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ANGIKA BASANT

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Abstract

In metazoans, cytokinesis is triggered by activation of the GTPase RhoA at the equatorial plasma membrane. ECT-2, the guanine nucleotide exchange factor (GEF) required for RhoA activation, is activated by the centralspindlin complex that concentrates on spindle midzone microtubules. However, these microtubules and the plasma membrane are not generally in apposition, and thus the mechanism by which RhoA is activated at the cell equator remains unknown. Our data provide a molecular mechanism for RhoA activation by the centralspindlin complex at the equatorial cortex. We demonstrate that centralspindlin localizes to this sub-cellular site by virtue of its ability to oligomerize and to directly interact with the plasma membrane. The Chromosome Passenger Complex subunit Aurora B promotes cortical RhoA activation by antagonizing the inhibitor of centralspindlin oligomerization, PAR-5/14-3-3. Our study places the CPC as a direct activator of centralspindlin in the pathway leading to contractile ring assembly and find that this function is conserved in both *C. elegans* and human cells. We have designed optogenetic probes to spatiotemporally control Aurora B kinase and test whether its activity is sufficient to generate RhoA-directed contractility via centralspindlin oligomers. We also find that the localization of centralspindlin on the membrane depends on active RhoA and myosin levels on the cortex. The former is governed by a direct interaction between CYK-4 and RhoA. The nature of the interaction between myosin and centralspindlin is yet to be determined.

Chapter 1: Introduction

Based on the original discoveries of Robert Remak, in 1855 Rudolf Virchow popularized the phrase *Omnis cellula e cellula,* promoting the idea that all cells come from pre-existing cells. This rejected the prevailing notion that living cells are spontaneously generated from non-living matter. Some years later in the 1870s, Walther Flemming extensively investigated various fixed tissues and described his observations of dividing animal cells with detailed illustrations of changes in cell shape and in particular, morphology of the chromatin during mitosis (Paweletz 2000). His pioneering work laid the foundation for research in cell division, the complex underlying mechanisms of which continue to intrigue biologists today.

In animal cells, segregation of chromosomes during anaphase is followed by the formation of a cleavage furrow in the cell equator that splits a single cell into two daughter cells. This process is known as cytokinesis. The position of the furrow is tightly spatially and temporally controlled to ensure that new-born cells receive a full complement of cellular material. During asymmetric division, the position of the division plane permits unequal inheritance of cell fate determinants (Griffin 2015; Connell et al. 2011). In cells dividing within intact tissues (Guillot and Lecuit 2013; Founounou, Loyer, and Le Borgne 2013) or in migrating epithelial cells (Campinho et al. 2013), the position of the cleavage furrow has a bearing on tissue integrity, and is influenced by polarity proteins and extrinsic cues such as inter-cellular tension. These and several other context-dependent factors can impact the position of the division plane, but a core mechanism for cytokinesis is largely conserved among metazoans. The cytokinetic

furrow is driven by a contractile ring comprised of dynamic actin and myosin filaments that localize on the equatorial cell cortex and fuel ingression of the plasma membrane to generate a new cell boundary (Schroeder 1973; Schroeder 1968). Successful cytokinesis requires precise regulation of contractile ring assembly in space and time.

1.1 Positioning the division plane in animal cells: role of the mitotic spindle

The mitotic spindle is a microtubule-based, temporary structure that assembles during metaphase and drives segregation of chromosomes in anaphase. Three types of microtubules originate from centrosomes (or spindle poles) of bipolar spindles. Kinetochore fibers reach towards the center of the cell with their plus ends contacting proteins at the centromeres of chromosomes. At the poles of a dividing cell, astral microtubules emanate from the centrosomes and their plus ends interact with the cortex (Figure 1). And finally, inter-polar microtubules overlap at the cell center, while their minus ends are focused at the poles. During anaphase as chromosomes are pulled polewards, the spindle rearranges dramatically to generate a high concentration of interdigitated, anti-parallel microtubules between segregating chromosomes. This structure, known as the central spindle or the spindle midzone, was identified and illustrated by Josef Spek in 1916 (Spek 1918)(Figure 1). Minus-ends of the central spindle microtubules lose interactions with the spindle poles later in anaphase and are splayed, unlike kinetochore fibers that are clustered to a point.

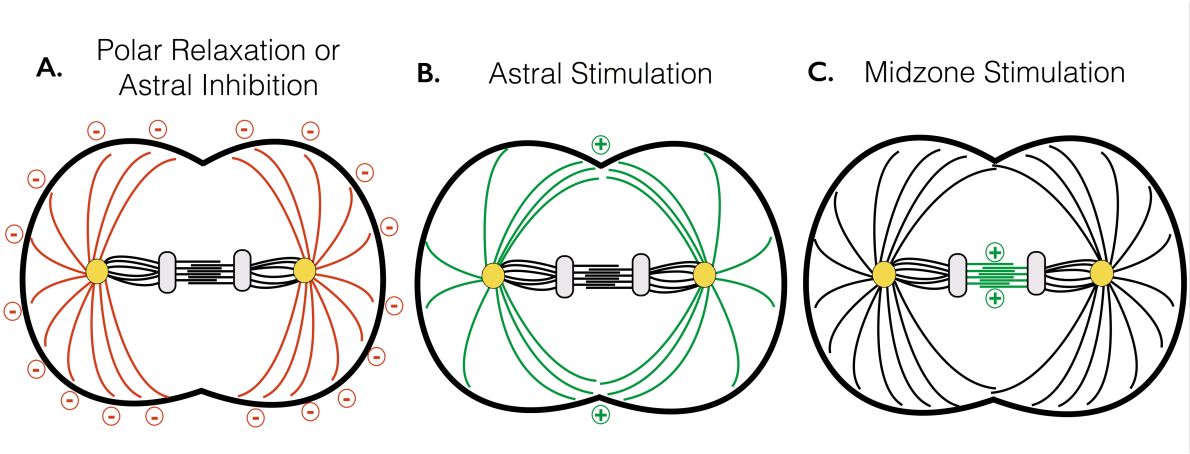


Figure 1. Models for division plane positioning in animal cells. A. The polar relaxation or astral inhibition model, strongly favored by Wolpert, posits that spindle asters are responsible for relieving cortical tension (red negative signs) in the poles, resulting in a furrow in the equatorial region. B. The astral stimulation model, based on experiments by Ray Rappaport, proposes that spindle asters deliver a cue (green plus signs) to the equatorial cortex that designates a cleavage furrow. C. The midzone stimulation model, based on genetic and cell biological evidence that the central spindle structure is required (green plus signs) for cytokinesis, states that antiparallel microtubules between the chromosomes signal to the overlying cortex to induce a furrow.

Experiments in the 1940s and 1950s established that the mitotic apparatus (spindle) was required for formation of a cleavage furrow (K. Dan and Dan 1947; Hiramoto 1956; J. M. Mitchison 1953). However, what aspect of the spindle is critical to furrow formation remained unknown. A prevailing model in the field proposed by Wolpert (Wolpert 1966) argued that astral microtubules signal relaxation of cortical tension at the poles resulting in contraction in the equatorial region (Figure 1A). This idea is supported by evidence that in some cell types, new membrane is added at the poles early in mitosis and this requires astral microtubules (Gudejko, Alford, and Burgess 2012). Contradicting this concept of “polar relaxation”, using micromanipulations of sand dollar embryos, Ray Rappaport discovered that pairs of astral microtubules can generate a stimulatory signal (Figure 1B) leading to furrow formation at the equator, in the absence of chromosomes or a midzone (Rappaport 1961; Rappaport 1969). He also found that placing small

barriers between the central spindle and the cell cortex did not prevent cleavage furrow formation (Rappaport 1973), suggesting that midzone microtubules do not regulate furrow formation. However, analogous experiments in smaller tissue culture cells have emphasized that the spindle midzone is crucial for furrow induction (Wheatley and Wang 1996; Eckley et al. 1997). In fact analysis of the *asterless* mutant in *Drosophila* spermatocytes suggests that asters are dispensable for cytokinetic signaling (Bonaccorsi, Giansanti, and Gatti 1998), and in the absence of an intact central spindle the contractile ring fails to assemble (Adams et al. 1998) (Figure 1C). These results led to considerable debate regarding which aspect of the mitotic spindle is most crucial in furrow induction.

It is now apparent in many model systems that parallel pathways can direct the formation of a furrow (Werner, Munro, and Glotzer 2007; Bringmann and Hyman 2005)(Figure 1). The central spindle microtubules promote furrow formation, whereas tips of astral microtubules appear to inhibit recruitment of contractile ring components to the membrane at the poles (Werner, Munro, and Glotzer 2007). Ray Rappaport's observations could reflect the phenomenon of astral inhibition, the exact molecular mechanism of which remains poorly understood (section 1.8). Alternatively, overlapping antiparallel asters from opposite poles could function as a spindle midzone beneath the plasma membrane (Su et al. 2014) resulting in "astral stimulation" to generate "Rappaport furrows".

Following Rappaport's pioneering experiments, several molecular details underlying cytokinesis, including the stimulatory signal provided by the spindle midzone, have been documented, and are discussed in detail in this chapter.

1.2 A small GTPase RhoA is the central regulator of cytokinesis

The formation of a cleavage furrow during cytokinesis requires a small GTPase called RhoA, a central component of the pathway leading to contractile ring formation.

Small GTPases are a family of monomeric hydrolase enzymes, analogous to the alpha subunit of heterotrimeric G-proteins, that can function independently to bind and hydrolyze GTP to GDP. They are required for an array of cellular processes such as intracellular trafficking (Wandinger-Ness and Zerial 2014), cell signaling during differentiation and proliferation (Downward 2003; Drosten et al. 2010), and morphology changes such as adhesion, migration and cytokinesis (Ridley 2015).

The *rho* family of GTPases was first identified 30 years ago in the marine snail *Aplysia* (Madaule and Axel 1985). RhoA is one of the best studied members of this family along with Rac and Cdc42. Soon after its discovery, RhoA was shown to be ribosylated by a bacterial C3 ribosyltransferase which rendered the GTPase inactive (Aktories et al. 1989; Paterson et al. 1990). Thus, this enzyme provided a specific way to inhibit RhoA *in vivo*, and together with other strategies it helped to demonstrate that RhoA is a key regulator of the actin cytoskeleton in cultured mammalian cells (Aktories et al. 1989; Paterson et al. 1990; Ridley and Hall 1992). Importantly, inhibition of RhoA *(rho* p21*)* function in *Xenopus* embryos was found to completely abolish the induction of a cleavage furrow, thus establishing one of the first molecular requirements for cytokinesis

(Kishi et al. 1993).

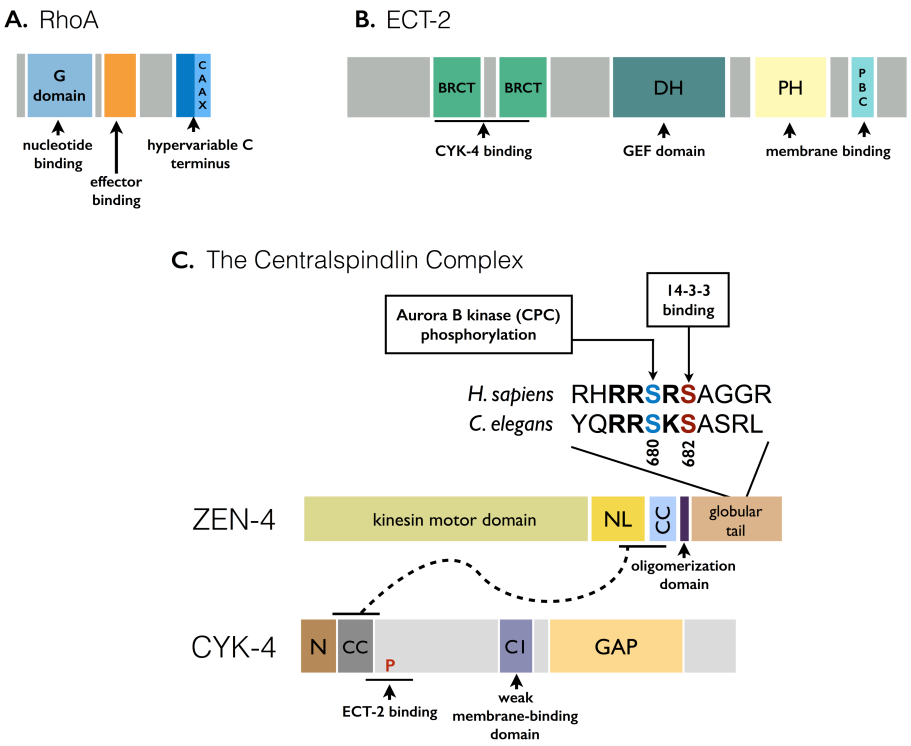


Figure 2. Schematic showing domain organization of RhoA, ECT-2 and centralspindlin. A. RhoA is a small protein that consists of a GTP/GDP binding domain, an effector binding domain, a hyper-variable region and a CAAX motif at the very C terminus to which a lipid binding motif is post-translationally attached. B. The ECT2 protein consists of two tandem BRCT domains to which CYK-4 binds, a DH domain involved in GEF activity, a PH domain and a poly-basic cluster (PBC) that contribute to membrane binding. C. The Centralspindlin Complex consists of a kinesin-6 motor ZEN-4 and a RhoGAP protein CYK-4. ZEN-4 consists of a N-terminal motor domain, a long neck linker (NL) region, a coiled-coil (CC) domain followed by a 16 amino acid oligomerization domain and a globular tail that contains a 14-3-3 binding site and a Aurora B phosphorylation site. CYK-4 has a short N-terminal domain, a coiled-coil (CC) that interacts with ZEN-4, a weak membrane-binding C1 domain and a C terminal GAP domain.

RhoA is a 21kD protein comprising a core G domain followed by a short “insert” region and a hypervariable C-terminus (Schaefer, Reinhard, and Hordijk 2014). The very C-terminus contains a CAAX box to which a lipid anchor is post-translationally attached, permitting membrane localization (Klooster and Hordijk 2007) (Figure 2A). Guanine nucleotide binding is mediated by the G domain, in particular the switch I and switch II

regions. Loss of the γ-phosphate following GTP hydrolysis leads to relaxation of these regions into the GDP-bound conformation (Wittinghofer and Vetter 2011; Vetter and Wittinghofer 2001). This confers the property of a molecular “switch” that cycles between an “on” and “off” state. In most cases, only the GTP-bound state is able to successfully interact with downstream effectors to elicit function.

During cytokinesis, RhoA activates specific effector proteins to induce furrow formation (schematic of the pathway leading to cortical contractility is shown in Figure 3A). In its GTP-bound form, RhoA directly binds the N-terminus of the actin polymerizing protein formin. In the absence of an active GTPase, the N- and C-terminal domains (called DID and DAD, respectively) of formin engage in an intramolecular, auto-inhibitory interaction. RhoA activity thus directly promotes actin nucleation by formin (Otomo et al. 2005). RhoA also indirectly promotes phosphorylation of the regulatory light chain of non-muscle myosin II by activating the kinases ROCK and citron kinase (Matsumura 2005). This facilitates assembly of myosin filaments on F-actin. Finally, RhoA binds to the C-terminus of the scaffold protein anillin and is required for its cortical recruitment (Piekny and Glotzer 2008). Anillin also directly interacts with actin and myosin (Oegema et al. 2000; Straight, Field, and Mitchison 2005), as well as the plasma membrane (Sun et al. 2015) and is required for the stability of the contractile ring (Somma et al. 2002; Piekny and Maddox 2010). Together these proteins generate a functional contractile ring that likely contracts due to a combination of actin depolymerization and myosin motor activity (Mendes Pinto, Rubinstein, and Li 2013), inducing deformation in the overlying plasma

membrane. Anillin appears to stabilize the ring and restrict it to the equatorial region of the cell in most cell types (Straight, Field, and Mitchison 2005).

In the absence of active RhoA, these downstream effectors fail to accumulate on the plasma membrane (Kishi et al. 1993). Based on the Rho binding domains of these effectors, biosensors have been developed in several model systems (Yoshizaki et al. 2003; Tse et al. 2012; Bement, Benink, and Dassow 2005; Piekny, Werner, and Glotzer 2005). This has led to a consensus that active RhoA accumulates prior to contractile ring components and marks the location of the future cleavage furrow. Recently our lab has demonstrated that local activity of RhoA is sufficient to direct the formation of a cleavage furrow in human cells (Wagner and Glotzer 2016a). In fact, there appears to be little spatial and temporal regulation of the pathway downstream of Rho-GTP (Wagner and Glotzer 2016a). Therefore, to understand how a cell positions its division plane accurately, upstream spatiotemporal regulation of RhoA must be investigated in detail.

1.3 Activation of RhoA by ECT-2 during cytokinesis

Formally, a Rho GTPase can execute the entire GTP hydrolysis cycle but is slow at each step except initial GTP loading (Gibbs et al. 1984; Neal et al. 1988). In cells, Rho GTPases are assisted in the “off” to “on” transition by guanine exchange factors (GEFs) that stabilize the empty form to promote the release of product and allow reloading of GTP present in high concentrations in the cell. GTPase activating proteins (GAPs)

traditionally accelerate the hydrolysis reaction, thereby turning Rho “off”. Rho GDIs (GDP Dissociation Inhibitors) are chaperone proteins that usually maintain Rho GTPases in an inactive conformation, sequestering them from membrane association and effector binding.

While several GEFs can activate RhoA (Cherfils and Zeghouf 2013; Snyder et al. 2002), ECT-2 (epithelial cell transforming sequence 2), originally identified as an proto-oncogene (Miki et al. 1993), activates RhoA during cytokinesis in diverse organisms (Tatsumoto et al. 1999; Prokopenko et al. 1999; Dechant 2003) (pathway schematic in Figure 3A). In the absence of ECT-2 or upon its inactivation, active RhoA and downstream components fail to accumulate on the membrane (Yüce, Piekny, and Glotzer 2005; Chalamalasetty et al. 2006). The ECT-2 protein consists of tandem BRCT domains in its N-terminus followed by a Dbl homology (DH)-type GEF domain, a pleckstrin homology (PH) domain and a polybasic cluster in the C terminus (Figure 2B). The DH-PH domain induces transition of RhoA from the GDP to GTP bound state (Kim, Billadeau, and Chen 2005) and contributes to membrane localization. ECT-2 is thought to normally exist in an inactive conformation owing to an intramolecular interaction between BRCT domains with DH-PH domains (Kim, Billadeau, and Chen 2005). To induce a cleavage furrow, localization and activation of ECT-2 in the division plane during anaphase is necessary. This role is performed by the centralspindlin complex and is discussed in detail in sections 1.8 and 1.9.

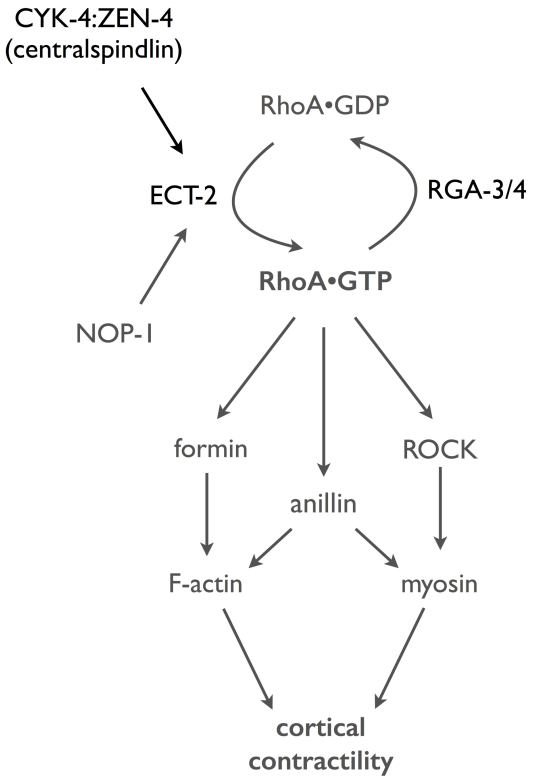


Figure 3. Known pathway leading to RhoA activation and cortical contractility during cytokinesis. NOP-1 is a *C. elegans*-specific ECT-2 activator.

1.4 The Centralspindlin Complex: identification and structure

The centralspindlin complex plays a role at all stages of cytokinesis and provides the vital link between the mitotic spindle, ECT-2 and RhoA. It is a well-conserved complex present in all metazoans consisting of two molecules of a kinesin-6 motor protein ZEN-4 (*C. elegans* nomenclature; MKLP1 in mammalian cells and *pavarotti* in *Drosophila*) and two molecules of a RhoGAP protein CYK-4 (*C. elegans* nomenclature; MgcRacGAP in mammalian cells and *tumbleweed* in *Drosophila*) (Figure 2C).

MKLP1 (mitotic kinesin like protein 1) was first identified in mammalian cells where it was found to stably associate with spindle midzone microtubules during anaphase (Sellitto and Kuriyama 1988; Nislow et al. 1990) and proposed to bundle antiparallel microtubules. Orthologs of MKLP1 were later discovered in other organisms (Raich et al. 1998; Powers et al. 1998; Adams et al. 1998). ZEN-4/MKLP1 consists of an N-terminal motor domain, a long neck linker region, a parallel coiled-coil and a globular C terminal domain (Figure 2C). Prior to anaphase, Cdk1 phosphorylation of the motor domain (T9 in ZEN-4 and T8 and T450 in MKLP1) prevents microtubule binding and kinesin activity (Mishima et al. 2004). Conventional kinesins contain a 13-15 amino acid linker (Wade and Kozielski 2000), whereas ZEN-4 has a 85 residue linker region following its motor domain (Mishima, Kaitna, and Glotzer 2002). In the absence of this unusual kinesin, the spindle midzone is disrupted (Adams et al. 1998) and cells display early (Adams et al. 1998) and late (Raich et al. 1998) cytokinesis defects in *Drosophila* and *C. elegans* embryos.

CYK-4 was discovered in human cells as a RhoGAP protein (Touré et al. 1998). Forward genetics in *C. elegans* revealed a role for CYK-4 in cytokinesis and its functional interaction with ZEN-4 {JantschPlunger:2000ww}. One-cell *C. elegans* embryos homozygous for a temperature-sensitive allele of *cyk-4* at the restrictive temperature phenocopied the loss of ZEN-4 {JantschPlunger:2000ww}. Moreover these two molecules co-localize at the midzone and are inter-dependent for their localization {JantschPlunger:2000ww}. The CYK-4 protein has a short N-terminal region followed by a parallel coiled coil, a C1 domain and a RhoGAP domain in the C terminus (Figure 2C).

The very N-terminus of CYK-4 (including the coiled coil) and the central region of ZEN-4 (encompassing the neck linker and coiled coil domains) directly bind each other (Mishima, Kaitna, and Glotzer 2002) to form a stable, stoichiometric 2:2 complex (Mishima, Kaitna, and Glotzer 2002; Pavicic-Kaltenbrunner, Mishima, and Glotzer 2007). The binding of CYK-4 to ZEN-4 induces a large conformational change in the motor, stabilizes the neck linker regions relative to one another, greatly enhances the ability of the kinesin to bundle microtubules and slows motor activity of ZEN-4 (E. A. White et al. 2013; Davies et al. 2015).

Centralspindlin is critical in three major aspects of cytokinesis. First, owing to its ability to bundle antiparallel microtubules, centralspindlin is required for the proper assembly of the central spindle (Adams et al. 1998; Powers et al. 1998). Second, CYK-4 can directly bind to and recruit ECT-2 to the spindle midzone during anaphase (Yüce, Piekny, and Glotzer 2005) thereby playing a crucial role in RhoA activation. These two functions are discussed in detail in the following sections.

In addition, centralspindlin mediates abscission, the concluding stage of cytokinesis. The final scission event of cell division is executed by the ESCRT-III (endosomal sorting complex required for transport) machinery (Agromayor and Martin-Serrano 2013; McCullough, Colf, and Sundquist 2013). Once the cleavage furrow fully ingresses, the spindle midzone matures into a stable midbody that connects the cytoplasm of the two daughter cells. The midbody contains densely packed microtubules where centralspindlin remains in high concentration, however it is rearranged into a dense cortical ring (Hu, Coughlin, and Mitchison 2012). The endosomal GTPase ARF6 binds

to the C terminal tail of ZEN-4 and ensures the stable accumulation of centralspindlin at the midbody (Joseph et al. 2012). Centralspindlin is required for abscission presumably because it indirectly recruits components of the ESCRT-III complex. Centralspindlin directly binds CEP-55 which interacts with subunits of the ESCRT I complex that in turn recruit ESCRT-III proteins (W.-M. Zhao, Seki, and Fang 2006).

1.5 The role and regulation of centralspindlin oligomers in central spindle assembly

The central spindle is a key structure that instructs the position and formation of the cytokinetic ring (Wheatley and Wang 1996; Eckley et al. 1997; Bonaccorsi, Giansanti, and Gatti 1998). Failure in proper central spindle assembly leads to defects in cytokinesis. During anaphase, centralspindlin precisely localizes at the tips of anti-parallel microtubules in the spindle midzone and is required for the integrity of this structure (Mishima et al. 2004; Mishima, Kaitna, and Glotzer 2002), however it is not sufficient. Protein regulator of cytokinesis 1 (PRC1), also known as SPD-1 in *C. elegans,* is another key regulator of this process. In the absence of PRC1, centralspindlin, though still bound to the spindle midzone, fails to strongly concentrate at the middle (Vernì et al. 2004; Mollinari et al. 2005) leading to defects in spindle elongation and cytokinetic failure in some cell types. Conversely, in the absence of centralspindlin, PRC1 localization and microtubule bundling in the midzone are heavily compromised (Raich et al. 1998; Adams et al. 1998; Verbrugghe and White 2004).

CYK-4 and ZEN-4 are individually incapable of accumulating at the central spindle; an intact complex is required {JantschPlunger:2000ww}. Moreover, the heterotetrameric unit must form higher order oligomers to travel to microtubule plus ends and stably accumulate there (Nishimura and Yonemura 2006; Hutterer, Glotzer, and Mishima 2009). The property to oligomerize is conferred by a conserved 16 amino acid domain in the structure of MKLP1/ZEN-4 (Figure 2C) (Hutterer, Glotzer, and Mishima 2009). In the absence of this oligomerization domain, centralspindlin associates only transiently with the spindle midzone (Hutterer, Glotzer, and Mishima 2009). The oligomerization of centralspindlin is tightly regulated (Douglas et al. 2010).

In human cells, 14-3-3 proteins inhibit centralspindlin oligomerization. 14-3-3 proteins are highly helical, dimeric, multi-functional proteins that bind to specific interactors via conserved phosphoserine motifs (Yaffe 1997; Aitken 2006). Prior to anaphase, centralspindlin is bound by specific isoforms of 14-3-3 proteins that block the ability of the complex to oligomerize (Douglas et al. 2010). However during anaphase Chromosome Passenger Complex (CPC), phosphorylates MKLP1/ZEN-4 (Guse, Mishima, and Glotzer 2005; Kaitna et al. 2000; Severson et al. 2000), which prevents 14-3-3 binding (Douglas et al. 2010). Thus the CPC promotes central spindle assembly by inducing oligomerization of centralspindlin. Incidentally, analogous to cells lacking the MKLP1/ZEN-4 oligomerization domain, *C. elegans* embryos depleted of CPC components fail to stably accumulate centralspindlin at the midzone (Kaitna et al. 2002).

1.6 The Chromosome Passenger Complex (CPC): identification, structure and role in mitosis

The Chromosome Passenger Complex (CPC) performs numerous essential roles during cell division. It consists of four proteins - an enzymatic subunit Aurora B kinase, a scaffold module INCENP and two non-enzymatic components survivin and borealin (also known as dasra and CSC-1) (Figure 4). Inner Centromeric Protein (INCENP) was the first of these subunits to be identified in a monoclonal antibody screen to determine components of mitotic chromosomes (Cooke, Heck, and Earnshaw 1987). INCENP, along with other members of the CPC, localizes on centromeres during metaphase and on the central spindle and equatorial cortex during anaphase (Cooke, Heck, and Earnshaw 1987). Aurora kinases are a family of Ser/Thr kinases. Aurora A, was discovered in a *Drosophila* genetic screen and

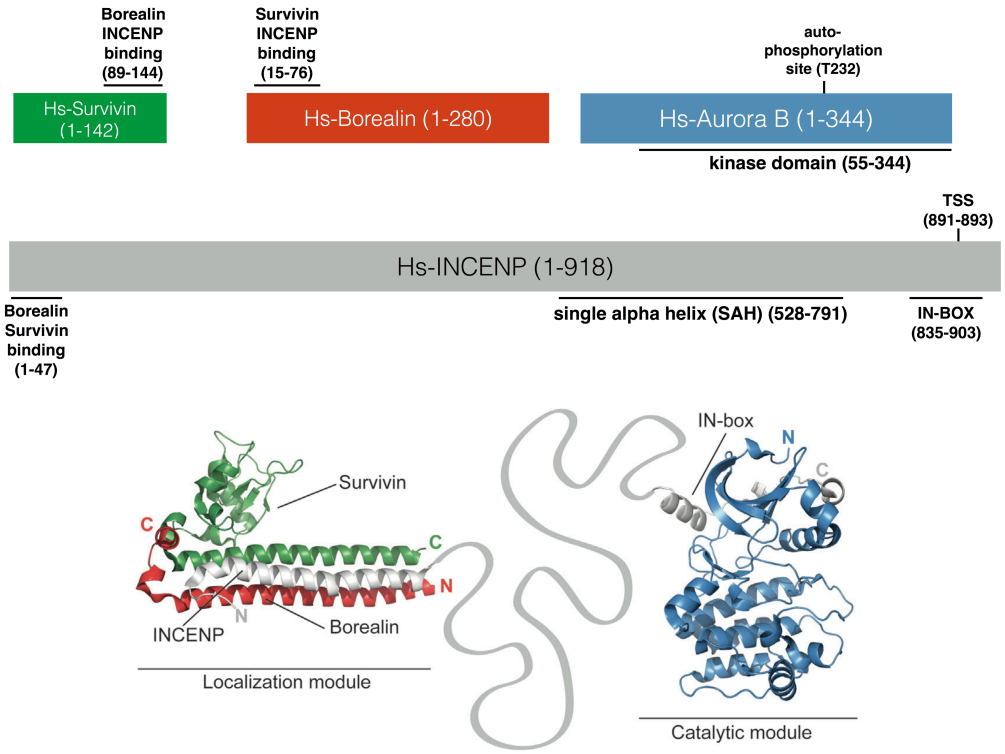


Figure 4. Schematic showing components and domain organization of the Chromosome Passenger Complex (CPC). The CPC consists of four proteins - Survivin, Borealin, INCENP and Aurora B kinase. Here the domains and interactions between the human proteins are illustrated, together with a crystal structure of the assembled complex. (adapted from *Krenn and Musacchio, Frontiers in Oncology, 2015*).

named so because in mutant cells chromosomes are arranged in an “aurora” around the pole (Glover 1989; Glover et al. 1995). The second member of the Aurora family was was discovered in a genetic screen for chromosome segregation defects in budding yeast where it was named Ipl1 (Increase in ploidy 1) (Chan and Botstein 1993) and as AIM-1 (Aurora and Ipl1-like midbody-associated protein) in rats (Giet and Prigent 1999). It came to be known as Aurora B. In contrast to the centrosomal localization of Aurora A, Aurora B localizes at the cell equator - at the centromeres in metaphase, and later at the spindle midzone and midbody (Giet and Prigent 1999), much like INCENP. The C-termini of Aurora B and Aurora A are highly similar but their N-termini are divergent

which permits their distinct localization pattern, protein interactions and function (J. Fu et al. 2009; Hochegger, Hégarat, and Pereira-Leal 2013; S. Li et al. 2015). A functional and biochemical interaction between INCENP and Aurora B kinase was soon established in *Xenopus* (Adams et al. 2000) and *C. elegans* (Kaitna et al. 2000) which, along with previous studies (Mackay et al. 1998), established a role for this complex in chromosome segregation, central spindle assembly and cytokinesis. INCENP and Aurora B were later found to associate with other proteins of the complex, survivin and borealin, in a variety of cell types (Ambrosini, Adida, and Altieri 1997; Gassmann et al. 2004; Sampath et al. 2004; Romano et al. 2003).

