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Aurora B Kinase Promotes Cytokinesis by Inducing Centralspindlin Oligomers that Associate with the Plasma Membrane

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SUMMARY

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. Here we report that a regulated pool of membrane-bound, oligomeric centralspindlin stimulates RhoA activation. The membrane-binding C1 domain of CYK-4, a centralspindlin component, promotes furrow initiation in *C. elegans* embryos and human cells. Membrane localization of centralspindlin oligomers is globally inhibited by PAR-5/14-3-3. This activity is antagonized by the chromosome passenger complex (CPC), resulting in RhoA activation at the nascent cleavage site. Therefore, CPC-directed centralspindlin oligomerization during anaphase induces contractile ring assembly at the membrane.

Graphical abstract

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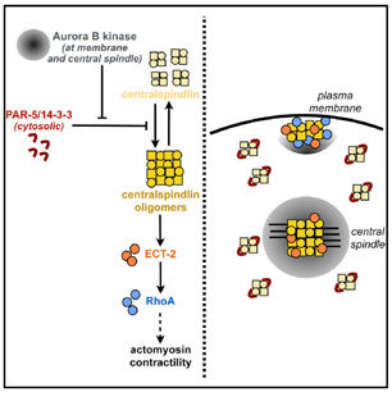
AUTHOR CONTRIBUTIONS

A.B. and M.G. conceived and designed the project. A.B. performed the majority of the experiments. S.L. performed the cultured cell experiments, Y.C.T. performed critical pilot experiments. D.Z. generated the CYK-4: :GFP transgenic lines, provided invaluable support by generating the *zen-4(S682A)* allele, and provided critical feedback on the manuscript; these authors each made significant contributions and are considered equal contributors and are listed alphabetically. K.L. provided support in generating *C. elegans* strains. M.P. supervised the cultured cell experiments. A.B. and M.G. wrote the manuscript.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, seven figures, and one movie and can be found with this article online at http://dx.doi.org/10.1016/j.devcel.2015.03.015.



INTRODUCTION

Cytokinesis physically partitions a single parent cell into two distinct daughters. The primary workhorse of this process is an actomyosin-based contractile ring localized at the cell cortex that drives ingress of the plasma membrane, generating a new cell boundary (Schroeder, 1968, 1973). The contractile ring must be positioned between segregating chromosomes so that the resulting progeny receives an equitable distribution of nuclear and cytoplasmic components. During asymmetric division, spatial regulation of the division plane permits an unequal inheritance of cell fate determinants.

The central regulator of contractility during cytokinesis is the small GTPase RhoA (Eggert et al., 2006; Barr and Gruneberg, 2007; Werner and Glotzer, 2008; Kishi et al., 1993). In its active GTP-bound form, RhoA directly activates the actin polymerizing protein formin (Otomo et al., 2005), indirectly promotes phosphorylation of the regulatory light chain of non-muscle myosin II (Matsumura, 2005), and is required for the cortical recruitment of the scaffold protein anillin (Piekny and Glotzer, 2008). Appearance of active RhoA on the cortex at the nascent division site is the key first step in cytokinesis, but the spatial regulation of RhoA remains poorly understood.

A conserved guanine nucleotide exchange factor (GEF), ECT-2, activates RhoA by inducing transition from the guanosine diphosphate (GDP)- to the guanosine triphosphate (GTP)-bound state (Kim et al., 2005). Cortical accumulation of active RhoA, its downstream effectors, and, consequently, cleavage furrow formation require ECT-2 (Tatsumoto et al., 1999). During anaphase, ECT-2 localizes to the cell cortex and to the interdigitated microtubules, the central spindle, midway between separated chromosomes. The latter localization is mediated by a direct interaction with the well conserved centralspindlin complex (Yüce et al., 2005; Wolfe et al., 2009; Petronczki et al., 2007; Burkard et al., 2009).

Centralspindlin is a heterotetramer of a dimeric kinesin-6 motor, ZEN-4/MKLP1, and a dimeric Rho family GTPase-activating protein (GAP), CYK-4/MgcRacGAP (Mishima et al., 2002; Pavicic-Kaltenbrunner et al., 2007). This complex performs a myriad of functions during cell division. It has microtubule bundling activity and is required for assembly of the central spindle. Central spindle assembly requires MKLP1 oligomerization, which increases the avidity of the complex for microtubules and promotes its stable localization to the

overlapping antiparallel microtubules in the center of the cell (Hutterer et al., 2009). Centralspindlin oligomerization is regulated by antagonistic interactions between 14-3-3 proteins and Aurora B kinase, and this limits the extent of centralspindlin binding to microtubules (Douglas et al., 2010). Therefore, centralspindlin oligomerization has one known function: to promote assembly of the central spindle.

Active RhoA must accumulate at the membrane, often at a distance from microtubules, to induce contractile ring assembly. Although considerable evidence demonstrates that the primary RhoA activator, ECT-2, concentrates at the central spindle in a complex with centralspindlin (Tatsumoto et al., 1999; Petronczki et al., 2007) and that this interaction is essential for RhoA activation in diverse metazoa (Yüce et al., 2005; Tse et al., 2012; Somers and Saint, 2003; Miller and Bement, 2009), how this complex leads to RhoA activation at the equatorial membrane is not understood. Spindle microtubules are not always directly adjacent to the equatorial cortex in many embryonic systems and can be dramatically attenuated without preventing cleavage furrow formation (von Dassow et al., 2009). It is not known where ECT-2 activation occurs, nor is it known how the exquisite spatial regulation characteristic of cytokinesis is achieved. 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.

In addition to its ability to accumulate on microtubules, the centralspindlin complex also has membrane-binding properties. The CYK-4/MgcRacGAP molecule contains a C1 domain that associates weakly with the plasma membrane. Its avidity for the membrane increases dramatically when the domain is present as tandem repeats (Lekomtsev et al., 2012). However, human cells expressing a CYK-4 variant lacking its C1 domain assemble fully ingressing cleavage furrows. Although they ingress fully, they fail to form a stable midbody and fail to abscise. Therefore, the C1 domain appears to be dispensable for RhoA activation, at least in this cell type.

