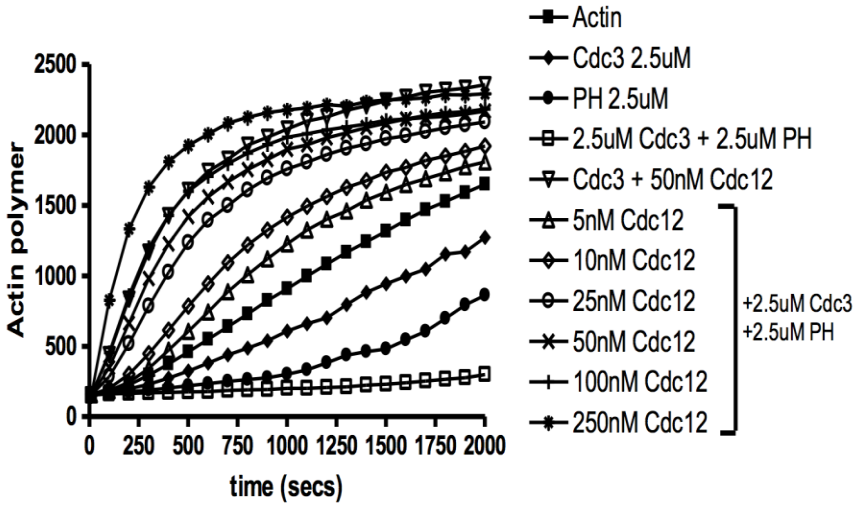


Figure 13: Inhibition of spontaneous actin assembly by the PH domain can be alleviated by Cdc12-Cdc3. A.) Various concentrations of Mid1 PH domain were incubated with 50 nM Cdc12 (formin), 2.5 μM Cdc3 (profilin), and 2.5 μM 20% pyrene labeled actin and fluorescence from spontaneously assembled actin filaments was measured. B.) 2.5 μM PH domain and 2.5 μM Cdc3 were incubated with various concentrations of Cdc12 and 2.5 μM 20% pyrene labeled actin. Spontaneous actin filamentation was measured by fluorescence.

The Mid1 PH domain bundles actin filaments

Since the Mid1 PH domain can bind actin and reduce its ability to form and elongate filaments, it is interesting to suggest that the PH domain may have other actin regulating properties. To test whether the Mid1 PH domain can bundle actin filaments, low speed cosedimentation assays were performed. When actin filaments are centrifuged at low speed, most of the filaments remain soluble in the supernatant. Once actin filaments have been bundled by addition of other proteins, centrifugation at low speed will cause the actin to pellet. As the concentration of the PH domain increased, more F-actin was pelleted until total actin filaments were bundled in the pellet (Figure 14A, from 1.0 μM to 5.0 μM PH domain, F-actin bands move completely into the pellet fraction). The known actin bundling protein alpha-actinin shows total actin filaments bundled in the pellet. BSA negative control shows actin in both supernatant and pellet, similar to that of actin alone. Surprisingly, not only does the PH domain bind actin and inhibit nucleation and elongation, it also has actin bundling function *in vitro*.

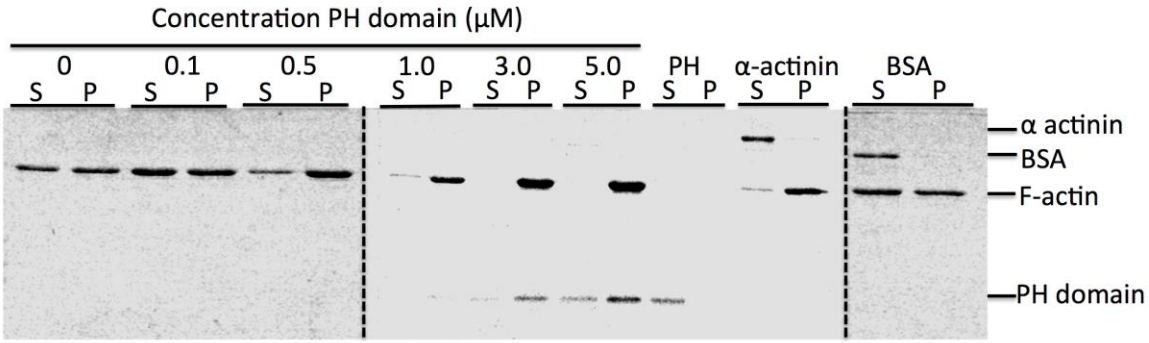
To determine the actin bundling efficiency of the PH domain, densities of F-actin bands from low speed co-sedimentation assays were used to determine the percent F-actin bundled as the concentration of the PH domain increased. The bundling efficiency of the PH domain was determined by finding the concentration of PH domain necessary to bundle half the maximum bundling of F-actin. Average values of three independent experiments were graphed with error bars representing standard deviation (Figure 14B). The bundling efficiency of the PH domain was determined to be 0.23 μM with a standard deviation of 0.11 μM calculated from the three independent experiments. The PH domain has bundling efficiency comparable to that of Rng2 (0.2 μM), which binds Mid1 and is recruited to the

