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Report

# The RhoGAP Domain of CYK-4 Has an Essential Role in RhoA Activation

Andy Loria,[1,2] Katrina M. Longhini,[1,2] and Michael Glotzer[1,*]
[1]Department of Molecular Genetics and Cell Biology, University of Chicago, Chicago, IL 60637, USA

## Summary

**Cytokinesis in animal cells is mediated by a cortical actomyosin-based contractile ring. The GTPase RhoA is a critical regulator of this process as it activates both nonmuscle myosin and a nucleator of actin filaments [1]. The site at which active RhoA and its effectors accumulate is controlled by the microtubule-based spindle during anaphase [2]. ECT-2, the guanine nucleotide exchange factor (GEF) that activates RhoA during cytokinesis, is regulated by phosphorylation and subcellular localization [3–5]. ECT2 localization depends on interactions with CYK-4/MgcRacGAP, a Rho GTPase-activating protein (GAP) domain containing protein [5, 6]. Here we show that, contrary to expectations, the Rho GTPase-activating protein (GAP) domain of CYK-4 promotes activation of RhoA during cytokinesis. Furthermore, we show that the primary phenotype caused by mutations in the GAP domain of CYK-4 is not caused by ectopic activation of CED-10/Rac1 and ARX-2/Arp2. However, inhibition of CED-10/Rac1 and ARX-2/Arp2 facilitates ingression of weak cleavage furrows. These results demonstrate that a GAP domain can contribute to activation of a small GTPase. Furthermore, cleavage furrow ingression is sensitive to the balance of contractile forces and cortical tension.**

## Results

The RhoGAP domain of CYK-4 is predicted to negatively regulate a Rho family GTPase. In vitro, the GAP domain of CYK-4 is more active toward Rac and Cdc42 as compared to RhoA [7, 8]. However, RhoA is the sole GTPase that is required for cytokinesis, though other Rho family members could be involved in a nonessential manner [8–12]. Moreover, genetic analyses of the CYK-4 GAP domain have failed to converge on a common mechanism of action. In *Drosophila* neurons, substitution of the catalytic arginine does not prevent cytokinesis [13]. In *Drosophila* embryonic cells, a small deletion in the GAP domain surrounding the catalytic residue results in an apparent failure of furrow ingression [14]. Similarly, in chicken B-lymphocytes, alanine substitution of the catalytic arginine appears phenotypically silent, but deletion of the GAP domain prevents cytokinesis [15]. In *Xenopus* embryos, the substitution of the catalytic arginine results in hyperaccumulation of RhoA·GTP and no secondary effects on other GTPases [16]. In contrast, overexpression of a catalytically inactive version appears to increase the signal from a FRET-based Rac1 reporter in mammalian cells [17]. In *C. elegans* embryos, two separate mutations in the GAP domain of CYK-4 prevent completion of cytokinesis [18], yet depletion of Rac GTPase or their downstream effector, ARX-2, a component of the Arp2/3 complex, allow embryos with GAP domain mutations to complete cytokinesis. These diverse outcomes from highly similar experiments indicate that the conserved function of this central regulator of cytokinesis remains to be elucidated.

To gain insight into this question, we first examined the degree to which cytokinesis in *C. elegans* embryos is sensitive to the dosage of RHO-1/RhoA and CED-10/Rac1. By using strains coexpressing mCherry-tagged histone and either GFP::PH (from PLC∂1) [19] or GFP-tagged nonmuscle myosin (NMY-2) [20], we measured the rate of furrow ingression and the accumulation of cortical myosin at the cell equator after anaphase onset. To reduce the function of CED-10, we used a strong loss-of-function allele, *ced-10(n1993)* [21], and we used RNAi to deplete RhoA. Progressive depletion of RHO-1 resulted in a progressive decrease in the rate of furrow ingression and a decrease in the amount of cortical myosin (Figures 1A–1C). In contrast, *ced-10(n1993)* embryos undergo cytokinesis at wild-type rates (Figures 2A and 2B). Furthermore, the amount of cortical myosin in *ced-10(n1993)* embryos did not differ markedly from that of control embryos (Figures 2C and 2D). Embryos depleted of the Arp2/3 subunit ARX-2 also cleaved at wild-type rates and with similar myosin recruitment to controls (Figures S1A and S1B). Thus, RHO-1 is a dosage-sensitive regulator of cytokinesis, and CED-10/Rac1 does not make a detectable contribution to cytokinesis.