Here we elucidate a complex regulatory mechanism that controls the localization of a cortical pool of centralspindlin that promotes RhoA activation in multiple biological systems. Using genetics and live imaging of *C. elegans* embryos, we demonstrate that PAR-5/14-3-3 globally inhibits membrane localization of centralspindlin and centralspindlin-dependent RhoA activation by preventing oligomerization of the complex. This function of PAR-5 is antagonized by Aurora B kinase, permitting formation of a cytokinetic furrow. We also demonstrate that the CYK-4 C1 domain contributes to RhoA activation via centralspindlin in both *C. elegans* embryos and human cells. It is apparent that furrow induction during cytokinesis is subject to multiple layers of regulation, as appropriate for a critical cellular process. Based on our findings, we propose a mechanism for activation of RhoA at the membrane in wild-type cells.

RESULTS

PAR-5/14-3-3 Depletion Leads to Enhanced Centralspindlin-Dependent Cortical Contractility

We began by characterizing a previously identified but poorly understood negative regulator of contractility called PAR-5, a 14-3-3 protein. Following fertilization, the one-cell *C. elegans* embryo undergoes a stereotyped RHO-1- and ECT-2-dependent contractility pattern while establishing anterior-posterior polarity. This culminates in the formation of a pseudocleavage furrow (RHO-1 is the *C. elegans* ortholog of the GTPase RhoA; hereafter, we will use the more general term RhoA). Following polarization, the cell enters anaphase and undergoes cytokinesis. Depletion of PAR-5/14-3-3 by RNAi results in increased contractility during both pseudocleavage and cytokinesis in one-cell *C. elegans* embryos (Morton et al., 2002). To visualize this change in contractility, 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 (Figures 1A and 1B) and exhibit an increase in cortical contractility.

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, and, as expected, we found that the hypercontractile phenotype was suppressed when PAR-5 was depleted from hypomorphic *ect-2* mutant embryos (Zonies et al., 2010; Figures 1C and 1D).

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 a partial redundancy with NOP-1 at this stage (Figure 1E; Tse et al., 2012). Surprisingly, although centralspindlin is dispensable for pseudocleavage in otherwise wild-type embryos, the exaggerated hypercontractility during pseudocleavage characteristic of PAR-5 depletion was completely suppressed by loss-of-function mutations in CYK-4 or ZEN-4 (Figures 1A, 1C, and 1D; Figure S1A). On the other hand, loss of NOP-1 function did not abolish this hypercontractility (Figures 1C and 1D). The genetic requirements for hypercontractility during cytokinesis are identical to those during pseudocleavage. Therefore, PAR-5 is a specific, negative regulator of centralspindlin-dependent RhoA activity (Figure 1E).

PAR-5 is a known regulator of cell polarity. Hypercontractility is not a common phenotype among polarity-defective mutants; therefore, the hypercontractility cannot 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 S1B). Polarity establishment and inhibition of centralspindlin-dependent contractility are therefore independent functions of PAR-5/14-3-3.

Loss of PAR-5 Increases Cortical Levels of Active RhoA and the Centralspindlin Complex

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 2A; Figure S2A, 7 of 7 embryos). Subsequently, the RhoA biosensor is observed in the equatorial region and at the ingressing furrow of the dividing cell (data not shown). Therefore, 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 of 7 embryos) and ZEN-4 (8 of 8 embryos). This accumulation was often observable on the lateral cortex prior to furrow ingression in PAR-5-depleted embryos (Figure 2B; Figure S2B). CYK-4 and ZEN-4 are interdependent for their stable association with the cortex (Figure 2C; Figure S2E) because they are at the central spindle. The cortical accumulation is accompanied by a slight decrease in centralspindlin accumulation at the midzone (Figure S2D). 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 strikingly at the tips of ingressing furrows (Figure S2H). This localization is lost as the cell enters mitosis and reappears during anaphase. 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 centralspindlin. When embryos undergo division from four to six 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 of 10 embryos) at an earlier stage than in wild-type embryos (Figure S2I).

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 2D; Figures S2F and S2G) does not impede cortical localization of CYK-4 or ZEN-4. This suggests that, unlike its localization at the central spindle, there is a microtubule-independent mechanism for membrane recruitment of centralspindlin. These observations suggest that the increase in cortical levels of a RhoA activator, the centralspindlin complex, might cause the increased contractility in *par-5* embryos and that this function must be independent, at least in part, of enhanced microtubule binding by oligomeric centralspindlin.

The Aurora B Kinase AIR-2 Antagonizes PAR-5/14-3-3 Inhibition of Centralspindlin-Directed RhoA Activation

In human cells, MKLP1 directly binds ζ and γ isoforms of 14-3-3 via S710 phosphorylation. 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), 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 (Guse et al., 2005; Kaitna et al., 2000). We hypothesized that an analogous mechanism could regulate cortical activation of RhoA. In a wild-type embryo, cytosolic PAR-5 may globally inhibit centralspindlin oligomer formation by directly binding ZEN-4. The CPC could disrupt PAR-5 inhibition by Aurora B kinase-mediated phosphorylation of ZEN-4 to locally induce oligomerization, membrane association, and contractility. We tested this model genetically during the first division of *C. elegans* embryos. The model (Figure 3B) predicts that, if the centralspindlin-independent NOP-1 pathway and Aurora B kinase (AIR-2) are inactivated simultaneously, then furrows should not form during pseudo-cleavage or cytokinesis. We imaged early embryos defective in NOP-1 and expressing a temperature-sensitive Aurora B kinase (Severson et al., 2000) using differential interference contrast (DIC) microscopy at the non-permissive temperature. Consistent with the model, no contractility is observed in *nop-1(it142); air-2(or207ts)* embryos (Figure 3A; Figure S3; 9 of 9 embryos). The model also 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 3A; Figure S3; 11 of 12 embryos). Although the cytokinetic furrow is restored, the cells rarely divide. The restored furrows exhibit cortically associated CYK-4 (data not shown). These data strongly support a model in which CPC promotes centralspindlin-directed cortical RhoA activation by inactivating the inhibitor PAR-5.