contractile ring (Takaine et al., 2003). Although similar to Rng2, Mid1 has a much lower efficiency than Fim1 (0.04 μM), which is also involved in contractile ring condensation (Takaine et al., 2003, Wu et al., 2001). Because there are more than one bundling protein localized to the cytokinesis nodes, differences in bundling capability may indicate cooperation of these proteins to bundle the contractile ring.

To view the bundling activity of the Mid1 PH domain, actin filaments were allowed to form before addition of different concentrations of Mid1 PH domain. Filaments were stained with rhodamine-phalloidin and imaged by fluorescence microscopy (Figure 15). Representative images are indicated in Figure 10. At concentrations of 1.5 μM to 3.5 μM PH domain, actin filament bundles can be seen. Bundled filaments cen be identified by the presence of more than one parallel filament in close proximity and incorporation of individual filaments into a single actin structure (Figure 15, 2.5 μM and 3.5 μM zoomed in images).

Given that actin filaments must be bundled and condensed during contractile ring maturation in fission yeast (Wu et al., 2003), it is possible that the Mid1 PH domain may aid in bundling actin filaments in the contractile ring during mitosis. Further studies suggested in the Discussion section will clarify the importance of the actin bundling function of the PH domain *in vivo*.

A.)



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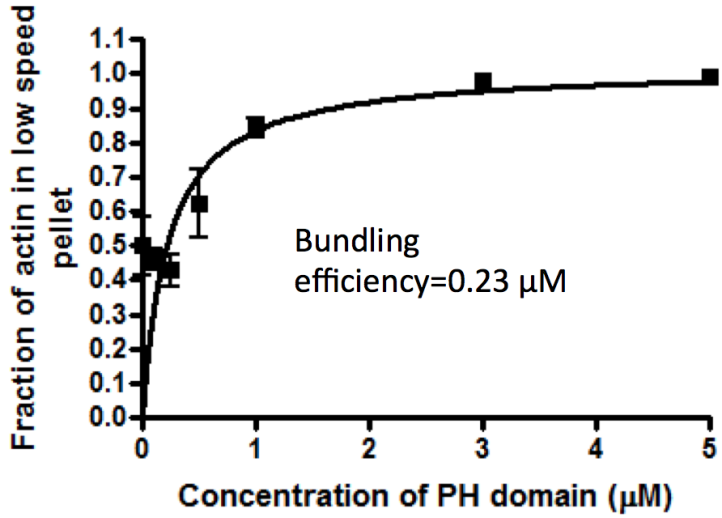

Figure 14: The Mid1 PH domain bundles actin filaments. A.) 3 μM of actin monomers were assembled into filaments for 1 hour. Increasing concentrations of Mid1 PH domain were added to the actin filaments and samples were centrifuged at 10,000 x g. Supernatants (S) were removed, pellets (P) were resuspended, and all samples were run on a 15% SDS gel. Gels were stained with Coommassie Blue. B.) The fraction of actin bundled in the pellet across concentrations of PH domain was used to create the bundling efficiency curve (y=x/Kd + x). Data set is an average of 3 independent experiments and error bars represent standard deviations.