We next examined the rate of cleavage furrow ingression and the amount of cortical myosin in *cyk-4(or749)* embryos that contain a mutation (E448K) in the GAP domain (Figure S2). The defective protein accumulates at comparable levels to wild-type CYK-4, it limits spindle elongation like the wild-type protein, and it allows proper accumulation of ZEN-4 on the central spindle (Figure S2) [18]. As previously shown [18], furrow ingression in *cyk-4(or749)* embryos is both incomplete and significantly slower than that of control embryos (Figures 2A and 2B) and this reduced rate of ingression correlates with a decrease in the amount of equatorial cortical myosin during furrow ingression (Figures 2C and 2D). These phenotypes are similar to those seen in embryos partially depleted of RHO-1, raising the possibility that the cytokinesis defect in *cyk-4(or749)* embryos results from a failure to fully activate RHO-1/RhoA.

To examine whether the inability of CYK-4 mutant embryos to rapidly complete cytokinesis is due to ectopic CED-10/Rac1 activation as previously proposed [18], we examined *cyk-4(or749)*; *ced-10(n1993)* embryos. We first confirmed that loss of function of CED-10/Rac1 allows *cyk-4(or749)* embryos to complete cytokinesis, demonstrating that impairment of this Rac1 ortholog alone is sufficient to allow cytokinesis to complete when the CYK-4 RhoGAP domain is mutated (Figures 2A and 3Bd′). To quantitatively compare these embryos, we measured the rate of cleavage furrow ingression and the amount of cortical equatorial myosin in *ced-10(n1993)* and *cyk-4(or749)*; *ced-10(n1993)* embryos. Although loss of CED-10/Rac1 function increases the extent of cleavage furrow ingression in *cyk-4(or749)* embryos, the rate of cleavage furrow ingression remains significantly slower than in control embryos (Figure 2B). Inactivation of CED-10/Rac1 function

[2]These authors contributed equally to this work
*Correspondence: mglotzer@uchicago.edu

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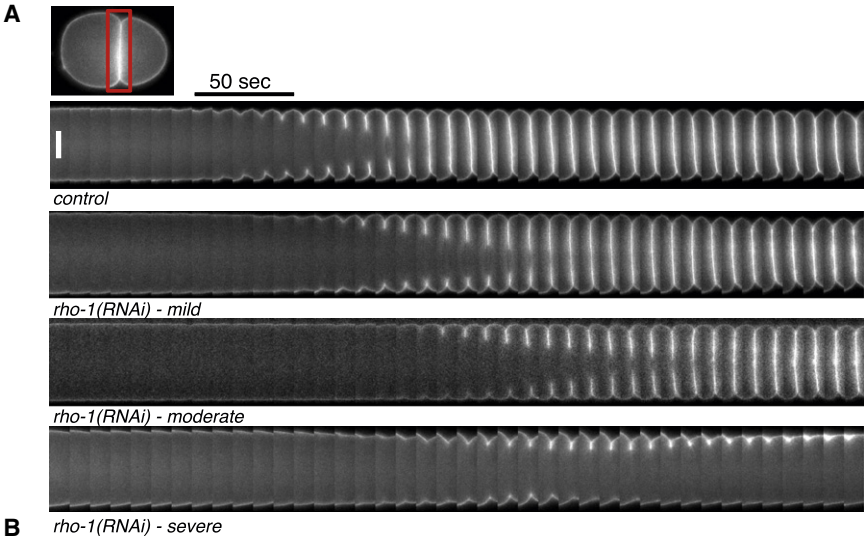