Astral Microtubules Pattern Active RhoA

According to the proposed 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 (Figures 1B and 3A). Astral microtubules that emanate from the spindle have been reported to negatively regulate cortical accumulation of RhoA effectors (Werner et al., 2007; Tse et al., 2012). 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 to generate embryos with small, posteriorly localized spindles. 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 et al., 2007). If astral inhibition can act on the hypercontractility resulting from depletion of PAR-5, then contractility should be restricted to anterior 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 the position of the spindle. Embryos depleted of both PAR-5 and ZYG-9 exhibit enhanced contractility in the anterior compared with embryos depleted of ZYG-9 alone (Figure 4; Figure S4). In a *nop-1* mutant background, *zyg-9(RNAi)* embryos primarily exhibit a posterior furrowing (3 of 5 embryos, no anterior furrow; 2 of 5 embryos, weak anterior furrow), whereas co-depletion of ZYG-9 and PAR-5 generates additional contractility in the anterior (5 of 5 embryos). *nop-1(it142); air-2(or207ts)* mutant embryos demonstrate little contractility at anaphase at the restrictive temperature, regardless of spindle position (4 of 5 embryos, no furrows; 1 of 5 embryos, weak posterior furrow). However, depletion of PAR-5 in these double mutants restores contractility, and the cortex appears quiescent only in regions enriched in astral microtubules (5 of 5 embryos). Cortical centralspindlin upon PAR-5 depletion was also confined to the anterior of the cell, away from the asters (data not shown), akin to the localization seen upon PAR-5 depletion in nocodazole-treated embryos (Figure 2D). Therefore, astral microtubules suppress contractility resulting from PAR-5 depletion, and the equatorial furrow likely forms because of local relief from this inhibitory effect, much like the formation of an equatorial furrow in a centralspindlin-deficient embryo (Werner et al., 2007).

The CYK-4 C1 Domain Promotes RhoA Activation in *C. elegans* Embryos

Next we addressed how centralspindlin associates with the membrane. 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 found that embryos expressing only CYK-4ΔC1: :GFP underwent furrow ingression but failed cytokinesis, whereas CYK-4: :GFP fully rescued the *cyk-4(RNAi)* phenotype (data not shown). This phenotype appears to be 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 toward 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. Embryos with these genotypes were filmed to visualize the extent of furrowing during cytokinesis. We found that, although 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 of 5), but cell division was completed successfully in embryos expressing the wild-type (8 of 8 embryos) transgene (Figure 5A). The membrane-binding property of the C1 domain is therefore essential for centralspindlin-dependent furrow induction.

CYK-4 and ZEN-4 can localize to the cortex in a microtubule-independent manner (Figure 2D). Because 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 and

CYK-4ΔC1 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 2B), full-length CYK-4 localizes to the equatorial cortex in the absence of PAR-5 (9 of 10 embryos). However, CYK-4ΔC1 was not detectable at the cortex (11 of 11 embryos) (Figure 5B; Figure S5A), and no hypercontractility was observable (data not shown). Finally, to determine whether the C1 domain is responsible for membrane recruitment of CYK-4 in wild-type embryos, we expressed CYK-4ΔC1: :GFP and full-length CYK-4: :GFP along with a membrane marker. We found that, in an otherwise wild-type embryo, full-length CYK-4 was observed at the tips of the ingressing furrow. However, CYK-4ΔC1 was not detectable (Figure 5C; Figure S5B).

A *zen-4* Allele Predicted to Be Defective in PAR-5 Binding Phenocopies PAR-5 Depletion

Our data so far demonstrate that a membrane-localized pool of centralspindlin contributes to RhoA activation and consequent induction of the cytokinetic furrow. Furthermore, this function of centralspindlin is antagonized by PAR-5/14-3-3. In human cells, MKLP1 directly binds 14-3-3 proteins via S710 phosphorylation, which 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 6A) to prevent oligomer formation and membrane recruitment of centralspindlin. In the absence of PAR-5/14-3-3, ectopic centralspindlin oligomers on the membrane are proposed to cause the observed hypercontractility. If this hypothesis is correct, then abolishing ZEN-4 S682 phosphorylation should mimic PAR-5 depletion. To stringently test this, we took advantage of the clustered regularly interspaced short palindromic repeats (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 S6B), but they are viable, and we were able to obtain embryos homozygous for *zen-4(S682A)*. At the one-cell stage, these embryos demonstrate a striking similarity to *par-5(RNAi)* embryos (Figures 6B and 6C; Movie S1). Pseudocleavage furrows are often unilateral, and, in a small fraction of embryos (2 of 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. To further test this model, 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 of 6 embryos) and anaphase (10 of 10 embryos) (Figure 6D; Figure S6A). These 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.

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 apparent contradiction to our observations in *C. elegans* embryos, the cytokinetic furrow ingresses deeply with essentially wild-type kinetics in the absence of the

C1 domain of HsCyk4 in human cells, although it ultimately regresses (Lekomtsev et al., 2012). This suggests that the membrane tether provided by HsCyk4 is not 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 human cells in the absence of an intact spindle. Cells depleted of endogenous HsCyk4 by small interfering RNA (siRNA) were arrested in mitosis and released into anaphase in the presence of a low dose of nocodazole (Figure 7A). 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 7B; Figure S7), and RhoA and its effectors are not recruited to the membrane (Figure 7C). These data suggest that inactivation of microtubule-dependent mechanisms of cortical centralspindlin recruitment renders the C1 domain of HsCyk4 crucial for RhoA activation. Moreover, the ability of HsCyk4 to activate RhoA and generate contractility is also lost when its MKLP1 binding, N-terminal domain is deleted (Figure 7B; Figure S7). These data suggest that MKLP1 plays a role in RhoA activation by HsCyk4 at the membrane and that this function of centralspindlin is conserved in *C. elegans* and human cells.

DISCUSSION

The work presented in this manuscript provides 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 (Figure 3B).