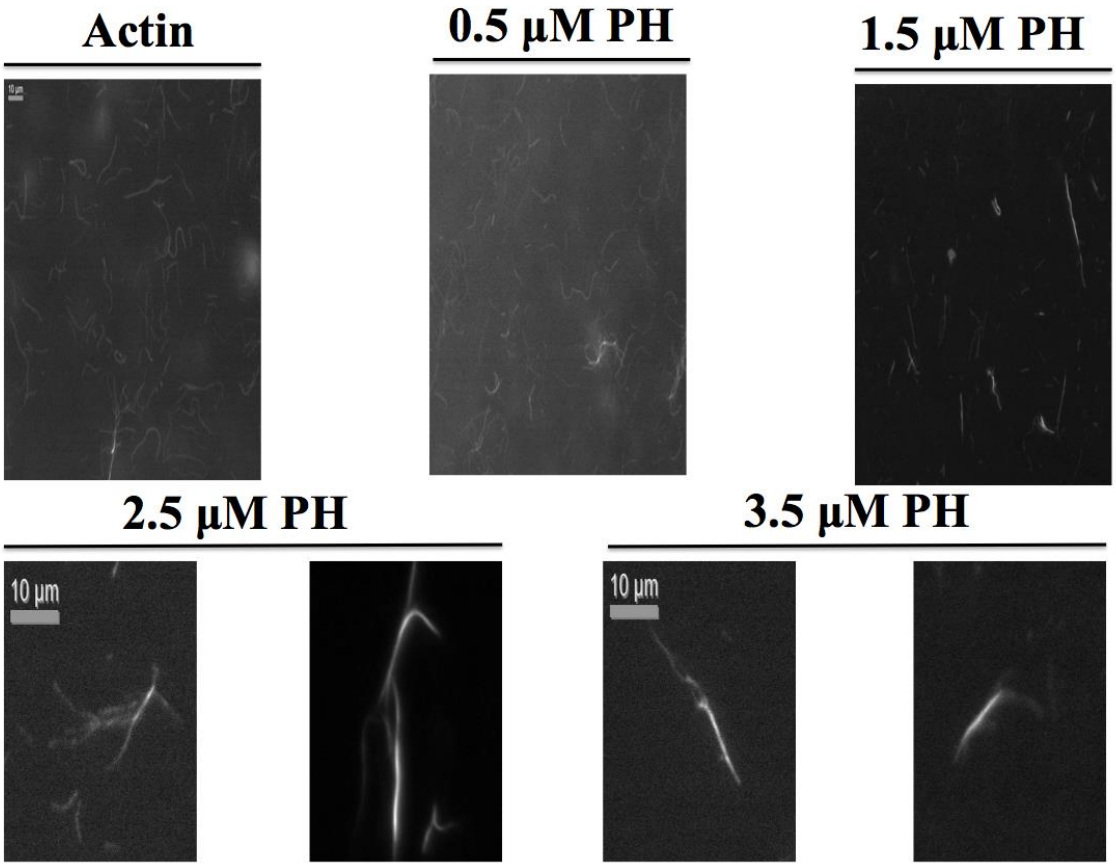

Figure 15: The Mid1 PH domain bundles actin filaments. Actin monomers were allowed to polymerize for 2.5 hours. Increasing concentrations of PH domain were added and filaments were immediately stained with rhodamine-phalloidin and imaged for fluorescence. Representative images are shown.

Interaction of Rho1 and Mid1

Because Mid1 can bind and bundle F-actin, other actin regulating proteins found at the contractile ring are of great interest. Anillin analogues in metazoans also bind and bundle F-actin (Oegema et al., 2000). Actin filament formation in these cells is regulated by the RhoA GTPase that also physically binds anillin. Because of these functional similarities, the interaction of Mid1 and Rho1 was investigated. To identify if Rho1 and Mid1 physically interact *in vivo*, a *rho1-GFP mid1-myc S. pombe* strain was created (Refer to Yeast strain section of Materials and Methods). Co-immunoprecipitation was performed with these cells to determine whether Rho1 and Mid1 bind. A western blot of the co-immunoprecipitation indicated that Mid1-myc was efficiently immunoprecipitated from yeast cell lysates, but when these immunoprecipitates were blotted with a GFP antibody, Rho1 was not detected (Figure 16). GFP tag integration into the Rho1 caused non-specific localization of Rho1 because it did not localize to growing cell tips and septa as seen in previous publications (Figure 17). Failure of localization could be due to failed incorporation of the GFP tag into the endogenous *rho1* site, or the presence of the tag causes Rho1 to lose some functions. The co-immunoprecipitation blots do not indicate whether Rho1-GFP was not detected because of inaccurate incorporation of the GFP tag or because Rho1-GFP does not associate with Mid1-myc. To determine localization changes in Rho1 protein in the absence of *mid1*, *rho1-GFP mid1Δ* strain was created. Determination of GFP tag incorporation into the *rho1* endogenous locus is currently being investigated by Western blot for Rho-GFP in denatured lysates of the *rho1-GFP* strain and *rho1-GFP mid1Δ* strain.