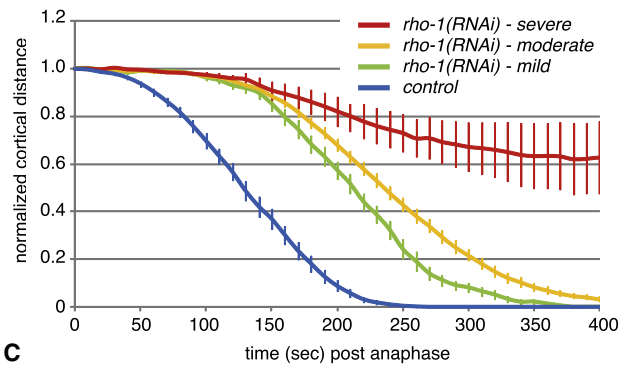


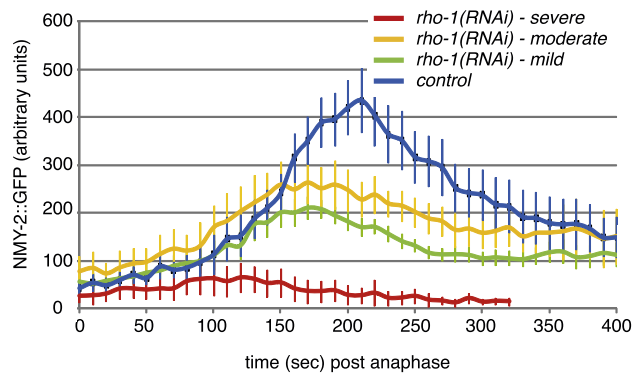


Figure 1. RHO-1/RhoA Is a Dosage-Sensitive Regulator of Cytokinesis

*C. elegans* embryos depleted of RHO-1. The extent of RHO-1 depletion was classified phenotypically (mild, pseudocleavage failure; moderate, pseudocleavage and second division failure; and severe, pseudocleavage, first and second cell divisions failure).

(A) Kymographs of the equatorial region of representative GFP::PH-expressing embryos. Scale bar represents 10 μm.

(B) Kinetics of furrow ingression (n = 7 embryos for mild, n = 7 for severe, n = 20 for moderate, n = 30 for control).

(C) Cortical myosin accumulation in the furrow region (n = 8 for mild, n = 5 for severe, n = 6 for moderate, n = 11 for control).

Error bars represent SEM. See also Figure S1.

does not correct the initial, prominent defect in accumulation of equatorial cortical myosin observed in *cyk-4(or749)* embryos (Figures 2C and 2D), though a modest, late, increase in myosin accumulation is detected in *cyk-4(or749); ced-10(n1993)* embryos as the furrows complete ingression. Identical results were obtained in *cyk-4(or749)* embryos in which the CED-10/Rac1 effector ARX-2 was depleted by RNAi (Figures 2C and S1A).

To further investigate whether *cyk-4(or749)* embryos are defective in RHO-1 activation or CED-10/Rac1 inactivation, we examined the levels of f-actin. The former model would predict a reduction in f-actin accumulation in the furrow because of reduced activation of the cytokinetic formin. In contrast, the latter model would predict an increase in f-actin because of the activation of Arp2/3 complex by active CED-10/Rac1. We used MOE::GFP to quantify accumulation of f-actin and found that mutation of the GAP domain of CYK-4 results in a dramatic decrease in f-actin levels (Figure 2E). Another RhoA effector, anillin, also accumulates at greatly reduced levels in *cyk-4(or749)* embryos as compared to controls (Figure 2F). Thus, mutation of the GAP domain of CYK-4 impairs the recruitment of several independent RhoA effectors [22]. We infer that RhoA activation is itself compromised.

