

CYK-4 regulates Rac, but not Rho, during cytokinesis

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ABSTRACT Cytokinesis is driven by constriction of an actomyosin contractile ring that is controlled by Rho-family small GTPases. Rho, activated by the guanine-nucleotide exchange factor ECT-2, is upstream of both myosin-II activation and diaphanous formin-mediated filamentous actin (f-actin) assembly, which drive ring constriction. The role for Rac and its regulators is more controversial, but, based on the finding that Rac inactivation can rescue cytokinesis failure when the GTPase-activating protein (GAP) CYK-4 is disrupted, Rac activity was proposed to be inhibitory to contractile ring constriction and thus specifically inactivated by CYK-4 at the division plane. An alternative model proposes that Rac inactivation generally rescues cytokinesis failure by reducing cortical tension, thus making it easier for the cell to divide when ring constriction is compromised. In this alternative model, CYK-4 was instead proposed to activate Rho by binding ECT-2. Using a combination of time-lapse *in vivo* single-cell analysis and *Caenorhabditis elegans* genetics, our evidence does not support this alternative model. First, we found that Rac disruption does not generally rescue cytokinesis failure: inhibition of Rac specifically rescues cytokinesis failure due to disruption of CYK-4 or ECT-2 but does not rescue cytokinesis failure due to disruption of two other contractile ring components, the Rho effectors diaphanous formin and myosin-II. Second, if CYK-4 regulates cytokinesis through Rho rather than Rac, then CYK-4 inhibition should decrease levels of downstream targets of Rho. Inconsistent with this, we found no change in the levels of f-actin or myosin-II at the division plane when CYK-4 GAP activity was reduced, suggesting that CYK-4 is not upstream of ECT-2/Rho activation. Instead, we found that the rescue of cytokinesis in CYK-4 mutants by Rac inactivation was Cdc42 dependent. Together our data suggest that CYK-4 GAP activity opposes Rac (and perhaps Cdc42) during cytokinesis.

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Abbreviations used: AO, anaphase onset; *cyk-4*, cytokinesis defect 4; GAP, GTPase-activating protein; GEF, guanine-nucleotide exchange factor; lof, loss of function; ts, temperature-sensitive; Utr^{ABD}, utrophin (actin-binding domain).

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INTRODUCTION

Rho-family small GTPases (Rho, Rac, and Cdc42) function as molecular switches: when GDP-bound, they are inactive, and when GTP-bound, they interact with cytoskeletal effectors to choreograph the cell shape changes required for complex cellular events such as cell motility, phagocytosis, and cytokinesis (Hall, 2012; Jordan and Canman, 2012; Mao and Finnemann, 2015; Ridley, 2015). Cytokinesis—the physical division of one cell into two—is driven by constriction of an actomyosin contractile ring, directed to form at the division plane after anaphase onset (AO) via Rho-family GTPase signaling. In most animal cells, assembly and constriction of the actomyosin contractile ring is downstream of Rho (O'Connell et al., 1999; Bement et al., 2005; Kamijo et al., 2006), which is activated at the division plane by

the guanine-nucleotide exchange factor (GEF) epithelial cell transforming-2 (ECT-2), a human oncogene (Tatsumoto *et al.*, 1999; Kimura *et al.*, 2000; Kamijo *et al.*, 2006; D'Avino *et al.*, 2015). Once activated, Rho promotes both filamentous actin (f-actin) nucleation by a diaphanous-family formin (CYK-1 in *Caenorhabditis elegans*; hereafter, formin) and nonmuscle myosin-II (NMY-2 in *C. elegans*; hereafter, myosin-II) motor activation, which drive contractile ring assembly and power ring constriction (for review, see Pollard, 2010; Green *et al.*, 2012; D'Avino *et al.*, 2015). Rho activation during cytokinesis is opposed by the GTPase-activating protein (GAP) MP-GAP (RGA-3/4 in *C. elegans*), which promotes Rho's hydrolysis of GTP to GDP (Zanin *et al.*, 2013).

The role(s) for the Rho family GTPase Rac and the Rac GAP CYK-4 during cytokinesis are under debate. CYK-4 (HsCYK4/MgcRacGAP in humans, RacGAP50c in flies; hereafter, CYK-4) is essential for cytokinesis in nearly all cell types (Jantsch-Plunger *et al.*, 2000; Hirose *et al.*, 2001; Goldstein *et al.*, 2005), forms a complex called Centralspindlin with the kinesin MKLP-1/ZEN-4 (Mishima *et al.*, 2002; Pavicic-Kaltenbrunner *et al.*, 2007), and has been proposed to both inhibit Rac activation and promote Rho activation at the division plane via its GAP domain (Jantsch-Plunger *et al.*, 2000; D'Avino *et al.*, 2004; Canman *et al.*, 2008; Miller and Bement, 2009; Bastos *et al.*, 2012; Loria *et al.*, 2012; Cannet *et al.*, 2014; Zhang and Glotzer, 2015). In vitro, CYK-4 inactivates Rac and Cdc42 by promoting GTP hydrolysis but has little effect on the activity of Rho (Toure *et al.*, 1998; Jantsch-Plunger *et al.*, 2000; Bastos *et al.*, 2012). In vivo, CYK-4 has been proposed to inhibit Rac activity (D'Avino *et al.*, 2004; Yoshizaki *et al.*, 2004; Canman *et al.*, 2008; Bastos *et al.*, 2012; Cannet *et al.*, 2014), promote Rho-GTP turnover (or flux; Miller and Bement, 2009), and promote the activation of Rho by stimulating the GEF activity of ECT-2 during cytokinesis (Zhao and Fang, 2005; Loria *et al.*, 2012; Tse *et al.*, 2012; Zhang and Glotzer, 2015). Thus there is no consensus view on the mechanisms by which CYK-4 and/or Rac activity regulate cytokinesis.

To support the model in which CYK-4 negatively regulates Rac during cytokinesis in worms, flies, and cultured human cells, mutational disruption of the CYK-4 GAP domain leads to cytokinesis failure that can be rescued by reducing Rac activity (D'Avino *et al.*, 2004; Canman *et al.*, 2008; Bastos *et al.*, 2012; Cannet *et al.*, 2014). Cytokinesis failure in CYK-4 GAP-domain mutants can also be rescued by depletion of the Arp2/3 complex, a Rac effector, and branched f-actin nucleator in worms and cultured mammalian cells (Canman *et al.*, 2008; Cannet *et al.*, 2014). Moreover, in cultured mammalian cells, cytokinesis failure caused by CYK-4 GAP disruption can be rescued by depleting Rac effectors that promote cell substrate attachment such as PAK1 and ARHGEF7 (Bastos *et al.*, 2012) and the upstream Rac GEF Trio (Cannet *et al.*, 2014). Thus CYK-4 GAP-mediated inactivation of Rac at the division plane has been proposed to function in parallel to Rho activation to allow contractile ring constriction and promote efficient cytokinesis.

In contrast, CYK-4 has also been proposed to function during cytokinesis through the promotion of Rho activation by facilitating GTP turnover (flux) or by activating the Rho GEF ECT-2 (Zhao and Fang, 2005; Miller and Bement, 2009; Loria *et al.*, 2012; Tse *et al.*, 2012; Zhang and Glotzer, 2015). Although, in vitro, CYK-4 is not effective at promoting the GTP turnover of Rho (Toure *et al.*, 1998; Jantsch-Plunger *et al.*, 2000; Bastos *et al.*, 2012), in vivo mutations in the GAP domain have a strong effect on the width and stability of the zone of active GTP-bound Rho at the division plane in *Xenopus* embryos (Miller and Bement, 2009). This could be due to a direct effect on Rho activation or an indirect effect by modulating other Rho family members that compete for effectors and upstream regu-

lators, such as GAPs, GEFs, and guanosine nucleotide dissociation inhibitor (GDIs; e.g., Tatsumoto *et al.*, 1999; Machacek *et al.*, 2009; Boulter *et al.*, 2010; Garcia-Mata *et al.*, 2011). CYK-4 has also been proposed to promote Rho activation by stimulating the Rho GEF ECT-2 (Zhao and Fang, 2005; Zhang and Glotzer, 2015). This hypothesis was based on the findings that CYK-4/MgcRacGAP can bind ECT-2 (Somers and Saint, 2003; Yuce *et al.*, 2005; Burkard *et al.*, 2009; Wolfe *et al.*, 2009; Zhang and Glotzer, 2015) and that mutational disruption of the CYK-4 GAP domain can be suppressed by hyperactivating mutations in the ECT-2 GEF domain (Zhang and Glotzer, 2015). The same group also reported that CYK-4 disruption prevents the enrichment of f-actin and myosin-II at the division plane, which would be expected if CYK-4 activates ECT-2 and thus Rho activity during cytokinesis (Loria *et al.*, 2012). However, other studies could not confirm that CYK-4 GAP disruption leads to decreased levels of myosin-II at the division plane (Canman *et al.*, 2008; Zhang and Glotzer, 2015). Moreover, in vitro analysis found no change in ECT-2 GEF activity toward Rho in the presence or absence of CYK-4 (Zhang and Glotzer, 2015), although this negative result cannot rule out that additional factors may be needed. Thus, whereas CYK-4 has been proposed to play an essential role in ECT-2 and Rho activation, much of the evidence for this model is indirect.

Furthermore, in models that support a role for CYK-4 in Rho activation via ECT-2 (Loria *et al.*, 2012; Zhang and Glotzer, 2015), the finding that cytokinesis failure resulting from CYK-4 GAP disruption can be rescued by Rac inactivation is attributed to a general or "bypass" role for Rac (Loria *et al.*, 2012) in regulating cortical tension and opposing contractile ring constriction, as was found in *Dictyostelium* (Zhang and Robinson, 2005). Support for this model relies on the finding that reducing Rac activity could also rescue cytokinesis failure in a hypomorphic *ect-2* (temperature-sensitive [*ts*]) mutant background (Loria *et al.*, 2012). Yet, in vitro ECT-2 GEF activity can activate both Rho and Rac (Tatsumoto *et al.*, 1999); thus Rac depletion might rescue cytokinesis when ECT-2 activity is weakened by simply reducing competition with Rho for the GEF domain. Moreover, Rac disruption does not rescue cytokinesis failure when Aurora B kinase activity is compromised, which also regulates contractile ring constriction in a pathway parallel to CYK-4 during cytokinesis (Lewellyn *et al.*, 2011). Thus a general role for Rac in opposing contractile ring constriction in animal cells is not well supported.