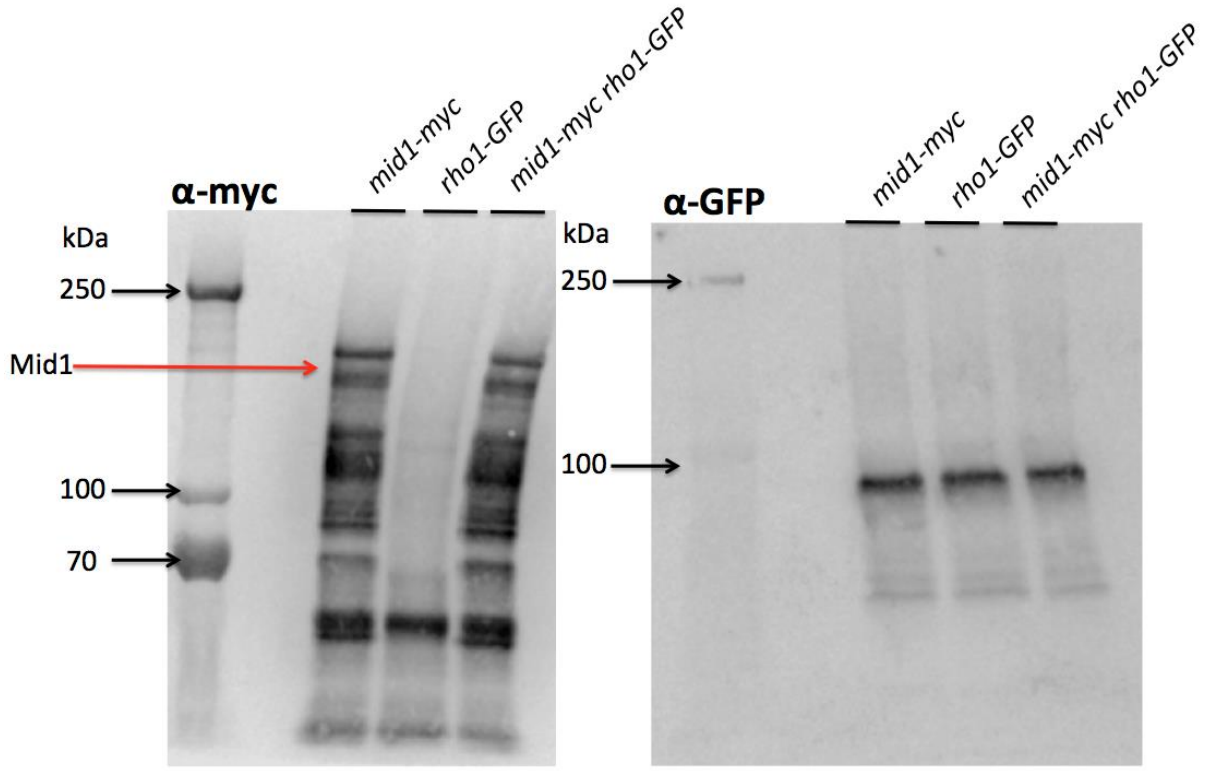

Figure 16: Co-immunoprecipitation of Mid1-myc with Rho1-GFP. Yeast strains 0052 *rho1-GFP:KanR ade6-M210 ura4-D18 leu1-32, h-*, 3136 *mid1-myc:ura4 ade6-M210 ura4-D18 leu1-32 h+, and* 0053 *rho1-GFP:KanR mid1-myc:ura4 ade6-M210, ura4-D18, leu1-32, h-*, were grown and lysed with glass beads. Lysates were incubated with Dynabeads coated with anti-myc antibody. Immunoprecipitates were resolved on Tris acetate gel, transferred onto a polyvinylidene difluoride (PVDF) membrane, blotted with myc antibody, and detected with goat anti-mouse IRDye-800W secondary antibody. Immunoprecipitates were also resolved on a 4-20% Tris glycine gel, transferred onto a PVDF membrane, blotted with GFP antibody, and detected with goat anti-mouse IRDye-800W secondary antibody. Membranes were viewed on an Odyssey Fc imager.