The central spindle promotes cleavage furrow formation. In human cells, RhoA activation requires binding of the RhoGEF ECT2 to phospho-CYK-4 [23, 24]. In several cell types including *C. elegans* embryos, cleavage furrows can form in central spindle-deficient embryos [8, 25–28], because of an astral microtubule-dependent mechanism that functions redundantly with the central spindle [29–32]. Central spindle-dependent furrowing can be assayed in embryos depleted of Gα [29]. Embryos solely depleted of Gα divide with high efficiency (Figure 3Ab); furrowing in these embryos requires the central spindle [29]. In stark contrast, *cyk-4(or749)* embryos depleted of Gα fail to form cleavage furrows altogether (Figure 3Ab′). Similar results were obtained with *cyk-4(or570)* embryos (data not shown). Central spindle-induced furrowing can also be assayed by spatial separation of central spindle- and aster-directed furrowing. Depletion of the microtubule-associated protein ZYG-9 results in shorter microtubules and assembly of the mitotic spindle near the posterior pole [33]. After anaphase onset in ZYG-9-depleted embryos, two furrows form, one in the posterior that depends on centralspindlin and an anterior furrow that is centralspindlin independent (Figure 3Ac) [31]. When ZYG-9 is depleted in *cyk-4(or749)* embryos, anterior furrow formation is not perturbed, but the posterior furrow is entirely absent (Figure 3Ac′). These data

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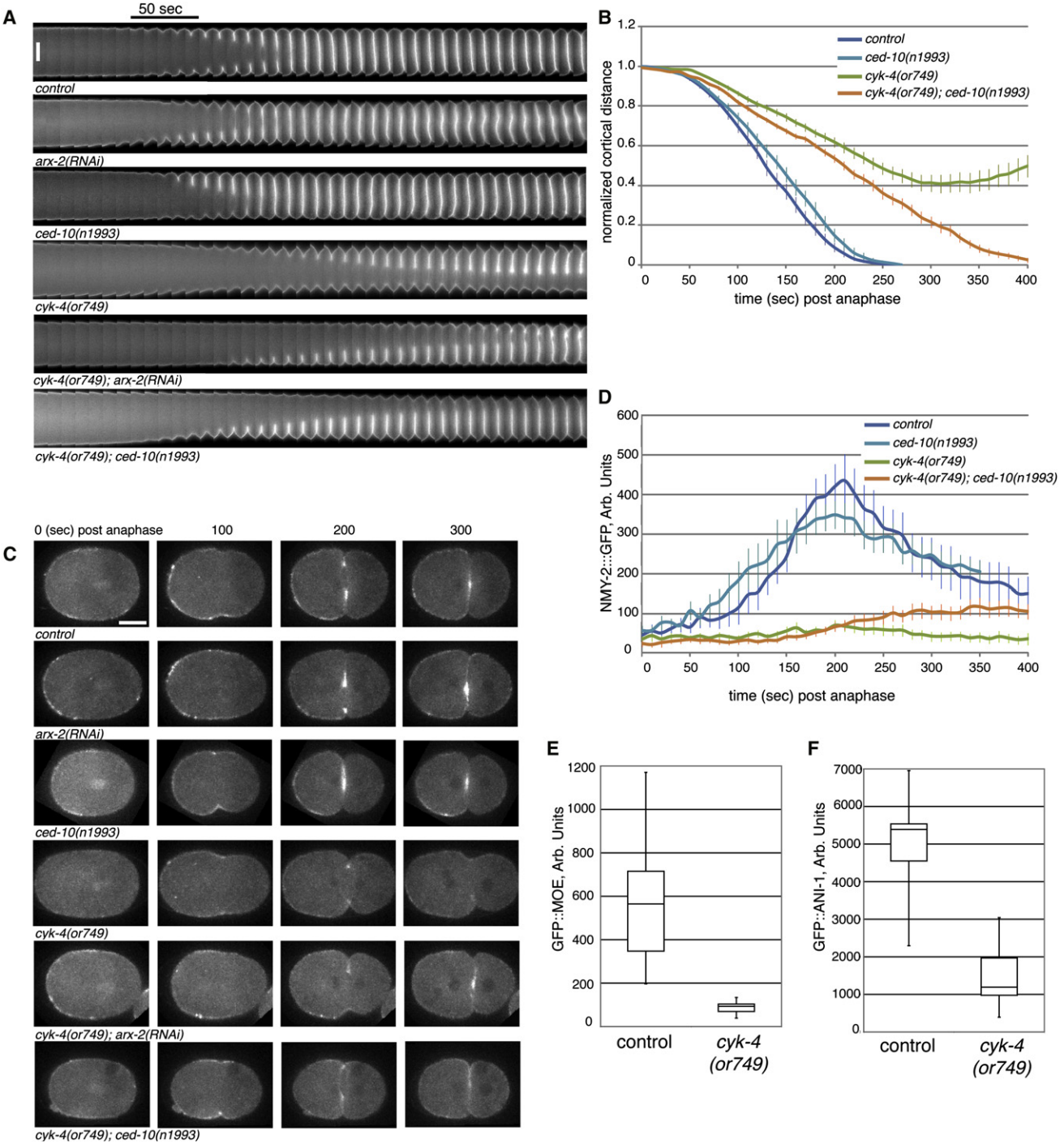