Spatial Regulation of Centralspindlin Oligomerization during Furrow Formation

PAR-5, a cytosolic 14-3-3 protein in *C. elegans*, negatively regulates ectopic activation of RhoA. In the absence of PAR-5, *C. elegans* embryos are hypercontractile during polarization and cytokinesis, which correlates with ectopic localization of centralspindlin to the membrane. The CPC antagonizes PAR-5 binding to centralspindlin and promotes RhoA activation (Figure 3). These data unequivocally show, in contrast to previous models (Lewellyn et al., 2011), that the primary role of the CPC in cytokinesis is to activate centralspindlin and, thereby, permit cleavage furrow formation. In wild-type cells, we anticipate that PAR-5 activity is locally inhibited by the CPC, particularly at the spindle midzone (Figures 7D and 7E). However, in addition to its strong recruitment at the central spindle, there are numerous reports of a second pool of the CPC that accumulates at the equatorial cortex (Earnshaw and Cooke, 1991; Cooke et al., 1987). This pool has no known function. In vitro reconstitution of cytokinetic signaling in *Xenopus* extracts supports a requirement for Aurora B kinase activity for the accumulation of RhoA-GTP at membranes overlying the spindle midzone (Nguyen et al., 2014). Our data provide a molecular

mechanism by which the CPC can induce RhoA activation. Cortically localized CPC could locally permit cortical association of centralspindlin by inhibiting PAR-5/14-3-3 binding, thereby promoting centralspindlin oligomerization. This model provides a mechanism for spatiotemporal control of RhoA activation at the cytokinetic furrow. The mechanism by which CPC localizes to this site is an important issue for future studies.

Numerous Functional Domains in Centralspindlin Are Required for RhoA Activation

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. To date, all phenotypes ascribed to inhibition of centralspindlin clustering have been assumed to result from defective central spindle assembly (Hutterer et al., 2009; Douglas et al., 2010). Here we define an early, independent role for centralspindlin oligomers in RhoA activation at the cell cortex (Figures 7D and 7E).

In the case of spindle midzone assembly, centralspindlin oligomerization facilitates interaction of the MKLP1/ZEN-4 motor domain with microtubules (Hutterer et al., 2009). In contrast, to induce cortical RhoA activation, the membrane-binding C1 domain of CYK-4 is required (Figures 5 and 7). 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). Therefore, membrane localization and spindle midzone accumulation of centralspindlin both require oligomerization, but they accumulate via distinct domains.

The plasma membrane pool of centralspindlin oligomers performs a critical role in cleavage furrow formation. Centralspindlin promotes ECT-2-mediated RhoA activation through the phosphoregulated binding of CYK-4 with the N-terminal autoinhibition domain of ECT-2 (Yüce et al., 2005; Wolfe et al., 2009; Burkard et al., 2009). This interaction has been thought previously to occur solely on overlapping microtubules (Wolfe et al., 2009; Petronczki et al., 2007). Centralspindlin localization to the plasma membrane provides an efficient mechanism for activation of RhoA at the membrane, the subcellular site where RhoA induces contractile ring assembly. The requirement for membrane accumulation is particularly acute in cell types where the central spindle is at a considerable distance from the membrane. Indeed, we demonstrate that this mechanism is dispensable for furrow ingression in human cells with properly organized bipolar spindles but essential in cells that enter anaphase with disorganized microtubule arrays (Figure 7). In sum, although centralspindlin has been detected previously 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 Role of Microtubules in Cortical Localization of Centralspindlin

Although the ability of centralspindlin to associate with the membrane is not strictly dependent on intact microtubule arrays (Figure 2D; Figures S2F and S2G), microtubule-mediated processes could facilitate plasma membrane localization of centralspindlin at different steps in cytokinesis. First, microtubules may regulate accumulation of the CPC on the equatorial membrane, where it would promote local centralspindlin oligomerization. Second, processive transport of centralspindlin along microtubules could promote cortical accumulation (Nishimura and Yonemura, 2006; Hutterer et al., 2009). Third, 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.

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. 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, in contrast, 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 4), 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, therefore, function at the level of RhoA or its effectors.

Multimerization: A General Mechanism for Cellular Spatiotemporal Regulation

PAR-5 and related 14-3-3 proteins regulate the oligomeric state of proteins other than centralspindlin (Benton and St Johnston, 2003; Rajagopalan et al., 2008). 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. 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 (Li et al., 2012) or via a protein scaffold to generate microdomains of high enzymatic activity. For instance, division plane positioning in *S. pombe* involves stable cortical nodes of Cdr2 kinase that recruit Mid1, an anillin ortholog that controls actomyosin (Almonacid et al., 2009). Assembly of

centralspindlin into oligomers promotes two separable functions: organization of the central spindle and generation of membrane-bound RhoA activating complexes.

Oligomerization induces cooperative binding, which could nucleate the assembly of distinct structures. Indeed, membrane-associated centralspindlin appears in domains rather than being distributed uniformly (Figure S2B). Centralspindlin oligomerization at the membrane constitutes part of the intricate pathway that leads to the generation of a well defined zone of RhoA activity characteristic of cytokinetic furrowing.

EXPERIMENTAL PROCEDURES

Strains

C. elegans strains (listed in Supplemental Experimental Procedures) were maintained on nematode growth medium (NGM) plates using standard procedures. Unless specified otherwise, all strains were obtained from the *Caenorhabditis* Genetics Center funded by the NIH National Center for Research Resources.

Transgene Insertion

Single-copy transgenic lines expressing CYK-4: :GFP and CYK-4ΔC1: :GFP (Figure 5) were integrated into the Mos element ttTi5605 on chromosome II using MosSCI (Frøkjaer-Jensen et al., 2008).

Genome Editing

The *zen-4(S682A)* mutation (Figure 6) was generated at the endogenous locus using a co-CRISPR strategy (Zhang and Glotzer, 2014). See Supplemental Experimental Procedures for further details.

RNAi

RNAi was administered by feeding nematodes with *E. coli* expressing the appropriate double-stranded RNA (dsRNA) (Timmons and 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 hr. RNAi plasmids were obtained a library produced by Kamath et al. (2003). See Supplemental Experimental Procedures for further details.

Microscopy

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 Axioplan II with a 100×/1.3 Plan-Neofluar objective. Images were captured with a charge-coupled device (CCD) camera (Imaging Source) 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 63×/1.4 numerical aperture (NA) oil immersion lens on a Zeiss Axiovert 200M equipped with a Yokogawa CSU-10 spinning disk unit (McBain)

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

Drug Treatment of Embryos

In experiments where microtubules were depolymerized (Figure 2D; Figure S2F), gravid adults were dissected into egg salt buffer containing 50 μg/ml nocodazole and mounted and imaged as described above. The absence of pronuclear migration in early embryos was indicative of microtubule depletion.