The N termini of INCENP and borealin are associated with the C terminus of survivin in a 3-helix bundle (Jeyaprakash et al. 2007), while the carboxy terminus of INCENP (termed the IN-BOX) binds to the N terminal lobe of Aurora B kinase (Sessa et al. 2005; Kaitna et al. 2000; Adams et al. 2000) (Figure 4). The latter interaction is the first of a complex, multistep process leading to Aurora B activation. The C-terminus of INCENP is sufficient to induce Aurora B activity *in vitro* in the absence of other factors (Sessa et al. 2005; Kaitna et al. 2000; Adams et al. 1998; Honda, Körner, and Nigg 2003). INCENP contains multiple Aurora B phosphorylation sites, however complete activation of the kinase requires phosphorylation of two serine residues in a conserved TSS motif in the INCENP tail (Bishop and Schumacher 2002; Honda, Körner, and Nigg 2003). A third requirement for full activation is auto-phosphorylation of Aurora B at T232 (human numbering) (Sessa et al. 2005; Yasui et al. 2004). Based on structural considerations (Sessa et al. 2005) phosphorylation of INCENP is likely to occur in trans, as is the auto-

phosphorylation of Aurora B. This is consistent with observations where artificially clustering the INCENP-Aurora B complex using an antibody (Kelly et al. 2007) or an exogenous dimerization domain (Tseng et al. 2010) dramatically enhances activity, and suggests that high local concentrations would greatly stimulate Aurora B activity in the cell. The very N-terminus of INCENP has been suggested to assist multimerization of the complex but the mechanism by which this is occurs is unknown (van der Horst et al. 2015).

The best described function of Aurora B is at the centromeres in promoting chromosome bi-orientation during metaphase. The CPC localizes at the centromeres primarily via an interaction between survivin and Histone H3 that is phosphorylated by a Ser/Thr kinase, Haspin at Thr3 (Krenn and Musacchio 2015). However, all components of the CPC are required for stable localization of the complex (Klein, Nigg, and Gruneberg 2006). Currently Aurora B is thought to promote proper assembly of the metaphase spindle in two ways. One, it locally destabilizes microtubule-kinetochore interactions that do not support chromosome bi-orientation (error-correction (EC)). Second, it is postulated to participate in the spindle assembly checkpoint (SAC) that sends out a “global” signal preventing mitotic exit in the presence of a single unattached or mis-attached kinetochore (Krenn and Musacchio 2015). The latter function was uncovered by use of small molecule inhibitors that poison Aurora B kinase activity (Hauf et al. 2003; Gadea and Ruderman 2005). It is proposed that when the spindle is properly bi-oriented and under tension, Aurora B substrates on the outer kinetochore become physically separated from the kinase that concentrates at the inner kinetochore

(Krenn and Musacchio 2015). These substrates consequently do not activate EC and SAC responses, allowing the cell to proceed into anaphase.

Central spindle localization of the CPC during anaphase is independent of its prior presence on chromosomes (making the term “passenger” a misnomer). The CPC concentrates on the central spindle even in cells that lack chromosomes (Bucciarelli et al. 2003). Furthermore, a specific survivin mutant has been identified that cannot localize to centromeres but can localize to the central spindle (Vader et al. 2006). As discussed in section 1.5, Aurora B phosphorylates MKLP1/ZEN-4 and promotes localization of the centralspindlin complex at the central spindle (Kaitna et al. 2000; Douglas et al. 2010). The CPC, PRC1 and centralspindlin are all required for central spindle assembly and loss of any of these components disrupts localization of the others. The mechanism by which the CPC localizes at the central spindle is complex and possibly variable across organisms. In mammalian cells and *Drosophila* neuroblasts, a kinesin-6 motor MKLP2/*subito* is required to localize Aurora B to the midzone microtubules (Gruneberg et al. 2004; Cesario et al. 2006). This protein is poorly conserved among other organisms. However, INCENP can directly interact with microtubules via a central single alpha helical (SAH) domain and this interaction is required to localize the CPC at the central spindle in human cells (Samejima et al. 2015; van der Horst et al. 2015; Tseng et al. 2010).

1.7 What is the role of the CPC in cytokinesis?

The CPC has been implicated to play a role in cytokinesis since the discovery of Aurora B kinase, but its exact function in the process is unclear. In the absence of the CPC or upon Aurora B inactivation, central spindle assembly is perturbed and centralspindlin does not stably accumulate in the mid-plane (Kaitna et al. 2000; Douglas et al. 2010). Because an intact midzone is required at least late in cytokinesis to execute abscission, loss of CPC function is associated with cytokinesis defects (Argiros et al. 2012). In the absence of proper spindle elongation, Aurora B kinase was shown to be required for furrow induction (Dechant 2003). However it is unclear if this role of Aurora B is an indirect consequence of failure to assemble a central spindle and concentrate ECT-2 in the midzone. The CPC also accumulates early at the equatorial cortex during anaphase prior to contractile ring components (Cooke, Heck, and Earnshaw 1987; Eckley et al. 1997; Murata-Hori and Wang 2002; Szafer-Glusman, Fuller, and Giansanti 2011; Kitagawa et al. 2013), however the function of the complex at this site has not been elucidated. It has been suggested (but never demonstrated) that the CPC may play a role in RhoA activation at this site but no mechanism has been proposed.

There is indirect evidence in several model systems that Aurora B activity may be required for RhoA activation, however a clear model has not emerged. In monopolar cytokinesis in human cells, Aurora B activity is required to spontaneously break symmetry and polarize microtubules, and for the accumulation of RhoA effectors at the cell cortex (Hu et al. 2008). In a cell-free reconstituted system using *Xenopus* extracts, it has been demonstrated that active RhoA accumulates on supported lipid bilayers overlaying spindle midzones and that this requires Aurora B kinase activity (Nguyen et

al. 2014). Depletion of Kif23, the *Xenopus* ZEN-4 homolog, was shown to have no effect on accumulation of active RhoA in this system, and therefore concluded to be dispensable in RhoA activation (Nguyen et al. 2014). In *Xenopus* embryos, forced activation of the CPC using activating INCENP antibodies results in formation of ectopic cleavage furrows, suggesting ectopic activation of RhoA (Field et al. 2015). It is not apparent if these results reflect a requirement for Aurora B in RhoA activation during wild-type cytokinesis. Importantly, it remains to be determined whether the CPC would control contractile ring assembly indirectly through its function in central spindle assembly or via a yet undetermined mechanism.

1.8 RhoA activation during cytokinesis: centralspindlin-dependent and parallel pathways

Centralspindlin is a conserved regulator of RhoA activity during cytokinesis owing to its interaction with the Rho GEF ECT-2. A central region in CYK-4, carboxy-terminal to the coiled-coil, directly binds to BRCT domains of ECT-2 (Yüce, Piekny, and Glotzer 2005; Somers and Saint 2003) resulting in recruitment of the GEF to the midzone where it is required for furrow induction. The interaction between these proteins is cell-cycle regulated and permitted only during anaphase. Phosphorylation of ECT-2 by Cdk1 inhibits its interaction with CYK-4 during metaphase (Yüce, Piekny, and Glotzer 2005). In early anaphase, the N-terminus of CYK-4 is phosphorylated by the mitotic kinase Polo like kinase 1 (Plk1) creating a phosphoepitope recognized by the BRCT domains of ECT-2 (Wolfe, Takaki, Petronczki, and Glotzer 2009a; Petronczki et al. 2007). In the

absence of CYK-4 phosphorylation, these proteins do not stably bind each other *in vivo* and ECT-2 fails to accumulate at the central spindle (Wolfe, Takaki, Petronczki, and Glotzer 2009a). Microtubules and PRC1 are critical factors that promote this interaction (Wolfe, Takaki, Petronczki, and Glotzer 2009a). Because ECT-2 is autoinhibited via an intramolecular interaction between the BRCT and DH-PH domains (Kim, Billadeau, and Chen 2005), CYK-4 binding is likely to activate ECT-2 (Wolfe, Takaki, Petronczki, and Glotzer 2009a; Burkard et al. 2009). Collectively, these data suggest that the centralspindlin complex effectively functions as a RhoA activator by localizing and activating the RhoGEF ECT-2 in the division plane.

However, in some cell types, a cleavage furrow can be initiated midway between astral microtubules from opposing spindle poles even in the absence of a functional centralspindlin complex. The existence of these parallel pathways has been elucidated in numerous ways in *C. elegans* embryos. By using a laser to sever one spindle pole from midzone microtubules, the midpoint between the asters and the position of the central spindle were spatially separated (Bringmann and Hyman 2005). This resulted in the formation of two furrows that ultimately merged at the midzone (Bringmann and Hyman 2005). The precise nature of the aster-directed pathway is not known. In *C. elegans zen-4* mutants, centrosome separation during anaphase plays a crucial role in furrow induction (Dechant 2003). By genetically manipulating the extent of spindle elongation in embryos lacking functional centralspindlin it was demonstrated that at a critical distance between centrosomes, a local minimum of microtubule density is generated at the equator. This minimum correlates with formation of a furrow (Dechant 2003). This evidence is not consistent with Wolpert's astral relaxation model or

Rappaport's theory of astral stimulation (section 1.1), but it is supported by findings where depolymerization of microtubules leads to ectopic contractility (Kurz 2002; Canman, Hoffman, and Salmon 2000). Modifying the spindle position while simultaneously imaging myosin and spindle microtubules in *C. elegans* embryos reveals that myosin is antagonized by asters (Werner, Munro, and Glotzer 2007). This supports the notion that components of the contractile ring accumulate at sites of weak microtubule density.

The molecular details of a centralspindlin-independent pathway for RhoA activation are not fully understood. In *C. elegans* embryos ECT-2 is primarily activated by centralspindlin during cytokinesis, but there is partial redundancy with a protein called NOP-1 at this stage. NOP-1 is a nematode specific protein, and has been identified to function as a global activator of ECT-2 via the astral pathway (Tse et al. 2012)(Figure 3A). *C. elegans* embryos in which centralspindlin function is disrupted, can form a cytokinetic furrow that ultimately regresses. However simultaneous loss of NOP-1 completely abolishes furrow induction and active RhoA does not accumulate on the membrane (Tse et al. 2012). The mechanism by which NOP-1 activates ECT-2 is currently unknown.

1.9 Centralspindlin is an unusual RhoGAP-containing complex

Activation of the GTPase RhoA is a curious function for a RhoGAP protein like CYK-4, which would ordinarily be predicted to turn the GTPase "off". Therefore the function of

the CYK-4 GAP domain has been a subject of considerable debate. Based on data from various model systems, an emerging view of CYK-4 function is summarized below. However it is quite likely that a single model for centralspindlin function does not apply to all experimental conditions and cell types.

In vitro the CYK-4 GAP domain promotes GTP hydrolysis by Rho family members, but has much stronger activity towards other Rho GTPases Rac and Cdc42 when compared to RhoA (Touré et al. 1998; Bastos et al. 2012). In some cell types such as *Xenopus* embryos, inactivating GAP function by abolishing the catalytic arginine finger increases the intensity and broadens the zone of RhoA activation during cytokinesis (Miller, Dassow, and Bement 2008). This is consistent with a conventional view of GAP function and favors a model where continuous GTPase flux is required for proper cytokinesis. However, deletion of the CYK-4 GAP domain in these embryos causes dramatic instability of the contractile ring (Miller, Dassow, and Bement 2008), suggesting that the GAP domain performs additional functions in cytokinesis. In other cell types, the CYK-4 catalytic arginine was found to be either dispensable for furrow induction (Yamada, Hikida, and Kurosaki 2006) or contrary to expectations for a conventional GAP, strictly required for furrow formation (Zavortink et al. 2005).

The precise role of the CYK-4 GAP domain during cytokinesis has been extensively tested in *C. elegans.* Temperature sensitive mutations close to the catalytic arginine in the CYK-4 GAP domain have been identified (Canman et al. 2008), though it is unknown whether these mutant proteins are GAP defective and if they have additional impairments. At the restrictive temperature these mutant embryos resemble CYK-4 or

ZEN-4 depletion conditions, with slow furrow ingression, reduced myosin accumulation at the cleavage site and ultimately failure in cytokinesis (Canman et al. 2008). Curiously, depletion of CED-10 (Rac1) protein in these embryos rescues the cytokinesis defect (Canman et al. 2008) leading to speculation that Rac1 inactivation, and not RhoA activation, by CYK-4 is essential for cytokinesis. However, further analysis revealed that Rac1 depletion can neither rescue the rates of furrow ingression nor loss of myosin accumulation in these GAP domain mutants (Loria, Longhini, and Glotzer 2012). In fact, Rac1 depletion causes a reduction in cortical stability (Loria, Longhini, and Glotzer 2012) which may make the membrane more compliant to furrow induction in the presence of weak activation. As described in section 1.8, in *C. elegans* embryos a centralspindlin-independent pathway can promote RhoA activation. In the absence of this parallel NOP-1-directed pathway, these GAP domain mutants completely lack the ability to induce furrows at the restrictive temperature (Zhang and Glotzer 2015). Similarly, in embryos where the catalytic arginine of CYK-4 is mutated and NOP-1 is simultaneously disrupted, no furrow is formed (Zhang and Glotzer 2015). In sum, these mutations in the GAP domain phenocopy centralspindlin depletion phenotypes in the early embryo. Importantly, in the absence of the NOP-1 pathway, Rac1 depletion can no longer rescue CYK-4 GAP domain defects in furrow formation (Zhang and Glotzer 2015). This suggests that suppression of Rac1 activity is unlikely to be an important function of the CYK-4 GAP domain during cytokinesis.

If the GAP domain of CYK-4 is indeed required for RhoA activation rather than Rac1 inactivation, then compensating for loss in RhoA activity should fully restore the

cytokinesis machinery. This was recently demonstrated in different ways. In the absence of the NOP-1 furrow induction pathway (Tse, Piekny, and Glotzer 2011; Werner, Munro, and Glotzer 2007; Bringmann and Hyman 2005), catalytically dead CYK-4 is unable to induce a cleavage furrow. Is this behavior due to impaired RhoA activation? Two highly similar proteins, RGA-3/4 function as conventional GAPs in the early *C. elegans* embryo (Schonegg et al. 2007; Schmutz, Stevens, and Spang 2007); therefore their depletion leads to an increase in RhoA activity. Quite surprisingly, depletion of RGA-3/4 fully restores the cytokinesis defect of catalytically dead CYK-4 mutants (Zhang and Glotzer 2015), indicating that counter intuitively, the GAP activity of CYK-4 is required for RhoA activation. Furthermore, using a suppressor screen in *C. elegans,* gain-of-function alleles in the DH-PH domain of *ect-2* were identified that restore wild-type furrow ingression rates and rescue the lethality of the GAP domain mutations (Zhang and Glotzer 2015). In fact, the GAP domain of CYK-4 and the DH-PH domain of ECT-2 have been found to directly bind each other (Zhang and Glotzer 2015). This suggests that CYK-4 plays an additional role in GEF activation, rather than simply relieving autoinhibition as was previously thought (Burkard et al. 2009; Wolfe, Takaki, Petronczki, and Glotzer 2009b). Taken together, these data support a model where the GAP domain of CYK-4 facilitates activation of RhoA via a direct interaction with the RhoGEF ECT-2.

1.10 How is precise spatiotemporal control of RhoA achieved during cytokinesis?

While the list of the molecules involved in RhoA activation during cytokinesis is largely complete and we know several details about their interactions, successful cytokinesis requires highly fine-tuned spatiotemporal control of RhoA activity. Our current knowledge of the mechanisms underlying cytokinesis does not fully explain how this is achieved. Active RhoA accumulates at the equatorial plasma membrane where it is required for contractile ring assembly (Kishi et al. 1993; Yoshizaki et al. 2003; Tse et al. 2012; Bement, Benink, and Dassow 2005; Piekny, Werner, and Glotzer 2005). When parallel pathways are inactivated and RhoA activation is solely centralspindlin-directed, cells are still able to position their cleavage furrow accurately (Tse et al. 2012; Werner, Munro, and Glotzer 2007; Bringmann and Hyman 2005; Bonaccorsi, Giansanti, and Gatti 1998). This is true even in large embryonic cells where the spindle midzone is 10-30 microns away from the equatorial plasma membrane (Tse et al. 2012; Bringmann and Hyman 2005; Dassow et al. 2009). Additionally in some experimental contexts, spindle microtubules can be dramatically attenuated without preventing cleavage furrow formation (Dassow et al. 2009; Bonaccorsi, Giansanti, and Gatti 1998). How is RhoA activated at such a large distance from where the centralspindlin-ECT-2 complex localizes (Figure 5)? This is the central question addressed in this thesis.

Several models could explain RhoA activation at the membrane. ECT-2 could be activated at the spindle and translocate to the cortex independent of centralspindlin. Alternatively, the active ECT-2/centralspindlin complex could assemble at the central spindle and diffuse or be delivered, by motor-dependent transport, to the equatorial cortex. And finally, the centralspindlin/ECT-2 complex may accumulate on the plasma

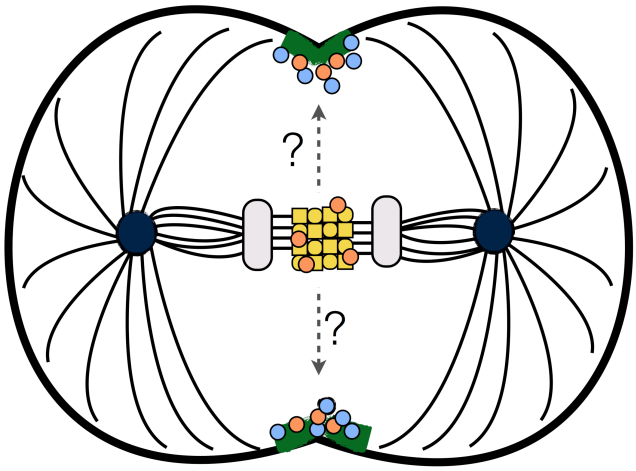


Figure 5. Open Question. The centralspindlin complex (yellow) accumulates at the spindle midzone and recruits ECT-2 (orange). ECT-2 and RhoA (blue) are detected on the equatorial cortex where they promote formation of an actomyosin-based contractile ring (green). How is RhoA activated at the plasma membrane if ECT-2 is activated by centralspindlin at the central spindle?

membrane independent of midzone assembly to locally activate RhoA. ECT-2 has two known membrane-binding domains (Su, Takaki, and Petronczki 2011), is readily detected on the plasma membrane prior to furrow ingression in several cell types and demonstrated to be essential for RhoA activation at this location (Tatsumoto et al. 1999; Prokopenko et al. 1999; Dechant 2003; Yüce, Piekny, and Glotzer 2005; Chalamalasetty et al. 2006). Is this pool of ECT-2 associated with and recruited by centralspindlin?

Although conventionally thought to be a microtubule-associated protein complex, centralspindlin has been detected on the cleavage furrow in some cell types (Adams et al. 1998; Verbrugghe and White 2007; Green et al. 2013). However the mechanism by

which it localizes, and whether this pool of centralspindlin performs any function in cytokinesis was unknown. The CYK-4 component of centralspindlin contains a weak membrane binding C1 domain (Figure 2C). In human cells, this domain plays a role late in cytokinesis linking the plasma membrane to the spindle microtubules as the midbody is formed (Lekomtsev et al. 2012). Because deletion of the HsCyk4 C1 domain does not impact cleavage furrow formation in these cells (Lekomtsev et al. 2012), it suggests that the membrane binding property of the C1 domain is not required for RhoA activation. Conversely, ectopic localization of the *Drosophila* homolog of CYK-4 on the plasma membrane in S2 cells leads to hypercontractility (D'Avino et al. 2006) suggesting that centralspindlin can function to activate RhoA at the membrane. However the relevance of this result in wild-type cytokinesis is obscure. It is plausible that the membrane-binding property of CYK-4 is dispensable for furrow induction in some cells, but crucial in other systems.

The results presented in this thesis provide evidence for a membrane-bound pool of centralspindlin that is required for RhoA activation in *C. elegans* embryos and human cells. We uncover mechanisms by which membrane localization of centralspindlin is positively and negatively regulated. Based on our data, we propose a model for activation of RhoA at the equatorial membrane to direct the formation of a single cleavage furrow. We also provide preliminary evidence for how centralspindlin may participate in feedback loops to tightly focus the zone of active RhoA on the membrane during cytokinesis.

Chapter 2: PAR-5/14-3-3 negatively regulates centralspindlin-directed RhoA activity in early *C. elegans* embryos

[This chapter is adapted from Basant et al., *Developmental Cell,* 2015. The *zen-4(S682A)* CRISPR line in Figure 8 was generated in collaboration with Donglei Zhang.]

2.1 Abstract

Localization of the centralspindlin complex on the plasma membrane offers a potential mechanism by which RhoA can be activated locally on the cell equator to direct cytokinesis. In this chapter we provide evidence in support of such a model. We performed genetic and cell biological analyses of a negative regulator of cortical contractility, PAR-5, in early *C. elegans* embryos. We find that PAR-5 globally inhibits RhoA activity by antagonizing centralspindlin localization on the plasma membrane. Our genetic analyses reveal that PAR-5 directs this inhibition by antagonizing centralspindlin oligomerization.

2.2 Introduction

Early *C. elegans* embryos are powerful model system used to understand cell polarity and cytokinesis. The embryo has a very obvious anterior-posterior axis determined early in development, at the one-cell stage. Entry of the sperm designates the future posterior

pole of the fertilized egg (Goldstein and Hird 1996). Following fertilization, the one-cell embryo undergoes a stereotyped RhoA- and ECT-2-dependent (Schonegg et al. 2007) contractility pattern while establishing anterior-posterior polarity; this culminates in the formation of a temporary pseudocleavage furrow (Rose et al. 1995). Our understanding of cytokinesis has benefited significantly by the analysis of polarization in the *C. elegans* embryo (Tse et al. 2012; Dechant 2003; Afshar et al. 2010). ECT-2 activity during pseudocleavage requires a nematode-specific protein called NOP-1 (Tse et al. 2012). In contrast, ECT-2 is primarily activated by centralspindlin during cytokinesis, but there is partial redundancy with NOP-1 at this stage (section 1.8, Figure 3) (Tse et al. 2012). Following polarization, the cell enters anaphase and undergoes cytokinesis resulting in a large anterior and a small posterior daughter cell. A group of widely conserved PAR proteins are essential for this asymmetry (Edwin Munro, Nance, and Priess 2004; Edwin M Munro 2006; Kemphues et al. 1988). While many polarity proteins localize in discrete membrane domains (Nance 2005; Edwin M Munro 2006), PAR-5 (formerly FTT-1) is cytosolic and homogeneously distributed in the cell (Morton 2002; Benton 2002). PAR-5 is a 14-3-3 protein. 14-3-3 proteins are highly helical, dimeric, multi-functional proteins that bind to specific interactors via conserved phosphoserine motifs (Yaffe 1997; Aitken 2006). Most species have several isoforms of 14-3-3 proteins, some of which are functionally redundant, making it challenging to characterize their cellular roles (H. Fu, Subramanian, and Masters 2000; Aitken 2006). PAR-5 is the only 14-3-3 protein expressed in the early *C. elegans* embryo (Morton 2002; W. Wang and Shakes 1997). In addition to polarity defects, depletion of PAR-5/14-3-3 by RNAi results in a striking

increase in contractility during both pseudocleavage and cytokinesis (Morton 2002). This implies that PAR-5 is a negative regulator of contractility. While the mechanism by which PAR-5 regulates polarity of the embryo has been addressed (Benton and St Johnston 2003), it remains completely unknown how PAR-5 regulates contractility. In theory, PAR-5 may antagonize proteins at various steps in the pathway leading to furrow formation (Figure 3) to achieve this. In this chapter we use genetic analysis in *C. elegans* embryos to assess the role of PAR-5 in cortical contractility and its implications for wild-type cytokinesis.

2.3 PAR-5/14-3-3 is an antagonist of centralspindlin: genetic analyses

We first quantified the change in contractility upon PAR-5 depletion, we acquired time-lapse movies of pseudocleavage and cytokinesis in wild-type and *par-5(RNAi)* embryos expressing a PH::GFP membrane marker. As shown in the representative images, embryos depleted of PAR-5 generate multiple furrows at both stages of contractility (Figure 6A and B) and exhibit an increase in cortical contractility. Following the first embryonic division, we did not detect strong hypercontractility in the absence of PAR-5 (not shown).

We next defined the genetic requirements for the hypercontractility resulting from PAR-5 depletion. All contractility in the one-cell stage embryo is dependent on RhoA activation by ECT-2. If PAR-5 inhibits cortical contractility upstream of ECT-2 in the pathway, then the phenotype observed in the absence of PAR-5 should be dependent on the RhoGEF.

We found that the hypercontractile phenotype was suppressed when PAR-5 was depleted in *ect-2* mutant embryos (Zonies et al. 2010)(Figure 6C and D). Because this *ect-2* mutation is hypomorphic (Zonies et al. 2010), furrow formation is not completely abolished during cytokinesis.

Next, we addressed whether PAR-5 inhibition is specific to one of the pathways that activates ECT-2 during cytokinesis. Surprisingly, we found that although centralspindlin is dispensable for pseudocleavage in otherwise wild-type embryos (Tse et al. 2012), the exaggerated hypercontractility during pseudocleavage characteristic of PAR-5 depletion was completely suppressed by loss-of-function mutations in CYK-4 or ZEN-4 (Figure 6C and D and 7A). On the other hand, loss of NOP-1 function did not abolish this hypercontractility seen in the absence of PAR-5 (Figure 6C and D and 7A). The genetic requirements for hypercontractility during cytokinesis were found to be identical to those during pseudocleavage. Thus, PAR-5 is a specific, negative regulator of centralspindlin-dependent RhoA activity (Figure 6E).

When PAR-5 is depleted in embryos where centralspindlin is compromised (Figure 6C), it is surprising that a NOP-1-dependent furrow is not formed during anaphase. However, in the absence of PAR-5, spindle elongation is also restricted, resulting in a high concentration of astral microtubules at the equatorial region which may inhibit NOP-1-dependent furrowing (Dechant 2003; Werner, Munro, and Glotzer 2007).

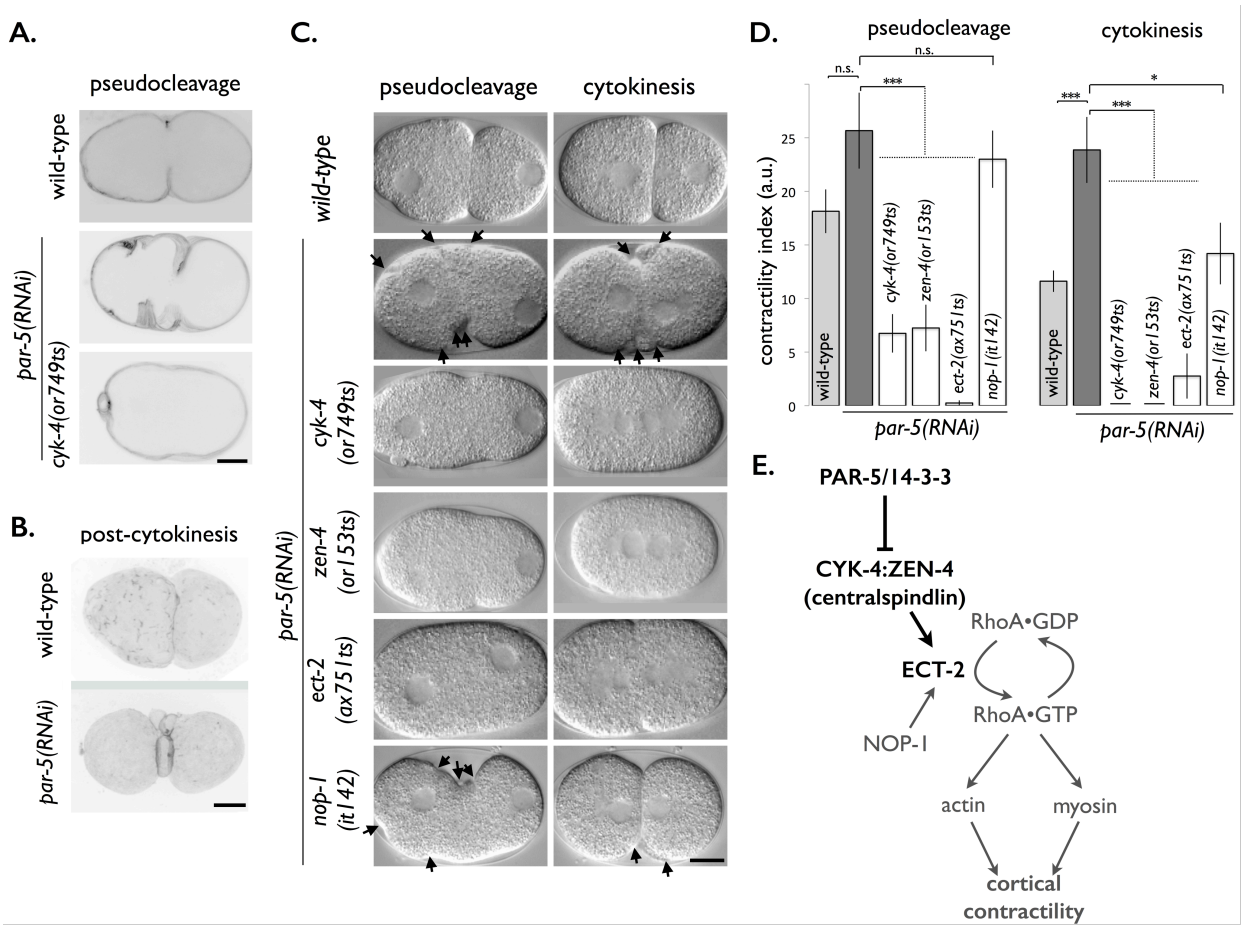


Figure 6. PAR-5/14-3-3 is a negative regulator of contractility and functions to inhibit centralspindlin: genetic analyses. A. Representative maximum intensity projections of time-lapse movies of one-cell *C. elegans* embryos expressing the membrane marker PH::GFP during polarity establishment. B. 3D maximum intensity projections of representative PH::GFP-expressing embryos after completion of cytokinesis. C. One-cell *C. elegans* embryos were filmed by DIC microscopy shortly after fertilization until completion of the first division. Shown are representative images of embryos of the indicated genotypes at pseudocleavage and late anaphase. *ect-2(ax751ts)* is a hypomorphic allele (Zonies et al., 2010). Arrows indicate ectopic membrane furrows. n>5. Scale bars, 10 mm. D. Embryos of the genotypes shown in C. were scored for the extent of contractility observed during pseudocleavage and cytokinesis (see methods for definition of the contractility index). Error bars indicate SEM. n.s. indicates that the populations are not significantly different at p = 0.05. ***p % 0.01 and *p % 0.05 (Mann-Whitney *U* test). a.u., arbitrary unit. E. Schematic illustrating a modified genetic pathway leading to RhoA activation and cortical contractility in *C. elegans* embryos.