Figure 2. CED-10/Rac1 Depletion Allows *cyk-4(or749)* Embryos to Complete Furrow Ingression without Restoring Furrow Ingression Rate nor Cortical Myosin

(A) Kymographs of the equatorial region of representative GFP::PH-expressing embryos of the indicated genotypes.

(B) Quantitation of the kinetics of furrow ingression of embryos of the indicated genotypes (n > 24 embryos for all genotypes).

(C) Cortical myosin (NMY-2::GFP) at a central plane of embryos of the indicated genotypes at various time points after anaphase onset.

(D) Quantitation of the amount of cortical myosin of embryos of the indicated genotypes (n $\geq$ 9 embryos for all genotypes).

(E) Box and whisker plot of the amount of equatorial cortical f-actin (GFP::MOE) of embryos of the indicated genotypes (n $\geq$ 10 embryos for each genotype; $p < .02$, Mann-Whitney U-test).

(F) Box and whisker plot of the amount of equatorial cortical anillin (GFP::ANI-1) of embryos of the indicated genotypes (n = 10 embryos for each genotype; $p < .02$, Mann-Whitney U-test).

Error bars represent SEM and scale bars represent 10 μm. See also Figure S2.

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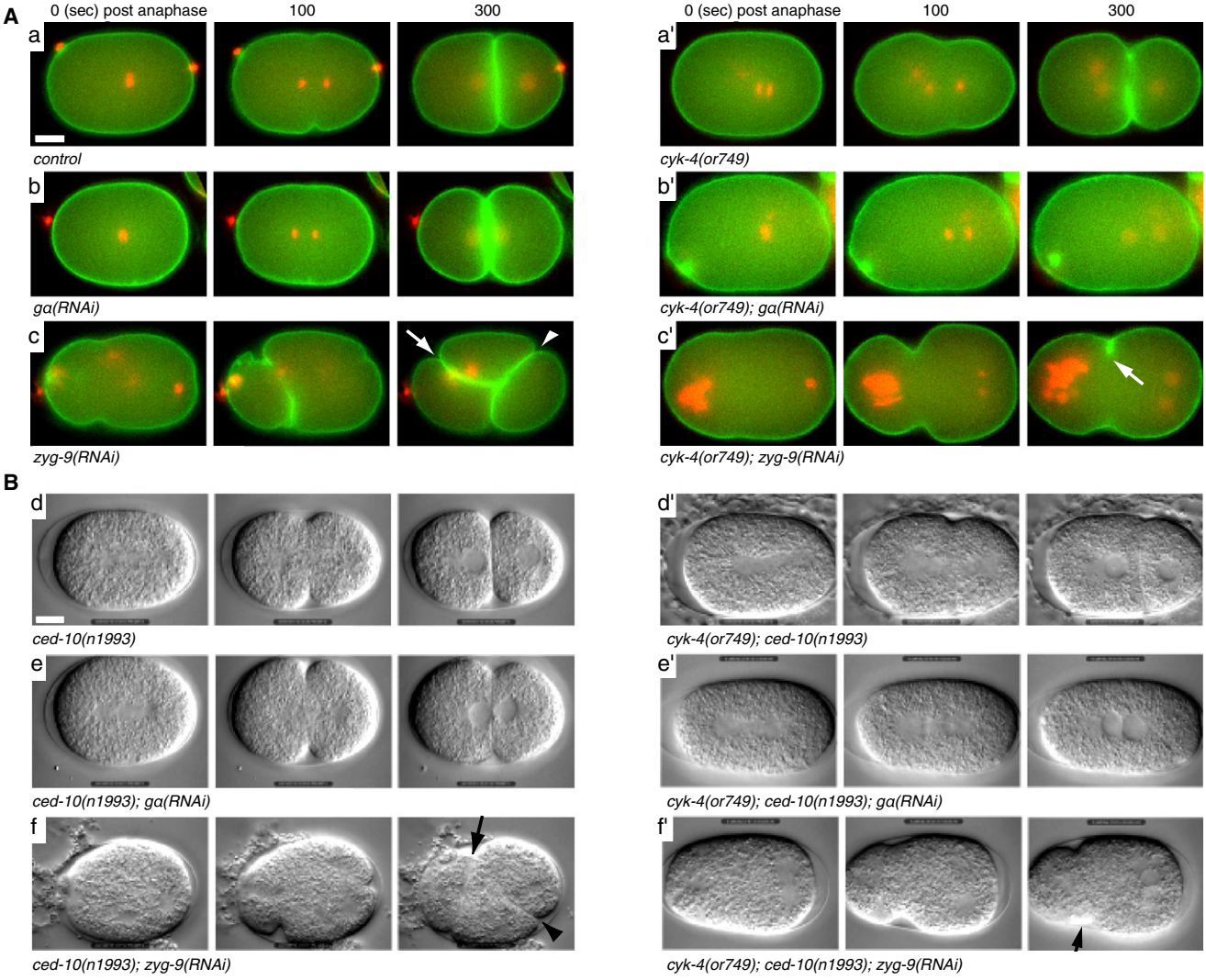