Cell Culture

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 and arrested in mitosis by addition of 25 ng/ml nocodazole (Sigma) for 4 hr prior to treatment with 20 μM flavopiridol (Sigma). To quantify the cytokinetic phenotypes in Figure 7B, 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 7C, cells were fixed for 16 hr at −20°C 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 previously (Lénárt et al., 2007) (see Supplemental Experimental Procedures for antibodies used). Images (Figure 7C) were acquired using a Olympus FV1000D (inverted microscope IX81) laser confocal scanning microscope equipped with a PlanApoN 60×/1.40 NA oil Sc objective lens and controlled by FV10-ASW software (Olympus).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- Centralspindlin oligomers promote RhoA activity at the membrane during cytokinesis
- Centralspindlin clustering and ectopic RhoA activation are inhibited by PAR-5/14-3-3
- Aurora B kinase promotes contractility by inhibiting PAR-5/14-3-3
- The CYK-4 C1 domain promotes membrane binding and RhoA activation by centralspindlin

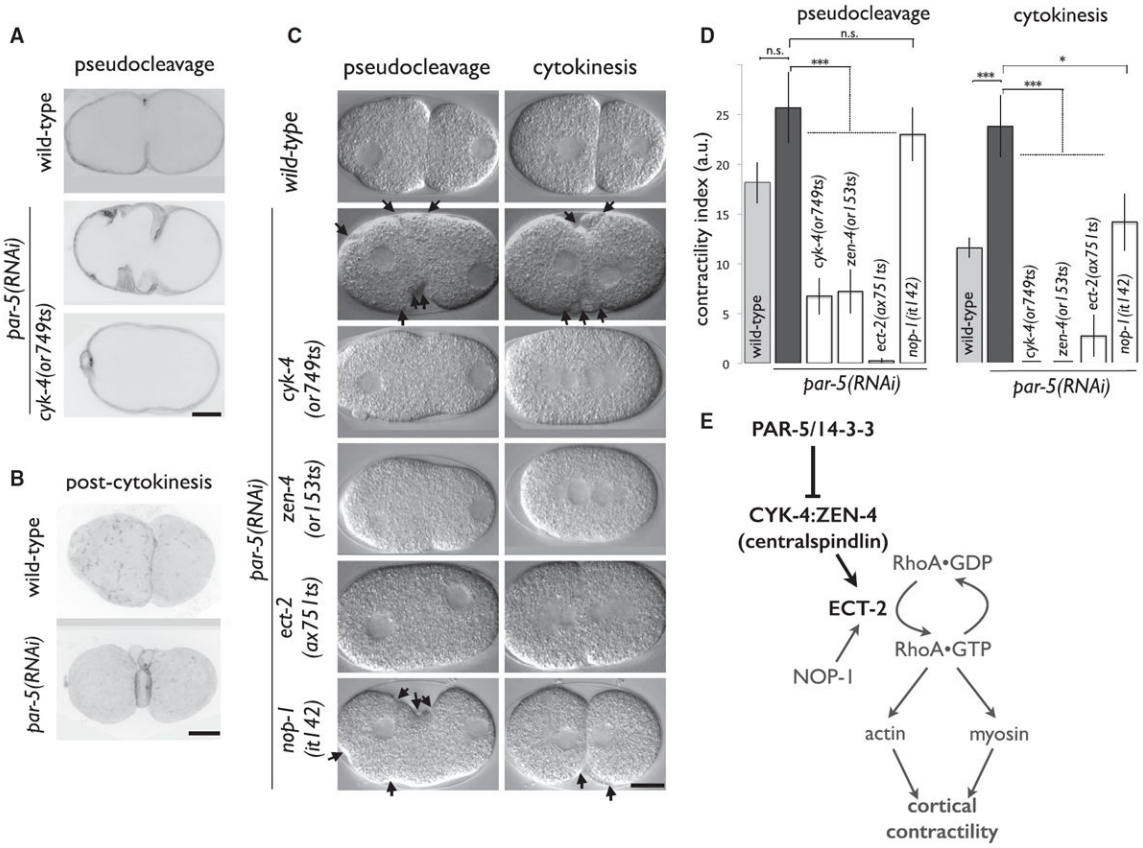

Figure 1. PAR-5/14-3-3 Depletion Leads to Enhanced Centralspindlin-Dependent Cortical Contractility

(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.

(D) Embryos of the genotypes shown in (C). were scored for the extent of contractility observed during pseudocleavage and cytokinesis (see Supplemental Experimental Procedures and Figure S1C for definition of the contractility index). Error bars indicate SEM. n.s. indicates that the populations are not significantly different at $p = 0.05$. ***$p \leq 0.01$ and *$p \leq 0.05$ (Mann-Whitney *U* test). a.u., arbitrary unit.

(E) Schematic illustrating the genetic pathway leading to RhoA activation and cortical contractility in *C. elegans* embryos. $n \geq 5$.

Scale bars, 10 μm.