The role for Cdc42 in animal cell cytokinesis is also controversial. Cdc42 is essential for polar body emission during meiotic cytokinesis in frog and murine embryos (Ma *et al.*, 2006; Zhang *et al.*, 2008; Wang *et al.*, 2013). However, disruption of Cdc42 does not block mitotic cytokinesis in most metazoan systems (Gotta *et al.*, 2001; Zhu *et al.*, 2011; Jordan *et al.*, 2016). Injection of constitutively active or dominant-negative forms of Cdc42 at very high levels (micromolar range) can disrupt mitotic cytokinesis in frog embryos (Drechsel *et al.*, 1997), and expression of constitutively active Cdc42 in dividing mammalian cultured cells leads to the assembly of a broader contractile ring but does not block cytokinesis (Zhu *et al.*, 2011). In asymmetrically dividing *C. elegans* embryos, Cdc42 regulates cell polarity and is required for robust contractile ring f-actin assembly, and its depletion leads to synthetic cytokinesis failure in embryos from a temperature-sensitive diaphanous-related formin mutant (Jordan *et al.*, 2016). Hence Cdc42 likely participates in animal cell cytokinesis but is not an essential player.

Here we examine the roles of Rac and the GAP CYK-4 in cytokinesis in the early *C. elegans* embryo. We find that, as shown previously, Rac inactivation rescues cytokinesis failure in a CYK-4 GAP mutant and to a lesser extent in a hypomorphic ECT-2 mutant. Rac disruption does not rescue the rate of contractile ring constriction

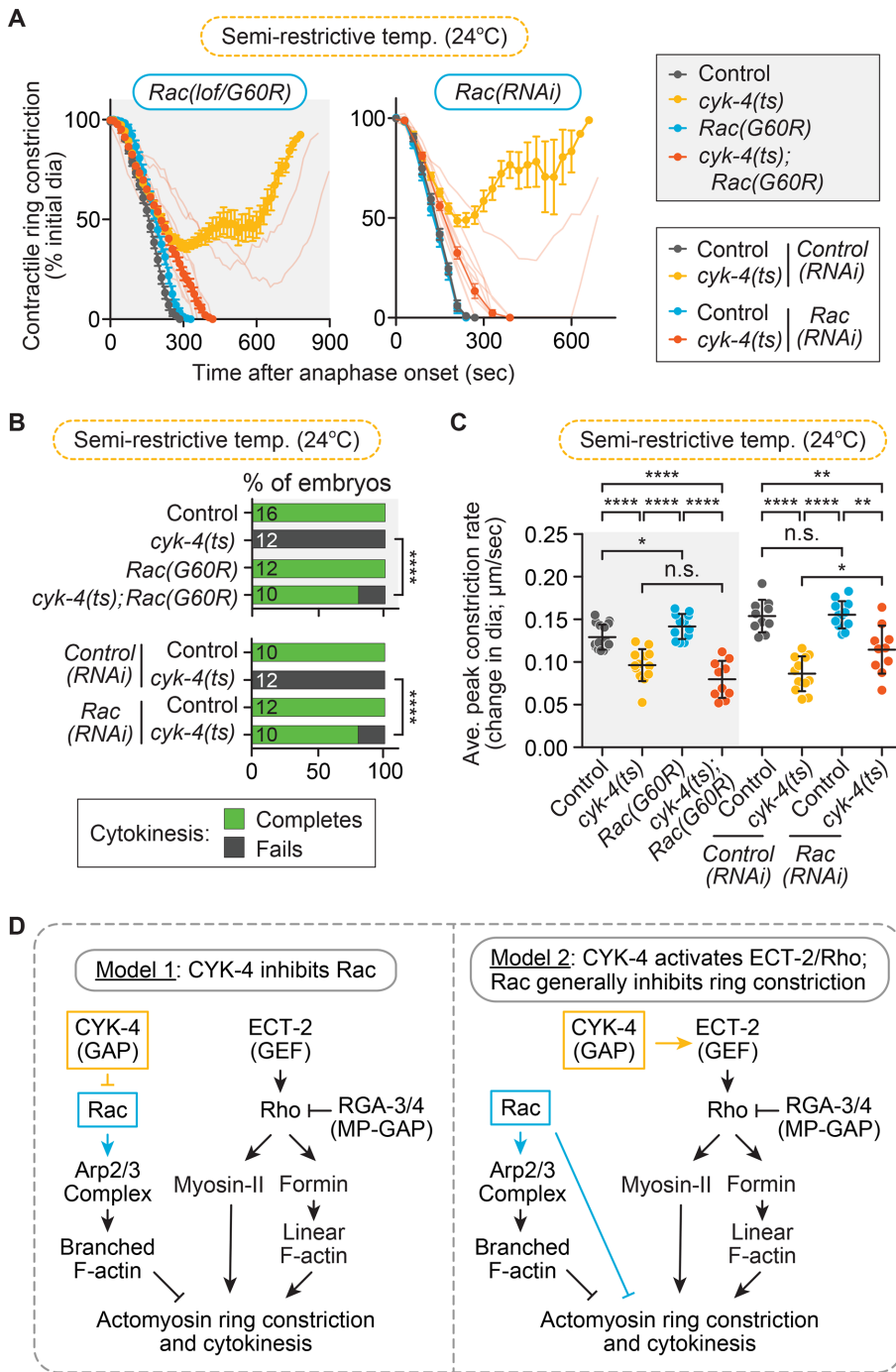


FIGURE 1: Rac disruption rescues cytokinesis failure in a CYK-4 GAP mutant. (A) Kinetic analysis of contractile ring constriction in control and *cyk-4(ts)* mutant 1-cell embryos at semirestrictive temperature (24°C) with and without *Rac(lox/G60R)* (left) and *Rac(RNAi)* (right). Mean ring diameter vs. time, with all replicates for the *cyk-4(ts)*; *Rac(G60R)* or *RNAi* shown in the background in a more transparent shade of the same orange. Error bars represent SEM. (B) Cytokinesis failure and success rates for different genotypes in A. The number of embryos per genotype is indicated on each individual bar; *p* values were obtained by both Fisher's and Barnard's exact tests (Supplemental Table S3). (C) Average peak rate of contractile ring constriction plotted as change in diameter (µm/s; see *Materials and Methods* for image analysis). Error bars represent SD; *p* values were obtained by an unpaired, two-tailed Student's *t* test (Supplemental Table S3). (D) Current genetic models for the function of CYK-4 and Rac during cytokinesis. n.s., *p* ≥ 0.05; **p* < 0.05; ***p* < 0.01; *****p* < 0.0001.

in CYK-4 or ECT-2 mutants, and we find that Rac disruption-mediated rescue of cytokinesis failure in CYK-4 mutants depends on Cdc42. We do not find evidence that Rac activity generally opposes contractile ring constriction, as Rac inactivation did not rescue the rate of cytokinesis failure in embryos with mutations in the Rho effectors diaphanous formin or the motor myosin-II. Furthermore, disrupting CYK-4 activity did not lead to a reduction in the total levels of contractile ring f-actin or myosin-II at the division plane, indicating that CYK-4 is unlikely to act upstream of ECT-2-mediated activation of Rho. Instead, we propose that, at least in the early *C. elegans* embryo, ECT-2 activity may promote Rac activation and thus in part negatively regulate cytokinesis. Together our data support a model in which CYK-4 functions to inhibit Rac activity (and potentially Cdc42) and does not participate directly in Rho activation; our data also do not suggest a nonspecific or bypass role for Rac in opposing contractile ring constriction.

RESULTS

We first sought to confirm that cytokinesis failure due to mutational disruption of the CYK-4 GAP domain could be rescued by reducing Rac activity. To do this, we performed time-lapse image analysis of cytokinesis in the one-cell *C. elegans* embryo, with and without Rac disruption, in a temperature-sensitive CYK-4 GAP-domain mutant (E448K) background (Canman et al., 2008), hereafter, *cytokinesis defect 4* (*cyk-4*) (*ts*). Imaging was done at the semirestrictive temperature (24°C), where contractile ring ingression progresses further in *cyk-4(ts)* mutant embryos than at fully restrictive temperature but cytokinesis fails 100% of the time in the *cyk-4(ts)* mutant alone (Figure 1, A and B). Rac activity was disrupted in two ways: 1) with feeding RNA interference (RNAi) and 2) with a loss-of-function (*lox*) Rac/CED-10 GTPase mutant (G60R; *ced-10(n3246)*; hereafter, *Rac(G60R)*; Reddien and Horvitz, 2000; Sun et al., 2012; Cabello et al., 2014) that, based on an identical G60R mutation in the closely related KRas, renders the small GTPase stuck in the GTP-bound state but unable to interact with effectors, GAPs, or GEFs (Gremer et al., 2011). As expected, 100% of control, *control(RNAi)*, *Rac(RNAi)*, and *Rac(G60R)* single-mutant embryos successfully completed cytokinesis (16 of 16, 10 of 10, 12 of 12, and 12 of 12 embryos divide, respectively), whereas 100% of *cyk-4(ts)* and *cyk-4(ts); control(RNAi)*

embryos failed in cytokinesis at this temperature (0 of 12 and 0 of 12 embryos divide, respectively; Figure 1, A and B). In contrast, cytokinesis was significantly rescued (Fisher's and Barnard's exact tests; *p* values in Supplemental Table S3) in *cyk-4(ts)*; *Rac(RNAi)* and *cyk-4(ts)*; *Rac(G60R)* double mutant embryos (8 of 10 embryos divide for both genotypes; Figure 1, A and B). To test whether this rescue is conserved in other cell divisions, we also investigated whether Rac disruption could rescue cytokinesis failure in the AB and P1 blastomeres of two-cell *cyk-4(ts)* embryos (Supplemental Figure S1C). We found that both AB and P1 blastomeres fail cytokinesis in *cyk-4(ts)* two-cell embryos at a lower rate than in one-cell embryos (4 of 19 AB cells and 12 of 20 of P1 cells divide at semirestrictive temperature, 25.5°C in this case) (Supplemental Figure S1C). Nonetheless, cytokinesis failure was significantly rescued (Fisher's and Barnard's exact tests; *p* values in Supplemental Table S3) in both blastomeres of *cyk-4(ts)*; *Rac(G60R)* double-mutant two-cell embryos (12 of 14 AB cells and 16 of 16 P1 cells divide successfully; Supplemental Figure S1C). Thus Rac inhibition rescues cytokinesis failure due to mutational disruption of the CYK-4 GAP domain in both one- and two-cell *C. elegans* embryos. This result supports a model in which CYK-4 GAP activity inhibits Rac activation during cytokinesis but does not rule out the possibility that Rac is a general inhibitor of contractile ring constriction (Figure 1D).