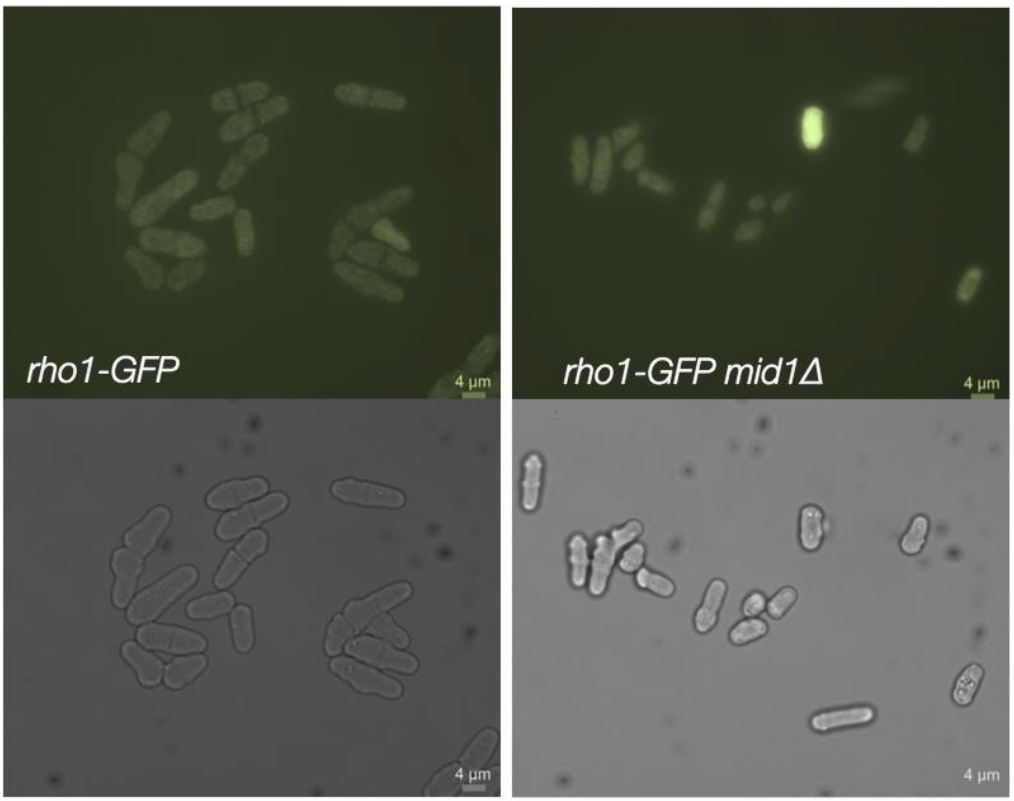

Figure 17: Fluorescence microscopy of Rho1-GFP yeast strain. Cells from strain 0052 *rho1-GFP* were methanol fixed and viewed by fluorescence microscopy. Dispersed localization of Rho1-GFP protein may indicate the incorrect insertion of the GFP tag into the endogenous *rho1* site, or the GFP tag incorporation affects the function of Rho1 in these cells.

To determine whether Mid1 and Rho1 interact *in vitro*, Rho1 cDNA was cloned into the pGEX vector resulting in its purification with a N-terminal GST tag (Refer to Plasmid section of Materials and Methods). GST-Rho1 will be combined with glutathione-Sepharose beads (binds GST) and incubated with native lysates from *mid1-myc* yeast strain (Strain 3136, Table 2). Low speed centrifugation will be used to pellet beads bound by GST-Rho1. Proteins that bind GST-Rho1 will be found in the pellet and unbound protein will remain in the supernatant. Proteins will be separated by SDS PAGE and the presence of Mid1-myc with GST-Rho1 will be compared to GST-Rho1 with myc alone to determine specific binding *in vitro*. If the Rho1GTPase-Mid1 interaction is dependent on the GTPase being

bound by GTP or GDP, Mid1 binding will not be seen in the assays with bacterially purified Rho1.This potential problem could be avoided by mutating the active site of Rho1 allowing it to be constitutively active (permanently bound to GTP) or unable to bind GTP (permanently bound to GDP). Mapping of the Rho1 GTP binding and hydrolysis domains and their consequent mutation phenotypes have been demonstrated in fission yeast by Nakano et al.,1997. These plasmids can be obtained and manipulated to incorporate His or TAP sequence tags and produced Rho1 mutant proteins will be collected by affinity purification. These tagged Rho1 mutant proteins will be combined with *mid1-myc* lysates and analyzed for specific binding by glutathione-Sepharose bead separation and SDS PAGE. Evidence of *in vitro* binding of Mid1 with Rho1 will determine whether Mid1 and Rho1 can directly bind and whether the interaction is independent of other proteins.

Interaction of Rho1 with Mid1 may provide a new mechanism for the regulation of actin formation in the contractile ring in fission yeast. It has been proposed that human anillin binds both RhoA, F-actin, and myosin II to scaffold the proteins in close proximity during contractile ring assembly (Oegema et al., 2000) This close association will allow RhoA to activate important actin polymerizing proteins and the myosin motors. Since Mid1 is also known as a scaffold protein important for organizing contractile ring components at cytokinesis nodes, it is possible that interaction of Mid1 and Rho1 serves a similar function. Evidence for the interaction and importance of Rho1 and Mid1 association in fission yeast will uncover another functional similarity between Mid1 and metazoan anillins that exceeds sequence homology.