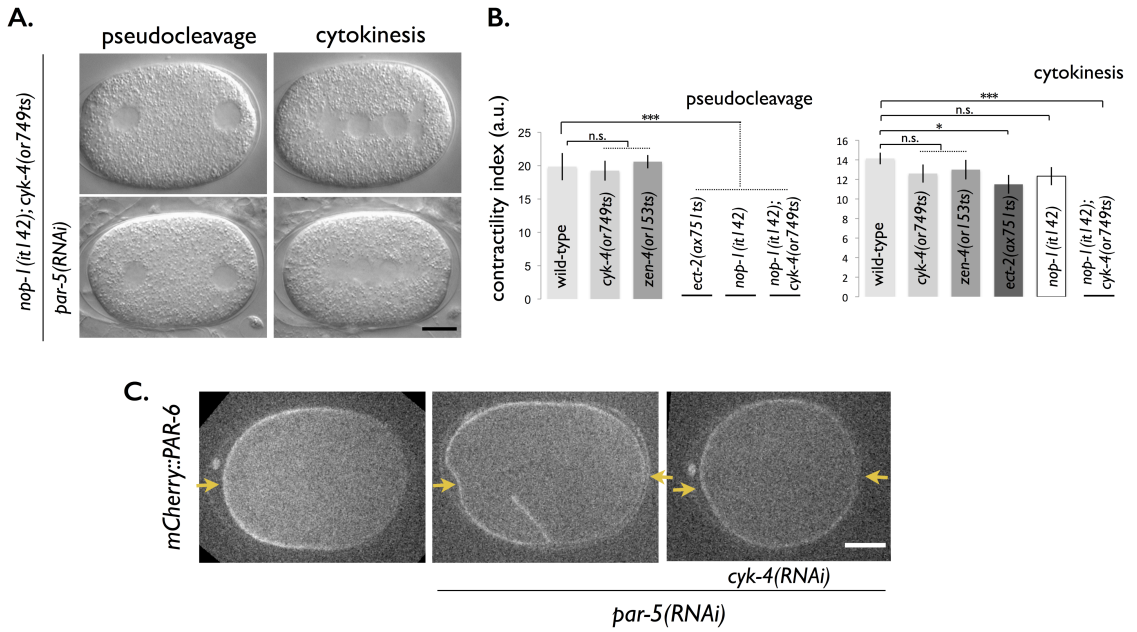


Figure 7. PAR-5/14-3-3 functions to inhibit centralspindlin independent of its role in polarity establishment. A. One-cell *C. elegans* embryos were analyzed by time-lapse DIC microscopy shortly after fertilization until completion of the first division. Shown here are representative images of embryos in the indicated genetic backgrounds at pseudocleavage and late anaphase. B. Embryos of the indicated genotypes were scored for the extent of contractility observed during pseudocleavage and cytokinesis, using the contractility index parameter (n≥ 5; error bars represent ±SEM; n.s. indicates that the populations are not significantly different at p=0.05, *** and * indicate significance at p≤0.01 and p≤0.05 respectively, Mann-Whitney- U). C. *C. elegans* embryos of the indicated genotypes expressing a mCherry::PAR-6 marker were filmed. Shown here are representative images of embryos shortly after the pronuclei are centered. Yellow arrows indicate presence of cortical PAR-6. B. Scale bars, 10µm.

PAR-5 is a known regulator of cell polarity. Hypercontractility is not a common phenotype among polarity defective mutants, therefore the hypercontractility can not be a consequence of the polarity defect. To determine whether PAR-5 has separable functions in polarity and cytokinesis, we assayed PAR protein localization in *par-5* embryos in the absence of CYK-4. Depletion of CYK-4 suppressed hypercontractility, but not the defect in PAR protein localization (Figure 7C). Polarity establishment and inhibition of centralspindlin-dependent contractility are therefore independent functions of PAR-5/14-3-3.

2.4 zen-4(S682A) phenocopies PAR-5/14-3-3 depletion

In human cells, MKLP1/ZEN-4 directly binds 14-3-3 proteins upon S710 phosphorylation and this binding prevents centralspindlin clustering (Douglas et al. 2010). We hypothesized that in *C. elegans*, PAR-5/14-3-3 may bind ZEN-4 at the analogous S682 position (Figure 2C) to prevent oligomer formation and membrane recruitment of centralspindlin. In the absence of PAR-5/14-3-3, we hypothesize that ectopic centralspindlin oligomers on the membrane cause the observed hypercontractility by activating RhoA. If this is true, then abolishing ZEN-4 S682 phosphorylation should mimic PAR-5 depletion. To test this, we took advantage of the CRISPR-associated nuclease Cas-9 system to generate a S682A substitution at the endogenous *zen-4* locus (Zhang and Glotzer 2014). Animals homozygous for this mutation are significantly less healthy than wild-type strains (Figure 8C), but they are viable and we were able to obtain embryos homozygous for *zen-4(S682A).* At the one-cell stage, these embryos demonstrate striking similarity to *par-5(RNAi)* embryos (Figure 8A and B). In contrast to PAR-5 embryos however, *zen-4(S682A)* embryos maintain their anterior-posterior polarity, and the cleavage furrow is asymmetrically positioned along this axis as in wild-type embryos (Figure 8A). Pseudocleavage furrows are often unilateral, and in a small fraction of embryos (2/15) these furrows fail to regress, leading to division of the zygote during polarization. Cells that enter anaphase undergo cytokinesis, but like PAR-5 depleted embryos, these cells exhibit ectopic furrows. In some cases, these embryos exhibit hypercontractility during subsequent divisions cycles (not shown).

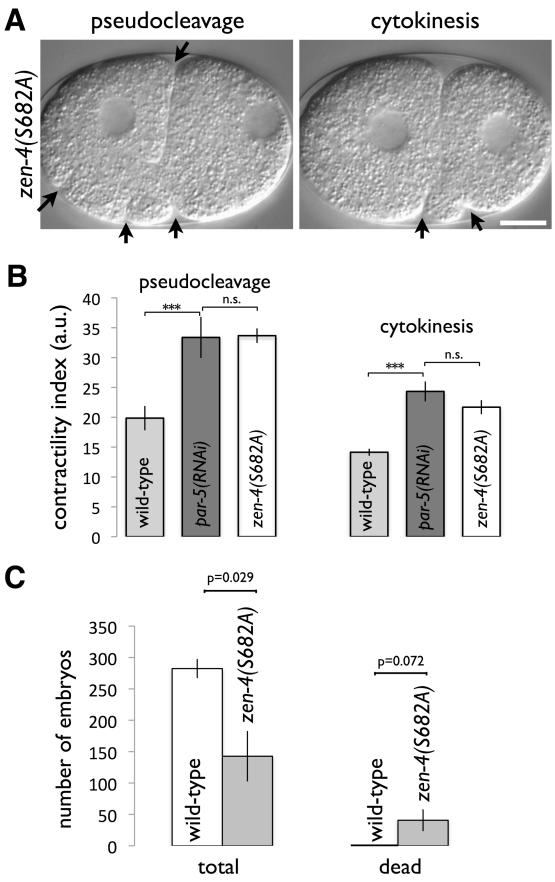


Figure 8. S682A mutation in the endogenous copy of *zen-4* phenocopies PAR-5 depletion - hypercontractility. A. One-cell *C. elegans* embryos homozygous for *zen-4(S682A)* at the endogenous locus were analyzed by time-lapse DIC microscopy shortly after fertilization until completion of the first division. Shown are representative images at pseudocleavage and cytokinesis. Arrows indicate membrane furrows. n = 13. B. Contractility observed in *zen-4(S682A)* was quantified (see methods) and plotted to compare with wild-type and *par-5(RNAi)* embryos (n>7). Error bars represent ± SEM. n.s. indicates that the populations are not significantly different at p = 0.05, ***p % 0.01, Mann-Whitney *U* test. C. *zen-4(S682A)* animals are less healthy than wild-type. Three L4 stage larvae of each strain were transferred to a plate. Animals were transferred to fresh plates every 8-16 hours. The total number of embryos laid were counted. Embryos that failed to hatch after 24 hrs were considered dead. Averages of three experiments are plotted. p values from a Student's t-test are indicated. Scale bars, 10µm.

2.5 PAR-5/14-3-3 negatively regulates cortical localization of centralspindlin and active RhoA

Our data thus far indicates that PAR-5/14-3-3 negatively regulates RhoA activity by antagonizing centralspindlin. To visualize the change in Rho activity in the absence of PAR-5, we used a GFP-tagged RhoA biosensor (Tse et al. 2012). During anaphase in

wild-type embryos, the biosensor accumulates on the cortex, primarily at the ingressing cleavage furrow (Tse et al. 2012). Depletion of PAR-5 leads to a striking, global increase in cortical levels of active RhoA upon anaphase onset, prior to furrowing (Figure 9A and D). Subsequently, the RhoA biosensor is observed in the equatorial region and at the ingressing furrow of the dividing cell (data not shown). Thus, PAR-5 negatively regulates RhoA activity in early embryos.

Because PAR-5 regulates RhoA activity specifically via centralspindlin, we assessed whether the subcellular localization of CYK-4 and ZEN-4 was altered in the absence of PAR-5. Strikingly, we observed an increase in cortical levels of CYK-4 (7/7 embryos) and ZEN-4 (8/8 embryos). This accumulation was often observable at the equatorial membrane prior to furrow ingression in PAR-5 depleted embryos (Figure 9B and C) and was accompanied by a mild decrease in levels of CYK-4 and ZEN-4 at the central spindle that was not found to be statistically significant (Figure 9E). We tested the effect of PAR-5 depletion at other stages in the early embryo. Despite release of PAR-5 inhibition, centralspindlin is not constitutively on the cortex but is recruited in a cell-cycle dependent manner. During pseudocleavage, centralspindlin is cytosolic in wild-type embryos, but CYK-4 and ZEN-4 can be detected on the cortex in *par-5* embryos, most at the tips of ingressing furrows (Figure 10A). This localization is lost as the cell enters mitosis and reappears during anaphase.

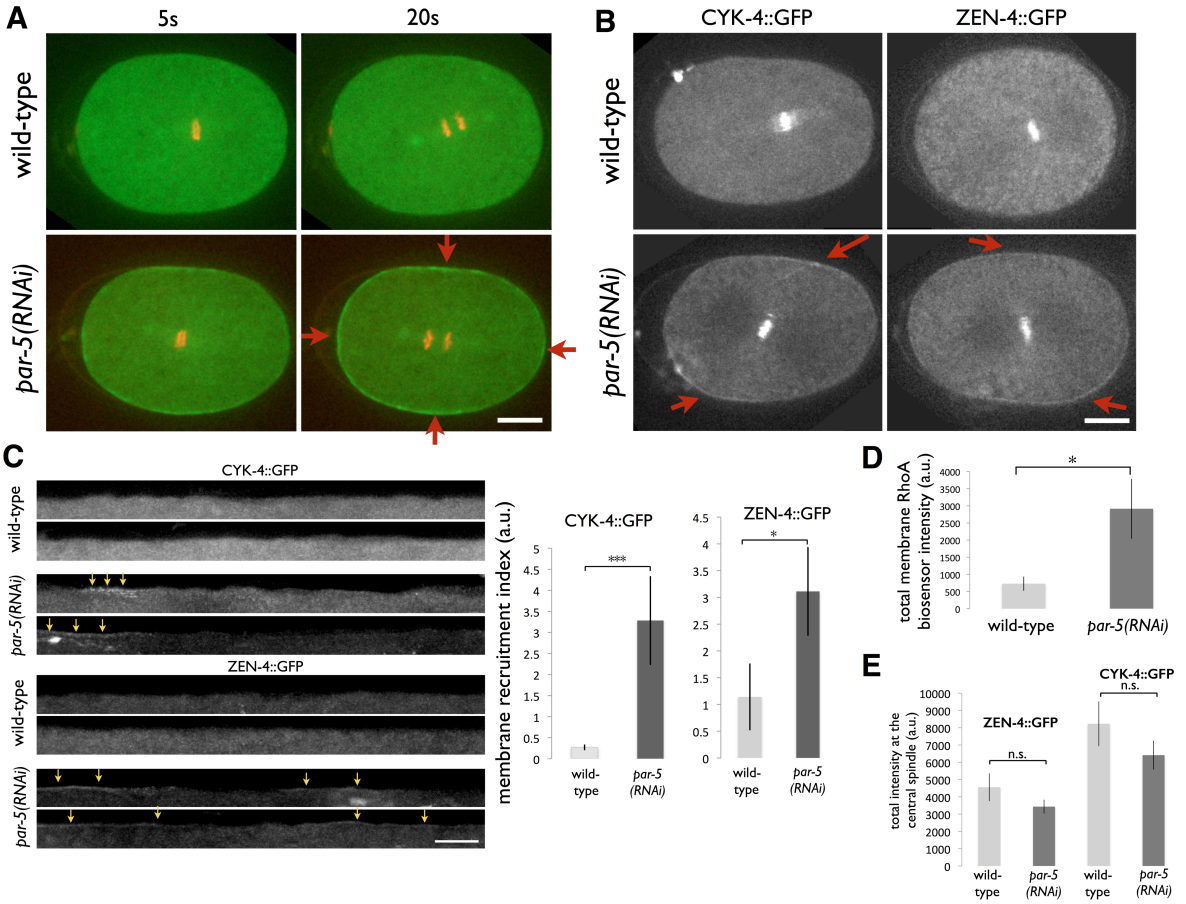


Figure 9. PAR-5/14-3-3 negatively regulates cortical localization of centralspindlin and active RhoA. A. One-cell *C. elegans* embryos expressing the chromatin marker mCherry::HIS and a GFP-tagged RhoA biosensor were filmed starting at metaphase. Representative images of wild-type and *par-5(RNAi)* embryos at the indicated times after anaphase onset are shown. B. Wild-type or PAR-5-depleted embryos expressing either CYK-4::GFP or ZEN-4::GFP were analyzed by time-lapse microscopy. Representative images of embryos 50s after anaphase onset are shown. n>5. Scale bars, 10 mm. C. Wild-type and *par-5(RNAi)* embryos expressing either CYK-4::GFP or ZEN-4::GFP were filmed during the first division cycle. Maximum intensity projections of the first 100s of anaphase were generated. 50 pixels of the cell edge was selected in these projections and straightened. Two examples of each genotype are shown. Yellow arrows indicate cortical recruitment of the fluorescent marker. D. Total membrane intensity of the GFP RhoA biosensor relative to a cytosolic average was measured over the first 100s of anaphase for each embryo and averaged across embryos (n≥6; error bars represent ±SEM; * represent $p < 0.05$, Student's t-test). E. Total intensity of the fluorescent probe at the central spindle was measured for the first 150s of anaphase. n.s. indicates that the populations are not significantly different at p=0.1, Mann-Whitney-U test.

Following the first embryonic division, we did not detect strong hypercontractility in the absence of PAR-5. However, there was an obvious change in membrane recruitment of

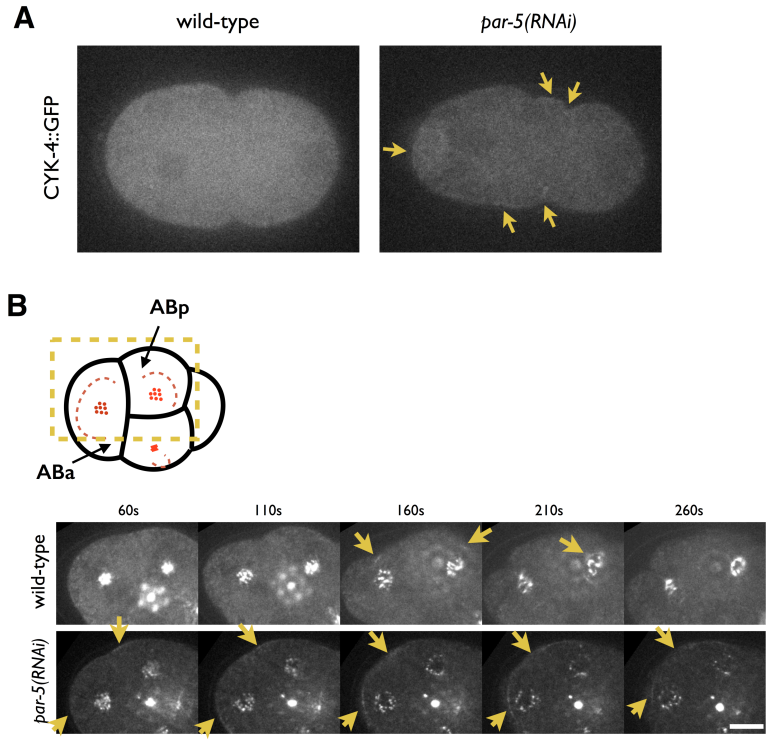


Figure 10. PAR-5/14-3-3 negatively regulates cortical localization of centralspindlin during pseudocleavage and later embryonic divisions. A. Wild-type and *par-5(RNAi)* embryos expressing CYK-4::GFP were imaged by time-lapse microscopy during polarization. Stills at maximum ingression during pseudocleavage are shown. Yellow arrows indicate membrane recruitment of CYK-4. Similar data was obtained for ZEN-4::GFP (not shown). B. The schematic on the top depicts a wild-type four-cell *C. elegans* embryo undergoing its divisions in the anterior blastomeres, ABa and ABp. The embryos are oriented such that the anteriormost blastomere (ABa) is to the left. Localization of centralspindlin is depicted in red dots and dashes. The mitotic spindle assembles perpendicular to the plane of imaging in ABp and ABa, whereas the contractile ring lies in plane. Embryos expressing CYK-4::GFP were filmed starting at metaphase in ABa. Representative images of the region boxed in yellow are shown for the indicated times after anaphase onset, n≥7. Centralspindlin on the spindle midzone appears as a cluster of dots. Upon PAR-5 depletion, CYK-4 is readily detect at the ingressing ring starting at the very edge of the cell. Yellow arrows indicate CYK-4::GFP on the contractile ring. Similar data was obtained for ZEN-4::GFP (not shown). Scale bar, 10µm.

centralspindlin. When embryos undergo division from 4 to 6 cells (ABa and ABp divisions), the contractile ring lies in the imaging plane. When PAR-5 is depleted, CYK-

4::GFP accumulates on the ring prior to its constriction (10/10 embryos) at an earlier stage than in wild-type embryos (Figure 10B).

If the role of PAR-5/14-3-3 binding is to antagonize recruitment of centralspindlin to the plasma membrane, increase in membrane centralspindlin levels should also be observed when the PAR-5 binding site on ZEN-4 is disrupted. To test this, we investigated whether the non-phosphorylatable ZEN-4(S682A) variant causes ectopic membrane recruitment of CYK-4::GFP. As predicted, *zen-4(S682A)* embryos expressing CYK-4::GFP demonstrate increased membrane localization of CYK-4 during pseudocleavage (6/6 embryos) and anaphase (10/10 embryos) (Figure 11).

The 14-3-3 binding site on ZEN-4/MKLP1 is generated through phosphorylation by Ndr kinases (Fesquet et al. 2015) and thus Ndr kinases indirectly regulate RhoA activity. Presently, it is unknown whether this is a conserved mechanism for centralspindlin regulation. In *C. elegans,* SAX-1 and WTS-1 are two Ndr kinases thought to be active in the early embryo. Depletion of both these kinases would be predicted to phenocopy the absence of PAR-5 or the *zen4 (S682A)* mutation. Thus far we have been unable to completely inactivate both Ndr kinases in *C. elegans* embryos simultaneously to test their role in RhoA activation.

Interestingly, an analogous mechanism is employed to regulate the activity of another GTPase, Cdc42. In fission yeast, the 14-3-3 protein Rad24 binds the Cdc42 activator Gef1 to prevent it from associating with cell tips, limiting Gef1 ability and Cdc42

activation (Das et al. 2015). The binding site on Gef1 is generated by the Ndr kinase Orb6 (Das et al. 2015).

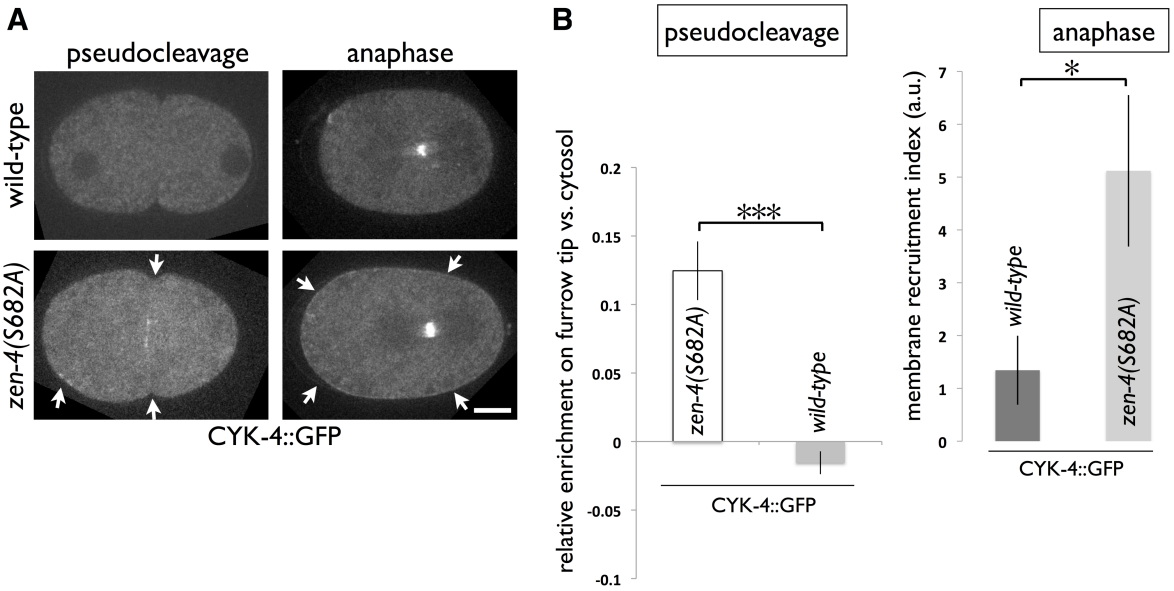


Figure 11. S682A mutation in the endogenous copy of *zen-4* phenocopies PAR-5 depletion - membrane levels of CYK-4. A. Wild-type and *zen-4(S682A)* embryos expressing CYK-4::GFP during pseudocleavage (at maximal ingression) and at 40 s after anaphase onset. Arrows indicate ectopic membrane recruitment of CYK-4::GFP. Pseudocleavage furrows in *zen-4(S682A)* embryos often ingress completely, and CYK-4::GFP can be detected on the furrow tips. n≥5. Scale bar, 10 µm. B. Wild-type and *zen-4(S682A)* embryos expressing CYK-4::GFP filmed during pseudocleavage and anaphase were analyzed for the extent of recruitment of GFP to the furrow tips (pseudocleavage) or to the membrane prior to ingression (anaphase), relative to a cytosolic background (see methods). Error bars represent ±SEM, *** indicates significance at p≤0.01, Student's t-test, * indicates significance at p≤0.05, Mann-Whitney-U test, n≥5.

2.6 Discussion

Our data strongly suggest that PAR-5/14-3-3 directly binds ZEN-4 to inhibit centralspindlin-directed RhoA activation by preventing centralspindlin clustering and membrane localization. Spatiotemporal control of protein oligomerization is a robust strategy to regulate cellular function. While such regulation of centralspindlin

oligomerization by 14-3-3 proteins has previously been reported (Douglas et al. 2010), our data point to a novel function of centralspindlin oligomers in RhoA activation. Additionally, our findings support a role for this multimeric complex at the plasma membrane where its function has previously not been reported.

The generation of protein clusters with increased avidity for a subcellular substrate is a regulatory mechanism exploited by biological systems in a number of contexts and is achieved in a variety of ways. Aggregation may be achieved by phase transitions at a critical concentration of the multivalent complex, resulting in a supramolecular structure that phase-separates from the bulk solution (P. Li et al. 2012), or via a protein scaffold to generate domains of high enzymatic activity. During division plane positioning *S. pombe* employs a strategy where stable cortical nodes of Cdr2 kinase assemble to recruit Mid1, an anillin ortholog that controls actomyosin and facilitates formation of a contractile ring (Almonacid et al. 2009).

PAR-5 and related 14-3-3 proteins regulate the oligomerization of complexes other than centralspindlin. In *C. elegans* embryos, PAR-1, a posteriorly localized kinase, phosphorylates the anterior protein PAR-3 (Bazooka) to generate two 14-3-3 binding sites (Benton and St Johnston 2003). PAR-5 binding inhibits PAR-3 oligomerization and its ability to bind to aPKC thus providing a mechanism to maintain distinct polarity domains (Benton and St Johnston 2003). Despite the large number of 14-3-3 targets, all the hyper contractility seen in *par-5* embryos is due to PAR-5/14-3-3 function vis a vis centralspindlin. Its function in polarization is likely due to effect on PAR-3 (Figure 7). Interestingly in another context, mammalian 14-3-3 isoforms ε and γ, function to

promote multimer formation. In *in vitro* assays 14-3-3 dimers have been demonstrated to directly bind dimers of p53 through a phosphoserine motif. This promotes formation of p53 tetramers that stably interact with DNA (Rajagopalan et al. 2008).

Cell biological and genetic data indicate that centralspindlin oligomers have two defined functions - one in central spindle assembly and a second in RhoA activation at the plasma membrane. However, certain aspects of the latter function remain uninvestigated. First, it would be important to directly establish in *C. elegans* that the oligomerization domain of ZEN-4 is required for furrow ingression in the absence of NOP-1. Secondly, though it has been demonstrated by photobleaching experiments that the spindle-bound pool of centralspindlin is oligomeric (Hutterer, Glotzer, and Mishima 2009), similar data for membrane-bound centralspindlin is lacking. We do not know the stability and dynamics of centralspindlin at the plasma membrane. Fluorescence Correlation Spectroscopy measurements of CYK-4::GFP molecules adjacent to the plasma membrane are likely to shed some light on this question. Further, structural details of the centralspindlin heterotetramer and oligomer are currently lacking. Structural studies and super-resolution microscopy of centralspindlin oligomers *in vivo* and *in vitro* would provide further understanding of their function and the configuration in which they engage with the plasma membrane. Finally, reconstituted systems such as those previously described (Nguyen et al. 2014; Coyle and Lim 2016) could be beneficial to determine if oligomerization of centralspindlin has a direct bearing on the ability of the complex to activate RhoA in addition to membrane binding.

Chapter 3: Requirements for centralspindlin localization to the plasma membrane

[This chapter is adapted from Basant et al., *Developmental Cell,* 2015. The CYK-4::GFP and CYK-4ΔC1::GFP lines in Figure 16 and 17 were generated by Donglei Zhang. The experiments in Figure 18 were performed by Sergey Lekomtsev in the laboratory of Mark Petronczki (Cancer Research UK, London Research Institute).]

3.1 Abstract

Our results thus far reveal a potential role of centralspindlin on the plasma membrane. In this chapter we address the mechanism by which the centralspindlin complex localizes at this site. Using live imaging of fluorescent probes in early *C. elegans* embryos, we find that the complex cannot accumulate on the membrane in the absence of either CYK-4 or ZEN-4. Strong depletion of microtubules does not impair membrane localization of centralspindlin, in fact its accumulation on the membrane is negatively regulated by spindle asters. Finally, we performed structure function analysis in CYK-4 to reveal that the membrane binding C1 domain in CYK-4 is required for RhoA activation in both human cells and *C. elegans* embryos and for membrane accumulation of CYK-4 on wild type furrows in the latter.

3.2 Introduction

The mechanism by which centralspindlin associates with and bundles microtubules is known. ZEN-4 binds microtubules via its N-terminal motor domain (Mishima et al. 2004). Bundling of microtubules by ZEN-4 requires CYK-4 {JantschPlunger:2000ww} and is stabilized by the ability of ZEN-4 to form oligomers (Hutterer, Glotzer, and Mishima 2009). Similar information is unavailable for the membrane-bound pool of centralspindlin. Can centralspindlin directly interact with the plasma membrane or is an adaptor protein required? CYK-4 contains a weak membrane-binding C1 domain (Lekomtsev et al. 2012) which is required late in cytokinesis in human cells. However, it is not known whether this function of the C1 domain is conserved and if it has a bearing on RhoA activation early during cytokinesis for furrow induction. Another likely mechanism is that microtubule bundles adjacent to the plasma membrane assist membrane binding of centralspindlin. However in contrast, it is also known that microtubules can antagonize RhoA activity (Werner, Munro, and Glotzer 2007; Canman, Hoffman, and Salmon 2000). Here we test the role of microtubules and of the CYK-4 C1 domain in centralspindlin localization at the membrane.

3.3 CYK-4 and ZEN-4 are inter-dependent for their cortical localization

At the central spindle, CYK-4 and ZEN-4 are interdependent for their stable association {JantschPlunger:2000ww}. The presence of CYK-4 induces a large conformational change in ZEN-4 (E. A. White et al. 2013; Davies et al. 2015) and this interaction appears to be crucial in microtubule bundling. We observe a similar interdependence in

the localization of the two proteins at the cortex in the absence of PAR-5/14-3-3 (Figure 12).

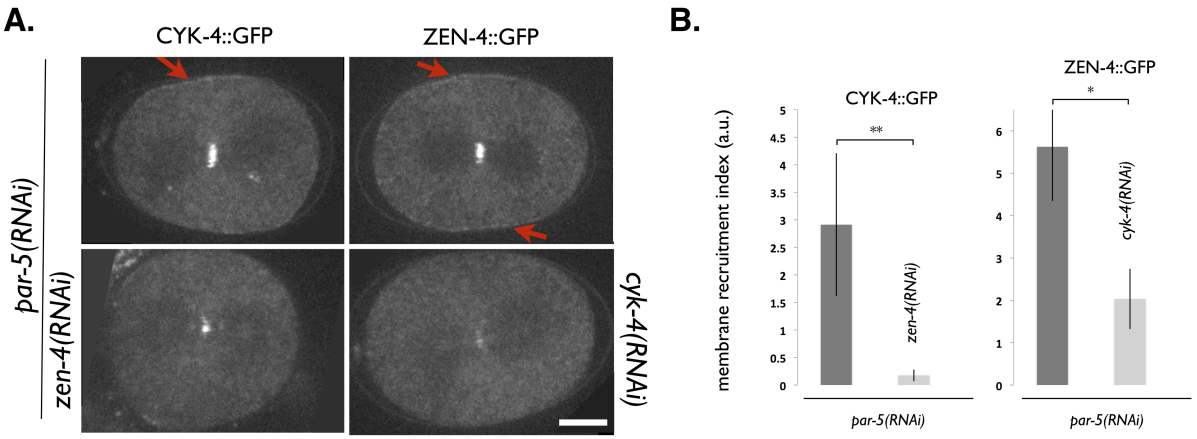


Figure 12. CYK-4 and ZEN-4 are interdependent for their localization to the plasma membrane. A. PAR-5 depleted embryos expressing labeled centralspindlin subunits were analyzed by time-lapse microscopy. Bottom, PAR-5 was depleted in combination with CYK-4 or ZEN-4 as indicated. B. Membrane recruitment of the GFP marker was scored as described in methods. ** and * indicate significance at p≤0.025 and p≤0.05 respectively, Mann-Whitney-U test.