We next sought to determine whether Rac disruption can also rescue the slowed rate of contractile ring constriction observed in one-cell *cyk-4(ts)* embryos. Rac disruption by RNAi or the G60R mutation either did not significantly change or only mildly increased the rate of contractile ring constriction in both control and *cyk-4(ts)* embryos (Figure 1C; unpaired Student's *t* test; *p* values in Supplemental Table S3). Thus Rac disruption rescues the extent of contractile ring constriction but overall does not significantly rescue the rate of ring constriction in *cyk-4(ts)* mutants (Supplemental Table S3; Loria et al., 2012). This result suggests that CYK-4 (and/or Rac) could play an independent role in regulating the rate of ring constriction, potentially by further activating ECT-2 (and thus Rho) at the division plane, as proposed in this system (Figure 1D; Tse et al., 2012; Zhang and Glotzer, 2015). This result could also support a model in which Rac disruption rescues cytokinesis in *cyk-4(ts)* mutant embryos via a non-specific or "bypass" pathway (e.g., Loria et al., 2012; Zhang and Glotzer, 2015).

The main argument proposed against Rac as a specific target of CYK-4 is twofold: first, that Rac is a general inhibitor of ring constriction, and its depletion might nonspecifically rescue cytokinesis failure; and second, that CYK-4's true target is ECT-2. In the first part of the argument, Rac disruption has been proposed to allow successful cytokinesis in *cyk-4(ts)* embryos via a "bypass" mechanism that generally reduces cortical tension and makes it easier to complete cytokinesis when the contractile ring is weakened (Loria et al., 2012; Figure 1D). We set out to resolve whether Rac disruption can generally rescue cytokinesis failure when ring constriction is weakened, by combining *Rac(RNAi)* or *Rac(lox)* with other mutants in the ECT-2/Rho pathway that would also compromise the integrity of the contractile ring. If disrupting Rac generally facilitates contractile ring constriction, then *Rac(lox)* should rescue cytokinesis failure when downstream targets of ECT-2/Rho are disrupted to weaken the contractile ring. Thus we next tested whether Rac disruption could rescue cytokinesis failure in a fast-acting *ts* mutant that affects the *C. elegans* diaphanous-related formin actin-nucleator CYK-1 (hereafter, *formin(ts)*), which is required to nucleate f-actin in the contractile ring and is activated downstream of Rho (Jordan and Canman, 2012; Jordan et al., 2016; Davies et al., 2014; Figure 1D). For this experiment, *formin(ts)* 1-cell embryos were imaged at the semi-

restrictive temperature (23.5°C), at which *formin(ts)* mutants alone can undergo partial contractile ring constriction but fail in cytokinesis 100% of the time (0 of 11 embryos divide; Figure 2, A and C; Jordan et al., 2016). Of importance, at this semirestrictive temperature, cytokinesis failure in *formin(ts)* embryos can be fully rescued by depletion of other negative regulators of contractile ring constriction (e.g., septin; Jordan et al., 2016). We found that at this temperature, *formin(ts)*; *Rac(G60R)* mutants still failed in cytokinesis 100% of the time (0 of 10 embryos divide) and underwent even less contractile ring constriction than in the *formin(ts)* mutant alone (Figure 2, A and C). This result suggests that disrupting Rac activity does not significantly rescue cytokinesis failure in *formin(ts)* mutant embryos (Fisher's and Barnard's exact tests; *p* values in Supplemental Table S3). We next examined whether Rac disruption could enhance the rate of cytokinesis failure in *formin(ts)* mutant embryos at the semipermissive temperature (18.5°C), at which all *formin(ts)* mutant embryos successfully complete cytokinesis (13 of 13 embryos divide; Figure 2, B and D; Jordan et al., 2016). At semipermissive temperature, *formin(ts)*; *Rac(G60R)* embryos exhibited mild cytokinesis failure (10 of 12 embryos divide; Figure 2, B and D), although this was not statistically significant (Fisher's and Barnard's exact tests; *p* values in Supplemental Table S3). The rate of contractile ring constriction was significantly slower in *formin(ts)* mutant embryos than in control embryos at both semirestrictive and semipermissive temperatures, but this rate was not affected by the *Rac(G60R)* mutation (Figure 2, E and F; unpaired Student's *t* test; Supplemental Table S3). Thus perturbing Rac activity does not rescue cytokinesis failure when the contractile ring is weakened by reduced formin/CYK-1 activity.

We next tested whether Rac disruption could rescue cytokinesis failure when the actin-based motor NMY-2 is weakened, which drives contractile ring constriction downstream of Rho (Figure 1D). For this experiment, we used a fast-acting *myosin-III(ts)* allele (Liu et al., 2010; Davies et al., 2014) and imaged at the semirestrictive temperature (25.5°C), at which *myosin-III(ts)* single-mutant one-cell embryos can undergo some contractile ring constriction but fail in cytokinesis 100% of the time (0 of 13 embryos divide; Figure 3, A and C; Jordan et al., 2016). Using the *Rac(G60R)* mutation, we found that at this temperature, all *myosin-III(ts)*; *Rac(G60R)* embryos fail in cytokinesis (0 of 10 embryos divide) and undergo reduced contractile ring constriction relative to the *myosin-III(ts)* mutants alone (Figure 3, A and C). This result suggests that disrupting Rac activity does not significantly change the rate of cytokinesis failure in *myosin-III(ts)* mutant embryos (Fisher's and Barnard's exact tests; *p* values in Supplemental Table S3). We next examined the effect of Rac disruption on *myosin-III(ts)* mutant embryos at the semipermissive temperature (22.5°C), where the *myosin-III(ts)* mutant embryos undergo contractile ring constriction more slowly but cytokinesis is always successful (13 of 13 embryos divide; Figure 3, B and D; Jordan et al., 2016). At semipermissive temperature, most *myosin-III(ts)*; *Rac(G60R)* embryos complete cytokinesis (9 of 10 embryos divide; Figure 3, B and D). We also found that the rate of contractile ring constriction was significantly slower in *myosin-III(ts)* mutant embryos than in control embryos at both semirestrictive and semipermissive temperatures, and this constriction rate was not affected by the *Rac(G60R)* mutation (Figure 3, E and F; unpaired Student's *t* test; Supplemental Table S3). Thus perturbing Rac activity does not significantly rescue cytokinesis failure or the rate of contractile ring constriction when myosin-III activity is weakened (Supplemental Table S3). Together with a lack of rescue upon Rac disruption when formin (Figure 2) or Aurora B (Lewellyn et al., 2011) activity is weakened, these data suggest that Rac disruption is not a simple bypass mechanism that