Chapter IV: Discussion

Mid1 PH domain functions

The discovery that the Mid1 PH domain can bind filamentous actin has many different implications for the regulation of contractile ring formation and cytokinesis in fission yeast. At the onset of mitosis, Mid1 translocates from the nucleus and acts as a scaffold for other proteins at the cell cortex (Celton-Morizur et al., 2004). Mid1 localization to the medial plane and attachment to the cell membrane has been attributed to multiple different factors. The amphipathic helix of Mid1 integrates into the cell membrane and is necessary for its stabilization (Celton-Morizur et al., 2004) Mid1 localization is also connected to cues from polarity proteins that coincide with the cell cycle. The polarity protein Pom1 is distributed in a gradient, concentrated at the cell tips (Celton-Morizur et al., 2006, Moseley et al., 2009). Pom1 targets Cdr2 kinase to the cell cortex, which binds to Mid1 and facilitates localization to the cell membrane (Morrell et al., 2004, Almonacid et al., 2009). Cdr2 attaches to the cell membrane and provides a second anchor for Mid1 (Almonacid et al., 2009). The nuclear export of Mid1 also regulates division site placement since Mid1 localizes at the equator of the nucleus when it is artificially displaced (Paoletti and Chang, 2000, Almonacid et al., 2009). This consistent localization is independent of Cdr2 influence (Almonacid et al., 2009). Although most reports determine the Mid1 PH domain to be unnecessary for overall Mid1 function, some interesting data suggests that it may also be important for localizing and stabilizing Mid1 at the cell cortex. In the absence of Cdr2, cortical localization of fluorescent Mid1 was reduced to about 35% of Mid1 detected at the cell cortex in wild type cells (Lee and Wu, 2012). Removal of the Mid1 PH domain in the Cdr2 deleted background, further reduced detection of Mid1 at the cell cortex worsens to

about 5% of wild type (Lee and Wu, 2012). This result suggests that the PH domain is important for Mid1 cortical localization and has overlapping function to that of Cdr2. FRAP data from the same study show that Mid1 protein missing the PH domain has a significantly reduced half-life at the cell cortex (2 fold decrease compared to full-length Mid1) suggesting that the PH domain is also important for stabilizing Mid1 at the cell cortex (Lee and Wu, 2012). *In vitro* lipid binding assays show that the PH domain has a weak affinity for select lipids found in the cell membrane (Lee and Wu, 2012).

Results presented in this thesis suggest that the interaction of the PH domain with F-actin provides another mechanism for localizing and stabilizing Mid1 at the cell cortex (Figure 18). The interactions of the human and *Drosophila* analogues of Mid1, anillin provide further evidence to support this idea. Although anillin and actin are recruited to the medial plane of these cells independently, F-actin depletion causes broad spatial localization of anillin at the medial plane and the timing for total recruitment of anillin to the cell cortex is reduced. (Oegema et al., 2000). Similarly, F-actin depletion by LatA treatment in fission yeast causes more dispersed localization of Mid1 at the cell cortex instead of in a tight ring at the cell equator (Wu et al., 2003). Taken together, the ability of the Mid1 PH domain to bind actin could ensure the anchoring of Mid1 and the stability of Mid1 at the medial cortex of the cell much like the interaction of anillin with actin in humans and *Drosophila*.

Mid1 regulation of actin filament elongation

The ability of the Mid1 PH domain to inhibit actin filament elongation is a role that is different from the function of the anillins since there is no evidence that anillins inhibit actin elongation. However, this divergent function of Mid1 and anillin can be attributed to the

differences in contractile ring assembly in yeast versus animal cells. The cell division site in human cells is determined by location of microtubules of the mitotic spindle, while nuclear position and the Mid1 cytokinetic node platform determine the position of the contractile ring in fission yeast (Sohrmann et al., 1996, Chang et al., 1996, Almonacid et al., 2009). Mid1 begins to translocate to the cell cortex about an hour before the onset of mitosis (Wu et al., 2003). During the G2/M transition, other cytokinesis node proteins are recruited. Two minutes after the beginning of spindle pole body separation, the formin Cdc12 localizes to the cytokinesis nodes (Wu et al., 2003). From the arrival of Cdc12 to 10 minutes post spindle pole body separation, actin filaments are polymerized from the nodes (Wu et al., 2003). Because Mid1 arrives at the medial plane much earlier than other cytokinesis proteins, it may be important for it to inhibit spontaneous actin filament elongation at the center of the cell before the cytokinesis nodes are completely assembled (Figure 18). Although Mid1 PH domain can inhibit F-actin elongation, addition of Cdc12 and Cdc3 can alleviate the inhibition and polymerize F-actin with optimal efficiency. Following this hypothesis, recruitment of Cdc12 to the cytokinesis nodes allows for actin polymerization and elongation to proceed for contractile ring assembly.