Figure 3. Mutations in the GAP Domain of CYK-4 Abrogate Central Spindle-Dependent Furrowing, Independent of the Activation of RAC-1/Rac1

(A) Control (>20, 100%), *gα(RNAi)* (11, 100%), *zyg-9(RNAi)* (14, 100%), *cyk-4(or749)* (>20, 100%), *cyk-4(or749);gα(RNAi)* (8, 12.5%); *cyk-4(or749);zyg-9(RNAi)* (18, 0%) embryos expressing GFP::PH and mCherry::H2B are shown at the indicated times after anaphase onset. *gα(RNAi)* refers to codepletion of GOA-1 and GPA-16. Numbers in parentheses represent number of embryos and the percent of embryos with ingressing cleavage furrows.

(B) Nomarski images of representative embryos of the indicated genotypes are shown at the indicated times after anaphase onset ($n \geq 9$ for all genotypes, 100% embryos exhibited the phenotype shown).

Scale bars represent 10 μm.

suggest that mutations in the GAP domain of CYK-4 abrogate the ability of the central spindle to induce cleavage furrow formation.

To test whether inappropriate activation of CED-10/Rac1 in *cyk-4(or749)* embryos is responsible for the inactivation of central spindle-induced furrowing, we examined whether loss-of-function mutations in *ced-10* could restore central spindle-induced furrowing in *cyk-4(or749)* embryos. Central spindle-induced furrowing is absent in both *cyk-4(or749)*; *ced-10(n1993)*; *gα(RNAi)* and *cyk-4(or749)*; *ced-10(n1993)*; *zyg-9(RNAi)* embryos (Figures 3Be′ and 3Bf′). We draw four conclusions from this series of experiments: (1) loss of CED-10/Rac1 function does not cause cytokinesis defects—even in highly sensitized backgrounds; (2) mutations in the GAP domain of CYK-4 prevent central spindle-induced furrowing; (3) the defect in central spindle-induced furrowing is not a consequence of ectopic CED-10/Rac1 activation; and (4) the increased extent of furrow ingression observed in CED-10/Rac1-depleted embryos is caused by a bypass suppression mechanism rather than suppression of the primary defect.