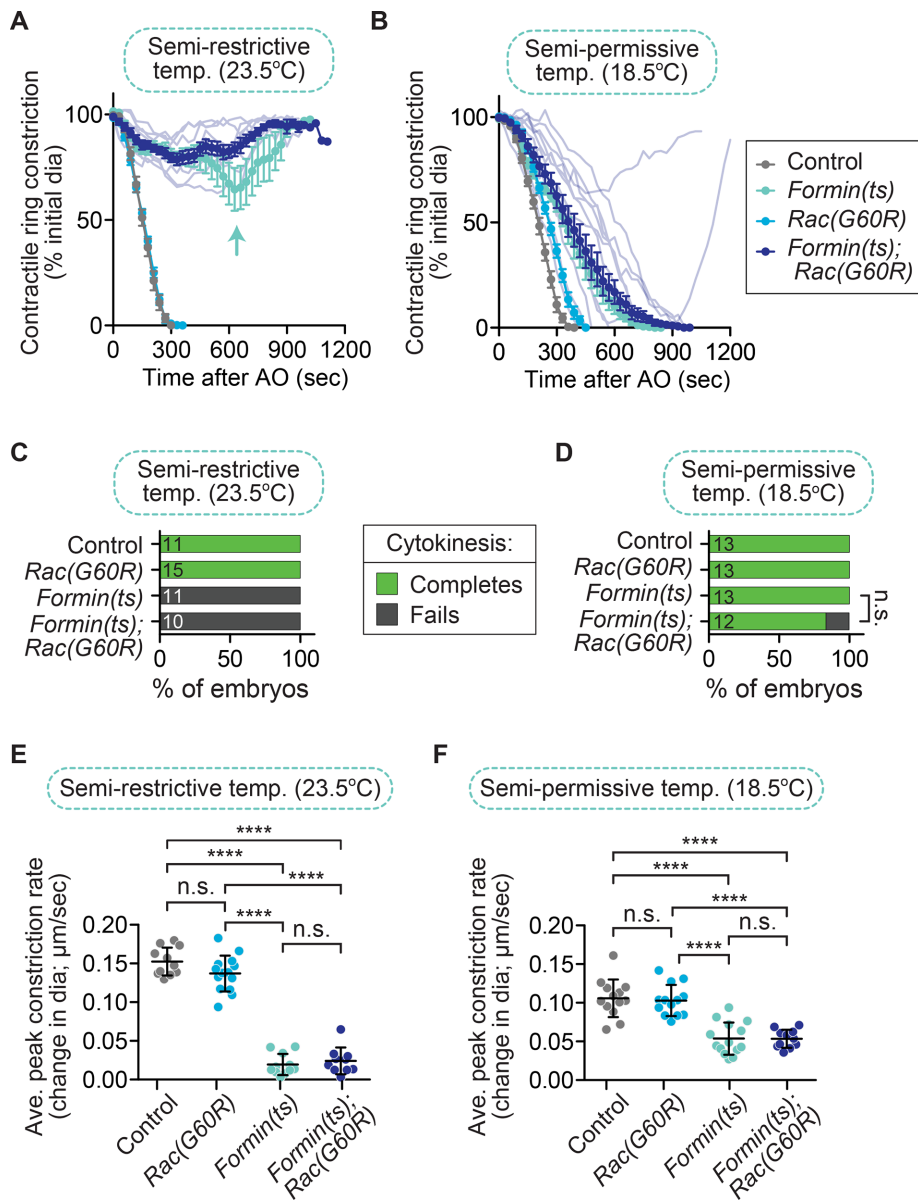


FIGURE 2: Rac disruption does not rescue cytokinesis failure in *formin(ts)* embryos. (A, B) Kinetic analysis of contractile ring constriction in control and *formin(ts)* mutant one-cell embryos with and without *Rac(G60R)*, dividing at (A) semirestrictive (23.5°C) and (B) semipermissive (18.5°C) temperature. Mean ring diameter vs. time, with all replicates for the *formin(ts); Rac(G60R)* double mutant in a more transparent shade of the same dark blue. Error bars represent SEM. The aqua arrow in A shows the more persistent attempt at contractile ring constriction seen in *formin(ts)* single-mutant embryos but not in *formin(ts); Rac(G60R)* double-mutant embryos. (C, D) Cytokinesis failure and success rates for different genotypes at (C) semirestrictive and (D) semipermissive temperature. The number of embryos per genotype is indicated on each individual bar; *p* values were obtained by both Fisher's and Barnard's exact tests (Supplemental Table S3). (E, F) Average peak rate of contractile ring constriction plotted as the change in diameter (µm/s) when the rate of ingression peaks from ~90–50% ring constriction (or the point of maximum contractile ring constriction when embryos did not constrict to 50% of the initial cell diameter) at (E) semirestrictive and (F) semipermissive temperature. Error bars represent SD; *p* values were obtained by an unpaired, two-tailed Student's *t* test (Supplemental Table S3). n.s., $p \geq 0.05$; **** $p < 0.0001$.

facilitates completion of contractile ring constriction. Instead, our and others' genetic results suggest that Rac disruption specifically suppresses cytokinesis failure when CYK-4 (or ECT-2) is reduced (D'Avino *et al.*, 2004; Canman *et al.*, 2008; Bastos *et al.*, 2012; Loria *et al.*, 2012; Cannet *et al.*, 2014).

To support the hypothesis that Rac disruption rescues cytokinesis failure via a non-specific mechanism, it was reported that Rac inhibition was also able to rescue cytokinesis failure of a slow-acting *ect-2* allele (*ax751ts*; hereafter, *ect-2(ts)*; Loria *et al.*, 2012); we next sought to confirm this result. Loria *et al.* (2012) reported that *ect-2(ts)* mutant one-cell embryos fail in cytokinesis at a high frequency (~97%) after a prolonged (8+ h) upshift to restrictive temperature and that this cytokinesis failure rate was reduced to ~35% in *ect-2(ts); Rac(V190G)* double-mutant embryos. This hypomorphic *ect-2(ts)* mutation is not a fast-acting *ts* allele and results from a single-residue substitution in the PH domain of ECT-2 (Zonies *et al.*, 2010). Moreover, embryos from this *ect(ts)* allele were first published to have a low frequency of cytokinesis failure after prolonged upshift to restrictive temperature, although the precise cytokinesis failure rate frequency was not reported (Zonies *et al.*, 2010). We determined the rate of cytokinesis failure in this *ect-2(ts)* mutant background by monitoring the success or failure of cytokinesis in one-cell embryos after prolonged upshifts (12+ h) of *ect-2(ts)* mutants to the restrictive temperature (26°C). In our hands, one-cell embryos from this *ect-2(ts)* mutant failed in cytokinesis at a rate of only 38% (8 of 13 embryos divide; Figure 4, A and C). *ect-2(ts)* mutant embryos that failed in cytokinesis exhibited a significantly slower rate of contractile ring constriction than *ect-2(ts)* mutant embryos that successfully completed cytokinesis, but the overall rate of contractile ring constriction in *ect-2(ts)* mutants was unchanged by *Rac(RNAi)* (Figure 2B; unpaired Student's *t* test; Supplemental Table S3). Although the rate of cytokinesis failure in *ect-2(ts)* mutant embryos was low, in agreement with the results of Zonies *et al.* (2010), we confirmed that cytokinesis failure could be partially suppressed by Rac disruption (Loria *et al.*, 2012), as *ect-2(ts); Rac(RNAi)* embryos failed in cytokinesis at a rate of 17% (10 of 12 embryos divide; Figure 4, A and C). Based on either a Fisher's exact test or a Barnard's exact test, this rescue was not statistically significant (*p* values in Supplemental Table S3). We also investigated whether Rac disruption could rescue cytokinesis failure in the AB and P1 blastomeres of two-cell *ect-2(ts)* embryos with and without Rac depletion (Figure 4E). We found that 100% of both AB and P1 blastomeres successfully completed cytokinesis in *ect-2(ts); control(RNAi)* and *ect(ts); Rac(RNAi)* embryos, even after a 12-h upshift to restrictive temperature (12 of 12 of both AB and P1 cells divide in *ect-2(ts); Control(RNAi)* embryos; 14 of 14 AB cells and 16 of 16 P1 cells divide in *ect-2(ts); Rac(RNAi)* two-cell embryos, respectively; Figure 4E). The lack of cytokinesis failure in

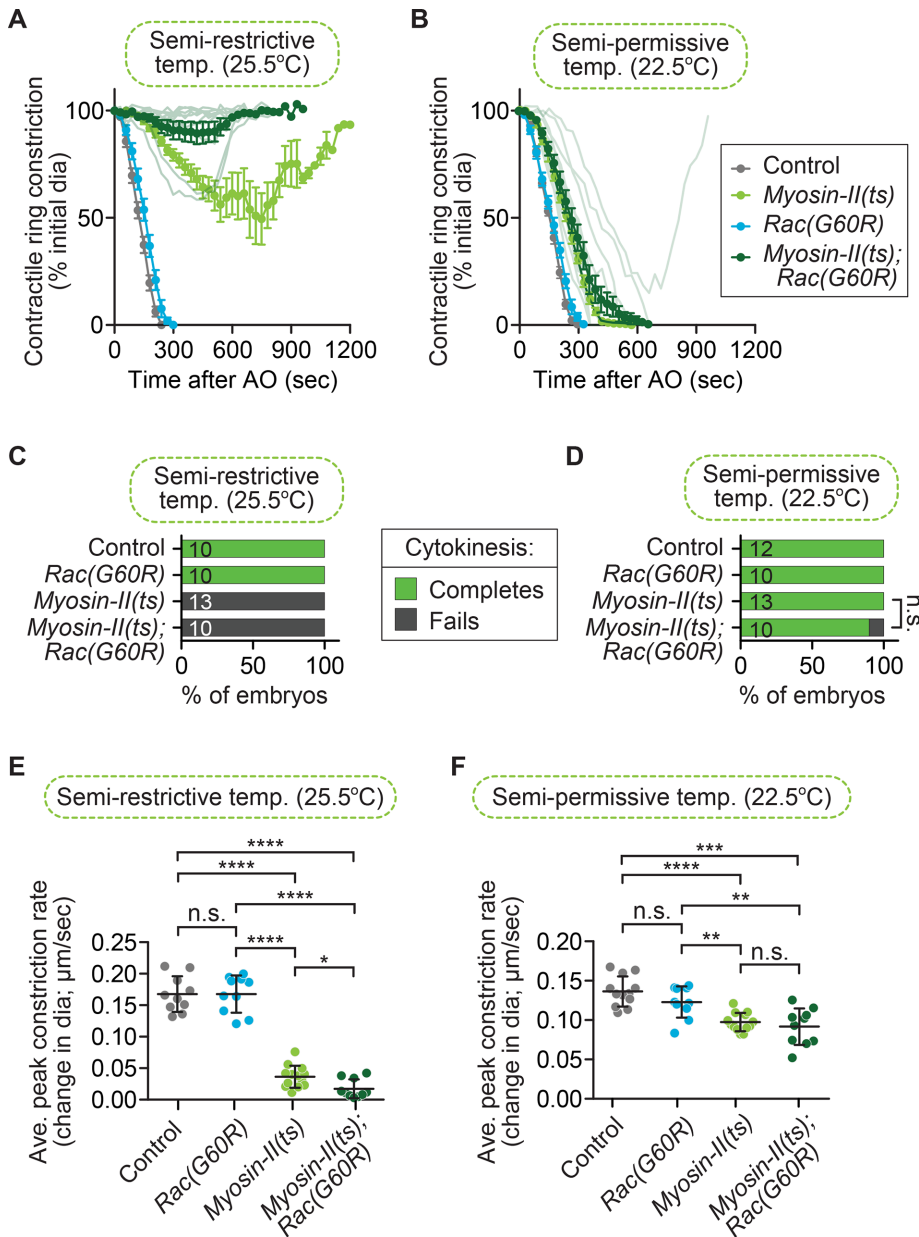


FIGURE 3: Rac disruption does not rescue cytokinesis failure in *myosin-II(ts)* embryos. (A, B) Kinetic analysis of contractile ring constriction in control and *myosin-II(ts)* mutant one-cell embryos with and without *Rac(G60R)*, dividing at (A) semirestrictive (25.5°C) and (B) semipermissive (22.5°C) temperature. Mean ring diameter vs. time, with all replicates for the *myosin-II(ts)*; *Rac(G60R)* double mutant in a more transparent shade of forest green. Error bars represent SEM. (C, D) Cytokinesis failure and success rates for different genotypes at (C) semirestrictive and (D) semipermissive temperature. The number of embryos per genotype is indicated on each individual bar; *p* values were obtained by both Fisher's and Barnard's exact tests (Supplemental Table S3). (E, F) Average peak rate of contractile ring constriction, plotted as the change in diameter (μm/s) at (E) semirestrictive and (F) semipermissive temperature. Error bars represent SD; *p* values were obtained by an unpaired, two-tailed Student's *t* test (Supplemental Table S3). n.s., $p \geq 0.05$; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

two-cell *ect-2(ts)* embryos is likely due to the hypomorphic nature of this allele (Zonies *et al.*, 2010), although we cannot rule out the possibility that ECT-2 may play less of a role during cytokinesis in two-cell embryos than it does in one-cell embryos. Thus our data suggest that depletion of Rac by RNAi can partially rescue cytokinesis failure in one-cell embryos from *ect-2(ts)* mutants, although this rescue is not statistically significant (Supplemental Table S3).