3.4 Centralspindlin can localize to the cortex in the absence of intact microtubules

Because 14-3-3 proteins regulate microtubule bundling by centralspindlin (Douglas et al. 2010), depletion of PAR-5 could induce microtubule-dependent localization of centralspindlin which could in turn promote cortical localization. Therefore we assessed whether cortical recruitment of centralspindlin was microtubule dependent. Disruption of cortical microtubules either via tubulin depletion (data not shown) or nocodazole treatment (Figure 13) does not impede cortical localization of CYK-4 or ZEN-4 in the absence of PAR-5/14-3-3. This suggests that, unlike its localization at the central spindle, there is a microtubule-independent mechanism for membrane recruitment of

centralspindlin. Interestingly however, recruitment of centralspindlin to the membrane is inhibited near the spindle poles (Figure 13A).

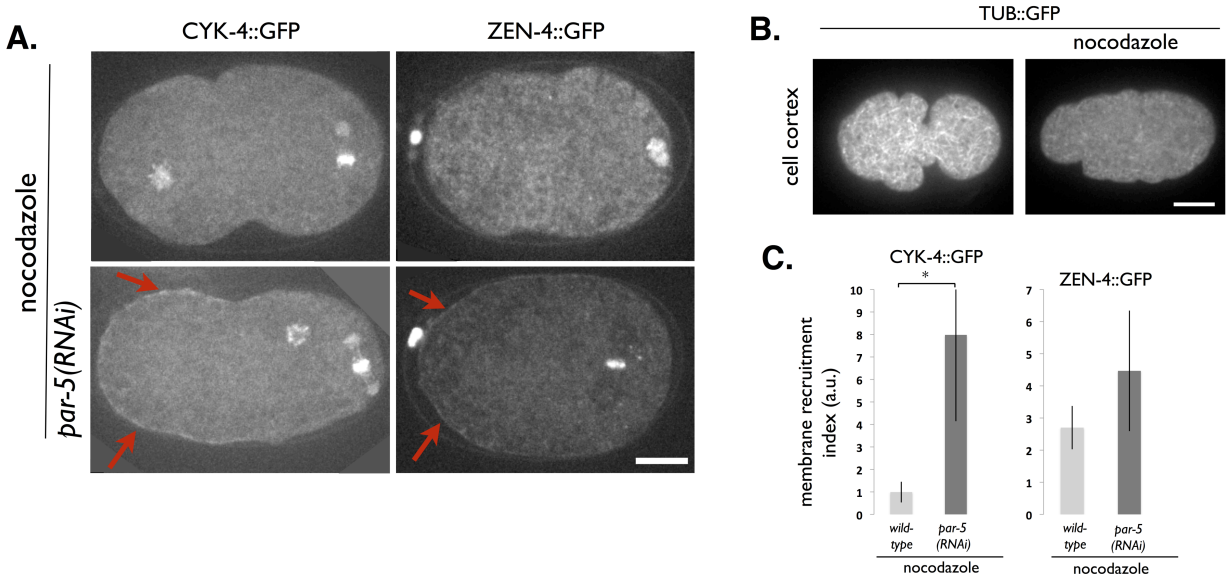


Figure 13. **In the absence of PAR-5, CYK-4 and ZEN-4 can localize in the absence of intact cortical microtubules but are antagonized by astral microtubules.** A. Cortical localization of centralspindlin does not require microtubules, and it is inhibited in regions of the cortex proximal to spindle poles. Wild-type and *par-5(RNAi)* embryos were treated with 50 µg/ml nocodazole. The pronuclei do not meet under these conditions, and the embryo sets up a small posterior spindle during anaphase. Arrows indicate cortical localization of the GFP-tagged protein. n>5. Scale bars, 10 µm. B. Embryos expressing GFP-tagged β- tubulin were filmed during polarity establishment with or without the microtubule depolymerizing drug nocodazole (50µg/mL). Representative images of the cell cortex are shown to demonstrate microtubule depolymerization in the presence of the drug. C. Membrane recruitment of the GFP marker was scored as described in methods. ** and * indicate significance at p≤0.025 and p≤0.05 respectively, Mann-Whitney-U test.

3.5 Astral microtubules negatively regulate cortical centralspindlin and RhoA activity

According to the emerging model, relief of PAR-5 inhibition would lead to global RhoA activation and contractility through ectopic localization of centralspindlin oligomers. Contrary to this prediction, we observe that *par-5(RNAi)* embryos exhibit furrow ingression principally in the equatorial region (Figure 6C). The centralspindlin recruited to the membrane in the absence of PAR-5/14-3-3 is also restricted to regions away from

the spindle poles (Figure 9B and 13A). Astral microtubules that emanate from the

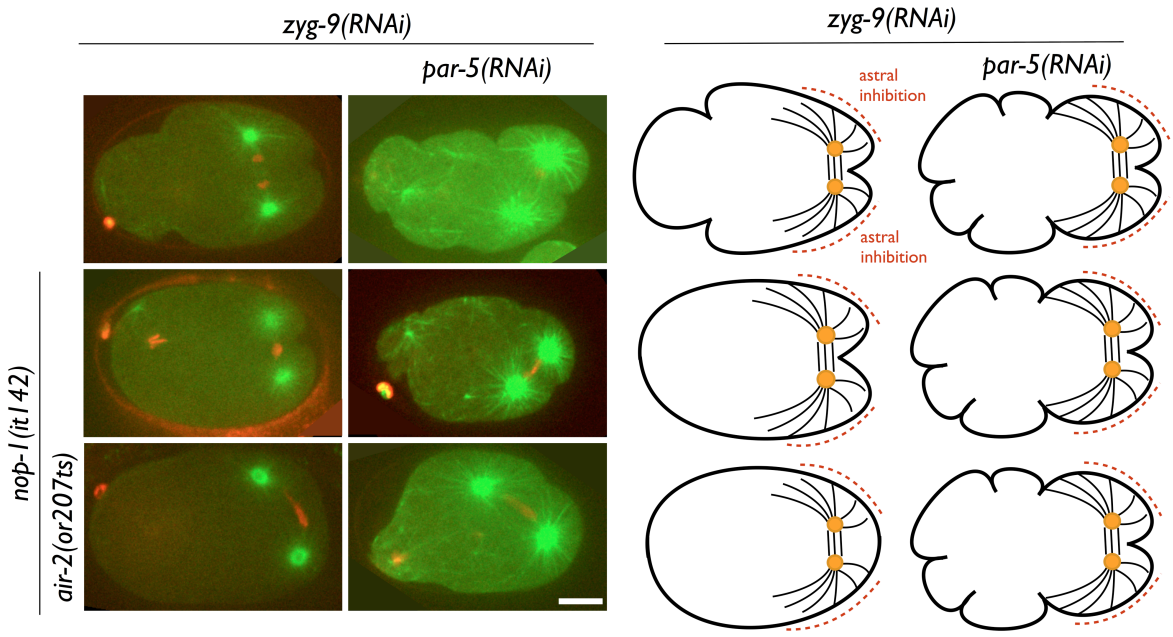


Figure 14. Astral microtubules negatively regulate RhoA activity. One-cell stage *C. elegans* embryos expressing TUB::GFP and mCherry::HIS were depleted of the microtubule-associated protein ZYG-9, resulting in the formation of a small posterior spindle. With the exception of the posterior, midzone-directed furrow, cortical contractility is inversely correlated with the position of the asters. Representative images of cells of the indicated genotypes at anaphase are shown. *air-2* is Aurora B kinase in *C. elegans*. A schematic of the observed cortical contractility is depicted for each condition. The yellow circles represent centrosomes in the posterior spindle. Scale bar, 10 µm.

spindle have been reported to negatively regulate cortical accumulation of RhoA effectors (Tse et al. 2012; Werner, Munro, and Glotzer 2007). Therefore, we determined whether astral microtubules play a role in dictating the site of contractility by manipulating the architecture of the mitotic spindle. We depleted the microtubule associated protein ZYG-9 in order to generate embryos with small, posteriorly localized, spindles, similar to those seen upon nocodazole treatment (Figure 13A). At anaphase,

this results in a centralspindlin-dependent furrow in the posterior and a second, NOP-1-dependent furrow in the anterior, at a distance from the mitotic spindle (Werner, Munro, and Glotzer 2007). If astral inhibition can act on the hypercontractility resulting from depletion of PAR-5, then

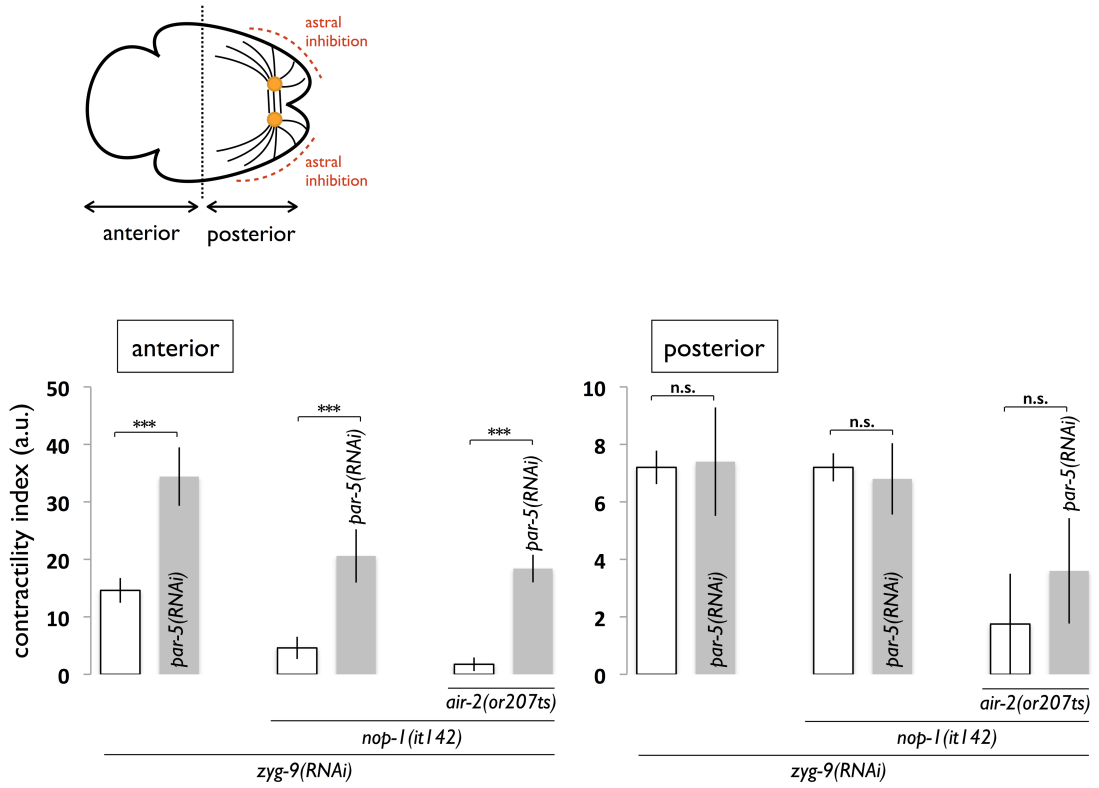


Figure 15. Astral microtubules negatively regulate RhoA activity - quantitation. Embryos of the indicated genotypes were scored for the extent of contractility observed in the anterior and posterior regions, using a parameter we define as contractility index (see methods) (n=5; error bars represent ±SEM, n.s. indicates that the populations are not significantly different at p=0.05, *** indicates significance at p≤0.01, Mann-Whitney-U test).

contractility should be restricted to regions of the cortex where astral inhibition is absent. We depleted embryos expressing GFP-tagged tubulin and mCherry-tagged histone of either ZYG-9 alone or both ZYG-9 and PAR-5. These embryos were filmed to assess

contractility relative to position of the spindle. Embryos depleted of both PAR-5 and ZYG-9 exhibit enhanced contractility as compared to embryos depleted of ZYG-9 alone (Figures 14 and 15). In a *nop-1* mutant background, *zyg-9(RNAi)* embryos primarily exhibit a posterior furrowing (3/5 embryos, no anterior furrow; 2/5 embryos, weak anterior furrow), whereas co-depletion of ZYG-9 and PAR-5 generates additional contractility in the anterior (5/5 embryos). *nop-1(it142); air-2(or207ts)* mutant embryos where both NOP-1 and Aurora B kinase are inactivated, demonstrate little contractility at anaphase at the restrictive temperature, regardless of spindle position (4/5 embryos, no furrows; 1/5 embryos, weak posterior furrow). However, depletion of PAR-5 in these double mutants restores anterior and posterior contractility, and the cortex appears quiescent only in regions enriched in astral microtubules (5/5 embryos). Cortical centralspindlin upon PAR-5 depletion was also confined to the anterior of the cell, away from the asters (Figure 13A). Thus, astral microtubules suppress contractility resulting from PAR-5 depletion and the equatorial furrow under these conditions likely forms due to local relief from this inhibitory effect, much like the formation of an equatorial furrow in a centralspindlin-deficient embryo (Werner, Munro, and Glotzer 2007).

3.6 CYK-4 C1 domain is required for cortical centralspindlin and RhoA activation

Thus far it is apparent that centralspindlin membrane localization is not promoted by microtubules in the absence of PAR-5. Therefore an alternate mechanism must exist. CYK-4 contains a membrane binding C1 domain. In human cells, a single C1 domain

from HsCyk4 has a weak affinity for the membrane, but synthetic tandem C1 repeats interact strongly with the membrane (Lekomtsev et al. 2012). This suggests that centralspindlin oligomers could stably associate with the membrane via the C1 domain to achieve RhoA activation. We first tested whether the C1 domain is required for cytokinesis in *C. elegans*. We generated strains expressing either RNAi-resistant CYK-4ΔC1::GFP or full-length CYK-4::GFP. Upon depletion of endogenous CYK-4, we find that embryos expressing only CYK-4ΔC1::GFP undergo furrow ingression but fail cytokinesis, whereas CYK-4::GFP fully rescues the *cyk-4(RNAi)* phenotype (Zhang and Glotzer 2015). This phenotype appears similar to the role described for the CYK-4 C1 domain in human cells (Lekomtsev et al. 2012). However, because *C. elegans* embryos have a centralspindlin-independent pathway for RhoA activation (Tse et al. 2012), we sought to determine the contribution of the C1 domain towards furrow induction when contractility was entirely centralspindlin-dependent. We generated *nop-1(it142)* strains expressing the RNAi-resistant CYK-4ΔC1::GFP or full-length CYK-4::GFP transgenes.

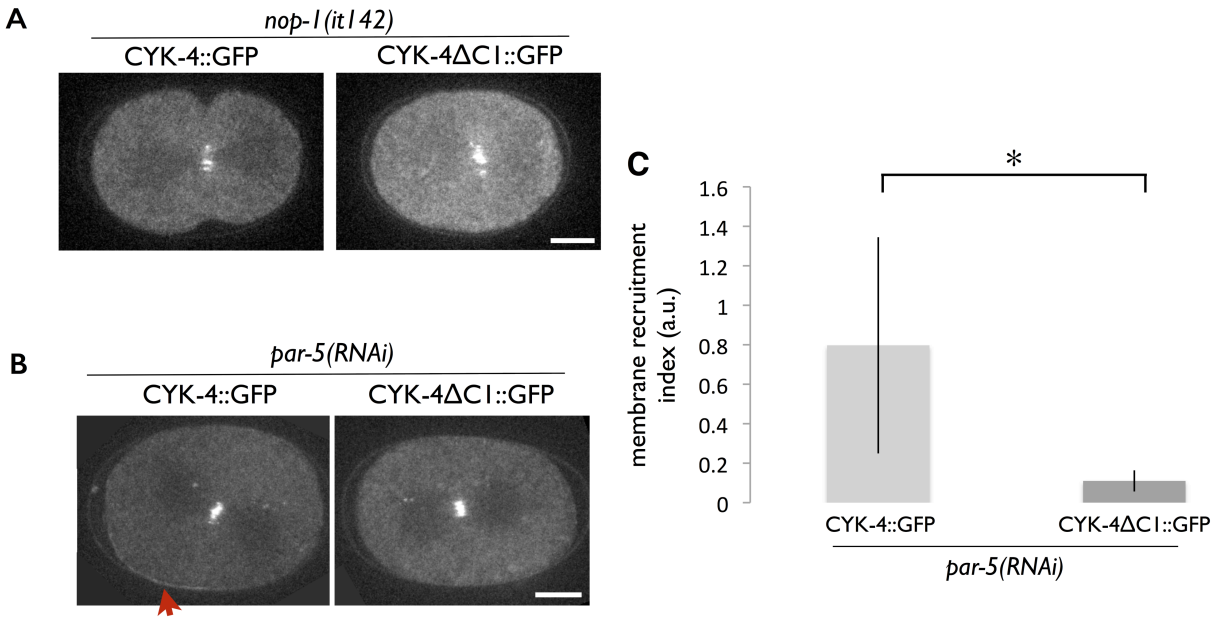


Figure 16. CYK-4 C1 domain is required for cortical centralspindlin and RhoA activation in early *C. elegans* embryos in the absence of PAR-5. A. *nop-1(it142)* embryos expressing RNAi-resistant wild-type CYK-4::GFP or CYK-4ΔC1::GFP transgenes were depleted of endogenous CYK-4 by RNAi and filmed during the first division. Representative images of embryos 200 s after anaphase onset are shown. No cleavage furrow is formed in the absence of the C1 domain though the central spindle assembles normally. n>5. B. *C. elegans* embryos expressing RNAi-resistant wild-type CYK-4::GFP or CYK-4ΔC1::GFP were simultaneously depleted of endogenous CYK-4 and PAR-5. The arrow indicates cortical CYK-4::GFP. n≥10. C. Embryos expressing CYK-4::GFP or CYK-4ΔC1::GFP filmed under the indicated conditions were scored for the extent of membrane recruitment of the GFP marker during the first 100s of anaphase (see methods). Error bars represent ±SEM, * indicates significance at p≤0.05, Mann-Whitney-U test, n≥10.

Embryos with these genotypes were filmed to visualize the extent of furrowing during cytokinesis and CYK-4 localization. We find that while the spindle localization of both transgenes appears normal in the absence of endogenous CYK-4, furrowing is blocked entirely in *nop-1(it142);* CYK-4 ΔC1::GFP embryos (5/5), but cell division completes successfully in embryos expressing the wild-type (8/8 embryos) transgene (Figure 16A). The membrane-binding property of the C1 domain is therefore essential for

centralspindlin-dependent furrow induction.

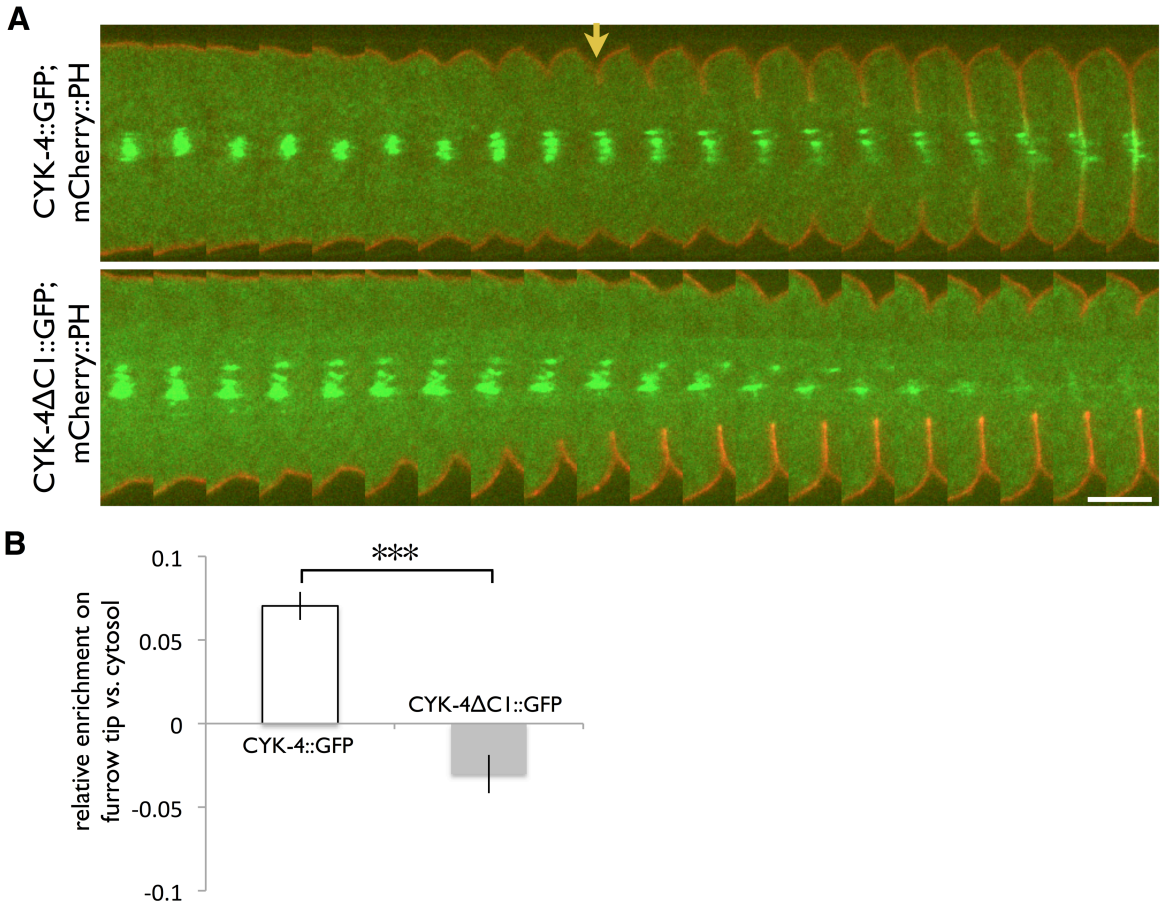


Figure 17. CYK-4 C1 domain is required for cortical centralspindlin and RhoA activation in wild-type *C. elegans* embryos. A. *C. elegans* embryos expressing a mCherry::PH membrane marker with an RNAi-resistant wild-type CYK-4::GFP or CYK-4ΔC1::GFP transgene were depleted of endogenous CYK-4 by RNAi. These embryos were filmed starting at metaphase in the first division cycle. Shown are montages of the equatorial region as the cell divides (n≥5). The arrow indicates the appearance of cortical CYK-4::GFP under wild-type conditions. Scale bar, 10μm. B. Embryos expressing CYK-4::GFP or CYK-4ΔC1::GFP were scored for the extent of recruitment of the GFP marker to the ingressing furrow tip during anaphase, relative to a cytosolic background (see methods). Error bars represent ±SEM, *** indicates significance at p≤0.01, Student's t-test, n≥7.

CYK-4 and ZEN-4 can localize to the cortex in a microtubule-independent manner (Figure 13). As the CYK-4 C1 domain contributes to cortical contractility, this domain could mediate recruitment of CYK-4 to the membrane. We assayed the ability of CYK-

4::GFP and CYK-4ΔC1::GFP to bind to the membrane upon PAR-5 depletion. Strains expressing RNAi-resistant CYK-4ΔC1::GFP and CYK-4::GFP transgenes were simultaneously depleted of endogenous CYK-4 and PAR-5 by RNAi. As seen previously (Figure 9B), full-length CYK-4 localizes to the equatorial cortex in the absence of PAR-5 (9/10 embryos), however CYK-4ΔC1 was not detectable at the cortex (11/11 embryos)(Figure 16B and C) and no hypercontractility was observable (not shown) while the accumulation at the central spindle was normal. Finally, to determine whether the C1 domain is responsible for membrane recruitment of CYK-4 in wild-type embryos, we expressed CYK-4ΔC1::GFP or full-length CYK-4::GFP along with a membrane marker. We find that upon depletion of endogenous CYK-4, full-length CYK-4::GFP is observed at the tips of the ingressing furrow, however CYK-4ΔC1::GFP is not detectable (Figure 17A and B).

3.7 The C1 domain and the ability of HsCyk4 to associate with MKLP1 promotes RhoA activation in human cells

Our results indicate that membrane-bound centralspindlin is critical for RhoA activation during cytokinesis in *C. elegans* embryos. In apparent contradiction to our observations, in human cells, the cytokinetic furrow ingresses deeply with essentially wild-type kinetics in the absence of the C1 domain of HsCyk4, although it ultimately regresses (Lekomtsev et al. 2012). This suggests that the membrane tether provided by HsCyk4 is not

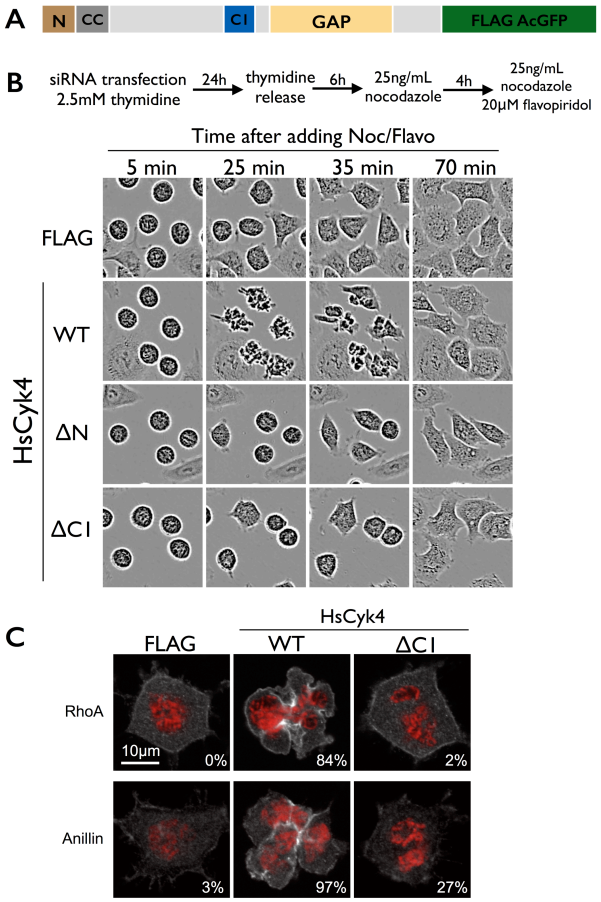


Figure 18. HsCyk4 C1 domain is required for RhoA activation in human cells lacking an intact spindle. A. Shown is the domain structure of the full-length HsCyk4 construct, truncations of which are used in B. N, N-terminal domain that interacts with MKLP1/ZEN-4. B. Schematic depicting conditions used for mitotic arrest and release of HeLa cells. HeLa cells transfected with siRNA targeting endogenous HsCyk4 and the indicated siRNA-resistant constructs (ΔN lacks the HsCyk4 N-terminus; ΔC1 lacks only the C1 domain) were imaged by DIC microscopy at different time intervals following nocodazole (Noc)/flavopiridol (Flavo) treatment. Cells expressing full length HsCyk4 are hypercontractile for close to an hour before flattening onto the substrate. ΔN and ΔC1 HsCyk4 expressing cells remain round until they respread onto the substrate. C. HeLa cells treated as described in A. were stained with anti-RhoA (white) and anti-anillin (white) antibody to visualize recruitment to the membrane. Percentages indicate the fraction of cells exhibiting cortical localization of each marker. Chromatin is marked in red.

essential for cortical RhoA activation early in cytokinesis. Because the central spindle microtubules in these cells lie relatively close to the equatorial membrane, we hypothesized that microtubule-dependent processes may permit delivery of the centralspindlin complex to the membrane thereby obscuring the role of the C1 domain in

RhoA activation. Therefore, we assessed RhoA activation in cells in the absence of an intact spindle. Cells depleted of endogenous HsCyk4 by siRNA were arrested in mitosis. Using a Cdk1 inhibitor flavopiridol, the cells were released into anaphase in the presence of a low dose of nocodazole (Figure 18A). When these cells are supplemented with wild-type, siRNA-resistant HsCyk4, they exhibit dramatic, global contractility. However, this contractility is absent when the C1 domain is deleted (Figure 18B) and RhoA and its effector anillin are no longer recruited to the membrane (Figure 18C). These data suggest that inactivation of microtubule-dependent mechanisms of cortical centralspindlin recruitment renders the C1 domain of HsCyk4 crucial for RhoA activation in human cells. Moreover, the ability of HsCyk4 to activate RhoA and generate contractility is also lost when its MKLP1 binding, N-terminal domain is deleted (Figure 18B). These data suggest that MKLP1 plays a role in RhoA activation by HsCyk4 at the membrane, and this function of centralspindlin is conserved in *C. elegans* and human cells.

3.8 Discussion

Our data indicate that the centralspindlin complex, previously known for its ability to bundle microtubules in the spindle midzone, can localize to the membrane via the CYK-4 C1 domain. This function is required for RhoA activation and is conserved between *C. elegans* and human cells. The C1 domain has weak membrane affinity (Lekomtsev et al. 2012), but is likely to associate stably with the plasma membrane once oligomerized,

a property conferred by ZEN-4. This would explain why both components of centralspindlin are interdependent for their membrane localization (discussed further in section 7.1).

Although the ability of centralspindlin to associate with the membrane is not strictly dependent on intact microtubule arrays in the absence of PAR-5 (Figure 13), in wild-type embryos the complex could simultaneously bind microtubules and the membrane, and microtubule-mediated processes could facilitate plasma membrane localization of centralspindlin at different steps in cytokinesis. Processive transport of centralspindlin along microtubules could promote cortical accumulation. In addition, the accumulation of centralspindlin at the midzone would generate a local pool that enhances equatorial - as opposed to global - accumulation of centralspindlin. This pool could diffuse to the membrane or, as the cleavage furrow ingresses, ECT-2 associated with centralspindlin on the spindle midzone could be brought into direct contact with the plasma membrane where it would enhance RhoA activation and sustain continued ingression of the furrow. Finally as discussed on section 1.8, microtubules are required for phosphorylation of CYK-4 by Plk1 (Wolfe, Takaki, Petronczki, and Glotzer 2009a), making them indispensable for a functional interaction between CYK-4 and ECT-2.

This spindle midzone accumulation of ECT-2-associated centralspindlin could underlie the differential requirements for the C1 domain in *C. elegans* embryos and human cells. In human cells, where the equatorial plasma membrane is adjacent to the midzone, the spindle midzone accumulation of the centralspindlin-ECT-2 complex and the two membrane-binding domains present in ECT-2 could suffice to locally concentrate and

activate RhoA in the absence of the Cyk4 C1 domain. Our data suggests that the relative sizes of the cell and mitotic spindle are likely to determine the extent to which these parallel mechanisms contribute to furrow induction.

Astral microtubules appear to play an inhibitory role in patterning the sites of cortical contractility. Specifically, in the absence of PAR-5/14-3-3, contractility during anaphase is restricted to the equatorial region of the cell. What provides the spatial cue in these embryos to direct equatorial furrowing, as centralspindlin would be predicted to oligomerize throughout the embryo? By repositioning the mitotic spindle to the posterior in PAR-5 depleted embryos (Figure 14), we found that contractility was widespread with the exception of cortical regions proximal to the asters. This regulation is directly analogous to the spatial regulation of NOP-1-dependent activation of RhoA by astral microtubules (Tse et al. 2012). This suggests that astral microtubules inhibit RhoA-dependent contractility independent of the activating pathway, and perhaps functions at the level of RhoA or its effectors. However, our lab recently demonstrated that active RhoA can accumulate and generate ectopic cleavage furrows at the spindle poles (Wagner and Glotzer 2016a). Additionally, we have observed that centralspindlin localization at the membrane is inhibited by astral microtubules (Figures 13 and 16). Do astral microtubules deliver a molecule that inhibits centralspindlin binding the membrane? Alternatively, it is plausible that spindle asters antagonize ECT-2 function or localization resulting in the disruption of the centralspindlin-ECT-2 complex. A possible model is that asters deliver a phosphatase that antagonizes Plk1 function, and thereby

disrupting the interaction between ECT-2 and CYK-4, however this remains to be tested.