There are other examples of temporal regulation of the actin cytoskeleton by sequential recruitment of actin binding proteins in fission yeast. After the contractile ring assembles, the capping protein Acp2 arrives to the site (Wu et al., 2003). Acp2 binds to barbed ends of actin filaments and prevents them from being elongated by Cdc12 (Kovar et al., 2005). This association seems to prepare the filaments in the contractile ring to be disassembled during the constriction of the contractile ring since Acp2 deletions cause delays in actin depolymerization and septation (Kovar et al., 2005). Deletion of the Mid1 PH

domain does not cause early polymerization of actin filaments at the medial plane (Paoletti and Chang, 2000). Cdc12 is necessary to assemble filaments in the contractile ring so inhibition of actin formation early in mitosis will be undetectable (Kovar et al., 2003). Therefore, evidence that the inhibition of actin dynamics by the PH domain is important for timing of contractile ring assembly will be difficult to study *in vivo*. The interruption of actin assembly by LatA provides a background in which overexpression of the Mid1 PH domain may cause an extended delay of actin filament assembly into the contractile ring. Earlier assembly of the contractile ring when the PH domain is deleted in the presence of LatA would also suggest a function for the PH domain in temporal regulation of contractile ring assembly. Further exploration will be necessary to determine the function of the Mid1 PH domain inhibition of actin elongation *in vivo*.

Mid1 actin bundling activity

Not only does the Mid1 PH domain bind and inhibit actin elongation, it can bundle filamentous actin. This result establishes another functional similarity between Mid1 and metazoan anillins, which contain canonical actin bundling domains (Oegema et al., 2000). Although the contribution of the bundling capability of anillins to the contractile ring in human and *Drosophila* has not been established, this conserved function of anillins in different species suggests its importance. In fission yeast, the crosslinking and bundling proteins Ain1 and Fim1 contribute to contractile ring maturation (Wu et al., 2001) Both Ain1and Fim1 localize to the contractile ring in an actin-dependent manner (Wu et al., 2001). However, deletion of either Ain1 or Fim1 does not affect cell morphology or actin contractile ring assembly (Wu et al., 2001). Under high salt and low temperature stress conditions, Ain1 deletion prevents actin contractile rings from forming. In the same conditions, Fim1 deletion

prevents actin redistribution from patches at the tips of the cell to the contractile ring during its formation (Wu et al., 2001). Deletion of Ain1 and Fim1 together was lethal to the cells indicating their important roles in regulating actin dynamics (Wu et al., 2001). Since deletion of either bundling protein does not abolish actin contractile ring maturation, another protein could be involved in this process or fulfill the role of actin bundling in their absence. Mid1 is present throughout actin contractile ring maturation and leaves the ring just prior to its constriction (Wu et al., 2003). The bundling activity of the PH domain may cooperate with other actin modifying proteins to develop the complete actin contractile ring (Figure 18). This idea may explain why removal of the PH domain or deletion of either actin bundling protein does not damage the contractile ring individually. To test this hypothesis, yeast strains with *ain1* deleted and *fim1* deleted will be crossed with a strain containing Mid1 lacking the PH domain. Double mutations may reveal the requirement of more than one bundling protein for actin organization and maturation within the contractile ring. This will elucidate the importance of Mid1 PH domain bundling activity *in vivo*.

Roles for Mid1 and Rho1 in actin cytoskeleton

The prospective interaction of Mid1 and Rho1 would mirror similar interactions of human anillin with RhoA during mitosis. Similar to Rho1, RhoA localizes to the medial plane of the cell during contractile ring formation (Arellano et al., 1997, Piekney et al., 2008). In this way, both RhoA and Rho1 proteins co-localize with anillin and Mid1 at the contractile ring in their respective cells (Arellano et al., 1997, Piekney et al., 2008). The human anillin C-terminus can physically bind RhoA in human cells (Piekney et al., 2008). RhoA binding is necessary for anillin localization to the cell cortex while anillin is important for the stability of RhoA at the cell cortex (Piekney et al., 2008). RhoA is necessary for

activating formins and myosin to assemble the contractile ring in human cells (Piekney et al., 2005). Anillin has an actin binding domain, a myosin binding domain, and is required for optimal myosin activation at the cell equator (Oegema et al., 2000, Piekney et al., 2009) These data suggests that anillin functions to scaffold RhoA in close proximity to myosin and actin to activate them and promote contractile ring assembly (Piekney et al., 2009).