Because we saw a lower rate of cytokinesis failure in the *ect-2(ts)* mutant and a lower rate of rescue after Rac disruption by RNAi than observed by Loria *et al.* (2012), we obtained the strains they used to test whether there could be strain variation between two labs. We found that their *ect-2(ts)* mutants still completed cytokinesis at a higher rate in our hands when upshifted to restrictive temperature for at least 12 h (52%, or 25 of 48 embryos divide here vs. ~3% in their work; Figure 4D), again more similar to the low cytokinesis failure rate of this mutant strain originally reported by Zonies *et al.* (2010). We also confirmed that the rate of cytokinesis failure was partially rescued in *ect-2(ts)*; *Rac(V190G)* double mutants (14 of 19 embryos divide; Figure 4D). *Rac(V190G)* is a mild lof allele (*n1993*) that affects the CAAX box and thus likely affects Rac association with the plasma membrane (Kinsella *et al.*, 1991; Reddien and Horvitz, 2000; Shakir *et al.*, 2006; Roberts *et al.*, 2008; Steffen *et al.*, 2013). Although we do not observe the same high rate of cytokinesis failure in the *ect-2(ts)* mutant after prolonged upshifts to restrictive temperature as reported by Loria *et al.* (2012), possibly due to environmental effects, we observed a similar rescue of cytokinesis failure rates in *ect-2(ts)*; *Rac(RNAi)* and *ect-2(ts)*; *Rac(V190G)* embryos. Thus reducing Rac activity can partially rescue cytokinesis failure when ECT-2 activity is compromised, although to a lesser extent than in *cyk-4(ts)* mutants (e.g., Figure 1; Canman *et al.*, 2008; Loria *et al.*, 2012) and not to statistically significant levels (Supplemental Table S3).

The second part of the argument against Rac as a specific target of CYK-4 is that the main target of CYK-4 is ECT-2, which then activates Rho. This is largely based on the findings that 1) CYK-4 and ECT-2 from various species can interact in biochemical assays (Somers and Saint, 2003; Yuce *et al.*, 2005; Burkard *et al.*, 2009; Wolfe *et al.*, 2009; Kim *et al.*, 2014; Zou *et al.*, 2014; Zhang and Glotzer, 2015), and 2) a *C. elegans* gain-of-function ECT-2 mutation can rescue cytokinesis failure caused by a lof CYK-4 GAP mutation (Figure 1D; Zhang and Glotzer, 2015). If CYK-4 is upstream of ECT-2, then reducing CYK-4 activity should lead to a reduction in ECT-2-mediated Rho activation and Rho effector accumulation at the division plane. To test this, we quantified the levels of downstream Rho targets green fluorescent protein (GFP::myosin-II [GFP::NMY-2; Munro *et al.*, 2004]) and a reporter for f-actin, GFP::utrophin^{actin-binding domain} (GFP::Utr^{ABD}; Burkel *et al.*, 2007; Tse *et al.*, 2012) at the division plane during cytokinesis in one-cell embryos at 150 s after anaphase onset. At this time point, most control, but not *cyk-4(ts)*, embryos have

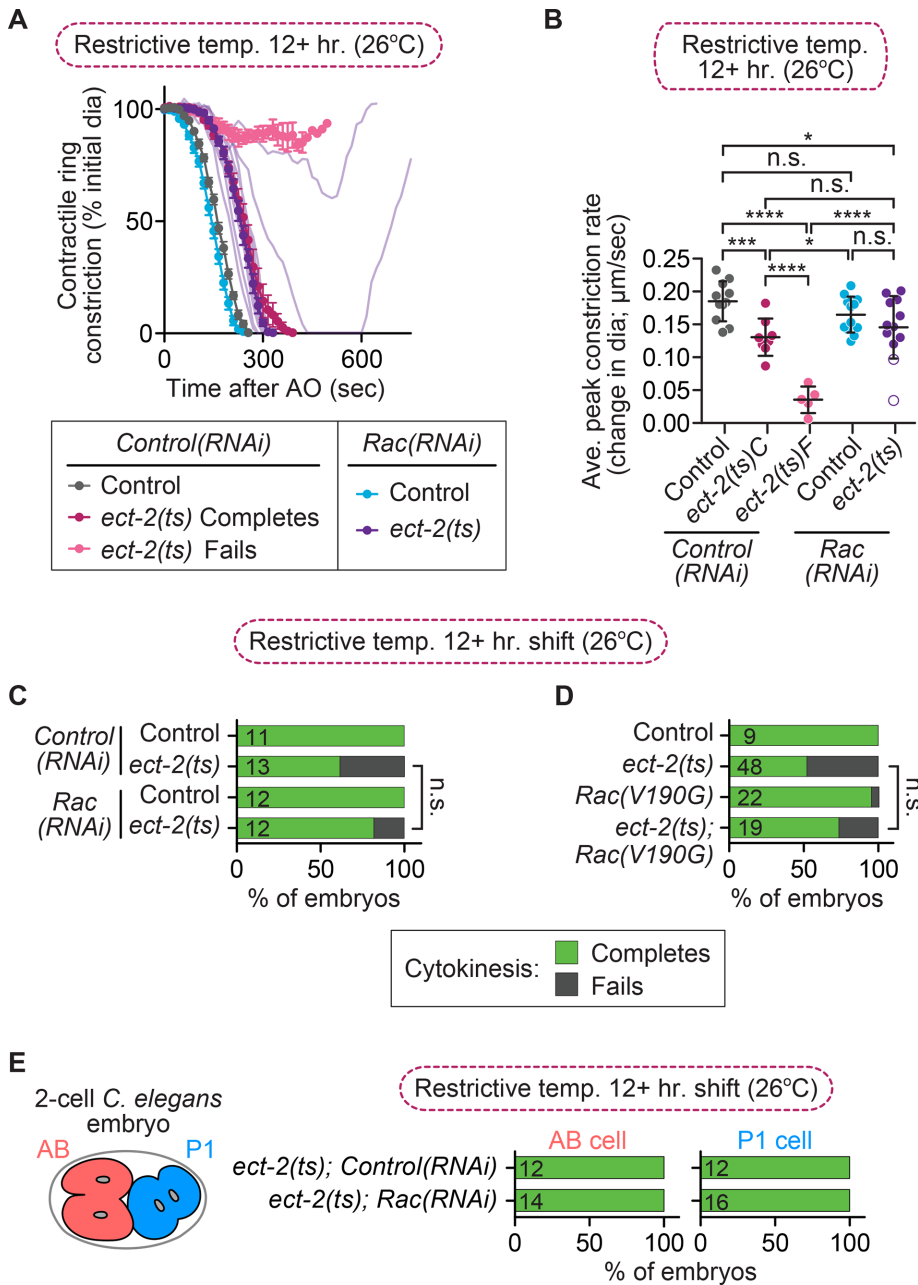


FIGURE 4: Rac disruption partially rescues cytokinesis failure in *ect-2(ts)* embryos. (A) Kinetic analysis of contractile ring constriction in control and *ect-2(ts)* mutant one-cell embryos at restrictive temperature (26°C) with and without *Rac(RNAi)*. Mean ring diameter vs. time, with all replicates for the *ect-2(ts); Rac(RNAi)* double mutant in a more transparent shade of deep purple. Error bars represent SEM. (B) Average rate of peak contractile ring constriction, plotted as the change in diameter (μm/s) for embryos in A. C, cytokinesis completes; F, cytokinesis fails. The white dots with a purple outline represent the *ect-2(ts); Rac(RNAi)* embryos that failed in cytokinesis. Error bars represent SD; *p* values were obtained by an unpaired, two-tailed Student's *t* test (Supplemental Table S3). (C) Cytokinesis failure and success rates for the embryos in A and (D) cytokinesis failure and success rates for one-cell embryos from *ect-2(ts)* mutant strains obtained from Loria *et al.* (2012). The number of embryos per genotype is indicated on each individual bar; *p* values were obtained by both Fisher's and Barnard's exact tests (Supplemental Table S3). (E) Schematic of the *C. elegans* two-cell embryo and graphs showing cytokinesis failure and success rates in the AB and P1 cell divisions for *ect-2(ts)* mutant embryos with or without *Rac(RNAi)*. n.s., *p* ≥ 0.05; **p* < 0.05; ****p* < 0.001; *****p* < 0.0001.

formed a double membrane at the furrow tip or initiated "furrow involution" (Lewellyn *et al.*, 2010; Supplemental Figure S1, A and B), as contractile rings in *cyk-4(ts)* embryos are less constricted

Sugimoto, 2006; Schonegg and Hyman, 2006). CYK-4 was also proposed to play a critical role in regulating cell polarity in the early embryo by functioning within the sperm to trigger polarity

than in controls (Figure 1, A and C). We found no significant difference in the levels of GFP::myosin-II or GFP::Utr^{ABD} at the division plane in *cyk-4(ts)* mutants relative to control embryos during this early stage of contractile ring constriction (Figure 5, A–C). Although in *cyk-4(ts)* embryos, contractile ring f-actin appeared to be less tightly bundled, likely due to the reduced constriction rate in *cyk-4(ts)* embryos relative to in controls (Figure 1, A and C), the total levels of f-actin at the division plane did not significantly differ from those in controls (Figure 5, B and D, and Supplemental Table S3). Thus the levels of f-actin and myosin-II, two downstream effectors of ECT-2 and Rho activation (Figure 1D and Supplemental Figure S2), are not significantly changed at the contractile ring upon functional disruption of CYK-4 GAP activity (unpaired Student's *t* test; Supplemental Table S3). This result suggests that CYK-4 is not upstream of ECT-2/Rho activation, but it does not exclude the possibility that ECT-2 is upstream of another function of CYK-4, such as modulation of Rac activity, or that CYK-4 may regulate another small GTPase (e.g., Cdc42).