Although these data reveal that CED-10/Rac1 inhibition is not the primary function of the CYK-4 GAP domain, mutation of *ced-10*/Rac1 and inhibition of ARP-2/3-dependent actin nucleation allow *cyk-4(or749)* embryos to complete cytokinesis. To examine the basis for this phenotypic suppression, we followed the position of the plasma membrane during cytokinesis and quantitated the changes in cortical dynamics. Embryos depleted of ARX-2/Arp2 and *ced-10(n1993)* embryos exhibit increased cortical instability as compared to control embryos (Figures 4A and 4B) [34]. We also imaged cortical F-actin in live embryos by using a GFP fusion to the actin binding domain of Moesin. During cytokinesis in control

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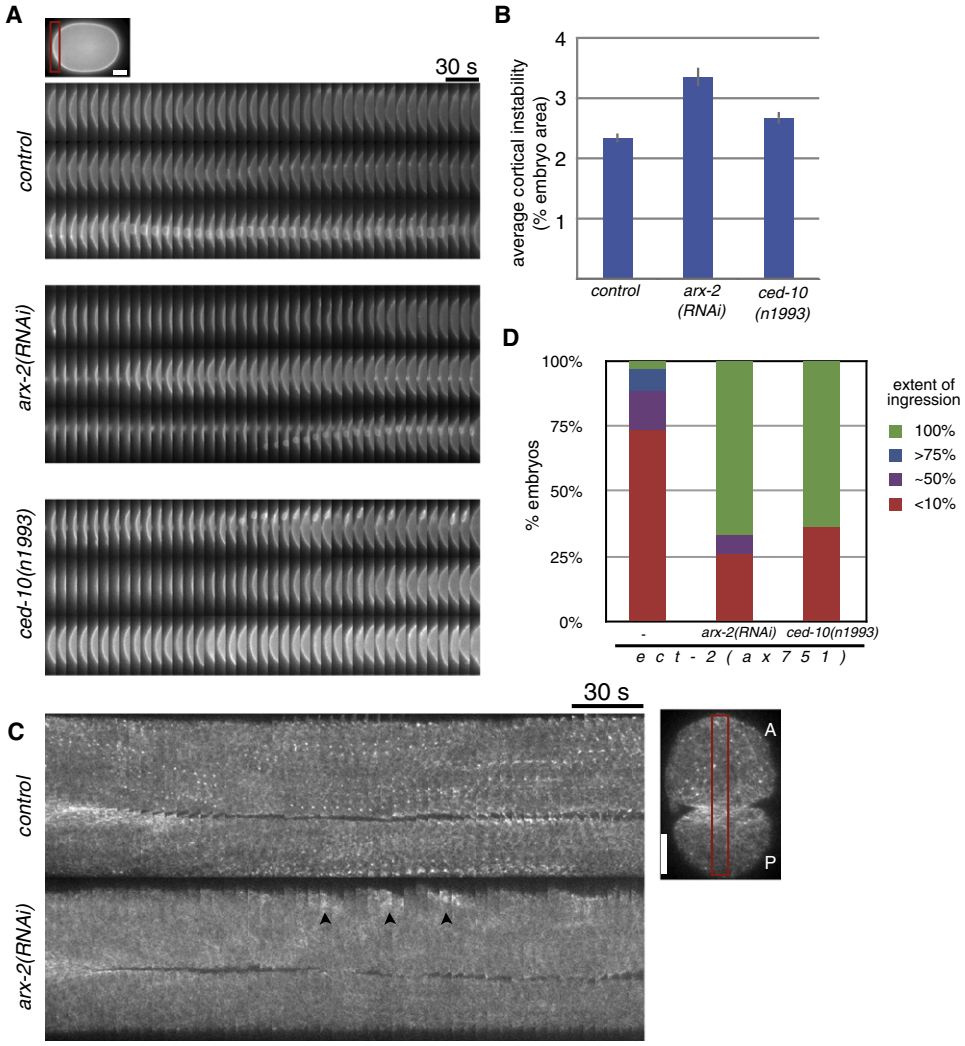


Figure 4. Mutation of *ced-10/rac1* and Depletion of ARX-2/Arp2 Induce Cortical Instability and Improve Cytokinesis in *ect-2*(-) Embryos

(A) Kymographs of the anterior region of the central plane of embryos expressing GFP::PH for control, *arx-2(RNAi)*, and *ced-10(n1993)* embryos from anaphase onset.

(B) The extent of cortical instability was quantitated by subtracting sequential frames (40 planes, every 10 s after anaphase onset) and measuring the area of the subtracted image (see Supplemental Experimental Procedures for details).