The Cdc12 formin in fission yeast is also necessary for actin polymerization at the contractile ring but an activating protein has not yet been identified (Kovar et al., 2003). It is possible that Rho1 has a function in contractile ring assembly by interacting with Mid1 at the cytokinesis nodes. Similar to anillin, Mid1 is important for recruiting myosin to the cytokinesis nodes where Cdc12 also accumulates (Wu et al., 2003). Therefore, the interaction of Rho1 and Mid1 may also be important to scaffold Rho1 to the cytokinesis nodes for activation of proteins that assemble the contractile ring (Figure 18). To test this hypothesis, the physical interaction of Rho1 and Mid1 must be confirmed *in vitro* and *in vivo*. Preliminary results identified Rho1 in complex with Mid1 purified from mitotic yeast extracts. Co-immunoprecipitation studies will be performed to validate whether Rho1 and Mid1 bind *in vivo*. If confirmed, Rho1 and Mid1 binding will be evaluated *in vitro* to determine whether the interaction is direct. Since Mid1 does not contain a canonical Rho1 binding site, it may be that Rho1 and Mid1 interact through a complex. Other *in vivo* studies will provide evidence that Mid1 and Rho1 interaction has important function for contractile ring assembly. The effect of Mid1 deletion on Rho1 localization will indicate whether Rho1 recruitment to the contractile ring is dependent on Mid1. Similarly, analysis of the localization of Mid1 when Rho1 is depleted may also gain insight into the function of the interaction. Further study of the possible interaction of Mid1 and Rho1 may provide another

evolutionary link between the functions of Mid1 and human anillin during contractile ring assembly. Exploration of Mid1 and Rho1 interaction may reveal the role of Rho1 in activation of contractile ring proteins in fission yeast.

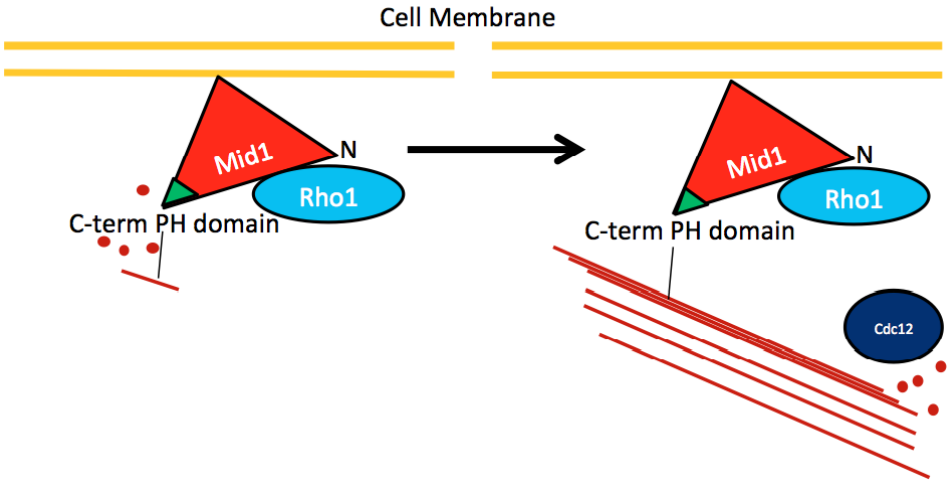

Figure 18: Model for the role of Mid1 in actin filament assembly in the contractile ring. Mid1 is a scaffold protein that recruits Rho1 to cytokinesis nodes. Mid1 PH domain binds and inhibits actin filaments to enhance Mid1 stability at the membrane and regulate the timing of contractile ring assembly. Once the formin Cdc12 arrives at the nodes, it overcomes the inhibition caused by the PH domain and allows for efficient actin filament elongation. Mid1 PH domain cooperates to bundle actin filaments in the contractile ring.

Broader implications of understanding Mid1 interactions

The regulation and formation of actin filaments during mitosis is extremely important for the fate of the cell and its progeny. Dysregulation of F-actin polymerization is a feature in many different cancers. In humans, anillin is upregulated in many different tumor cells (Hall et al., 2005) and Rho proteins are also implicated in different cancer types (Ridley 2004). Gene expression profiling of non-small cell lung cancers revealed an upregulation of anillin, which interacts with RhoA to enhance the migration of these cancer cells through actin regulation (Suzuki et al., 2005). The impact of anillin, RhoA, and actin on cancer cell types alludes to their important roles in cell growth and development. Actin cytoskeletal

rearrangement is essential for cell growth, division, migration, and responses to stimuli (Alberts et al., 2008). RhoA and anillin are both tightly involved in cytoskeletal organization. The study of the direct interactions of Mid1 with F-actin and the actin regulatory protein Rho1GTPase could uncover other conserved functions of Mid1 and anillin, which can lead to clarification of the regulation of these proteins during development. In addition, the effect of Mid1 interactions with Rho1GTPase and F-actin can lead to possible mechanisms or targets for loss of regulation in different cancer types.

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