The foregoing results, as well as the finding that we did not rescue the reduced rate of contractile ring constriction in *cyk-4(ts)* mutants by disrupting Rac activity (Figure 1C), could indicate a secondary positive role for CYK-4 or Rac during cytokinesis. In support of a possible positive role for Rac in cytokinesis, we saw a slight (although nonsignificant) enhancement of cytokinesis failure after depletion of Rac in both *formin(ts)* and *myosin-III(ts)* mutant embryos. However, Rac depletion on its own did not lead to any delays or obvious defects in cytokinesis in most of our experiments (Figures 1–4). In vitro, CYK-4 can also promote the GTP hydrolysis of Cdc42 (CDC-42 in *C. elegans*; hereafter, Cdc42; Toure *et al.*, 1998; Jantsch-Plunger *et al.*, 2000; Bastos *et al.*, 2012). Thus it is possible that CYK-4 GAP activity may also regulate Cdc42 during cell division. Depletion of Cdc42 by RNAi leads to a short but reproducible delay in the onset of contractile ring constriction (Jordan *et al.*, 2016). At this early stage of *C. elegans* development, Cdc42 plays an essential role in maintaining anterior–posterior polarity, which leads to asymmetric cell division and results in a larger anterior and smaller posterior blastomere (Gotta *et al.*, 2001; Kay and Hunter, 2001; Aceto *et al.*, 2006; Motegi and

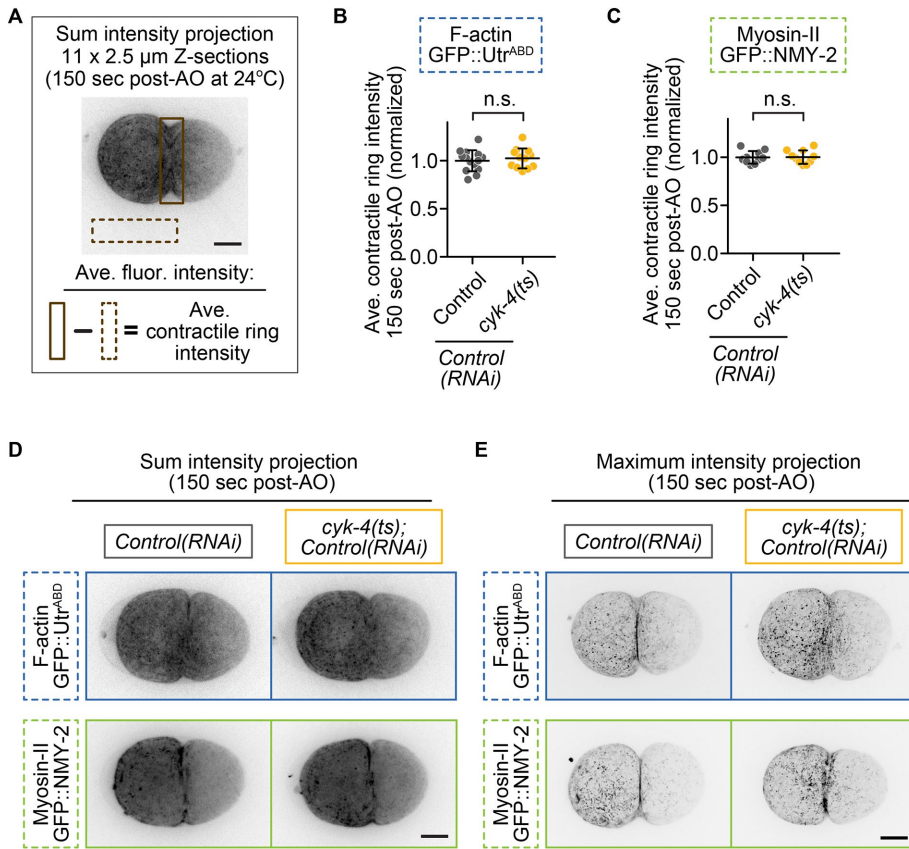


FIGURE 5: Downstream targets of Rho, f-actin, and myosin-II are not reduced at the division plane in *cyk-4(ts)* mutants. (A) Schematic showing how quantitative image analysis was performed for B and C. (B, C) Quantification of contractile ring levels of (B) f-actin (GFP::Utr^{ABD}) and (C) myosin-II (NMY-2::GFP) at 150 s post-AO at semirestrictive temperature (24°C). Error bars represent SD; *p* values were obtained by an unpaired, two-tailed Student's *t* test (Supplemental Table S3). (D) Representative sum projections of f-actin (GFP::Utr^{ABD}) and myosin-II (NMY-2::GFP) in control and *cyk-4(ts)* embryos at 150 s post-AO. (E) Representative higher-Z-resolution maximum projections of f-actin and myosin-II at 150 s post-AO. Scale bars, 10 μm. n.s., *p* ≥ 0.05.

establishment upon fertilization (Jenkins *et al.*, 2006). However, we do not observe a dramatic increase in embryonic lethality or decrease in brood size when the sole sperm used for fertilization is homozygous for the *cyk-4(ts)* mutation, even at restrictive temperature, which would be expected if CYK-4 GAP activity plays this essential role in cell polarity (Figure 6A; unpaired Student's *t* test; Supplemental Table S3). Furthermore, we do not see a significant loss in the asymmetric position of the site of furrowing or daughter cell size in *cyk-4(ts)* single-mutant embryos (Figure 6B; unpaired Student's *t* test; Supplemental Table S3). Thus, if CYK-4 regulates Cdc42 in this system, it is likely not a major player in anterior–posterior cell polarity.

We recently identified a role for Cdc42 during cytokinesis in promoting the robustness of contractile ring constriction (Jordan *et al.*, 2016). One possibility is that CYK-4 regulates Cdc42 specifically during cytokinesis. We tested whether disrupting Cdc42 by RNAi could further rescue the rate of cytokinesis failure in one-cell *cyk-4(ts)*; *Rac(1of)* double-mutant embryos. To ensure that we were able to obtain sufficient embryos from all genetic backgrounds, we used a partial Cdc42 depletion that reduced but did not eliminate cell polarity, as evidenced by the significant reduction in anterior–posterior daughter size asymmetry (Figure 6B; unpaired Student's *t* test; Supplemental Table S3). *Cdc42(RNAi)* did not significantly change the success rate or constriction kinetics of cytokinesis

in control, *Rac(G60R)*, or *cyk-4(ts)* embryos, and, as expected, *cyk-4(ts)*; *Rac(G60R)*; *control(RNAi)* embryos failed in cytokinesis only 20% of the time (12 of 15 embryos divide; Figure 6, C and D, and Supplemental Table S3). In contrast, *cyk-4(ts)*; *Rac(G60R)*; *Cdc42(RNAi)* triple embryos failed in cytokinesis 100% of the time (0 of 11 embryos divide; Figure 6, C and D). Thus Cdc42 depletion significantly enhances the rate of cytokinesis failure relative to *cyk-4(ts)*; *Rac(G60R)*; *control(RNAi)* embryos (Fisher's and Barnard's exact tests; Supplemental Table S3), indicating that the rescue of cytokinesis failure in *cyk-4(ts)* mutants after *Rac* disruption is dependent on Cdc42. This is consistent with a role for CYK-4 in regulating Cdc42 during cytokinesis, although, due to the known role of Cdc42 and the PAR polarity proteins in cytokinesis (Jordan *et al.*, 2016) and lack of an effect of *Cdc42(RNAi)* on cytokinesis in the *cyk-4(ts)* mutant, it is difficult to draw any firm conclusions (Figure 6, C and D). In summary, our data support a model in which the predominant role for CYK-4's GAP activity during cytokinesis is to down-regulate *Rac* to allow for timely contractile ring constriction, at least in *C. elegans* one- and two-cell embryos (Supplemental Figure S2).

DISCUSSION

This work addressed the role of CYK-4 and the Rho-family small GTPase *Rac* in cytokinesis. Our results confirmed that, as was previously found, *Rac* inactivation partially rescued the rate of cytokinesis failure in a CYK-4 GAP mutant and a hypomorphic ECT-2 mutant (Canman *et al.*, 2008; Bastos *et al.*, 2012; Loria *et al.*, 2012; Cannet *et al.*, 2014; Zhang and Glotzer, 2015). However, we did not find evidence that *Rac* inhibition is a nonspecific “bypass” mechanism that generally facilitates contractile ring constriction, as was proposed by others (Loria *et al.*, 2012). Although *Rac* disruption did not rescue the rate of contractile ring constriction in *cyk-4(ts)* mutants, suggestive of another role for CYK-4 (or *Rac*) in cytokinesis, we did not find a role for CYK-4 upstream of ECT-2–mediated activation of Rho. We also did not find evidence that *Rac* activity changes the rate of or generally opposes contractile ring constriction; *Rac* inactivation did not rescue cytokinesis failure in strains with mutations in the Rho effectors diaphanous formin/CYK-1 and myosin-II/NMY-2. Furthermore, disrupting CYK-4 GAP activity did not affect the levels of f-actin or myosin-II in the contractile ring, indicating that CYK-4 is unlikely to act upstream of ECT-2–mediated activation of Rho. Our data instead suggest that CYK-4 negatively regulates *Rac* (and potentially Cdc42) during cytokinesis (Supplemental Figure S2).

There are at least three possible explanations for the mild rescue of ECT-2–mediated cytokinesis failure by loss of *Rac*: 1) *Rac* is a general suppressor of cytokinesis when the contractile ring is weakened, as was proposed (Loria *et al.*, 2012; Zhang and Glotzer, 2015), although our data showing a lack of rescue resulting from *Rac* disruption in *formin(ts)* and *myosin-II(ts)* mutants argue against this