(C) Kymograph analysis of the behavior of MOE::GFP labeling f-actin beginning 90 s after anaphase onset. Note the overall reduction of cortical actin and the transient behavior of remaining f-actin (arrowheads) in *arx-2(RNAi)* embryos.

(D) The progression of cytokinesis in embryos of the indicated genotypes at the restrictive temperature (n = 34, 15, and 11 embryos, respectively). Colors represent four bins representing the observed extents of furrow ingression.

Scale bars represent 10 μm.

embryos, we observed a combination of a network of actin filaments undergoing contractile behavior and bright puncta of F-actin. Depletion of ARX-2/Arp2 greatly attenuates both populations of F-actin and the remaining filaments are highly dynamic and appear unstable (Figure 4C). However, the cortical f-actin pools detectable with GFP::Moe in *ced-10(n1993)* embryos do not differ as drastically from control embryos as ARX-2-depleted embryos, suggesting that cortical destabilization does not require extensive disruption of the actin microfilament network (not shown).

We suggest that *cyk-4(or749)* embryos fail cytokinesis because of an inability to fully activate RhoA and that depletion of CED-10/Rac1 allows cytokinesis completion via a bypass mechanism. If generally true, this would predict that loss-of-function mutants in CED-10/Rac1 would be more resistant to cytokinesis defects caused by incomplete RhoA activation. Indeed, *ect-2(ax751)* embryos, which express a temperature-sensitive allele of the RhoA GEF ECT-2 [35], fail cytokinesis with high frequency at the restrictive temperature. When ARX-2/Arp2 was depleted from *ect-2(ax751)* embryos, the frequency of cytokinesis failure was greatly diminished (Figure 4D); similar results were obtained with *ect-2(ax751); ced-10(n1993)* embryos (Figure 4D). We infer that Rac1- and Arp2/3-dependent actin filaments generate cortical tension that antagonizes furrow ingression. This tension does not significantly delay furrow ingression under normal conditions but becomes consequential when levels of RhoA are attenuated.

## Discussion

Full activation of RhoA involves the central spindle and the intact GAP domain of CYK-4. Although activation of RhoA is a paradoxical function for a GAP domain, previous findings have demonstrated that CYK-4 directly interacts with ECT2 [5, 6, 24]. We suggest that the GAP domain also contributes to RhoA activation by ECT2. Our results also demonstrate that cytokinesis is robust; it can proceed to completion under a range of conditions. Partial reduction in the level of active RhoA slows furrow ingression but does not block cytokinesis; more severe reductions block ingression altogether. Furthermore, in several cases including some shown here, cytokinesis completion occurs in the apparent absence of the canonical GAP activity of CYK-4. This does not imply that the GAP activity is always dispensable; indeed, cells that divide with low levels of active RhoA may not require RhoGAP activity to complete cytokinesis. Previous, divergent results concerning the requirement for the GAP activity of CYK-4 are now easily reconciled. Mutations that affect GAP activity alone are predicted to allow full activation of RhoA and will inhibit completion of cytokinesis only if RhoA is hyperactivated; mutations that affect the structure of the GAP domain are predicted to prevent full activation of RhoA and impede furrow ingression [13–15].

We have also used a number of quantitative assays to examine the contribution of CED-10/Rac1 and ARX-2/Arp2 to cytokinesis. Reducing the activities of these components does not affect the rate of furrow ingression or the extent of myosin recruitment, nor does it render the cleavage furrow sensitive to perturbations that unmask stronger cytokinesis defects in other situations [36, 37]. However, reducing their activity does allow cytokinesis to proceed to a greater extent when the furrow is compromised. Antagonistic interactions between force generation in the contractile ring and the polar cortex occurs during cytokinesis in *Dictyostelium* cells; indeed, in *Dictyostelium*, reduced cortical tension increases the rate of furrow ingression [38, 39]. In contrast, in *C. elegans* embryos, the branched actin network impedes cytokinesis only when RhoA is incompletely activated.

### Supplemental Information

Supplemental Information includes Supplemental Experimental Procedures, two figures, and one table and can be found with this article online at doi:10.1016/j.cub.2011.12.019.

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