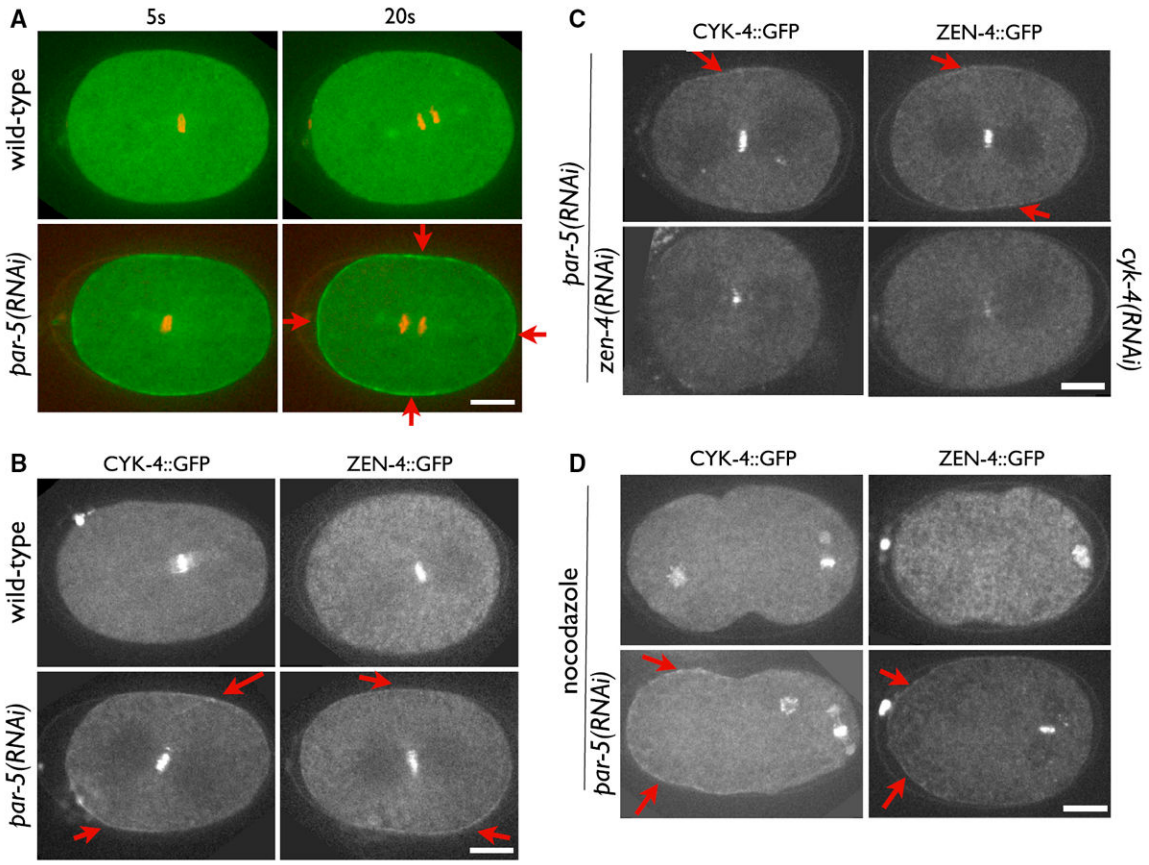

Figure 2. Cortical Levels of Active RhoA and Centralspindlin Increase upon PAR-5/14-3-3 Depletion

(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 50 s after anaphase onset are shown.

(C) 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.

(D) Cortical localization of centralspindlin does not require microtubules. 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. Quantitation of these data is shown in Figure S2.

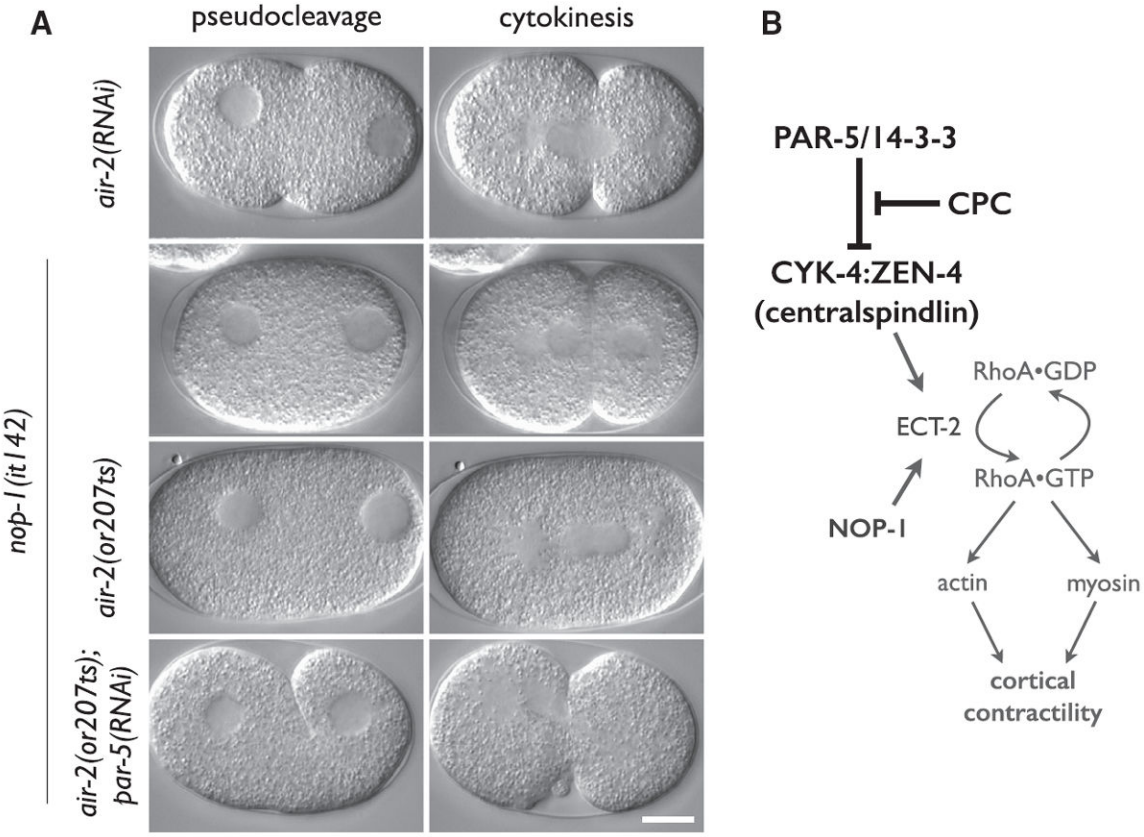

Figure 3. The Aurora B Kinase AIR-2, a CPC Component, Antagonizes PAR-5 Inhibition of Centralspindlin

(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. Changes in contractility are quantified in Figure S3. n ≥ 7. Scale bar, 10 μm.

(B) Schematic for the genetic pathway activating RhoA and generating cortical contractility in *C. elegans* embryos.