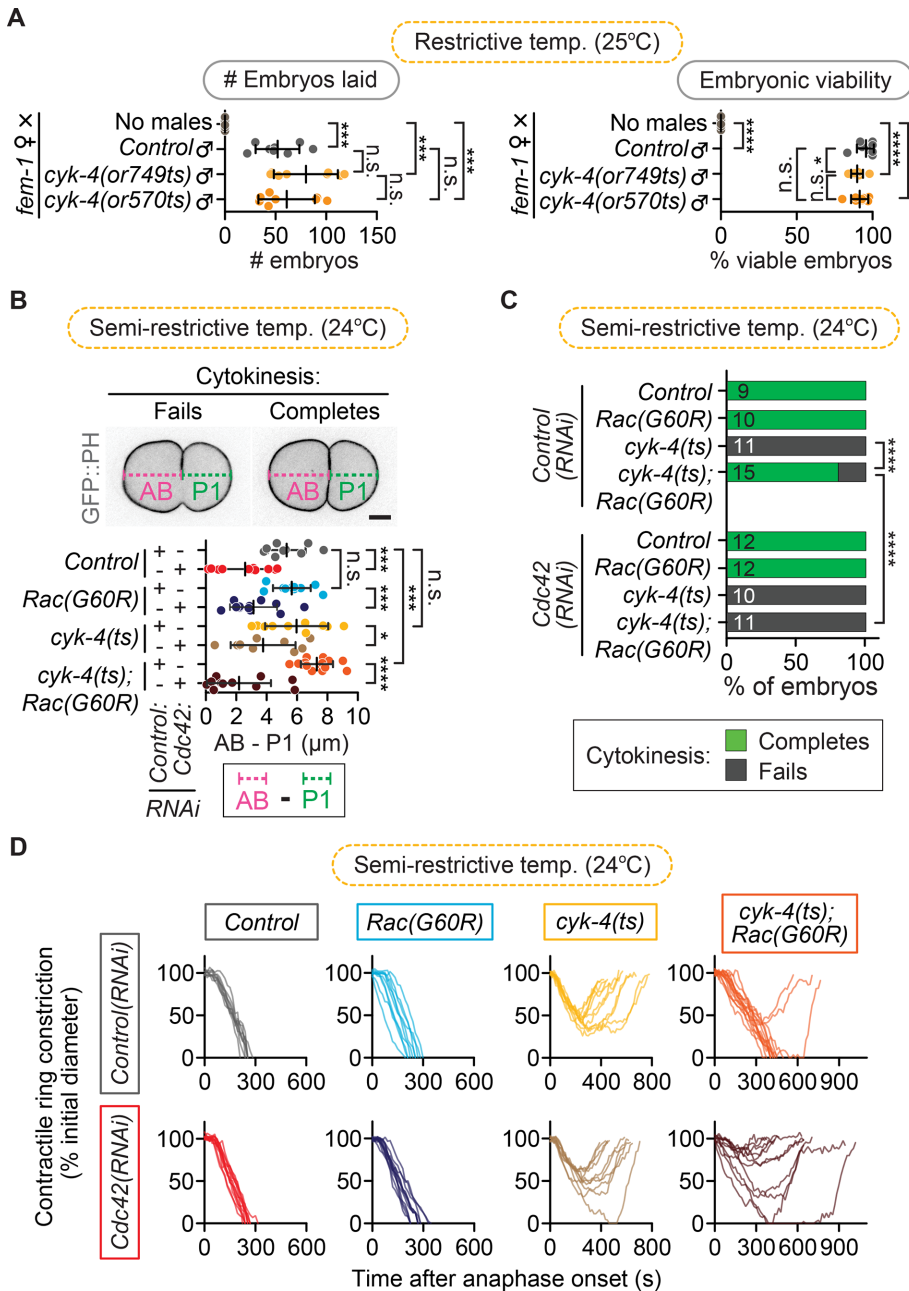


FIGURE 6: *cyk-4(ts)*; *Rac(G60R)* fail in cytokinesis when *Cdc42* is depleted. (A) Paternal requirement test for sperm-supplied CYK-4 GAP activity in ~24- to 30-h brood size (left) and embryonic viability (right) when *cyk-4(ts)* males are mated with non-sperm-producing feminized *fem-1(hc17ts)* hermaphrodites at restrictive temperature (25°C). The low embryonic lethality rate indicates that sperm-supplied CYK-4 GAP activity is not essential to establish and/or maintain cell polarity. Error bars represent SD; *p* values were obtained by an unpaired, two-tailed Student's *t* test (Supplemental Table S3). (B) Asymmetry of furrow position in one-cell embryos (when cytokinesis fails) or of AB vs. P1 daughter cell size (when cytokinesis completes) among different genotypes used in D. Error bars represent SD; *p* values were obtained by an unpaired, two-tailed Student's *t* test (Supplemental Table S3). (C) Cytokinesis failure and success rates for different genotypes in D. The number of embryos per genotype is indicated on each individual bar; *p* values were obtained by both Fisher's and Barnard's exact tests (Supplemental Table S3). (D) Kinetic analysis of contractile ring constriction in control, *cyk-4(ts)*, *Rac(G60R)*, and *cyk-4(ts); Rac(G60R)* double-mutant one-cell embryos with and without *Cdc42(RNAi)* at semirestrictive temperature (24°C). Embryos from each genotype are plotted on individual graphs to make it easier to see, and traces for all individual embryos are shown. Scale bar, 10 μm. n.s., *p* > 0.05; **p* < 0.05; ****p* < 0.001; *****p* < 0.0001.

model; 2) Rac and Rho are mutually inhibitory and/or compete for binding partners (e.g., RhoGDI, GEFs, GAPs, or effectors), so that perturbing Rac activity promotes more Rho activity, as can occur in other cell contexts (e.g., Machacek et al., 2009; Boulter et al., 2010; Garcia-Mata et al., 2011); or 3) the GEF activity of ECT-2 promotes Rac activation during cytokinesis either directly or via CYK-4 (Supplemental Figure S2), which is inhibitory to contractile ring constriction. In vitro, ECT-2 was found in one study to be an even more potent GEF for Rac and *Cdc42* than Rho (Tatsumoto et al., 1999); therefore this is indeed a possibility. This would explain why disruption of Rac activity can rescue cytokinesis failure in both *cyk-4(ts)* and, to some extent, *ect-2(ts)* mutant embryos but not *formin(ts)* or *myosin-II(ts)* embryos. Thus we propose that, in addition to its positive role in cytokinesis via Rho activation, ECT-2 (alone or through CYK-4) may function in a negative regulatory feedback loop to spatiotemporally control Rac activity and/or GTPase flux during cytokinesis (Supplemental Figure S2).

Our results on Rho-effector levels in the contractile ring after CYK-4 disruption conflict with the results reported by Loria et al. (2012) but agree with previous analyses of the effect of CYK-4/Centralspindlin disruption on myosin-II targeting to the contractile ring during ring constriction (Canman et al., 2008; Lewellyn et al., 2011), including myosin-II analysis presented more recently by the same lab that published the Loria et al. (2012) work (Zhang and Glotzer, 2015). One possible difference between these studies is the method used to quantify fluorescence intensity in the contractile ring. Loria et al. (2012) quantified the levels within a region of interest (ROI) around the division plane from the maximum projected images of 5 × 2.5 μm central Z-sections, applied a threshold with a minimal value of 1.25 times the background intensity, and then integrated and normalized to the background. Here we quantified the signal in a ROI around the entire division plane from the sum-projected images from 11 × 2.5 μm Z-sections, subtracted the sum-projected background signal from an identical-sized ROI, and then normalized to the levels in control embryos (Figure 5). Although our analysis reflects all of the GFP signal in the contractile ring, the arbitrary thresholding and maximum projections used in the other analysis would decrease detection of more diffuse cytoskeletal signal in the contractile ring. In the case of *cyk-4(ts)* mutants, a more diffuse signal

would be expected due to the reduced rate of contractile ring constriction (Figure 1C) and thus likely reduced rate of actomyosin bundling. Furthermore, limiting analysis to only $5 \times 2.5 \mu\text{m}$ central Z-sections during imaging (Loria *et al.*, 2012; vs. $11 \times 2.5 \mu\text{m}$ here; *C. elegans* embryos are $\sim 25\text{--}30 \mu\text{m}$ in diameter) would be more likely to miss regions of the equatorial cell cortex that contain peak levels of contractile ring proteins especially early in cytokinesis, as contractile ring constriction itself is asymmetric in this system and initiates from a single position along the equatorial cell cortex (Maddox *et al.*, 2007).

We also found that rescue of *cyk-4(ts)* GAP-defective mutants by Rac depletion is Cdc42 dependent. This could represent a role for CYK-4 in regulating Cdc42 activity during cell polarity maintenance and/or cytokinesis or general Rho-family imbalance due to Cdc42-mediated competition for inhibitory Rho-family effectors and/or regulators (e.g., GDIs; Tatumoto *et al.*, 1999; Machacek *et al.*, 2009; Boulter *et al.*, 2010; Garcia-Mata *et al.*, 2011). Cdc42 hyperactivation was found to inhibit the formation of a focused band of f-actin in the contractile ring in HeLa cells (Zhu *et al.*, 2011), and yet depletion of Cdc42 does not block contractile ring constriction in HeLa cells or *C. elegans* embryos (Zhu *et al.*, 2011; Jordan *et al.*, 2016). In the early *C. elegans* embryo, Cdc42 plays an important role in maintaining anterior–posterior (AP) polarity (Gotta *et al.*, 2001; Kay and Hunter, 2001), and we recently identified a role for Cdc42 (via a collaboration with the PAR polarity proteins) in promoting f-actin accumulation in the contractile ring (Jordan *et al.*, 2016). Thus it is possible that CYK-4 may regulate this function of Cdc42 during polarity maintenance. Sperm-supplied CYK-4 has also been implicated in regulating AP cell polarity in *C. elegans* embryos (Jenkins *et al.*, 2006), but we see either no difference or only a mildly significant difference in embryonic lethality and no significant difference in brood size when homozygous *cyk-4(ts)* males are mated with genetically feminized worms (*fem-1* mutant) at restrictive temperature, as would be expected if AP polarity was grossly disrupted. Furthermore, in *cyk-4(ts)* single mutants, we do not see a change in two strong indicators of a polarity disruption: 1) asymmetric cleavage furrow position along the AP axis and 2) daughter size asymmetry (Figure 6B). We do, however, see a significantly more posteriorized furrow position in *cyk-4(ts); Rac(lop)* double mutants (Figure 6). This is a similar phenotype to that seen after depletion of the Cdc42 GAP chimaerin 1, known to be a regulator of Cdc42 during cell polarity maintenance in this system (Kumfer *et al.*, 2010; Beatty *et al.*, 2013). Nevertheless, enhancement of cytokinesis failure alone cannot confirm a role for CYK-4 in Cdc42 regulation during contractile ring constriction. Taken together, our data support a model in which, at least in the early *C. elegans* embryo, CYK-4 functions to inhibit Rac activity and does not participate directly in Rho activation (Supplemental Figure S2).

MATERIALS AND METHODS

Strain maintenance

C. elegans were kept on standard Nematode Growth Medium (NGM) plates seeded with OP50 *Escherichia coli* bacteria as described (Brenner, 1974). All strain names and genotypes used in this study are listed in Supplemental Table S1.