Chapter 4: Role of the Chromosome Passenger Complex in promoting centralspindlin-directed RhoA activation

[Section 4.1 is adapted from Basant et al., *Developmental Cell,* 2015. The *C. elegans* strains in Figure 22 were generated by Katrina Longhini.]

4.1 Abstract

The Chromosome Passenger Complex (CPC) has a role in regulating the oligomerization of centralspindlin and in consequent central spindle assembly (Douglas et al. 2010; Kaitna et al. 2000; Guse, Mishima, and Glotzer 2005). It is unclear whether this is the only role performed by the CPC during cytokinesis and whether its known function has any bearing on RhoA activation at the plasma membrane. In this chapter we demonstrate that CPC activity is strictly required for centralspindlin-dependent RhoA activation and furrow formation in both *C. elegans* embryos and human cells during cytokinesis. Interestingly, in *C. elegans* embryos we also find that even though CPC activity is required for localizing centralspindlin at the cleavage furrow in wild-type cells, CPC components cannot be detected on the plasma membrane.

4.2 Introduction

In human cells, MKLP1 directly binds ζ and γ isoforms of 14-3-3 via S710 phosphorylation (position S682 in *C. elegans*)(Figure 2C). 14-3-3 binding prevents

centralspindlin oligomerization and its accumulation on microtubules (Douglas et al. 2010). Phosphorylation of MKLP1 by Aurora B kinase, a component of the Chromosome Passenger Complex (CPC) at S708 (position S680 in *C. elegans*) (Guse, Mishima, and Glotzer 2005), releases 14-3-3 binding and facilitates centralspindlin association with the spindle (Douglas et al. 2010). Indeed in *C. elegans* embryos, the CPC is required for stable accumulation of ZEN-4 at the spindle and completion of cytokinesis (Kaitna et al. 2002). We hypothesized that a similar mechanism could regulate cortical activation of RhoA. The CPC has been reported to accumulate on the equatorial membrane during anaphase prior to active RhoA (Cooke, Heck, and Earnshaw 1987; Eckley et al. 1997; Murata-Hori and Wang 2002; Szafer-Glusman, Fuller, and Giansanti 2011; Kitagawa et al. 2013), but no function has been assigned to this pool of the complex. It is likely that the CPC disrupts PAR-5 inhibition by Aurora B kinase-mediated phosphorylation of ZEN-4 to locally induce oligomerization, membrane association, and contractility. Here, we genetically test this model in *C. elegans* embryos and using small molecule inhibitors in human cells. In addition, we have developed tools to assess the localization of endogenously-tagged CPC components in *C. elegans* embryos.

4.3 The Chromosome Passenger Complex antagonizes PAR-5/14-3-3 inhibition on centralspindlin in C. elegans embryos

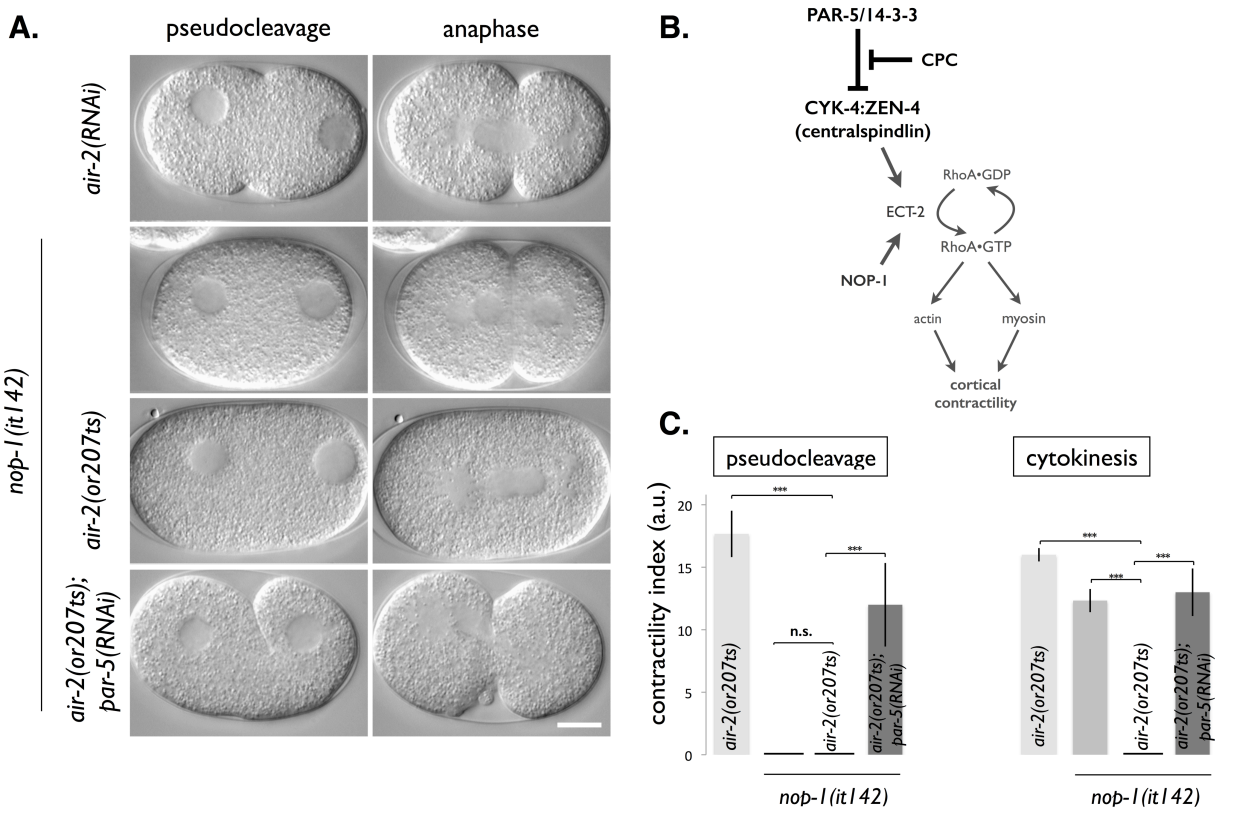


Figure 19. Aurora B kinase antagonizes PAR-5/14-3-3 inhibition on centralspindlin in *C. elegans* embryos. A. One-cell *C. elegans* embryos were analyzed by time-lapse DIC microscopy shortly after fertilization until completion of the first division. Shown are representative images of embryos of the indicated genotypes at pseudocleavage and late anaphase. The temperature-sensitive allele *air-2(or207ts)* abolishes Aurora B kinase activity (Severson et al., 2000). AIR-2 depletion/inactivation results in defects in chromosome segregation that are independent of its role in cytokinesis. n≥7. Scale bar, 10 µm. B. Schematic for a modified genetic pathway activating RhoA and generating cortical contractility in *C. elegans* embryos. C. Embryos of the indicated genotypes were scored for the extent of contractility observed during pseudocleavage and cytokinesis, using a parameter we define as contractility index (see methods) (n≥ 7; error bars represent ±SEM, n.s. indicates that the populations are not significantly different at p=0.05, *** indicates significance at p≤0.01, Mann-Whitney-U test).

Our model (Figure 19B) predicts that, if the centralspindlin-independent NOP-1 pathway and the CPC component Aurora B kinase (AIR-2) are simultaneously inactivated, furrows should not form during pseudocleavage or cytokinesis. We imaged early embryos defective in NOP-1 and mutant for a temperature-sensitive Aurora B kinase

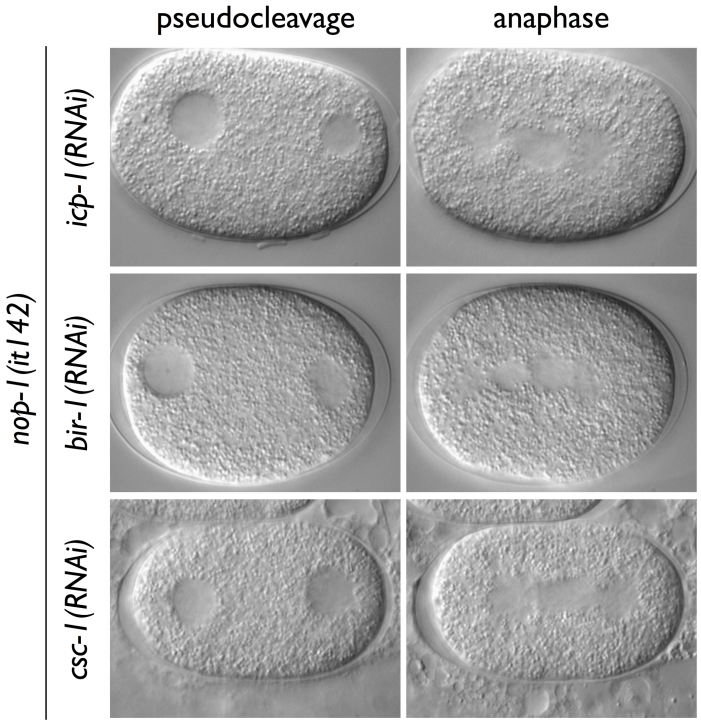


Figure 20. All components of the CPC are required in its function of promoting contractility in *C. elegans* embryos. One-cell *C. elegans* embryos were analyzed by time-lapse DIC microscopy shortly after fertilization until completion of the first division. Shown are representative images of embryos of the indicated genotypes at pseudocleavage and late anaphase. ICP-1 = INCENP, BIR-1 = Survivin, CSC-1 = Borealin. Depletion of CPC components results in defects in chromosome segregation that are independent of its role in cytokinesis. n≥4. Scale bar, 10 μm.

(Severson et al. 2000) using DIC microscopy at the non-permissive temperature. Consistent with the model, no contractility is observed in *nop-1(it142); air-2(or207ts)* embryos (Figure 19A and C, 9/9 embryos). This is also true when other components of the CPC are depleted by RNAi in *nop-1* mutant embryos (Figure 20). The model further predicts that depletion of PAR-5 in these double mutant embryos should restore both pseudocleavage and cytokinetic furrows. We depleted PAR-5 in *nop-1(it142); air-2(or207ts)* embryos by RNAi and observed that contractility was indeed restored at both stages (Figure 19A and C, 11/12 embryos). Although the cytokinetic furrow is restored,

the cells rarely divide. The restored furrows are associated with cortical CYK-4 (not shown). These data strongly support a model in which CPC promotes centralspindlin-directed cortical RhoA activation by inactivating the inhibitor PAR-5.

4.4 Aurora B kinase is required for accumulation of CYK-4 on the ingressing cytokinetic furrow in C. elegans embryos

Our experiments thus far establish a genetic role for the Chromosome Passenger Complex in centralspindlin-mediated RhoA furrow induction. Our data suggest that activity of Aurora B kinase promotes formation of centralspindlin oligomers in the equatorial region of a dividing cell and facilitate their localization to the plasma membrane to locally activate RhoA.

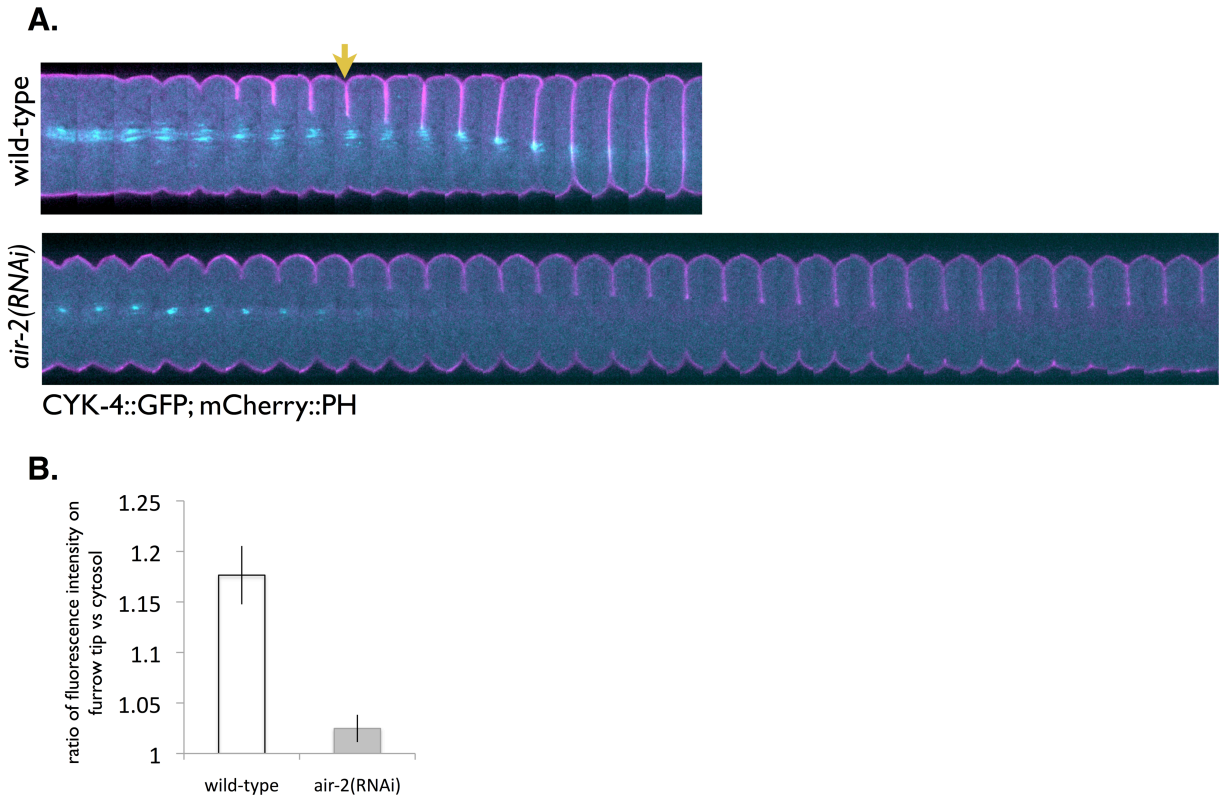


Figure 21. Aurora B kinase is required for accumulation of CYK-4 on the ingressing cytokinetic furrow in *C. elegans* embryos. A. *C. elegans* embryos expressing a mCherry::PH membrane marker (pink) with a CYK-4::GFP transgene (cyan) were depleted of endogenous Aurora B (AIR-2) by RNAi. These embryos were filmed starting at metaphase in the first division cycle. Shown are montages of the equatorial region as the cell divides (n≥5). The arrow indicates the appearance of cortical CYK-4::GFP under wild-type conditions. Scale bar, 10µm. B. Embryos expressing CYK-4::GFP were scored for the extent of recruitment of the GFP marker to the ingressing furrow tip during anaphase, relative to a cytosolic background (see methods). Error bars represent ±SEM, *** indicates significance at p≤0.01, Student's t-test, n≥5.

25We tested whether Aurora B was required for cortical accumulation of centralspindlin during wild-type cytokinesis. We depleted Aurora B (AIR-2) by RNAi in embryos expressing CYK-4::GFP and a membrane marker mCherry::PH. Along the lines of previous reports (Verbrugghe and White 2004), we find that compared to CYK-4 accumulation on the tips of ingressing wild-type furrows, upon AIR-2 depletion CYK-4 was not detectable on furrows ingressing to the same extent (Figure 21).

4.5 Localization of endogenously-tagged Chromosome Passenger Complex components in early C. elegans embryos

In several cell types analyzed, components of the CPC have been observed at the central spindle and equatorial cortex during anaphase. Our genetic and cell biological evidence in *C. elegans* provides a potential role for membrane-localized CPC in centralspindlin-directed RhoA activation. We wished to test whether a membrane localized pool of the CPC could be detected in the early *C. elegans* embryo. Endogenous copies of AIR-2 (Aurora B) and CSC-1 (borealin) were tagged with a neon green fluorescent probe using CRISPR/Cas9. We observed both these components of the CPC localize on chromatin, centromeres and the central spindle as expected and exhibit no obvious defects in cell division. However no membrane-bound pool could be

detected (Figure 22).

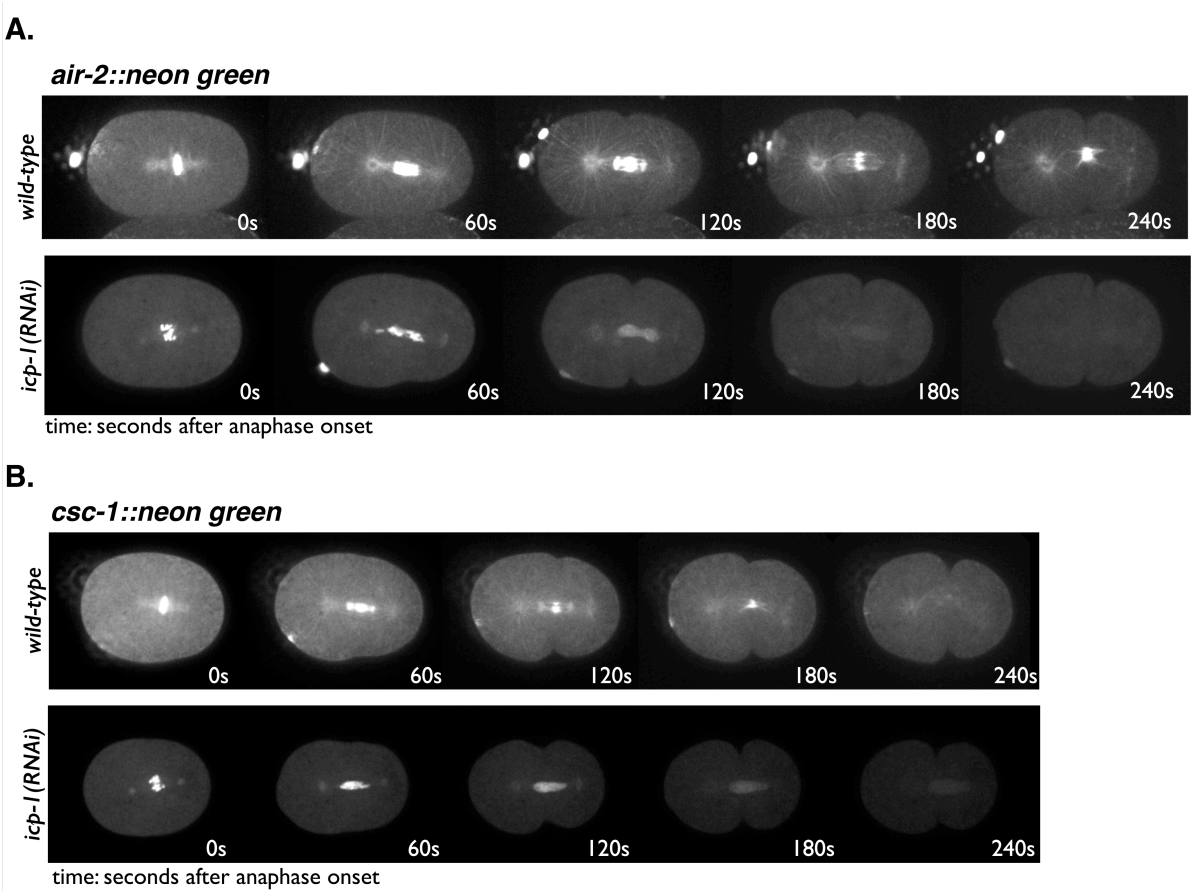


Figure 22. Localization of endogenously-tagged AIR-2 (Aurora B) and CSC-1 (Borealin) in early *C. elegans* embryos. *C. elegans* embryos expressing either endogenously tagged A. Aurora B (*air-2::nG*) or B. Borealin (*csc-1::nG*) were filmed starting at metaphase in the first division cycle. The embryos are either wild-type or depleted of endogenous INCENP (ICP-1) by RNAi. In wild-type embryos both tagged proteins can be detected on centromeres, central spindle and astral microtubules. In the absence of INCENP, cells fail to segregate their chromosomes accurately, and astral and central spindle microtubule localization of the fluorescent probes is lost. n≥4.

Unexpectedly, AIR-2 and CSC-1 also localized on astral microtubules. INCENP (ICP-1) of the CPC contains a microtubule binding domain (van der Horst et al. 2015; Samejima et al. 2015). The localization of CPC components observed on microtubules was lost in the absence of (ICP-1) (Figure 22)(4/4 embryos in each strain).

4.6 Aurora B kinase activity is required for centralspindlin-directed RhoA activity in human cells

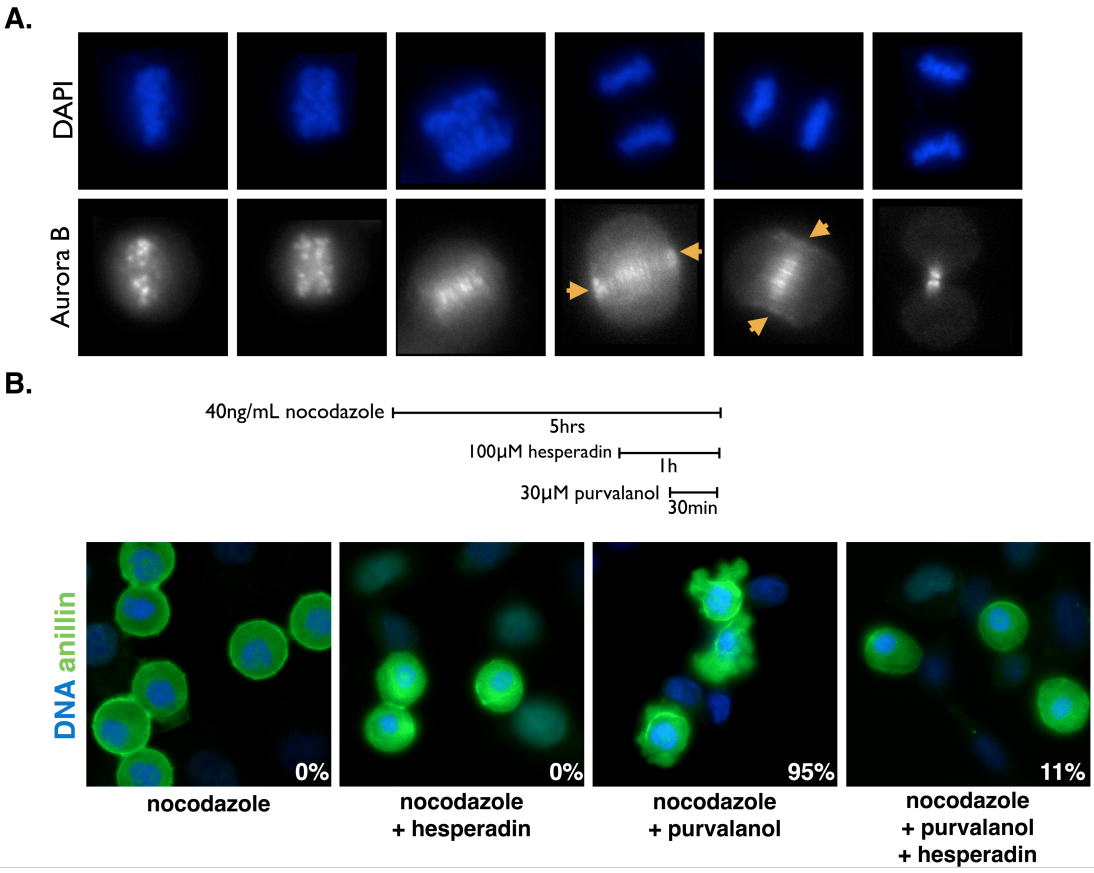


Figure 23. Localization of endogenous Aurora B and its requirement in centralspindlin-directed RhoA activity in human cells. A. HeLa cells were fixed with 4% paraformaldehyde and stained with anti-Aurora B antibody (grey) and DAPI (blue) to label chromatin. A montage of cells at various stages of cell division is shown. Arrows indicate a pool of Aurora B on the equatorial cortex that has previously been reported in several cell types. B. HeLa cells were arrested in metaphase using a low dose of nocodazole (40ng/mL) for four hours. In the presence of nocodazole, they were either treated with hesperadin (100μM), to inhibit Aurora B kinase for 1 hour, or with purvalanol (30μM), to inhibit Cdk1 for 30 minutes, or both. Following the indicated treatment, the cells were fixed with cold methanol and stained with DAPI (blue) and anti-anillin antibody (green). The percentage (white) of mitotic cells that displayed hypercontractility was scored in each case.

Our genetic evidence in *C. elegans* embryos establishes a clear role for Aurora B kinase in centralspindlin-mediated RhoA activation. It is unknown if this role is conserved in

other organisms and cell types. In human cells, it has previously been shown that Aurora B localizes at the equatorial cortex during anaphase (Figure 23A) however its function at this location is unknown. During bipolar cytokinesis, inactivation of Aurora B kinase or its depletion does not abolish cleavage furrow formation (Hauf et al. 2003). This may be due to the high concentration of centralspindlin on the midzone microtubules adjacent to the plasma membrane in these cells, that could promote RhoA activation. To assess whether a role for Aurora B kinase is obscured by the contribution of such a parallel furrow-induction pathway, we inactivated Aurora B in cells lacking an intact spindle. We arrested HeLa cells in metaphase using a low dose of nocodazole. When these cells are forced into anaphase using a Cdk1 inhibitor, they are hypercontractile presumably because in the absence of a mitotic spindle to direct furrow position, the centralspindlin-ECT-2 complex is now globally active and induces ectopic furrows. This hypercontractility is centralspindlin dependent (Lekomtsev et al. 2012) and Aurora B can be detected at the ectopic furrows generated (data not shown). We observed that if these cells are released into anaphase in the presence of an Aurora B inhibitor, they no longer display hypercontractility (Figure 23B). These results point towards a conserved role for Aurora B kinase in directing centralspindlin-dependent RhoA activation.

4.7 Discussion

The evidence provided in this chapter establishes that the primary role of the CPC during cytokinesis is to antagonize 14-3-3 inhibition of centralspindlin, thereby indirectly promoting RhoA activation at the plasma membrane. This function is conserved in both *C. elegans* and human cells, though obscured by parallel furrow induction pathways in both cases. These results conflict with previous models for CPC function during cytokinesis in *C. elegans* (Lewellyn et al. 2011). In this study it was found that depletion of septin proteins in *air-2* (Aurora B) temperature-sensitive mutants results in a rescue of the cytokinesis defect. Septins are GTP-binding proteins that form filaments and higher order structures and associate with actomyosin networks including the contractile ring (Bridges and Gladfelter 2015). Septin depletion could not rescue the cytokinetic defect observed in a *zen-4* temperature-sensitive allele. This was perplexing because if loss of Aurora B function completely eliminated centralspindlin activity, both mutants would be expected to behave identically. These results were interpreted to mean that the CPC and centralspindlin function independently in promoting contractile ring assembly. While both the *air-2* and *zen-4* mutants are defective in central-spindle assembly and midzone localization of centralspindlin, the *zen-4* allele is more severe because it prevents CYK-4 binding to ZEN-4, thereby completely abolishing function of the complex. In addition, the contribution of the NOP-1-dependent astral pathway for furrow induction was not taken into consideration. In contrast to these results, our data show that the CPC antagonizes PAR-5 binding to centralspindlin and promotes RhoA activation in *C. elegans* embryos (Figure 19). We unequivocally demonstrate that the primary role of the CPC in cytokinesis is to promote centralspindlin oligomerization and

thereby permit cleavage furrow formation, as the function of CPC can be suppressed by depletion of PAR-5. In the light of our results, we speculate that in wild-type cells, septin inhibits centralspindlin activity by either limiting its access to the membrane or perhaps competing for a limiting pool of RhoA molecules. In a sensitized background such as *air-2* mutants where Aurora B kinase activity is abolished, septin depletion could permit available centralspindlin complexes to rescue furrow ingression as observed by Lewellyn et al. However in a *zen-4* mutant where CYK-4 and ZEN-4 cannot bind each other, release of septin inhibition would be predicted to have no effect.

Our results also point to an important role for membrane-localized CPC during anaphase (Cooke, Heck, and Earnshaw 1987; Eckley et al. 1997; Murata-Hori and Wang 2002; Szafer-Glusman, Fuller, and Giansanti 2011; Kitagawa et al. 2013) in locally promoting centralspindlin oligomerization. However, a general mechanism for how the CPC localizes at this site is unavailable. Diverse cell types may utilize different mechanisms for CPC localization to execute its function. Determining which of these models is most widely applicable to a variety of cell types will be an important avenue for future studies. In cultured NRK epithelial cells, weak doses of nocodazole that disrupt astral microtubules but not the midzone, disrupt membrane localization of Aurora B (Murata-Hori and Wang 2002). Photobleaching of midzone Aurora B in these cells does not perturb the cortical protein (Murata-Hori and Wang 2002). This suggests aster-dependent delivery of the complex to the membrane, but the motor responsible for this function has not been not determined. In human cells both central spindle and membrane-bound CPC require a kinesin-6 protein MKLP2 (Kitagawa et al. 2013).

However, MKLP2 is poorly conserved in species where it is found when compared to MKLP1 (http://www.ncbi.nlm.nih.gov/homologene?Db=homologene&Cmd=Retrieve&list_uids=38093 vs http://www.ncbi.nlm.nih.gov/homologene/?term=kif23) and is absent in several systems such as *C. elegans.* Therefore other mechanisms for the localization of CPC proteins must exist. CPC localization has also been studied in a cell-free system using *Xenopus* egg cytosol and artificial centrosomes, where mitotic spindles were generated overlaying a supported lipid bilayer (Nguyen et al. 2014). The CPC was transported and focussed at the midzone by two kinesins Kif4A and a Kif20A paralog. It remains to be tested whether analogous proteins perform this function in other species.

Although positively charged motifs have been identified in CPC proteins (Jeyaprakash et al. 2007), there are no reports of phospholipid binding and an obvious membrane-binding motif has not been determined. However, the likelihood that the CPC directly associates with the plasma membrane cannot be ruled out. Another possibility is that the CPC associates with a component(s) of the cleavage furrow. There are reports of indirect interaction between CPC components and actomyosin (Kitagawa et al. 2013; Hu et al. 2008), but a conserved role for this association in cytokinesis has not emerged. This model is surprising in the context of our genetic model (Figure 19B) where the CPC lies upstream of contractile ring components. Actomyosin may stabilize association of the CPC with the plasma membrane but the initial accumulation may be triggered by another pathway such as astral microtubule delivery. Because Aurora B and ZEN-4 can interact and we have demonstrated that centralspindlin is a component of the cleavage

furrow, another complex model to consider is that the localization of centralspindlin and the CPC at the membrane are interdependent.

Finally, it is also likely that Aurora B kinase can exert its function in RhoA activation without locally concentrating at the site of furrow formation in some cell types. Given that the central spindle microtubules are at a distance of 15-20 microns from the plasma membrane in *C. elegans* embryos, one might anticipate a role for membrane-localized Aurora B in local RhoA activation in these cells. Curiously however, despite clear genetic evidence that the CPC is required for furrow formation, we do not detect accumulation of endogenously-tagged AIR-2 (Aurora B) or CSC-1 (Borealin) at the cleavage furrow (Figure 22). This may simply be due to a low level of accumulation that is not detectable under our imaging conditions. We do observe a striking localization on astral microtubules in addition to the spindle midzone. Unlike the Kif4a and Kif20A-dependent localization seen in *Xenopus* (Nguyen et al. 2014), this association is dependent on INCENP (Figure 22). Currently we do not have any evidence suggesting that this pool of the CPC travels to microtubule plus ends or activates centralspindlin oligomers in the equatorial region of the cell. It is possible that Aurora B kinase does not always accumulate at a specific site to exert a local effect. Aurora B kinase has previously been observed to execute function at the central spindle without locally concentrating there. Phosphorylation of MKLP1 by Aurora B kinase is required for accumulation of the former at the central spindle (Adams et al. 2000; Kaitna et al. 2000; Guse, Mishima, and Glotzer 2005). Both proteins co-localize at this site in wild-type anaphase, presumably facilitating MKLP1 phosphorylation. However, in the absence of

MKLP2 protein in human cells, Aurora B kinase cannot localize at the spindle midzone and remains on chromosomes (Gruneberg et al. 2004). Under these conditions, MKLP1 still accumulates at the central spindle microtubules in an Aurora B-dependent manner (Gruneberg et al. 2004). Similarly, it is plausible that in *C. elegans* embryos, a global or more diffuse Aurora B signal generates centralspindlin oligomers throughout the embryo, but their localization is focused on the equatorial membrane by another mechanism such as astral inhibition (section 3.3) or by a feedback mechanism at the cleavage furrow (Chapter 6) or a combination thereof.