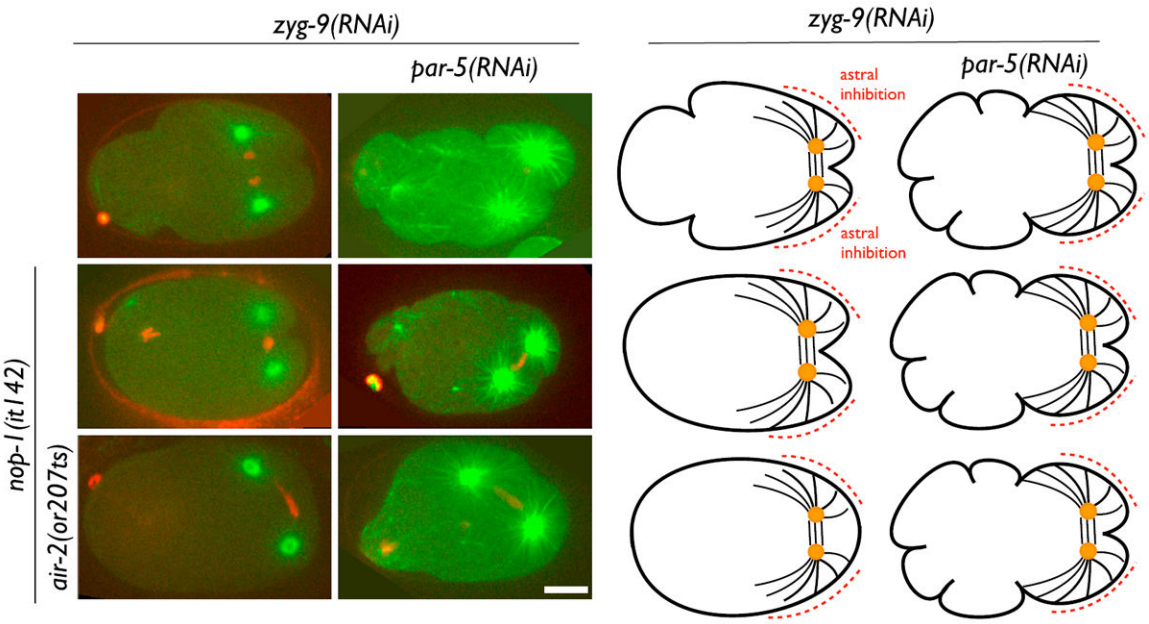

Figure 4. Astral Microtubules Pattern RhoA Activity and Furrow Position

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. 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. Changes in contractility are quantified in Figure S4.

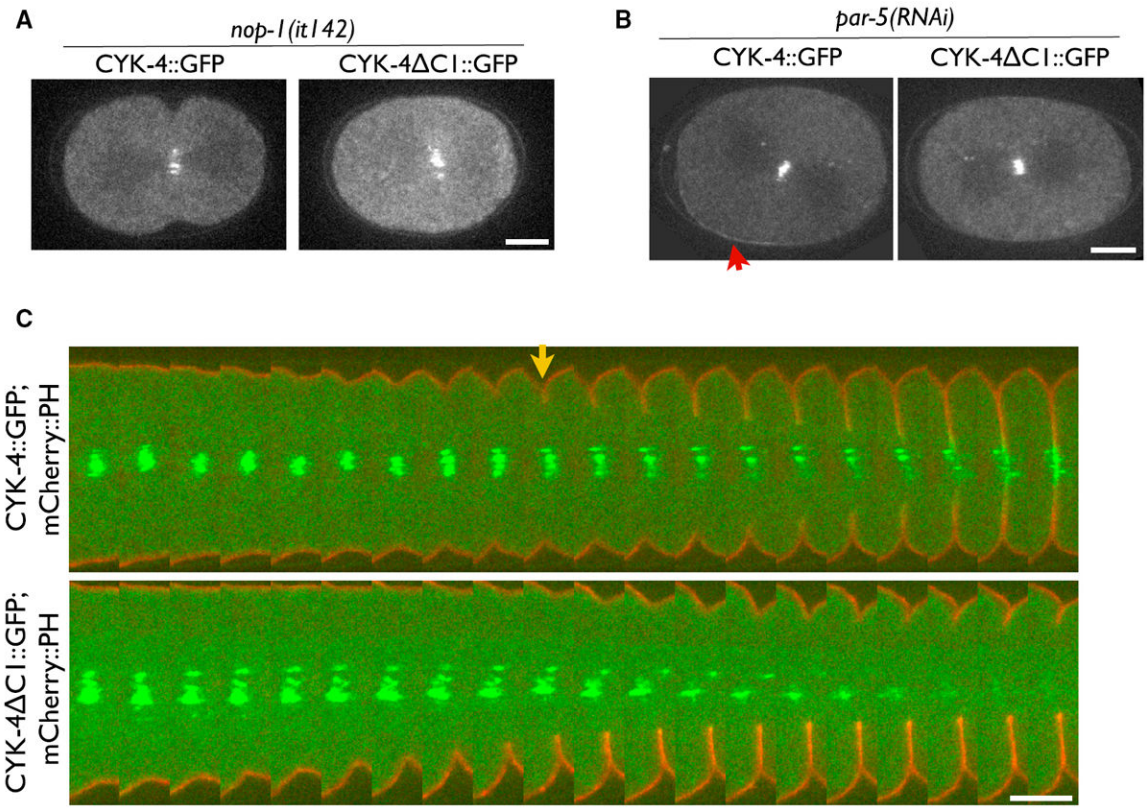

Figure 5. The CYK-4 C1 Domain Promotes RhoA Activation and Cortical Localization of Centralspindlin in *C. elegans* Embryos

(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. 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. Cortical recruitment of CYK-4: :GFP is quantified in Figure S5A. n ≥ 10.

(C) *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, 10 μm. Recruitment of the GFP-tagged protein to the furrow tip is quantified in Figure S5B.