Temperature control

All control and ts strains were raised in an incubator (Binder) kept at permissive temperature ($16 \pm 0.5^\circ\text{C}$), and all fast-acting ts strains were also dissected in cooled ($\sim 16^\circ\text{C}$) M9 buffer (3 g of KH_2PO_4 , 6 g of Na_2HPO_4 , 5 g of NaCl, 1 ml of 1 M MgSO_4 , and H_2O to 1 l and sterilized by autoclaving) until just before the one- or two-cell

division. For the non-fast-acting *ect-2(ts)* experiments (Figure 4), worms were raised at permissive temperature (16°C) until the L4 stage, at which time they were moved to a 26°C incubator (Binder) overnight for at least 12 h and dissected in a warmed room ($\sim 26^\circ\text{C}$) in $\sim 26^\circ\text{C}$ M9 buffer (Brenner, 1974).

Live-cell imaging was performed in a thermally controlled room. Room temperature was monitored with two thermometers attached directly to the objective and another thermometer attached to the stage via a glue gun to monitor the temperature of the specimen during filming. Wherever mentioned, experimental temperatures were calculated by averaging the values from the two thermometers attached to the objective and were as follows: *cyk-4(ts)* semirestrictive ($24.0 \pm 0.5^\circ\text{C}$); *ect-2(ts)* restrictive ($26.0 \pm 0.5^\circ\text{C}$); *formin(ts)* semipermissive ($18.5 \pm 0.5^\circ\text{C}$); *formin(ts)* semirestrictive ($23.5 \pm 0.5^\circ\text{C}$); *myosin-III(ts)* semipermissive ($22.5 \pm 0.5^\circ\text{C}$); and *myosin-III(ts)* semirestrictive ($25.5 \pm 0.5^\circ\text{C}$). Temperatures are defined for each individual mutant allele as follows: permissive temperature, all ts embryos complete cytokinesis with kinetics most similar to non-ts controls; semipermissive temperature, highest temperature at which all ts embryos complete cytokinesis; semirestrictive temperature, lowest temperature at which all ts embryos fail in cytokinesis; and restrictive temperature, all embryos fail in cytokinesis with a null-like phenotype (aside from the *ect-2(ts)* mutant, which often divides successfully even at the restrictive temperature of 26°C).

Live-cell imaging

Single-cell embryos were mounted on a 2% agar pad as described previously (Gonczy *et al.*, 1999; Jordan *et al.*, 2016). Imaging was performed on a Nikon Ti inverted microscope with $40\times/1.3$ numerical aperture (NA) and $60\times/1.4$ NA oil-immersion PlanApochromat objectives using a Yokogawa CSU-10 spinning-disk confocal equipped with Borealis (Spectral Applied Research) and a Hamamatsu Orca ER camera. Z-sectioning was done with a piezo-driven motorized stage (Applied Scientific Instrumentation), and focus was maintained using Perfect Focus (Nikon) before each Z-series acquisition. Solid-state 150-mW lasers were used for excitation at 488 and 561 nm for enhanced GFP and mCherry, respectively (Spectral Applied Research), and a filter wheel was used for emission wavelength selection (Sutter Instruments). The imaging system was controlled by MetaMorph software (Molecular Devices).

A central, transmitted light image and through-cell GFP and mCherry fluorescent Z-series were collected every 15 or 30 s to measure ingression kinetics (see later description). For data presented in Figures 1–4 and 6 and Supplemental Figure S1, embryos were imaged with the $40\times/1.3$ NA oil objective with 2×2 binning and 100-ms exposures. Laser power was tuned to 20% for the 488-nm laser and 50% for the 561-nm laser (except for Figure 6, B–D, where we looked at the effect of *Cdc42(RNAi)* on cytokinesis in *cyk-4(ts)* embryos with and without *Rac(G60R)*); the 561-nm laser intensity was 70%. For data presented in Figures 1, 3, and 4 (*cyk-4(ts)* kinetics with and without *Rac(G60R)* or *Rac(RNAi)*; *cyk-1(ts)* kinetics with and without *Rac(G60R)*; and *nmy-2(ts)* kinetics with and without *Rac(G60R)*), the same imaging parameters were used, except that embryos were imaged every 30 s. The Z-sectioning was as follows: $13 \times 2 \mu\text{m}$ steps (Figures 1–4 and 6, B–D, and Supplemental Figure S1C), with the exception of a few embryos filmed using $7 \times 2 \mu\text{m}$ steps in Figures 2 and 3 at the semirestrictive temperature, where the contractile ring closes in the central Z-plane. The embryos imaged using $7 \times 2 \mu\text{m}$ steps were acquired with the $60\times/1.4$ NA oil objective 2×2 binning (Figures 2 and 3). For analysis of the contractile ring levels of GFP::NMY-2 (Munro *et al.*, 2004) and GFP::Utr^{ABD}, a reporter for f-actin (Burkel *et al.*, 2007; Tse *et al.*, 2012), a single

11 × 2.5 μm or 65 × 0.5 μm Z-section image stack (see also *Image analysis* section) was taken 150 s after AO using a 60×/1.4 NA oil-immersion PlanApoChromat objective with 2 × 2 binning on a Hamamatsu Orca ER camera and 150-mW, 488- and 561-nm lasers at 50% laser power intensity with 100-ms exposures (Figure 5).

Image analysis

MetaMorph (Molecular Devices) and FIJI (ImageJ [Schindelin *et al.*, 2012; Schneider *et al.*, 2012]) software were used for all data analysis. Contractile ring constriction was measured from the time of metaphase (time point immediately before AO) until AO of the next division, or, in the case of cytokinesis failure, until complete contractile ring regression. The contractile ring diameter was measured at each time point in the Z-section where the ring was most open and displayed as a percentage of the initial diameter over time. The rate of ingression is plotted as micrometers/second when the rate of ingression peaks from ~90–50% of the initial cell diameter. When embryos did not ingress to 50% of the initial cell diameter, the point of deepest contractile ring constriction was used (Figures 1–4). Where histone markers were not available (in strains with *Rac(1of)* and *cyk-4(ts);GFP::NMY-2*), AO was determined by transmitted light.

The levels of f-actin and myosin-II (GFP::Utr^{ABD} and GFP::NMY-2) in the contractile ring in *cyk-4(ts)* embryos (at semirestrictive temperature, 24°C) were calculated as follows. All 11 × 2.5 μm Z-stacks were sum projected using FIJI (Figure 5, B–D). A region of fixed size (195 × 38 pixels) surrounding the entire division plane was used to measure the average pixel intensity, and measurements from a region of the same size in a nearby background area, outside of the embryo, were subtracted for each individual embryo, as schematized in Figure 5A. These data were normalized to controls and plotted as the average intensity at 150-s post-AO (Figure 5, B–D). Where higher-resolution images are shown (Figure 5E), a 65 × 0.5 μm Z-section stack acquired at 150 s post-AO and a maximum projection image for all 65 Z-sections were generated using FIJI.

The asymmetric differences in daughter cell size were calculated by measuring the length of the newly formed AB (anterior) and P1 (posterior) blastomeres using the time point immediately after completion of cytokinesis (Figure 6B, showing *cyk-4(ts)* with and without *Rac(G60R)* and *Cdc42(RNAi)*). The length of the posterior P1 cell was subtracted from the length of the anterior AB cell. Cell size measurement was done using a single central plane from the 488-nm GFP channel. When embryos failed to complete cytokinesis, a similar measurement was taken using the distance from the anterior and posterior poles to the cleavage furrow at the time of maximum contractile ring constriction. Analysis of the time from AO to double-membrane formation in Supplemental Figure S1 was done using the same embryos as in Figure 1A (kinetics of cytokinesis in *cyk-4(ts)* mutant embryos with and without *Rac(RNAi)*).

Embryonic viability and brood size

The *fem-1(hc17ts)* hermaphrodites were raised at 25°C to feminize them (by blocking sperm production) and then singled as L4s onto individual mating plates (35-mm NGM agar plates seeded with 10 μl of OP50; Brenner, 1974). The *cyk-4(ts)* males were also raised at 25°C from the ~L3/L4 stage. Seven males per genotype (except for the no-male controls) were added to each plate containing a single hermaphrodite and allowed to mate for ~15 h at 25°C. To ensure that the *fem-1(hc17ts)* hermaphrodites were feminized (and thus sterile), we did not add males to one set of *fem-1(ts)* hermaphrodites (no-male controls). The adults from each individual cross were transferred twice to a new plate every ~10 h (after laying ~50 embryos). The numbers of viable progeny and dead embryos per

feminized hermaphrodite (Figure 6A) were quantified for two plates per cross (representing ~24–30 h of egg laying) at 24 h after transferring off the adults (to allow all embryos to hatch) using a digital counting pen (Fisher) on a Nikon dissecting microscope.

Feeding RNAi

All primers and DNA template used for RNAi experiments are listed in Supplemental Table S2. Cloning was performed by inserting the *Rac/ced-10* sequence into the L4440 vector using standard cloning protocols and then transformed into HT115 *E. coli* (Timmons *et al.*, 2001). The empty L4440 vector in HT115 cells was used as a control. RNAi feeding bacteria were inoculated in Luria broth with 100 μg/ml ampicillin for either 7 h at 37°C or 16 h at 32°C. A 300-μl amount of this culture was plated on RNAi plates (NGM agar plates [Brenner, 1974] supplemented with 50 μg/ml ampicillin and 1 mM isopropyl-β-D-thiogalactoside). After drying, the plates were grown at 32°C for 24–48 h. L1 worms were transferred onto RNAi plates and then grown at 16°C until young adulthood, when they were dissected to obtain the embryos. RNAi knockdown for *Rac/ced-10* was confirmed by observing 1) deeper furrowing during pseudocleavage and 2) completion of cytokinesis in *cyk-4(ts)* mutant embryos at the semirestrictive temperature (24 ± 0.5°C). *Cdc42* (CDC-42) knockdown was confirmed by assaying embryonic lethality and loss of cell division asymmetry (Figure 6B).

Statistical analysis

Unpaired Student's *t* tests were conducted using GraphPad Prism software. n.s., *p* ≥ 0.05; **p* < 0.05; ***p* < 0.01; ****p* < 0.001; and *****p* < 0.0001. Error bars for traces of cell diameter over time during cytokinesis represent SEM. Error bars for contractile ring constriction rates, myosin-II and f-actin levels, embryonic lethality, brood size, and asymmetry of furrow position represent SD. Statistics for cytokinesis completion/failure bar graphs used a two-tailed Fisher's exact test, but this analysis was also done with a Barnard's exact test (Supplemental Table S3). See also statistical analysis in Supplemental Table S3 for all *p* values.

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