Chapter 5: Optogenetic regulation of Aurora B kinase activity in human cells

5.1 Abstract

Our data suggests a model where Aurora B kinase activity on the plasma membrane induces centralspindlin oligomers. To test this model we are employing an optogenetic system developed in our lab (Strickland et al. 2012; Wagner and Glotzer 2016b) to spatiotemporally control Aurora B kinase activity on the membrane. In this chapter we provide preliminary evidence for such a paradigm. In human cells, we demonstrate that we can control the membrane recruitment of Aurora B kinase and its activator INCENP during interphase and mitosis. We are currently developing strategies to test the activity of membrane-recruited Aurora B and whether that is sufficient to drive furrow formation.

5.2 Introduction

Based on the cell biological and genetic evidence collected so far, we hypothesize that local Aurora B activity should be sufficient to antagonize 14-3-3 binding to MKLP1/ZEN-4 and permit formation of centralspindlin clusters on the plasma membrane. Centralspindlin oligomers should activate ECT-2 and RhoA leading to actomyosin-directed contractility. Testing such a hypothesis requires conditional spatial and temporal control over Aurora B activity in cells. A suitable way to achieve this is using optogenetics to deliver either Aurora B or its activator to the cell cortex (Figure 24) (Guntas et al. 2015; Yazawa et al. 2009; Levskaya et al. 2009; Kennedy et al. 2010). A

light-directed sub-cellular protein recruitment developed in our lab (Strickland et al. 2012) would allow us to make such perturbations and test their biological consequences. This system relies on an increased binding affinity exhibited by two proteins in the light as compared to the dark. The light regulated protein used here is called the LOV domain. It alters its conformation when in the lit state such that it then favorably interacts with and binds an engineered PDZ (ePDZ) domain (Strickland et al. 2010). This provides the basis for the light-directed recruitment. The LOV peptide (LOVpep) can be localized to a certain sub-cellular site (eg. the plasma membrane, mitochondria, centromeres etc) by being covalently linked to a targeting protein. ePDZ fused with our protein of interest, can then be recruited by LOVpep to a defined site by shining focused light. Our lab has developed and successfully demonstrated the use of this system in controlling Cdc42 activity in budding yeast polarization (Strickland et al. 2012) and RhoA activity during cytokinesis in human cells (Wagner and Glotzer 2016a).

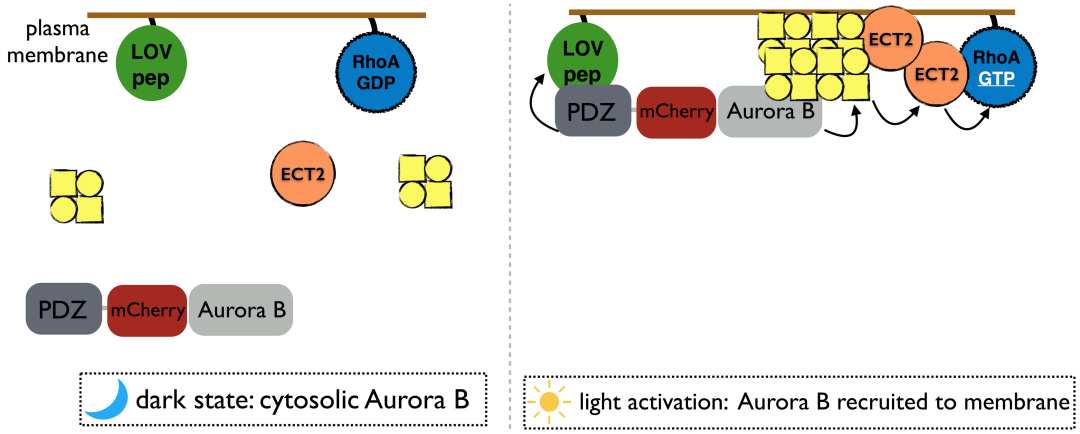


Figure 24. Schematic illustrating optogenetic control of Aurora B using the TULIPs system. Cells expressing membrane-tethered LOVpep (green) will be co-expressed with either Aurora B or its activating protein fused to the C-terminus of a PDZ-mCherry construct. In the absence of light, the PDZ construct is predicted to be cytosolic. In the presence of blue light, LOVpep should bind the PDZ domain thus recruiting Aurora B or its activator to the plasma membrane. Here, during anaphase, we anticipate that Aurora B will drive the oligomerization of centralspindlin (yellow) which will promote ECT-2 (orange) and RhoA (blue) activation at the membrane. At sufficiently high doses of Aurora B activity, we anticipate that local illumination of the plasma membrane will drive local changes in actin and myosin accumulation through this pathway and result in change in cell shape.

5.3 Development of a construct sufficient for Aurora B activation in human cells

INCENP, a key component of the CPC, comprises three major domains. The very N-terminus of INCENP is required for CPC localization at centromeres (Figure 4), the central region contains a single alpha helical (SAH) domain that directly binds microtubules and the C-terminus, known as the IN-box, directly binds the N-terminus of Aurora B (Figure 25A). *In vitro* the last interaction is sufficient to generate high levels of Aurora B activity. Based on these data, we sought to optogenetically control Aurora B activity in human cells by recruiting the INCENP C-terminus to the plasma membrane (Figure 25B). We generated a construct by appending a fragment of mouse INCENP C

terminus downstream of tandem PDZ domains fused to mCherry. This fragment included a part of the SAH microtubule binding domain.

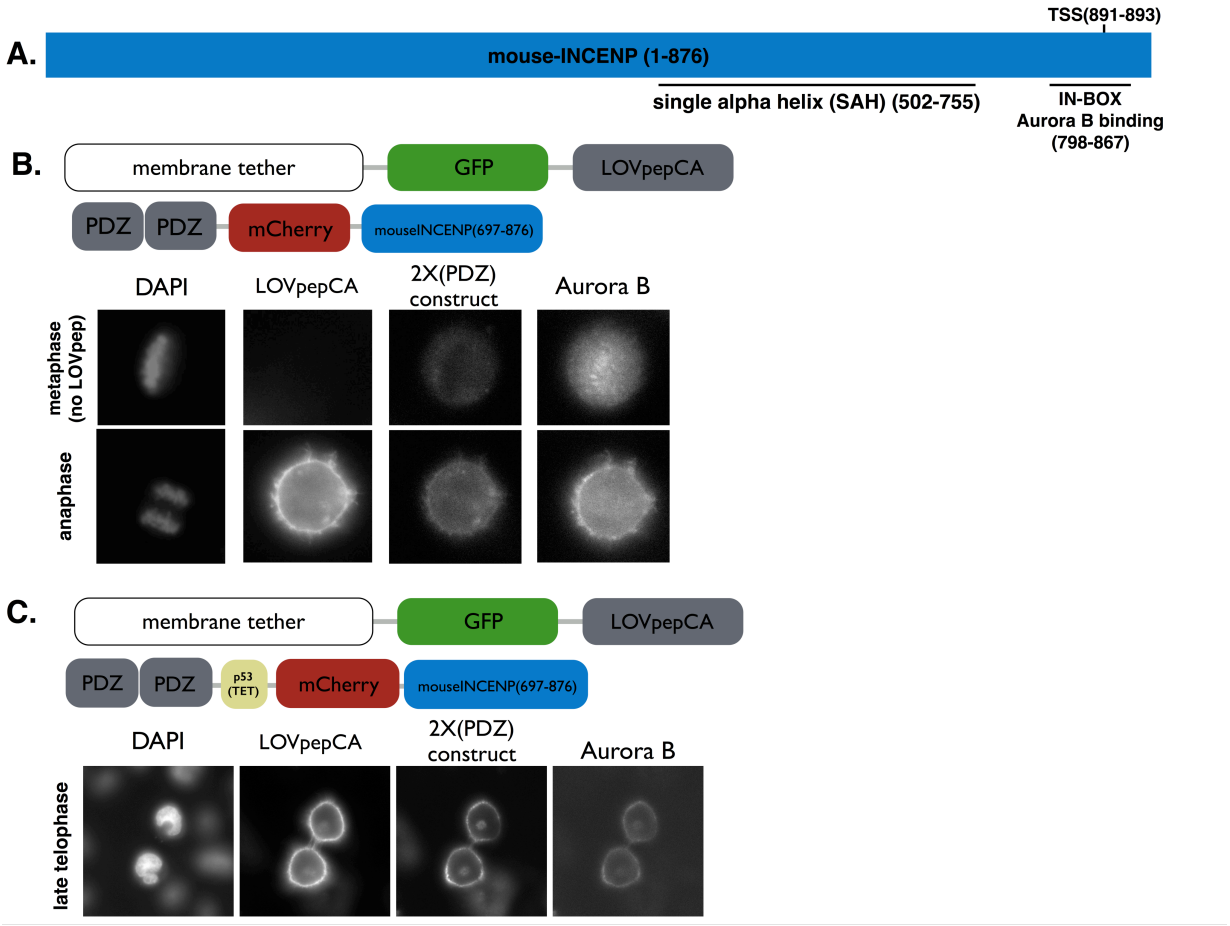


Figure 25. Optogenetic recruitment of Aurora B to the plasma membrane. A.Domain structure of mouse INCENP. B. A membrane-tethered constitutively active (CA) GFP-tagged LOVpep construct and a PDZ-mCherry construct fused to the indicated C-terminal region of INCENP were transiently transfected into HeLa cells. Cells were fixed with 4% paraformaldehyde and stained with anti-Aurora B antibody and DAPI. C. Experiment performed as in B. To induce clustering of INCENP, the tetramerization domain of p53 was included in the PDZ construct. INCENP can be recruited to the plasma membrane in all stages of the cell cycle (not all conditions are depicted here).

We transiently expressed these constructs in human cells along with membrane-tethered, constitutively active or light-sensitive LOVpep and analyzed the cells post-fixation. We find that INCENP can be recruited to the plasma membrane by LOVpep

(Figure 25A) and in a light-dependent manner (not shown). Moreover, this fragment of INCENP is at least partially functional because staining with Aurora B antibody reveals that endogenous Aurora B is recruited to the plasma membrane in response to exogenous INCENP localization (Figure 25A). Higher concentrations of membrane-recruited INCENP results in stronger localization of Aurora B on the membrane (not shown).

Another feature of Aurora B activation is that it requires auto-phosphorylation of the kinase. This appears to occur in trans and is facilitated by multimerization of the CPC (Sessa et al. 2005; Kelly et al. 2007; Tseng et al. 2010). Previously, using a chimeric, oligomeric INCENP construct spontaneous microtubule polymerization has been induced *in vitro* (Tseng et al. 2010). To potentiate the ability of our light-regulated construct to activate Aurora B, we introduced an exogenous tetramerization domain (from p53) N-terminal to mCherry (Figure 25B). Our results suggest that this construct efficiently recruits Aurora B to the plasma membrane (Figure 25B).

Curiously however, in several cells upon strong recruitment of Aurora B to the membrane via optogenetically-controlled INCENP, staining of endogenous Aurora B at the chromatin was diminished (Figure 25A). These results suggest, as recently reported (Gohard et al. 2014), that over-expressing a free INCENP fragment could act as a dominant-negative for CPC localization and function by sequestering the kinase. To address this issue and to activate Aurora B more potently, we designed new constructs based on a crystal structure of the *Xenopus* INCENP-Aurora B complex solved by the Musacchio group (Sessa et al. 2005). This structure of the activated complex reveals

that the INCENP C-terminal residue lies in close proximity to the N-terminus of Aurora B. Therefore in our design we covalently linked them. We appended full-length or truncated Aurora B C-terminal to a short optogenetically-controlled INCENP fragment (Figure 26A). We expect that in this format, INCENP and Aurora B will engage in an intra-molecular interaction, will not associate with endogenous CPC components, and thus preclude any potential dominant-negative effects. Supporting this idea, another group has independently constructed a similar fusion protein to control Aurora B kinase activity in human cells (Nunes Bastos et al. 2013; Bastos, Cundell, and Barr 2014). Such a strategy has also previously been used to control Cdk1 activity in fission yeast (Coudreuse and Nurse 2010). It is likely that over-expression of a highly active form of Aurora B kinase could cause adverse effects in cells. To circumvent this possibility we used an analog-sensitive variant of Aurora B developed by the Lens lab (Hengeveld et al. 2012). We expect that in the presence of an inhibitor the analog-sensitive, exogenous kinase would be inactive and inert whereas the endogenous protein would be unperturbed. The inhibitor can be removed from the media when optogenetic control of the kinase is desired.

5.4 Testing activity of constructs used for optogenetic control of Aurora B

We tested the activity of INCENP-Aurora B fusion constructs *in vitro* comparing them with Aurora B activity in the presence of free INCENP. We expressed and purified GST-tagged recombinant proteins (Figure 26B) from *E. coli* and assessed the ability of these

proteins to phosphorylate histone H3 at Serine 10 *in vitro*. The extent of phosphorylation was determined by analyzing Western blots probed with a phospho-histone antibody.

We found, as previously published (Sessa et al. 2005; Kaitna et al. 2000; Adams et al. 2000; Honda, Körner, and Nigg 2003), that activity of Aurora B is enhanced in the presence of the free C-terminus of INCENP (Figure 26C). Proteins where INCENP and Aurora B fragments are

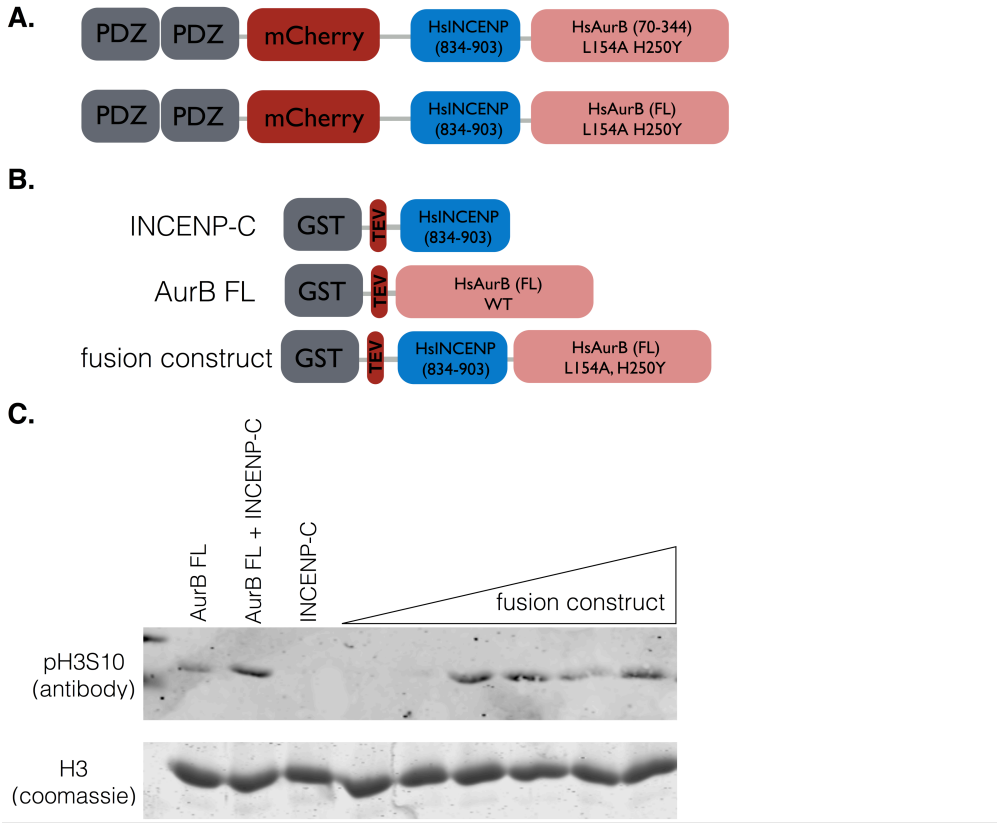


Figure 26. *In vitro* activity of INCENP-Aurora B fusion constructs generated for optogenetic control of Aurora B kinase in human cells. A. Schematic of fusion constructs built to control Aurora B activity using TULIPs in human cells. B. GST-tagged INCENP-Aurora B constructs to test activity of fusion proteins *in vitro.* C. The indicated proteins were expressed and purified from *E. coli* and incubated with histone H3 for 30 minutes in a kinase reaction (see methods). The extent of histone phosphorylation was determined by Western Blot.

fused were also able to phosphorylate H3S10 in a concentration dependent way.

However, the activity of this construct was comparable to or less than reactions in which

INCENP activated Aurora B in trans (Figure 26C), leading to the concern that these proteins may not be fully active in this configuration.

We wished to determine the activity of our corresponding light-regulated Aurora B constructs in human cells. The most frequently-used approach for detecting Aurora B *in vivo* is using FRET-based sensors that change intra-molecular FRET between CFP and YFP based on phosphorylation of an Aurora B substrate peptide from a kinesin 13 family (B. G. Fuller et al. 2008; E. Wang, Ballister, and Lampson 2011). This strategy is incompatible with TULIPs-based optogenetics because the CFP emission wavelength would lead to undesirable activation of LOVpep.

Using COS-7 cells, it is has been demonstrated that co-expression of the INCENP C terminus and Aurora B is sufficient to drive Aurora B into the nucleus and direct ectopic histone H3S10 phosphorylation prior to mitosis (Yasui et al. 2004). Our INCENP-Aurora B fusion constructs when expressed in human cells localize to interphase nuclei but we are unable to detect phospho-H3S10 staining (data not shown), suggesting that the kinase activity of these proteins is weak.

5.5 Optogenetic control of Aurora B activity in live human cells

We have demonstrated that the C-terminus of INCENP is sufficient to recruit endogenous Aurora B to the plasma membrane using the TULIPs system. Based on our genetic and cell biological evidence, we hypothesize that local Aurora B activity should

be sufficient to generate RhoA-mediated contractility. No dramatic changes in cell morphology were observed in response to global recruitment of Aurora B to the membrane in fixed cells (Figure 25). This could be because in such a scenario opposing contractile forces are balanced out. To test whether local recruitment of Aurora B can

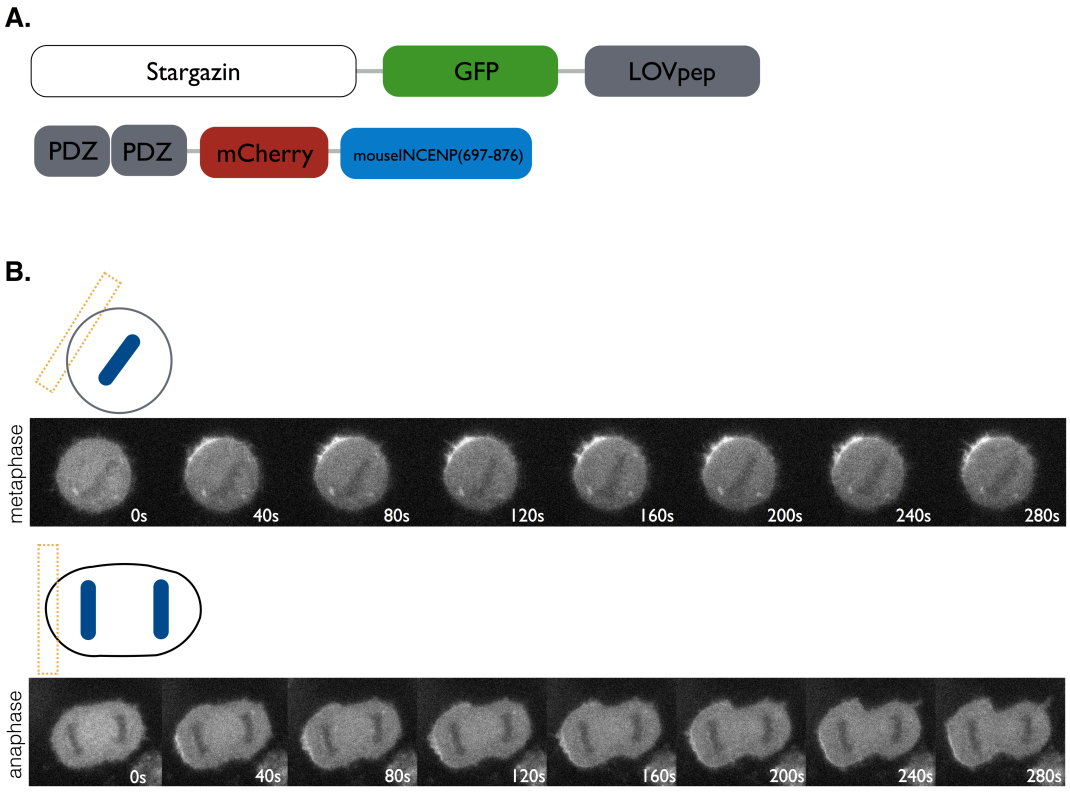


Figure 27. Optogenetic control of Aurora B activity in live human cells during anaphase. A. Schematic of constructs used. Stargazin is a membrane-localized protein. B. Membrane-tethered, light-inducible GFP-tagged LOVpep and a PDZ-mCherry construct fused to the indicated C-terminal region of INCENP were transiently transfected into HeLa cells. The schematics depict a metaphase and anaphase cell; the chromatin is in dark blue and the boxed regions indicates where the 488nm light was shone. Cells were locally illuminated with blue light (488nm) every 20s in the indicated region of the cell using a laser scanning confocal microscope. mCherry images were acquired pre- and post-488 excitation at each time point. Post-excitation images of the mCherry-tagged probe are shown at indicated time points. The first pre-excitation image is designated as t=0s.

drive cell shape change, we illuminated defined regions on the membrane of live cells expressing recruitable INCENP(C) and tracked cells as they progressed through cytokinesis. Unsurprisingly, when we locally recruit the C terminus of INCENP to the

plasma membrane during metaphase we do not observe any obvious change in cell shape (Figure 27B). During metaphase we do not expect Cyk4 and Ect2 to interact and therefore RhoA activation is unlikely. During anaphase however, centralspindlin should be competent to activate RhoA. We locally recruited INCENP(C) to the poles of dividing cells to determine whether an ectopic furrow could be generated. While we do not see the formation of a furrow in the poles, we observe that the cell membrane flattens where INCENP(C) is recruited (n=4/5 for this construct) (Figure 27B). Recently our lab demonstrated that ectopic RhoA activation at the poles efficiently competes with ingression of the endogenous furrow (Wagner and Glotzer 2016a). However in contrast, recruitment of INCENP(C) to the polar cortex rarely blocks endogenous cytokinesis.

5.6 Discussion

In our experiments so far we have been unable to drive strong, ectopic Aurora B kinase activity on the membrane in human cells. However our results thus far are not inconsistent with the hypothesis that local Aurora B activation is sufficient to induce RhoA via centralspindlin. We are currently working on strategies to improve the efficacy of our optogenetically controlled Aurora B kinase construct.

This weak change in cell morphology observed upon membrane recruitment of Aurora B during anaphase, could be due to a variety of reasons. It is likely that construct design for ectopic Aurora B activation results in very weak kinase activity. We are currently combining our optogenetics approach with a previously characterized INCENP-Aurora B

fusion construct (Nunes Bastos et al. 2013; Bastos, Cundell, and Barr 2014) to address this issue. Further, as we have demonstrated (section 3.3) and as shown by Wagner and Glotzer (2016), we expect that astral microtubules inhibit RhoA activity upstream of RhoA. This might occur by the action of phosphatases that either antagonize Aurora B or Plk1 kinase activity. This mechanism is likely to counteract any centralspindlin oligomers we may be generating via Aurora B function. To circumvent this issue, these experiments will be repeated under a low dose of nocodazole that reduces the density of astral microtubules and thereby weaken their inhibitory effect. In addition, we will recruit Aurora B kinase to the endogenous division site to determine whether the furrow ingression kinetics deviate from wild type in the presence of additional Aurora B protein.

Chapter 6: Regulation of centralspindlin localization at the cleavage furrow

[The *C. elegans* strains in Figure 29 were generated in collaboration with Katrina Longhini.]

6.1 Abstract

In the absence of PAR-5/14-3-3, centralspindlin is membrane localized only in the equatorial region of the cell, and only at certain stages of the cell cycle. In this chapter we investigate potential mechanisms contributing to this behavior that may reflect how the zone of active RhoA is tightly focused. Surprisingly, we find that components downstream of centralspindlin in the pathway to furrow induction can regulate its levels at the cleavage furrow. We observe that direct interaction between CYK-4 and RhoA is strictly required to recruit centralspindlin to the membrane, pointing to a potential positive feedback loop in RhoA activation. Rather interestingly, we also find that upon myosin depletion, levels of active RhoA are increased on the membrane in a centralspindlin-dependent way and that centralspindlin recruitment to the membrane is sensitive to actomyosin. We are currently investigating whether these behaviors have a direct consequence in wild-type cytokinesis.

6.2 Introduction

During cytokinesis active RhoA is precisely localized in the equatorial region of the plasma membrane, resulting in a tightly focused furrow. The sharpness of this zone of localization suggests that feedback mechanisms may be involved in its fine-tuning. Feedback loops have been implicated in cortical RhoA activation during cytokinesis (Bement et al. 2015; Goryachev et al. 2016), however direct cell biological evidence or mechanism is lacking.

There is precedent for such regulation of other GTPases. For instance, the activity of the Ras-specific GEF, Son of Sevenless (SOS) is dramatically enhanced when bound by Ras at a second binding site distal to the catalytic site (Boykevisch et al. 2006; Margarit et al. 2003; Sondermann, Zhao, and Bar-Sagi 2005). During yeast cell polarization, self-amplification of the Rho family GTPase Cdc42 activity is achieved by positive feedback. This mechanism involves the formation of a complex between a Cdc42 effector, Cdc42•GTP, a Cdc42 GEF and a scaffold protein (Irazoqui, Gladfelter, and Lew 2003; Butty et al. 2002; Kozubowski et al. 2008).

In our current view of cytokinesis, the mitotic spindle and its components unidirectionally control RhoA activity and localization of contractile ring components. Interestingly however, in meiotic divisions in *Drosophila* males, profilin (*chickadee*) and formin (*diaphanous*) mutants not only fail to generate intact contractile rings but also lack properly assembled central spindles (Giansanti et al. 1998; Gatti, Giansanti, and Bonaccorsi 2000). A similar phenotype was observed when contractile ring assembly was bluntly disrupted by cytochalasin B treatment (Giansanti et al. 1998). In *Drosophila* S2 cells, depletion of RhoA and myosin by RNAi yield similar effects and disrupt ZEN-4

(*pavarotti)* accumulation at the central spindle (Somma et al. 2002). In human cells, during monopolar (but not bipolar) cytokinesis, disrupting RhoA activity using C3 transferase or using small molecule inhibitors of actin and myosin assembly, abolishes the polarized organization of spindle midzone microtubules (Hu et al. 2008). These phenotypes, while not readily observed in all model systems, point to a potential feedback-based regulatory mechanism by which components of the contractile ring might signal to upstream activators in the pathway. These interactions may be important to properly focus the cleavage furrow.

Our results show that in *C. elegans* embryos, cortical localization of centralspindlin is negatively regulated by 14-3-3 proteins like PAR-5 (Figure 9). 14-3-3 inhibition is antagonized by Aurora B kinase that promotes centralspindlin multimerization (Figure 19). If centralspindlin oligomerization was sufficient to localize the complex to the plasma membrane, in the absence of PAR-5, CYK-4 and ZEN-4 should be constitutively on the cell cortex. However, upon PAR-5 depletion, increase in cortical centralspindlin is not constitutive and is only observed during phases of RhoA activation. This suggests that cortical recruitment of centralspindlin is cell-cycle regulated and may have additional requirements such as binding of CYK-4 to ECT-2, RhoA or to actomyosin. Consistent with this idea, during polarization, centralspindlin is detectable only on the anterior cortex in the absence of PAR-5, and during anaphase it is observable only in the equatorial region (Figure 10A). We have demonstrated that centralspindlin localization at the polar cortex is antagonized by astral microtubules (Figure 13A). Here we report additional mechanisms by which cortical centralspindlin localization is

regulated and speculate whether centralspindlin may serve as a nexus for feedback in cytokinesis.

6.3 Centralspindlin localization to the membrane depends on the interaction between the CYK-4 GAP domain and RhoA

Zhang and Glotzer (2015) demonstrated that the CYK-4 GAP domain plays a crucial role in RhoA activation by promoting activity of the GEF ECT-2. Interestingly, it was also observed that the GAP dead mutant of CYK-4 hyper-accumulates at the plasma membrane, presumably because it binds RhoA very tightly. Conversely, CYK-4 mutants that cannot bind RhoA are not detectable on the plasma membrane. These results suggest that active RhoA may recruit its activator centralspindlin to the membrane.

To test the model (Figure 28A) that binding to active RhoA facilitates the recruitment of centralspindlin to the plasma membrane, we analyzed early *C. elegans* embryos expressing CYK-4::GFP depleted of the conventional GAP proteins RGA-3/4. We observe a subtle but obvious increase in CYK-4 levels on the equatorial plasma membrane (Figure 28C), suggesting a positive correlation between active RhoA levels and membrane recruitment of centralspindlin.