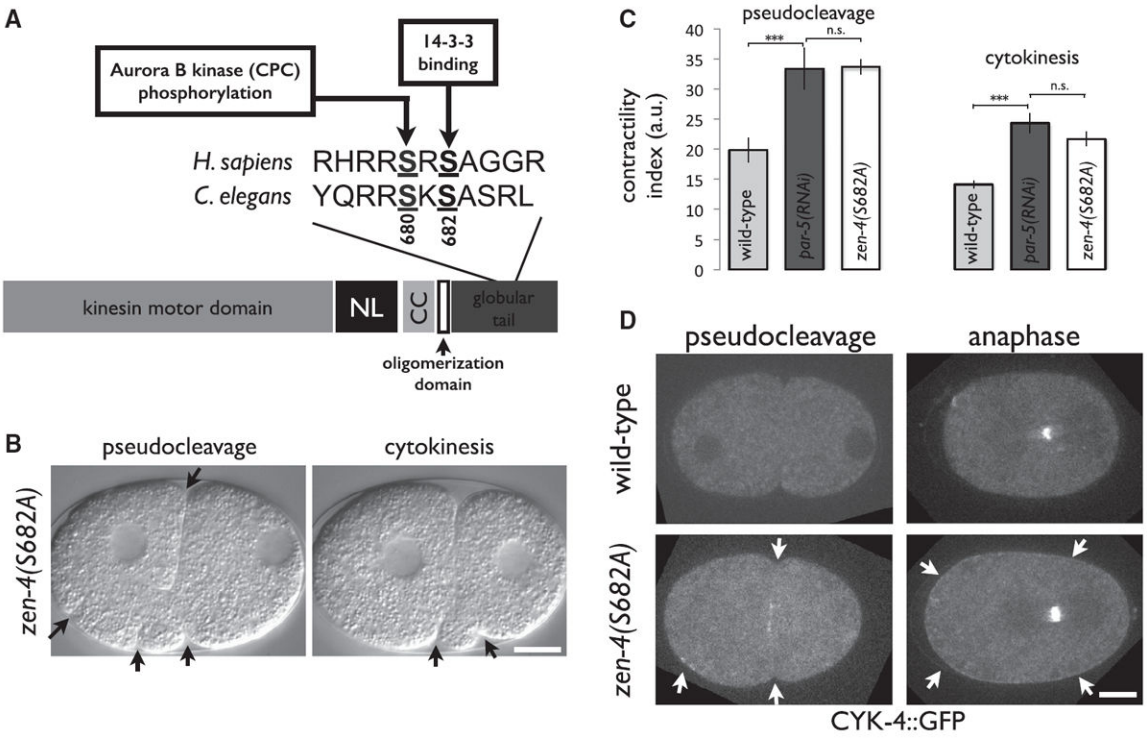

Figure 6. A *zen-4* Variant Predicted to be Defective in PAR-5 Binding Phenocopies PAR-5 Depletion

(A) Domain structure of the ZEN-4 protein showing its oligomerization domain and conserved sites in the globular tail that are predicted to regulate centralspindlin clustering. NL, neck linker; CC, coiled coil.

(B) 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. See also Movie S1.

(C) Contractility observed in *zen-4(S682A)* was quantified (see Supplemental Experimental Procedures) 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. (D) 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. Membrane recruitment of CYK-4: :GFP is quantified in Figure S6A.

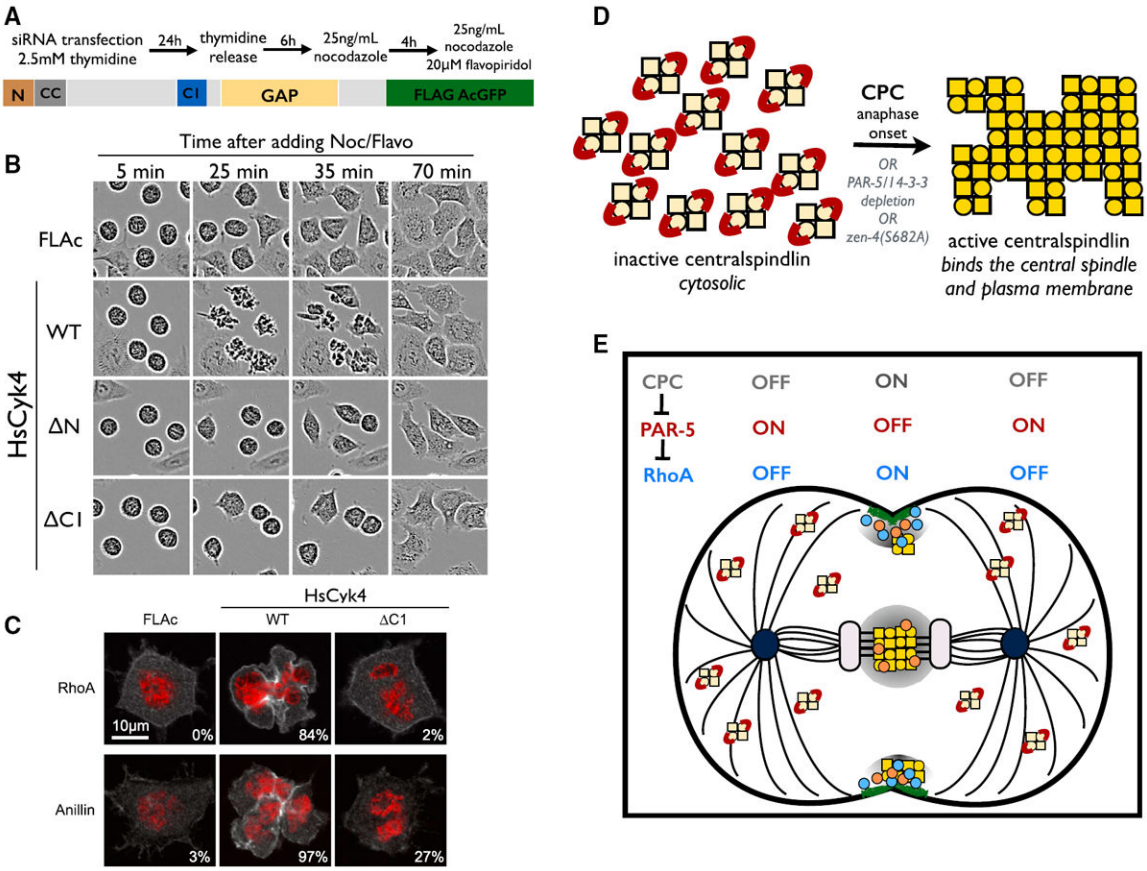

Figure 7. Membrane Association of Centralspindlin Promotes RhoA Activation in Human Cells

(A) Schematic depicting conditions used for mitotic arrest and release of HeLa cells. Shown is the domain structure of the full-length HsCyk4 construct, truncations of which are used in (B). N, N-terminal domain.

(B) HeLa cells transfected with siRNA targeting endogenous HsCyk4 and the indicated siRNA-resistant construct were imaged by DIC microscopy at different time intervals following nocodazole (Noc)/flavopiridol (Flavo) treatment. These data are quantified in Figure S7. FLAc, N-terminally tagged with a single FLAG epitope and AcGFP (*A. coerulescens* GFP).

(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.

(D and E) Working model for membrane activation of RhoA in wild-type cells.

(D) 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.

(E) 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.