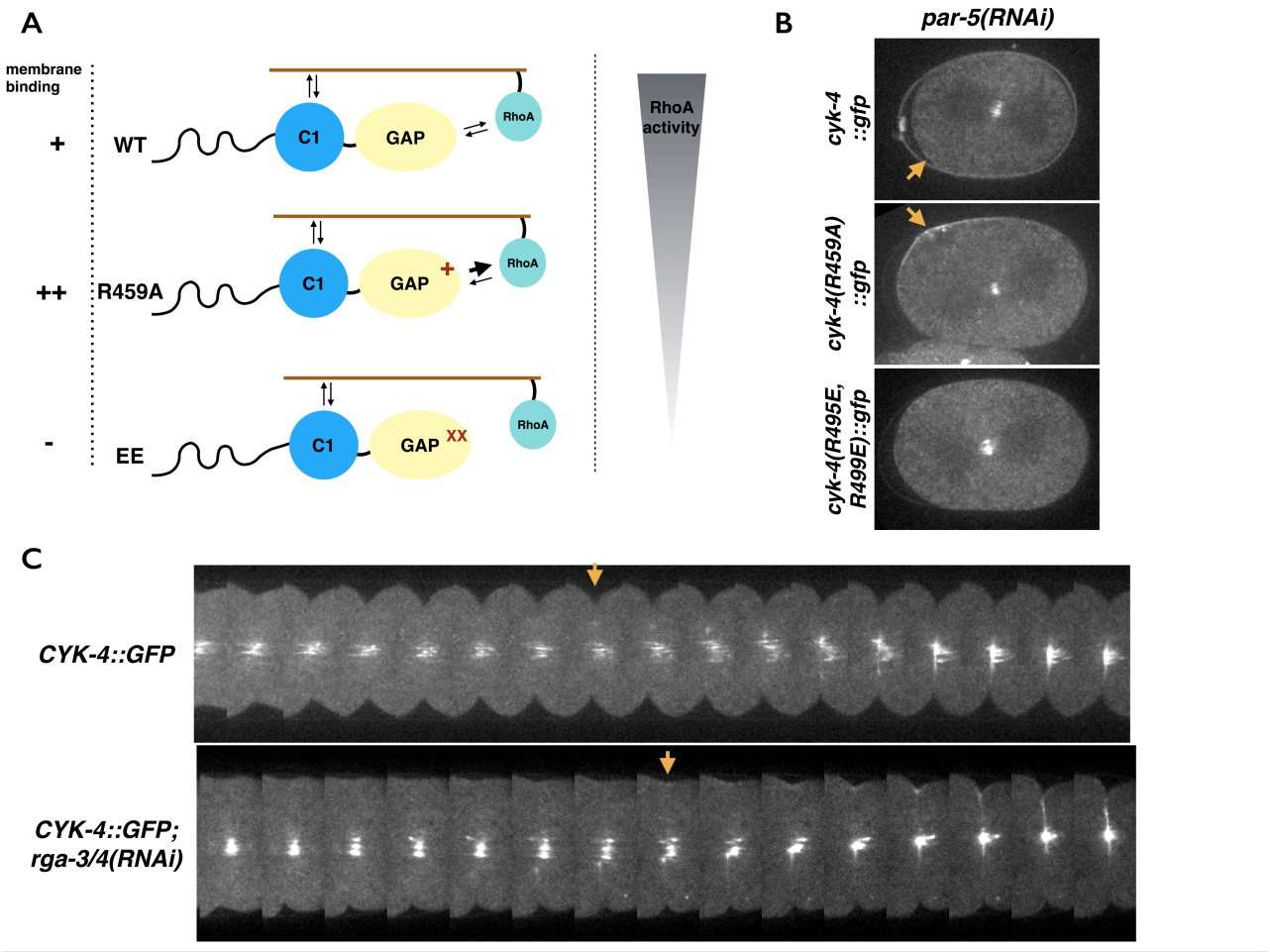


Figure 28. Centralspindlin localization to the membrane depends on the interaction between the CYK-4 GAP domain and RhoA. A. A schematic depicting the interactions of the CYK-4 C1 domain with the plasma membrane and the GAP domain with RhoA. Based on results from (Zhang and Glotzer, 2015), our model predicts that the GAP-defective R459A mutant, though weaker than WT at RhoA activation, will bind tightly to membrane-bound Rho and will accumulate strongly on the plasma membrane when oligomerized. In contrast, R495E,R499E (EE) mutations that abolish RhoA binding (Zhang and Glotzer, 2015) are predicted to fail to accumulate on the membrane, even if the centralspindlin complex is oligomerized. B. *C. elegans* embryos expressing a wild-type CYK-4::GFP, CYK-4(R459A)::GFP or CYK-4(R495E,R499E)::GFP transgene were simultaneously depleted of endogenous CYK-4 and PAR-5 by RNAi. Images of cells during anaphase are shown. Arrows indicate cortical accumulation of CYK-4. C. *C. elegans* embryos expressing a wild-type CYK-4::GFP transgene were simultaneously depleted of RGA-3/4 proteins by RNAi. Shown are montages of the equatorial region as the cell divides (n≥5). Arrows indicate membrane accumulation of CYK-4.

Further, we tested whether the interaction between RhoA and CYK-4 was crucial to membrane localization of centralspindlin even in the absence of PAR-5. *C. elegans*

embryos expressing RNAi-resistant forms of GAP dead (R459A) or RhoA binding defective (K495E, R499E) CYK-4 were depleted simultaneously of PAR-5 and endogenous CYK-4 by RNAi. When compared to wild-type CYK-4, the R459A mutant hyper-accumulates on the membrane (Figure 28B). Conversely, and rather strikingly, even in the absence of PAR-5, the K495E, R499E form of CYK-4 is incapable of stably localizing to the plasma membrane (Figure 28B).

So far our model for membrane localization of centralspindlin posited that the weak membrane-binding property of the CYK-4 C1 domain is potentiated by formation of centralspindlin oligomers. The above results demonstrate that the interaction of the CYK-4 GAP domain with RhoA is an additional requirement in membrane localization of centralspindlin. These data provide a molecular mechanism for a positive feedback loop by which RhoA can recruit its own activator. This mechanism would be in addition to a role for RhoA in promoting ECT-2 activation (Zhang and Glotzer 2015).

6.4 Depletion of the downstream effector NMY-2 leads to a centralspindlin-dependent increase in active RhoA levels on the plasma membrane

Active RhoA indirectly promotes assembly of myosin (NMY-2) mini-filaments on F-actin, and thus NMY-2 is a downstream effector of RhoA. In a linear pathway leading towards cortical contractility, alteration in levels of downstream effectors should have no impact on upstream players. However rather surprisingly, we observe that upon NMY-2

depletion, there is a measurable increase in the levels of a RhoA biosensor in the equatorial region of the cell during anaphase (Figure 29).

We sought to determine whether this increase in RhoA biosensor levels was dependent

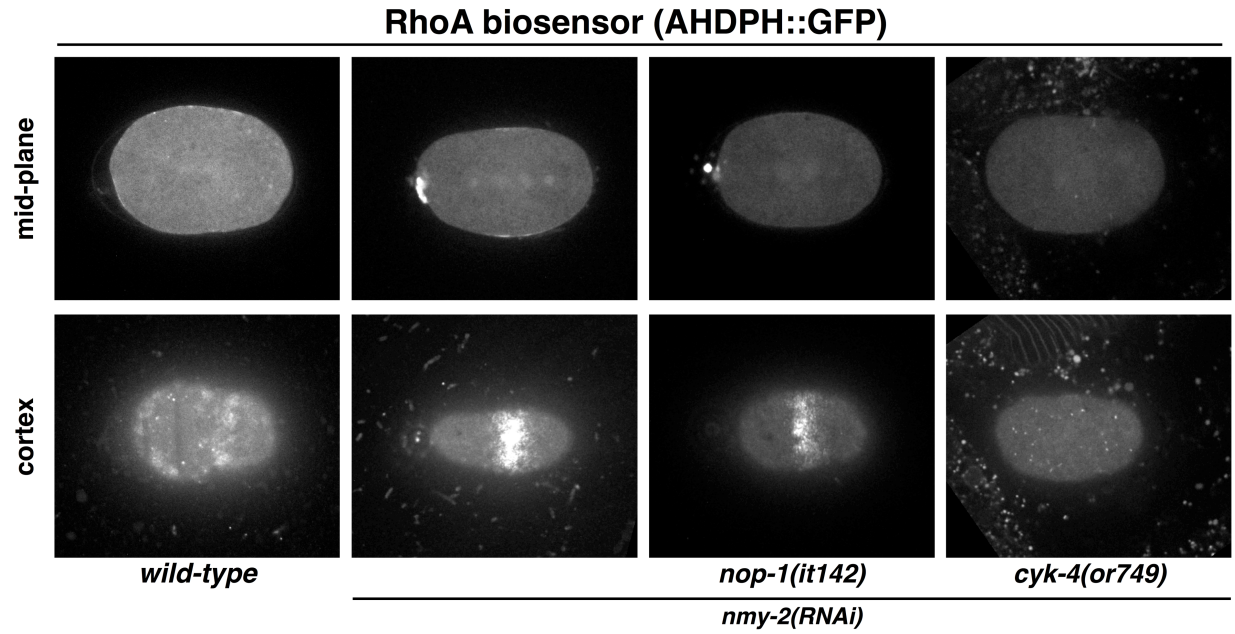


Figure 29. Depletion of the downstream effector NMY-2 leads to a centralspindlin-dependent increase in active RhoA levels on the plasma membrane. *C. elegans* embryos expressing AHDPH::GFP RhoA biosensor and endogenously-tagged mKate::NMY-2 (not depicted) were depleted of NMY-2 by RNAi. Images of the mid-plane and cortex of embryos of the indicated genotype during anaphase are shown. The extent of RhoA biosensor accumulation on the membrane dramatically increases when NMY-2 is depleted. This accumulation requires CYK-4 activity. However, the extent of biosensor accumulation does not appear to depend on the severity of NMY-2 RNAi (not shown).

on the pathway activating RhoA. We analyzed *nop-1(it142)* mutant embryos expressing the RhoA biosensor AHPH::GFP and endogenous NMY-2 labeled with mKate (not shown in micrographs). We find that as in the case of embryos where both ECT-2 activation pathways are functional, in *nop-1* mutant embryos AHPH::GFP levels also increase in the cell equator upon NMY-2 depletion (Figure 29). However, when an

identical experiment was performed in embryos where the centralspindlin pathway is impaired, namely a *cyk-4(or749ts)* mutant, no hyper accumulation was observed upon NMY-2 depletion (Figure 29). The severity of this phenotype does not seem to depend on the strength of NMY-2 depletion. These results indicate that NMY-2 directly or indirectly antagonizes active RhoA in a centralspindlin-dependent manner.

6.5 Levels of CYK-4 on the membrane are sensitive to the depletion of NMY-2

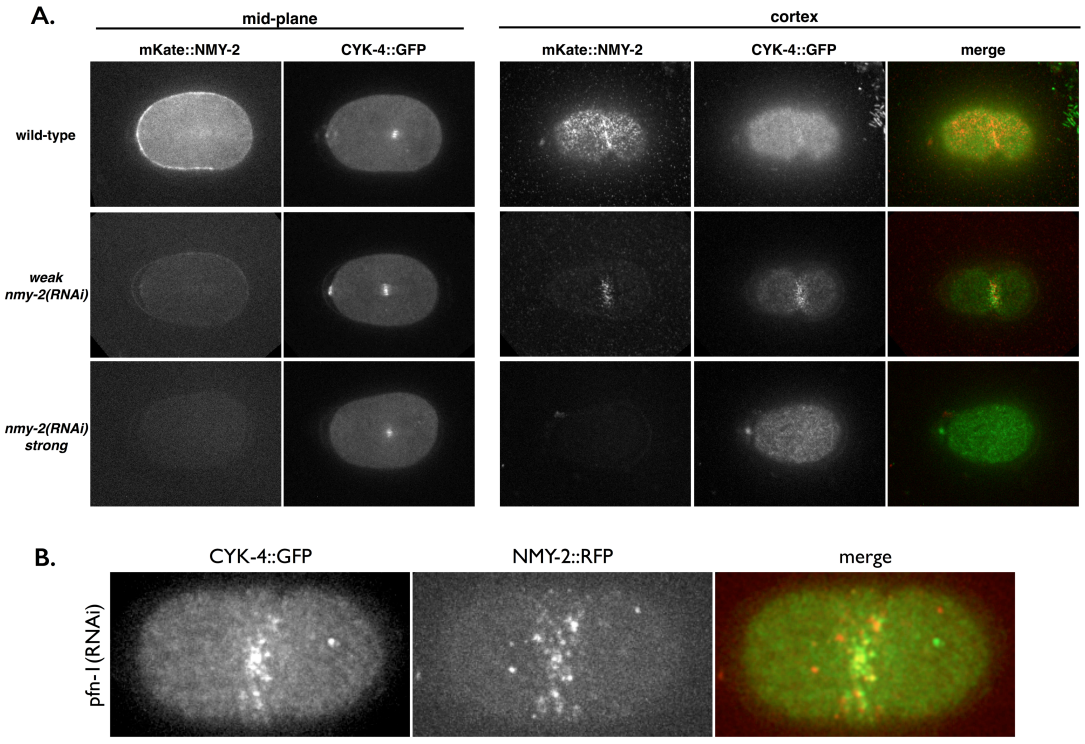


Figure 30. Levels of CYK-4 on the membrane are sensitive to the depletion of NMY-2 and profilin (PFN-1). A. *C. elegans* embryos expressing a CYK-4::GFP transgene and endogenously-tagged mKate::NMY-2 were depleted of NMY-2 by RNAi. The strength of RNAi is judged by the intensity of mKate signal. Images of the mid-plane and cortex of embryos during anaphase are shown. Under certain intermediate regimes of NMY-2 depletion, a strong accumulation of CYK-4 is observed on the membrane which co-localizes with NMY-2 at the equatorial furrow. Strong depletion of NMY-2 leads to a loss of this accumulation. The accumulation of CYK-4 on the central spindle appears unaffected by NMY-2 depletion. B. *C. elegans* embryos expressing CYK-4::GFP and NMY-2::RFP transgenes were depleted of PFN-1 (profilin) by RNAi. Images of the cell cortex of embryos of during anaphase are shown. CYK-4 accumulates in bright foci on the membrane and co-localizes with NMY-2.

We tested whether depletion of NMY-2 had any impact on cortical levels of centralspindlin. We depleted NMY-2 by RNAi in embryos expressing CYK-4::GFP transgene and endogenous NMY-2 labeled with mKate. In wild-type embryos, small quantities of CYK-4 are detectable on the plasma membrane during cytokinesis. However, when NMY-2 was strongly depleted, CYK-4 was no longer detectable on the plasma membrane (Figure 30A) even though the central spindle assembled normally. Interestingly, at intermediate levels of NMY-2 depletion, when cytosolic NMY-2 was largely absent but enough accumulated at the equatorial cortex to assemble a slowly ingressing furrow, CYK-4 hyper-accumulated and co-localized along with NMY-2, relative to wild-type embryos (Figure 30A). These results are surprising and suggest that membrane localization of centralspindlin is sensitive either to NMY-2 levels or to another component of the actomyosin network.

6.6 Levels and pattern of CYK-4 on the membrane is sensitive to the depletion of profilin (PFN-1)

Our analysis of CYK-4 on the membrane upon NMY-2 depletion, rather surprisingly revealed that centralspindlin localization is affected by perturbations in components of the contractile ring. We investigated whether a similar phenotype would be observed upon disruption of actin filaments. We depleted *C. elegans* embryos of profilin (PFN-1) which is required for formin mediated F-actin assembly. Following *pfn-1(RNAi)* CYK-

4::GFP localized on the membrane in bright, distinct foci during anaphase. We also imaged embryos co-expressing a NMY-2::RFP transgene under the same conditions, to find that these CYK-4 foci co-localize with NMY-2 on the plasma membrane (Figure 30B). Although in wild-type embryos centralspindlin can be detected on the cleavage furrow where the actomyosin-based contractile ring is present, these data support suggest a more complex link between localization of centralspindlin on the plasma membrane and the actomyosin network.

6.7 Discussion

Together, the results presented in this chapter suggest that Rho-GTP and its downstream effectors either directly or indirectly interact with the Rho activator centralspindlin, to fine-tune the pathway leading to cortical contractility. The observation that cortical recruitment of CYK-4 is dependent on RhoA activity may provide a mechanism for positive feedback in cytokinesis.

Accumulation of active RhoA on the plasma membrane during cytokinesis is not diffuse but tightly focused in a sharp zone. There is no mechanism known that fully explains this behavior. One way to generate high concentrations of protein in a distinct domain is via oligomerization. Oligomerization induces cooperative binding, which could nucleate assembly of distinct structures. Small amounts of a multimeric complex could trigger the accumulation of a large cluster to generate a sharp boundary of RhoA activity. Indeed,

membrane-associated centralspindlin appears in domains, rather than being uniformly distributed (Figure 9C).

An exciting and unexplored aspect of cytokinesis are feedback mechanisms between RhoA, its effectors and activators that could restrict the zone of RhoA activity to a defined region or enhance an initial, feeble asymmetry. The role of centralspindlin oligomers at the membrane only constitutes part of an intricate pathway leading to spatially regulated RhoA activation. The C-terminal GAP domain of CYK-4 plays an unexpected but crucial role in the activation of ECT-2 (Zhang and Glotzer 2015). RhoA promotes ECT-2 activation resulting in a positive feedback loop (Zhang and Glotzer 2015). In addition we find that membrane localization of centralspindlin is also dependent on the interaction between CYK-4 and RhoA (Figures 28), providing a second mode of positive feedback.

Some molecules of centralspindlin in a cluster may bind RhoA and promote membrane localization, others in the cluster could bind ECT-2 and activate it. These two mechanisms involve the same three players and therefore are intricately linked. To test whether RhoA-dependent CYK-4 accumulation on the membrane promotes furrow induction, we require an experimental system in which initial RhoA activation is independent of CYK-4. We are employing an optogenetic approach to address this question. Recent results from our lab (Wagner and Glotzer 2016a) demonstrate that recruitment of the DH domain of the RhoGEF LARG to the membrane is sufficient to generate ectopic RhoA activity and permit furrow formation. However these ectopic furrows often do not ingress to completion, and are not self-sustaining in the absence of

light. To test whether the presence of a Rho binding domain (RBD) would improve RhoGEF function and promote further furrow ingression, we have fused the RBDPH domains of anillin to the C terminus of LARG(DH). A similar GEF-effector fusion protein has been shown to strongly promote Ras activation under certain, albeit limited, regimes (Coyle and Lim 2016). Further, we have generated constructs where the RBDPH domains are replaced with the Cyk4 GAP domain. The prediction is that because these domains can directly bind RhoA, once recruited to the membrane by transient light excitation, these fusion proteins will remain membrane-bound and allow sustained furrow ingression. If this function is unique to Cyk4, other RhoGAP domains will be unable to promote Rho activity in this context.

Our experiments also demonstrate that the actomyosin cytoskeleton negatively regulates RhoA activity via centralspindlin. In addition, centralspindlin on the membrane appears to depend on cortical NMY-2. However it is unclear whether these proteins directly interact. Are these observations relevant to wild-type cytokinesis? Are they indicative of a negative feedback loop in cytokinesis?

Unexpectedly, our data also point to an interaction between the centralspindlin complex and actomyosin. At intermediate levels of myosin (NMY-2) depletion or when profilin (PFN-1) is depleted in *C. elegans* embryos, CYK-4 strongly co-localizes with myosin in foci at the equatorial cortex (Figure 30). We are currently investigating whether this behavior represents a functional interaction in wild-type cytokinesis. One putative model is that centralspindlin associates with a pool of disassembling actin in the wake of an ingressing cleavage furrow. For successful cytokinesis, it may be advantageous to

restrict RhoA activity to the tips of ingressing furrows, and the actomyosin-bound pool of centralspindlin may in fact be sequestered from activating RhoA outside of the furrow region, as a form of delayed negative feedback. When the contractile ring is disrupted by myosin and profilin depletion, we may generate an increase in disassembling actin and hence observe centralspindlin accumulate in myosin-rich foci. However, this is a highly speculative model that would require rigorous testing. An important question to address is whether centralspindlin directly interacts with actin or myosin. In human cells we find that centralspindlin fragments, when over-expressed, strongly associate with a fraction of actin cables in interphase cells (Figure 31). This interaction requires the globular tail of ZEN-4 (Figure 31). We are currently mapping the exact domains participating in this interaction. The results of our experiments may provide a way to unambiguously test if the interaction between centralspindlin and actomyosin is relevant in wild-type cytokinesis.

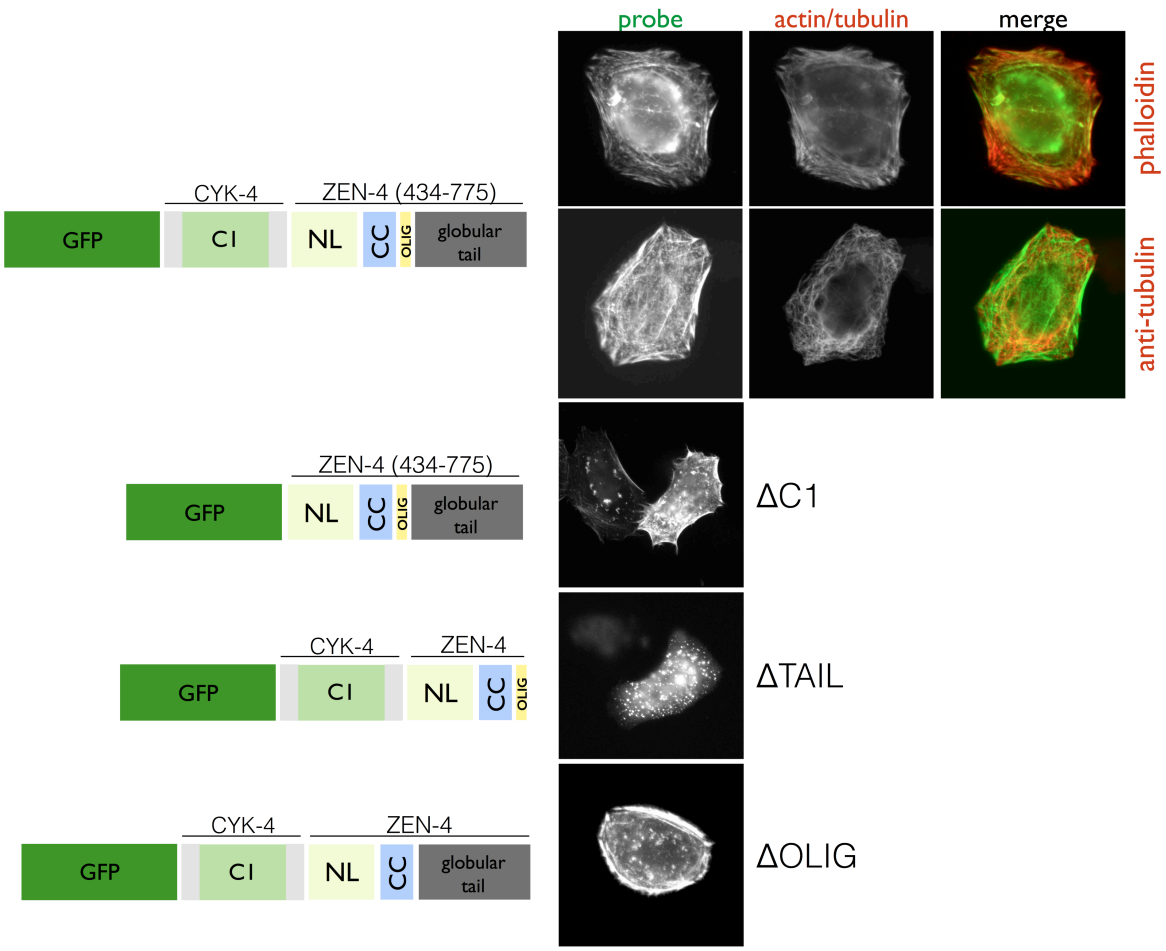


Figure 31. Actin co-localization of centralspindlin fusion proteins in HeLa cells. A GFP fusion construct containing the CYK-4 C1 domain, ZEN-4 neck-linker (NL), coiled-coil (CC), oligomerization domain (OLIG) and globular tail (*C. elegans* protein sequence, codon optimized for human cells) was expressed in HeLa cells. During interphase, this fusion protein when over-expressed, strongly localized with a subset of actin fibers (as judged by phalloidin staining) but not with microtubules. This localization was lost when the globular tail of ZEN-4 was deleted, resulting in aggregates in the cytoplasm.

Finally, it is curious that at strong depletions of NMY-2 when CYK-4 is no longer detectable on the membrane (Figure 30A), active RhoA still hyper-accumulates in a CYK-4-dependent fashion (Figure 29). This suggests that local, stable accumulation of CYK-4 may not be required for activating RhoA (discussed further in section 7.2).

Chapter 7: Discussion: Centralspindlin, a multi-tasking complex

During cytokinesis in metazoans a group of well-conserved proteins are employed to divide cells of various sizes and morphology in diverse physiological and developmental contexts. The mechanisms underlying cytokinesis therefore must be robust yet malleable in order to adapt to the requirements of any cell. In this section we discuss how the mechanisms we have discovered (Figure 32 and 34) can be applied to and refashioned under different conditions, redundant pathways that may obscure the roles performed by centralspindlin and some unexplored aspects of cytokinesis.

7.1 Distinct contributions of centralspindlin components in RhoA activation

While centralspindlin has previously been detected on the plasma membrane in diverse systems (Adams et al. 1998; Green et al. 2013; Verbrugghe and White 2004), our data provide a comprehensive mechanism for how the complex accumulates and what function it serves at this location. The requirement for membrane accumulation may be particularly acute in cell types where the central spindle is at a considerable distance from the membrane. The regulatory mechanism that controls membrane localization of centralspindlin is conceptually similar to, but independent of, and functionally distinct from, the mechanism that underlies microtubule bundling. In both cases, oligomerization enhances weak localization signals. However, the sites of localization are distinct and the localization motifs reside on different subunits of centralspindlin (the ZEN-4 kinesin motor domain for microtubule binding and the CYK-4 C1 domain for membrane

association). To date, all the phenotypes ascribed to inhibition of centralspindlin clustering have been assumed to result from defective central spindle assembly (Hutterer, Glotzer, and Mishima 2009; Douglas et al. 2010). Centralspindlin promotes ECT-2-mediated RhoA activation through the phosphoregulated binding of CYK-4 with the N-terminal autoinhibition domain of ECT-2 (Yüce, Piekny, and Glotzer 2005; Wolfe, Takaki, Petronczki, and Glotzer 2009a; Burkard et al. 2009). This interaction was previously thought to occur solely on overlapping microtubules (Wolfe, Takaki, Petronczki, and Glotzer 2009a; Petronczki et al. 2007). Here, we define an early, independent role for centralspindlin oligomers in RhoA activation at the cell cortex (Figure 32).

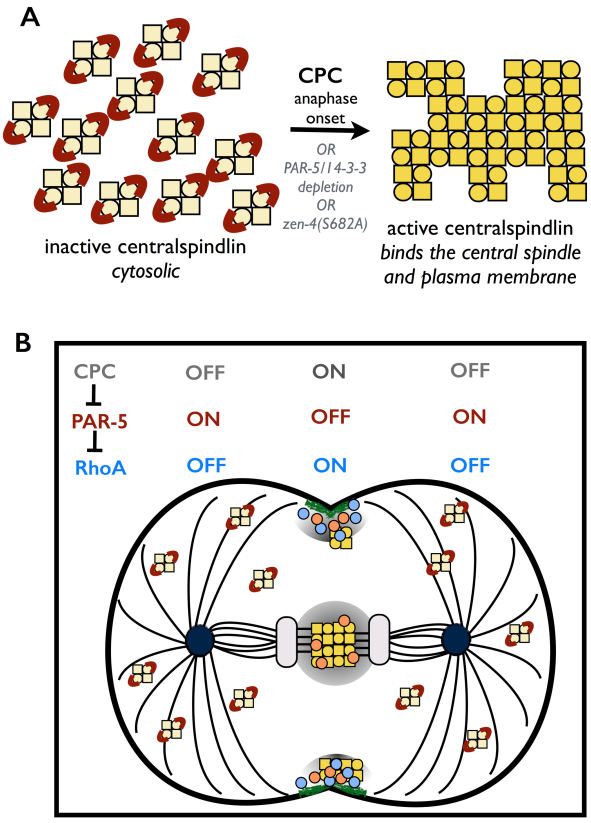


Figure 32. Model for spatiotemporal regulation of centralspindlin-directed RhoA activation by PAR-5/14-3-3 and Aurora B kinase. A. Our data indicate that PAR-5/14-3-3 proteins (red) act globally to prevent centralspindlin oligomerization and maintain the complex in its soluble, inactive form (pale yellow). During anaphase, Aurora B kinase (CPC) activity antagonizes PAR-5 activity, allowing centralspindlin (dark yellow) to form clusters that can bind microtubules and the plasma membrane with high avidity. B. Schematic to illustrate spatial regulation of RhoA activation during anaphase. The CPC (gray) is known to be active in the equatorial region of a dividing cell, specifically at the central spindle and the plasma membrane during anaphase. This could result in zones of centralspindlin activity where centralspindlin (dark yellow) locally forms clusters, allowing ECT-2 (orange) and RhoA (blue) activation at the equatorial membrane and subsequent recruitment of actomyosin (green) to generate the contractile ring. Centralspindlin is inactive (pale yellow) in other regions of the cell because of PAR-5/14-3-3 (red) inhibition.

In the case of spindle midzone assembly, centralspindlin oligomerization facilitates interaction of the MKLP1/ZEN-4 motor domain with microtubules (Hutterer, Glotzer, and Mishima 2009). In contrast, to induce cortical RhoA activation, the membrane-binding C1 domain of CYK-4 is required (Figures 16-18). Our study indicates that centralspindlin

oligomerization likely potentiates low-affinity interactions of the C1 domain via an avidity effect. This interpretation is consistent with the finding that artificially generated tandem repeats of the HsCyk4 C1 domain bind more strongly to the plasma membrane (Lekomtsev et al. 2012). Thus, membrane localization and spindle midzone accumulation of centralspindlin both require oligomerization, but they accumulate via distinct domains.

The extent to which each component of centralspindlin contributes to RhoA activation may vary between different experimental situations. For instance in human cells, depletion of CYK-4 by RNAi completely abolishes furrow formation (Yüce, Piekny, and Glotzer 2005) which indicates that RhoA activation in these cells is entirely centralspindlin-dependent. Yet, MKLP1/ZEN-4 depletion in these cells does not block furrowing (Yüce, Piekny, and Glotzer 2005). This may be because a) there is sufficient MKLP1 remaining to localize a small amount of CYK-4 to the membrane, b) ECT-2 activation via CYK-4 does not strictly require MKLP1-directed oligomerization in human cells or c) CYK-4 can promote RhoA activity by a redundant mechanism. In the absence of an intact spindle, MKLP1/ZEN-4 becomes essential for furrow induction (not shown). Similarly, the C1 domain of CYK-4 is dispensable for furrow ingression in human cells with properly organized bipolar spindles, but required in cells that enter anaphase with disorganized microtubule arrays (Figure 18). In contrast in *C. elegans*, centralspindlin-directed furrowing requires CYK-4, ZEN-4 and the CYK-4 C1 domain.

These results underscore how different cells use the same complex - centralspindlin - in non-identical ways to direct furrow ingression. A number of scenarios could explain

these cell type-specific differences. The cellular abundance of centralspindlin and other downstream components may vary across cell types and impact various loss-of-function phenotypes. Cultured human cells are also considerably smaller than *C. elegans* embryos and the spindle midzone in these cells lies a few microns from to the plasma membrane. In these cells, small amounts of microtubule-associated CYK-4 could productively associate with the membrane via other proteins. Apart from the C1 domain and MKLP1/ZEN-4-dependent oligomerization, other factors can regulate CYK-4 localization to the membrane. The GAP domain of CYK-4 binds RhoA which is membrane-tethered (Zhang and Glotzer 2015; Touré et al. 1998) and CYK-4 binds ECT-2 which contains two membrane binding motifs (Wolfe, Takaki, Petronczki, and Glotzer 2009a; Petronczki et al. 2007; Yüce, Piekny, and Glotzer 2005; Su, Takaki, and Petronczki 2011). Additionally, ECT-2 has been shown to bind anillin (Frenette et al. 2012) and CYK-4 may also interact with actomyosin (Chapter 6) to localize to the cleavage furrow.

7.2 The function and interaction of various centralspindlin pools during cytokinesis

During anaphase, centralspindlin can be cytosolic, associate with microtubule bundles, bind to the plasma membrane and perhaps associate with actomyosin (Figure 33). We have gained considerable insight into the requirements for centralspindlin localization at each of these sites (Figure 33) but we have little information regarding how and the extent to which these distinct pools of the complex exchange with each other, their

stability at each sub-cellular site and to what extent each pool participates in RhoA activation in the presence of the others. In fission yeast, competition between actin-binding proteins and distinct actin structures for a common pool of actin has been demonstrated to have important biological consequences (Suarez et al. 2015; Burke et al. 2014). Similar data is unavailable for centralspindlin and centralspindlin-associated structures.

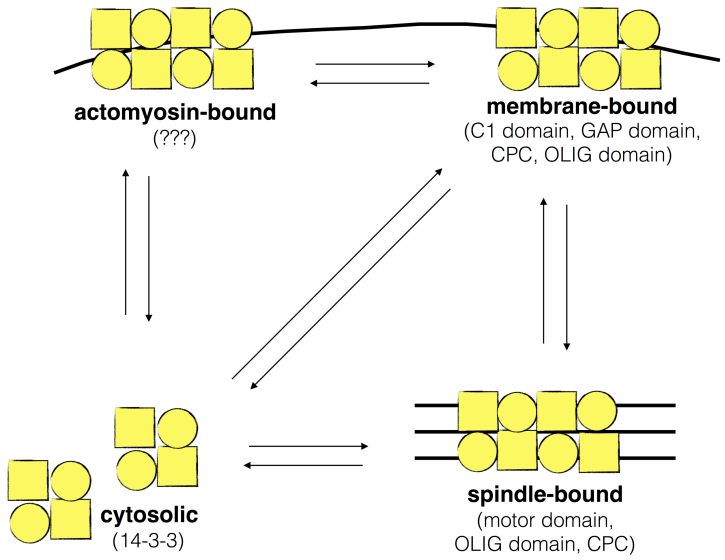


Figure 33. Distinct pools of centralspindlin. In the context of previous data, our results reveal that centralspindlin in an anaphase cell can be cytosolic (where it is likely present as individual heterotetramers bound by 14-3-3 proteins), associated with spindle microtubules as an oligomer (this requires the motor and oligomerization domains of ZEN-4 and activity of the CPC), associate with the membrane (this requires the C1 and GAP domains of CYK-4, oligomerization domain of ZEN-4 and activity of the CPC) or associated with actomyosin. The requirements for the latter interaction are currently unknown. It is also likely that the actin-bound pool of centralspindlin simultaneously binds the membrane. In theory all these pools of centralspindlin could exchange with each other. Future experiments will reveal the dynamics and interactions between them and their relevance in wild-type cytokinesis.

The central spindle-bound complex is highly stable and, once it accumulates, it does not appear to exchange much with cytosolic protein (Hutterer, Glotzer, and Mishima 2009). Therefore it seems unlikely that membrane-bound centralspindlin is derived from

spindle-associated complexes. The C1 domain is required in membrane binding of centralspindlin and RhoA activation. However it is unclear if this reflects a stable interaction with the plasma membrane or a dynamic one.

One of the most perplexing results of this work, presented in Chapter 6 (see section 6.7 and Figures 29 and 30), suggests that when myosin is strongly depleted, centralspindlin can promote RhoA activation without being stably localized at the plasma membrane. Is the membrane-binding property of CYK-4 still required for such a mechanism? This contrasts with our current model (section 7.3 Figure 34) where we observe that membrane accumulation of centralspindlin correlates with and is required for RhoA activation. To fully comprehend the mechanisms of RhoA activation by centralspindlin, it will be important to determine whether centralspindlin can activate RhoA “at a distance” or via fleeting, dynamic interactions.

Finally, an interesting unanswered question is whether the spindle-bound pool of centralspindlin is essential for furrow ingression in the presence of membrane-bound centralspindlin. This could be tested in *C. elegans* by simultaneously disrupting the spindle midzone (strong PRC1 depletion) and the NOP-1-dependent furrow induction pathway. Under these conditions one would need to test if a) the levels of membrane-bound centralspindlin are altered due to loss of the spindle-bound pool and b) if the membrane-localized complex is sufficient for furrow induction. A prevailing model for cytokinesis suggests that the mitotic spindle is required early to designate the division site and very late for abscission but once the contractile ring is assembled, it will contract without cues from the spindle. However, it is likely that centralspindlin on

microtubules provides continued RhoA activation as the furrow ingression and local concentration of the complex assists in sustained furrow ingression. Indeed our lab has demonstrated that accumulation of active RhoA on the plasma membrane is sufficient to initiate a cleavage furrow but not to complete cytokinesis (Wagner and Glotzer 2016a).

7.3 Features of a fully-functional centralspindlin complex

As summarized in Figure 34A, the regulation and function of centralspindlin in RhoA activation is complex. Viewed in the context of previous analyses (Wolfe, Takaki, Petronczki, and Glotzer 2009a; Loria, Longhini, and Glotzer 2012; Zhang and Glotzer 2015; Hutterer, Glotzer, and Mishima 2009), our work reveals that RhoA activation at the plasma membrane exploits numerous features of the centralspindlin complex requiring centralspindlin to productively engage in a large number of protein-protein interactions (Figure 34B). CYK-4 binds to ECT-2 via its N-terminal domain (Wolfe, Takaki, Petronczki, and Glotzer 2009a) and its C-terminal GAP domain (Zhang and Glotzer 2015). Both of these interactions appear crucial in activating the GEF. CYK-4 has a membrane-binding C1 domain, which we demonstrate is essential in RhoA activation and furrow induction in the absence of parallel pathways in both human cells and *C. elegans.* Importantly, in addition to the C1 domain, we find membrane localization of centralspindlin is also contingent on the ability of ZEN-4 to oligomerize as well as the interaction between CYK-4 GAP domain and RhoA.

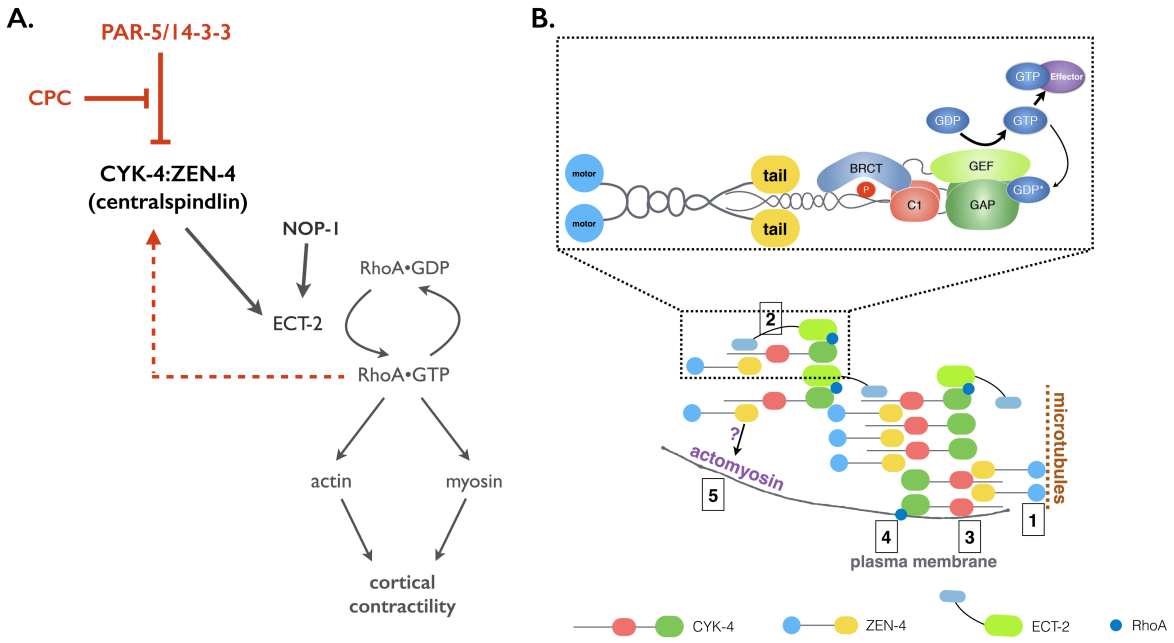


Figure 34. A revised view of the pathway leading to cortical contractility during cytokinesis and a model for an active, membrane-bound centralspindlin complex. A. The experiments in this thesis demonstrate that centralspindlin-directed RhoA activation is subject to upstream spatiotemporal regulation by 14-3-3 proteins and CPC activity. Recruitment of centralspindlin to the plasma membrane is dependent on the ability of CYK-4 to bind RhoA resulting in a positive feedback loop. B. A large centralspindlin oligomeric unit could simultaneously engage in diverse protein interactions unlike a single heterotetramer. The inset provides a detailed molecular view of the interactions between CYK-4, ZEN-4, ECT-2 and RhoA (adapted from Zhang and Glotzer, 2015). **1**. The ZEN-4 motor domains bind to microtubules **2**. CYK-4 binds to the BRCT and GEF domains of ECT-2. **3**. The CYK-4 C1 domain directly binds the plasma membrane. **4**. The CYK-4 GAP domain binds RhoA on the plasma membrane. **5**. Centralspindlin associates with actomyosin but the nature of this interaction is unknown. (ZEN-4 motor domain, light blue. ZEN-4 globular tail, yellow. CYK-4 C1 domain, pink. CYK-4 GAP domain, green. ECT-2 BRCT domain, steel blue. ECT-2 GEF domain, light green. RhoA, dark blue).

The latter interaction provides a possible mechanism allowing positive feedback to sustain high levels of active RhoA at the tips of an ingressing furrow. We conclude that to activate RhoA at the plasma membrane, the oligomeric form of centralspindlin must directly interact with the membrane, ECT-2 and RhoA. Finally, we are investigating a potential interaction between centralspindlin and actomyosin, which may yet be an additional feature of a fully-functional centralspindlin complex during cytokinesis. It is clear that centralspindlin performs functions in early, mid and late cytokinesis. While

local RhoA activity is sufficient to generate a cleavage furrow (Wagner and Glotzer 2016a), the stringent spatiotemporal regulation of furrow position and completion of cytokinesis require an intact centralspindlin complex. A single heterotetramer of centralspindlin that weakly localizes at a cellular site would likely be incapable of efficiently performing all the functions of centralspindlin. However, centralspindlin oligomers could provide a mechanism (Figure 34B) in which different molecules serve different roles generating an industrious and versatile multimeric unit that directs cytokinesis in metazoans.

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Appendix: Methods

C. elegans culture

Strains

C. elegans strains were maintained on nematode growth medium (NGM) plates using standard procedures. Unless otherwise specified, all strains were obtained from the *Caenorhabditis* Genetics Center funded by the National Institutes of Health (NIH) National Center for Research Resources. A list of strains used is provided later in this section.

Transgenic lines

Single copy transgenic lines expressing CYK-4::GFP and CYK-4ΔC1::GFP (Figures 16 and 17) were integrated into the Mos element ttTi5605 on chromosome II using MosSCI (Frøkjær-Jensen et al. 2008).

CRISPR lines and genome editing

The *zen-4(S682A)* mutation (Figure 8) was generated at the endogenous locus using a co-CRISPR strategy (Zhang & Glotzer 2014). To generate the sgRNA template, the following primers were used to amplify a region near *zen-4 S682:* MG4854(forward), ATCCAATAGACGAGATGCAGACTGTTTTAGAGCTAGAAATAGCAAGT and MG4855 (reverse), AGTCTGCATCTCGTCTATTGGATCAAGACATCTCGCAATAGG. The PCR product obtained was cloned into the pDD162 vector which also expresses the Cas9 enzyme (Dickinson et al. 2013). The oligo MG4853,

GTTAATCCAAAATATCAAAGAAGATCTAAGGCTGCATCTCGTCTATTGGATCATCAG CCA was co-injected as a repair template to generate *zen-4(S682A)*. This primer also contains a silent mutation to abolish the PAM site without altering S680.

The AIR-2:mNeonGreen:Flagx3 and CSC-1:mNeonGreen:Flagx3 transgenic strains (Figure 22) were generated by fusing the endogenous gene with mNeonGreen using the CRISPR protocol in Dickinson et al. 2015. The AIR-2:mNeonGreen^SEC^ Flagx3 (pKL263) and CSC-1:mNeonGreen^SEC^Flagx3 (pKL264)repair plasmids were built using parental vector pDD268. Homology arms were amplified from N2 worm lysate - the left arm contains 604bp of C-term ORF of AIR-2 and the right arm is 580bp of the AIR-2 3' UTR; the left arm contains 574bp of C-term ORF (plus a silent mutation of the targeted sgRNA PAM motif) of CSC-1 and the right arm is 519bp of CSC-1 3' UTR. These arms were inserted by Hifi DNA Assembly to flank the Self-Excising Cassette (SEC) using AvrII and SpeI sites per the protocol's C-term fusion directions. The sgRNA (pKL261- Peft-3:Cas-9; Pu6:AIR-2 sgRNA) was generated by overlapping the following primers: MG5461, GCGAGATGATTGAAAGAAGGAGTTTTAGAGCTAGAAATAGCAAGT and MG5462, CTCCTTCTTTCAATCATCTCGCAAGACATCTCGCAATAGG. The sgRNA (pKL262 - Peft-3:Cas-9; Pu6:CSC-1 sgRNA) was generated by overlapping the following primers: MG5467, GCGGGGGAAAAATTAAATCGGTTTTAGAGCTAGAAATAGCAAGT and MG5468, CCGATTTAATTTTTCCCCCGCCAAGACATCTCGCAATAGG. The PCR products were cloned into pDD162 by SLiCE.

RNAi

RNAi was administered by feeding nematodes with *E. coli* expressing the appropriate double-stranded RNA (dsRNA) (Timmons & Fire 1998). HT115 bacterial cultures were grown in Luria broth with 100 µg/ml ampicillin overnight at 37°C. Cultures (250 µl) were seeded on NGM plates containing 100 µg/ml ampicillin and 1 mM isopropyl β-d-1-thiogalactopyranoside and incubated at room temperature (~23°C) for 8 hours. RNAi plasmids were obtained a library produced by (Kamath et al. 2003).

L4 hermaphrodites were picked onto feeding plates at 25°C at least 24 hours prior to dissection. For mild RNAi conditions, embryos were imaged 12-16 hours after worms were transferred to feeding plates. For very strong depletion, embryos were hatched on feeding plates and gravid worms were dissected for imaging 40-48 hrs later. For experiments where two genes were simultaneously knocked down by RNAi, bacterial cultures of *E. coli* expressing the appropriate dsRNA were mixed in a 1:1 ratio seeded onto NGM plates containing 100 µg/ml ampicillin and 1 mM isopropyl β-d-1-thiogalactopyranoside and incubated at room temperature (~23°C) for 8 hours. If stronger depletion of one of the two genes was desired, embryos were first hatched onto feeding plates targeting the gene and L4 larvae were then transferred to fresh plates with bacteria expressing dsRNA against both genes.

Drug treatment of embryos

In experiments where microtubules were depolymerized (Figure 2D and Supplementary Figure 13), gravid adults were dissected into egg salt buffer containing 50µg/mL

nocodazole and mounted and imaged as described above. The absence of pronuclear migration in early embryos was indicative of microtubule depletion.

HeLa cell culture

Maintenance

HeLa cells were grown in Dulbecco's modified Eagle's medium (DMEM, Sigma-Aldrich) supplemented with 10% FBS (Hyclone; Thermo Fisher Scientific), and 1% penicillin/streptomycin (Invitrogen) at 37ºC, 5% CO_2.

Cell preparation for live imaging

For Figure 18, HeLa cells were transfected with MgcRacGAP siRNA (Lekomtsev et al. 2012) and, at the same time, treated with 2.5 mM thymidine (Sigma). After 24 hr cells were released for 6 hr, arrested in mitosis by addition of 25ng/ml nocodazole (Sigma) for 4 hr prior to treatment with 20 μM flavopiridol (Sigma). In Figure 18B, phase contrast images of cells were recorded every 5 min in normal medium using an IncuCyte FLR integrated live-cell imaging system (Essen Bioscience).

For Figure 27, HeLa cells were transfected with Stargazin-GFP-LOVpep (a variant that contains the substitutions T406A, T407A and I532A) and PDZ-PDZ-mCherry fused to the C terminus of INCENP. 24 hours later, the cells were arrested in prometaphase with 40ng/mL nocodazole (Sigma-Aldrich) for 3-6 hours. Cells were then washed with DMEM

3-4 times and incubated at 37ºC for 30-45 minutes in DMEM containing 10mM HEPES and 3% Oxyrase (Mansfield, OH) to permit exit into anaphase prior to imaging.

Drug treatment

For Figure 23B, as described in the legend, HeLa cells were arrested in metaphase using a low dose of nocodazole (40ng/mL) for four hours. In the presence of nocodazole, they were either treated with hesperadin (100µM), to inhibit Aurora B kinase for 1 hour, or with purvalanol (30µM), to inhibit Cdk1 for 30 minutes, or both. (Purvalanol was most effective when first diluted from stock in DMSO). Following the indicated treatment, the cells were fixed with cold methanol for 20 minutes at -20ºC prior to antibody staining.

Cell fixation and antibody staining

For Figure 18C, cells were fixed for 16 h at -20ºC methanol (for Anillin staining) or for 15 min on ice in 10% tricholoroacetic acid (for RhoA staining) before being processed for immunofluorescence microscopy as described (Lénárt et al. 2007). In Figure 18C. Rabbit anti-anillin (Piekny & Glotzer 2008) (immunofluorescence microscopy 1:2000) and mouse monoclonal anti-RhoA (26C4, Santa Cruz, immunofluorescence microscopy 1:75).

For Figure 23A and Figure 25, cells were fixed in 4% paraformaldehyde (PFA) for 20 minutes at 37ºC and subsequently washed with PBS. Cells on coverslips were incubated in a humidified chamber at room temperature with 5% NDS for 15 minutes and with Aurora B antibody (1:200, BD transduction) for 1 hour.

Following 3 washes with TBST, cells were incubated with the appropriate secondary antibody for 45 minutes at room temperature, washed thrice with TBST and incubated with DAPI (1:1000) for 5 minutes. The coverslips were rinsed with TBST 3 times and mounted on a glass slide using mounting media (Southern Biotech) for imaging. For Figure 23B, a similar protocol was followed, except the cells were fixed in cold methanol as described above and stained with anti-Anillin antibody (1:500, Glotzer lab). In Figure 31, cells were fixed with 4% PFA as described, and stained with Rhodamine-phalloidin (1:100) or anti-tubulin antibody (mouse, DMIA, 1:200).

Microscopy

C. elegans embryos

To prepare one-cell embryos for imaging, gravid hermaphrodites were dissected into egg salt buffer on coverslips, mounted onto 2.5% agar pads and sealed with vaseline. For Nomarski imaging, embryos were observed with a Zeiss (Thornwood, NY) Axioplan II with a 100X/1.3 Plan-Neofluar objective. Images were captured with a charge-coupled device (CCD) camera (Imaging Source, Charlotte, NC) controlled by Gawker (http://gawker.sourceforge.net). Images were acquired every 5 s and processed with ImageJ (http://rsbweb.nih.gov/ij). For confocal imaging, embryos were imaged with a 63X/1.4 numerical aperture oil-immersion lens on either a Zeiss Axiovert 200M equipped with a Yokogawa CSU-10 spinning-disk unit (McBain, Simi Valley, CA) and

illuminated with 50-mW, 473-nm and 20-mW, 561-nm lasers (Cobolt, Solna, Sweden), or a Zeiss Axioimager M1 equipped with a Yokogawa CSU-X1 spinning-disk unit (Solamere, Salt Lake City, UT) and illuminated with 50-mW, 488-nm and 50-mW, 561-nm lasers (Coherent, Santa Clara, CA). Images were captured on a Cascade 1K EM-CCD camera or a Cascade 512BT (Photometrics, Tucson, AZ) controlled by MetaMorph (Molecular Devices, Sunnyvale, CA). Image processing was performed with ImageJ. Time-lapse acquisitions were assembled into movies using Metamorph and ImageJ.

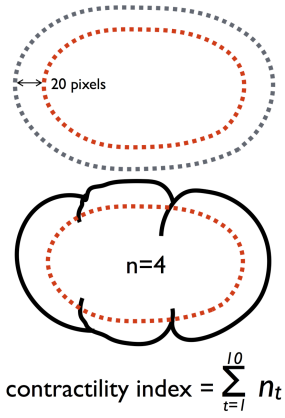
HeLa cells

Images in Figure 23 and 25 were acquired with a 63X/1.4 numerical aperture oil-immersion lens on a Zeiss Axioimager M1 equipped with a Yokogawa CSU-X1 spinning-disk unit (Solamere, Salt Lake City, UT) and illuminated with epifluorescence light.

Images in Figure 18 were acquired using a Olympus FV1000D (InvertedMicroscopeIX81) laser confocal scanning microscope equipped with a PlanApoN x60/1.40 NA Oil Sc objective lens and controlled by FV10-ASW software (Olympus).

Images in Figure 27 were acquired with a 63X/1.4 NA oil immersion lens on a Zeiss laser scanning confocal unit, illuminated with 488nm and 561nm lasers. ZEN acquisition software was used to control the microscope.

Image quantification



Contractility Index (DIC movies): To quantify the extent of contractility in various genotypes, sub-stacks of time-lapse movies acquired by DIC microscopy were generated. In Figure 6D, frames acquired every 35s from 280s to 70s prior to pronuclei meeting (pseudocleavage) or every 25s from 300s to 550s after pronuclei meeting (anaphase) were used for analysis. In Figures 7B, 8B, 15 and 19, frames acquired every 30s from 500s to 200s before (pseudocleavage) or every 35s from 100s to 450s after (anaphase) Nuclear Envelope Breakdown (NEBD) were used. Using ImageJ, an ellipse was drawn within the cell, 20 pixels away from the cell edge (as shown). In each frame, the number of furrows that ingress up to or beyond this ellipse were counted. The total number of furrows counted in each stack was considered the contractility index of the embryo for that phase of contractility.

Membrane recruitment (RhoA biosensor): To quantify the accumulation of GFP::AHPH on the membrane (Figure 9A,D), we measured the integrated intensity of pixels 1.45X above cytosolic background.

Membrane recruitment index (centralspindlin): To quantify membrane recruitment of CYK-4::GFP or ZEN-4::GFP, maximum intensity projections of the first 100s of anaphase were generated from time-lapse movies of embryos expressing either marker. For each embryo, a 50 pixel wide region of the cell edge was manually defined and straightened using ImageJ. A maximum intensity projection of a 15-pixel-wide box at the membrane was generated to obtain a line of 1-pixel width. A 5-pixel-wide line was drawn in the cell 50 pixels away from the membrane, to obtain intensity values in the cytoplasm. The membrane and cytosol values at each pixel were normalized to their respective averages across the embryo. 5-pixel running averages for normalized membrane and cytosol values were obtained, and these cytosolic values were subtracted from the corresponding membrane values for each pixel along the cell edge. The values below an arbitrary threshold of 0.07 were set to zero and the sum of the remaining differences was calculated. This number was considered the membrane recruitment index for each embryo.

Centralspindlin recruitment to ingressing furrows:

Embryos expressing CYK-4::GFP and a membrane marker (mCherry::PH) were filmed simultaneously by time-lapse confocal and DIC microscopy. In the mCherry::PH or the DIC images, the tip of the furrow was defined by an ROI in ImageJ at different stages of

ingression. The maximum intensity of CYK-4::GFP in the ROI was determined in the corresponding frames. Using the same ROI, a maximum intensity in a random region of the cytoplasm was obtained. The extent of recruitment to the furrow tip was calculated as (max. furrow intensity/max. cytosolic intensity - 1). These values were averaged across embryos of a given genotype.

Biochemistry

(Experiments described in Figure 26B).

Protein purification

Coding sequences for the indicated constructs were cloned into the GST expression vector pGEX-4T- tobacco etch virus (TEV). GST-tagged proteins were expressed in *E. coli* strain BL21 by adding 0.6 mM IPTG at OD600 reached 0.5–0.75 at 25ºC. Cells were grown for another 4 hr at 25ºC and collected. Frozen cells were thawed in lysis buffer (10mM HEPES pH 7.7, 1mM EGTA, 1mM $MgCl_2$, 250mM NaCl, 0.5mM PMSF, 1µg/ml leupeptin, 1µg/ml pepstatin A, 0.1% Triton X-100, 1mM DTT, 0.5mg/ml lysozyme) and lysed by sonication. The bacterial lysate was centrifuged at 18,000RPM at 4˚C for 20 min. Glutathione-Sepharose 4B beads (bioWORLD) were added to supernatant and incubated at 4ºC for 4 hr. The beads were washed 1X with lysis buffer containing PMSF and 2X with lysis buffer without PMSF. Protein-bound beads in were stored in 10mM HEPES pH 7.7, 1mM EGTA, 1mM $MgCl_2$, 1% Triton X-100, 1 mM DTT,

1µg/ml leupeptin, 1µg/ml pepstatin A, 25% glycerol at –20ºC. Supernatants and eluted proteins were stored in the same buffer but with 10% glycerol at -80ºC.

Kinase assay

In these reactions, commercially available Histone H3 protein (gift from Ruthenburg lab) was mixed at a final concentration of 200µg/mL in kinase buffer (25mM HEPES, 10mM $MgCl_2$, 0.5mM DTT, pH7.5) containing 2mM ATP with either full-length Hs Aurora B bounded to beads or soluble INCENP (C terminus) or both, or with varying concentrations of the INCENP-Aurora B fusion protein shown in the figure. Reactions were incubated at 30ºC for 15 minutes and quenched by adding sample buffer. Reactions were run on a 15% SDS-PAGE gel, and analyzed by Western blot (anti-pH3-S10 antibody, 1:1000, Cell Signaling 9706S).

List of *C. elegans* strains

Strain number	*Genotype*	*Figure*
N2	ancestral N2 Bristol strain; “wild-type”	
MG535	mCherry:HIS IV; PH::GFP	6
OD239 (MG501)	*cyk-4(or749ts)* ltIs38 [pAA1; *pie-1*/GFP::PH(PLC1delta1) *unc-119* (+)]III; ltIs37 [pAA64; pie-1/mCHERRY::his-58; unc-119 (+)]IV	6
MG520	*cyk-4(or749)*III*;* zuIs45[*nmy-2*::NMY-2::GFP + *unc-119*(+)]V; ltIs37[pAA64; *pie- 1*::mCHERRY::HIS-58; *unc-119* (+)]	6
EU554 (MG131)	*zen-4(or153ts)*(outcrossed 3x)IV	6
JH2754 (MG552)	*ect-2(ax751)*II	6
KK725 (MG400)	*nop-1(it142)* (outcrossed 10x)III	6, 19, 20
MG512	*cyk-4(or749) nop-1(it142)*III	7
JH2647 (MG591)	axIs1928 [mCherry::par-6] oxIs1182 [GFP::par-2]	7
MG644	mgSi5[cb-UNC-119 (+) GFP::ANI-1(AH+PH)]II; *unc-64(e246)*III]; mCherry:HIS IV	9
MG662	mCherry::HIS IV; xsIs7[Myo2::GFP;CYK-4::GFP-Pie-1 3’UTR;UNC-119(wt)]	9, 10, 12, 13
MG170	*zen-4(or153ts)*; *zen-4::GFP* complex array rescue construct in EU716	9, 12, 13
EU707 (MG640)	*air-2(or207) unc-13(e51)* I	19
MG666	*nop-1(it142)* III; *air-2(or207ts) unc-13(e51)* I	19
MG505	Tubulin:GFP	13
MG571	Tubulin:GFP; mCherry:HIS IV	14, 15
MG575	Tubulin:GFP; mCherry:HIS IV; *nop-1(it142)* III	14, 15
MG702	Tubulin:GFP; mCherry:HIS IV; *nop-1(it142)* III; *air-2(or207ts) unc-13(e51)* I	14, 15
MG694	*nop-1(it142)* III; mgSi[cyk-4::gfp rRNAi, cb-unc-119(+)] II	16

MG744	*nop-1(it142)*III; mgSi[cyk-4(ΔC1)::gfp rRNAi, cb-unc-119(+)] II; mCherry::PH	16
MG688	mgSi[cyk-4::gfp rRNAi, cb-unc-119(+)] II	11, 16
MG695	mgSi[cyk-4(ΔC1)::gfp rRNAi, cb-unc-119(+)] II	16
MG725	mgSi[cyk-4::gfp rRNAi, cb-unc-119(+)] II; unc-119(ed3) III; ltIs44 V, ltIs44pAA173; [pie-1p-mCherry::PH(PLC1delta1) + unc-119(+)].	17
MG726	mgSi[cyk-4(ΔC1)::gfp rRNAi, cb-unc-119(+)] II; unc-119(ed3) III; ltIs44 V, ltIs44pAA173; [pie-1p-mCherry::PH(PLC1delta1) + unc-119(+)].	17
MG831	zen-4(S682A) IV; GFP at NF-GFP site on IV	8
MG833	zen-4(S682A) IV; GFP at NF-GFP site on IV; mgSi[cyk-4::gfp rRNAi, cb-unc-119(+)] II	11
MG656	unc-119(ed3) III; ltIs44 V ltIs44pAA173; [pie-1p-mCherry::PH(PLC1delta1) + unc-119(+)]; xsIs7[Myo2::GFP;CYK-4::GFP-Pie-1 3'UTR;UNC-119(wt)]	21
MG944	AIR-2(xs115[AIR-2:mNeonGreen:flagx3]) I	22
MG948	CSC-1(xs119[CSC-1:mNeonGreen:flagx3]) II	22
MG685	mgSi43[cyk-4::gfp rRNAi, cb-unc-119(+)] II	28
MG716	mgSi16[cyk-4(R459A)::gfp rRNAi, cb-unc-119(+)] II	28
MG804	mgSi33[cyk-4(K495E, R499E)::gfp rRNAi, cb-unc-119(+)] II	28
MG930	NMY-2::mKate; GFP::AHPH (Rho biosensor)	29
MG939	NMY-2(endog)::mKate; mgSi5[cb-UNC-119 (+) GFP::ANI-1(AH+PH)]II; nop-1(it142) II	29
MG965	nmy-2(cp52[NMY-2::mKate])I; mgSi5[cb-UNC-119 (+) GFP::ANI-1(AH+PH)]II; cyk-4(or749)/unc-64(e246)III; mCherry:HIS IV	29
MG464	cyk-4::gfp;cyk-4;unc-119;myo-2-gfp;nmy-2::rfp	30
MG952	mgSi43[cyk-4::gfp rRNAi, cb-unc-119(+)] II; NMY-2::mKate	30

List of constructs

Plasmid number	*Construct*	*Figure*
EK772	Stargazin-GFP-LOVpep(CA)	25
*pAB65	2XPDZ-mCh-mouse INCENP(697-876)	25
pAB89	2XPDZ-p53(tetramerization domain)-mCh-mouse INCENP(697-876)	25
pAB122	GST-TEV-HsINCENP(834-903)	26
pAB120	GST-TEV-HsAurora B(WT, FL)	26
pAB126	GST-TEV-HsINCENP(834-903)-HsAurora B(FL, L154A, H250Y)	26
EK1259	Stargazin-GFP-LOVpep	27
*pAB88	2XPDZ-mCh-mouse INCENP(697-876)	27
pAB66	GFP-Ce-CYK-4(C1)-Ce-ZEN-4(NL-CC-OLIG-TAIL) (codon adapted)	31
pAB70	GFP-Ce-ZEN-4(NL-CC-OLIG-TAIL) (codon adapted)	31
pAB71	GFP-Ce-CYK-4(C1)-Ce-ZEN-4(NL-CC-OLIG) (codon adapted)	31
pAB72	GFP-Ce-CYK-4(C1)-Ce-ZEN-4(NL-CC-TAIL) (codon adapted)	31

*pAB65 has a few extra amino acids accidentally included in the C terminus prior to the STOP codon. pAB88 has the STOP in the right place.

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