Supporting Online Material for

Inhibition of Rac by the GAP Activity of Centralspindlin is Essential for Cytokinesis

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This PDF file includes:

Materials and Methods
Figs. S1 to S7
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Other supporting material includes:

Movies S1 and S2
Materials and Methods:

Mutant cloning and rescue experiments:

The cyk-4(or570ts) and cyk-4(or749ts) (CYK-4^{GAP(T546I)} and CYK-4^{GAP(E448K)}, respectively) mutants were isolated in EMS and ENU mutagenesis screens, respectively, for conditional (temperature-sensitive) embryonic-lethal mutants as described (S1, S2). The mutations were mapped to LG::III using strains MT3751 and MT464. Mutations were identified by sequencing, after refining their location by 3-factor visible marker mapping as described (S3) using strains DR104 and CB2053 for cyk-4(or570ts) and cyk-4(or749ts) respectively. To confirm that the cyk-4 mutations were the cause of the cytokinesis defect, we rescued the embryonic lethality by expression of a GFP fusion with CYK-4. To test for rescue, the cyk-4(or570ts) and cyk-4(or749ts) mutants were crossed with WH0279 non-Unc hermaphrodites. First, cyk-4(or749ts) and cyk-4(or570ts) were linked to unc-119(ed3) and homozygosed then, ojls12[cyk-4::GFP unc-119(+)]/+ (which is lethal when homozygous) progeny were screened for the ability to rescue embryonic lethality at restrictive temperature. Both cyk-4(or570ts) and cyk-4(or749ts) were back-crossed six times prior to mating into fluorescent protein expressing strains for phenotypic analysis (him-8(e1489) first cross, N2 remaining out-crosses). All analysis was done in non-Him strains to avoid possible phenotypic complications.

Temperature control:

Strains were maintained at the permissive temperature of 16°C. For all live imaging experiments, the temperature was controlled by heating the room to 26°C for at least one hour prior to filming or until the microscope temperature reached 26°C. Embryos were maintained at 16°C until immediately prior to filming. The temperature was continuously monitored during filming using a thermometer probe wedged into the microscope body near the stage. For some experiments, a heated stage was also used (20/20 Technology Inc, Wilmington, NC).
**Imaging and quantitative analysis:**

Live imaging was performed on newly fertilized embryos mounted on agarose pads as previously described (S4). Embryos were filmed using a spinning disk confocal as described (S5) using a 60x, 1.4 NA PlanApochromat lens with 2x2 binning. For all movies, Differential Interference Contrast (DIC) images and/or images of an RFP<sup>mCherry</sup> fusion with histone H2B were collected in parallel to monitor cell cycle progression. The rate of furrow ingress ion was measured in strains expressing a GFP labeled plasma membrane probe. A z-series consisting of 14 or 15 planes at 2.5µm intervals was collected every 20s and the data from the equatorial region of the embryo was isolated, rotated by 90°, and projected to generate an “end on” view that was used to measure furrow diameter. To test for suppression of the mutant phenotype, central plane images of mutant embryos depleted of the indicated protein by RNAi were collected at 10s intervals. GFP::AuroraB<sup>AIR-2</sup> was imaged by collecting 5 z-planes at 1.5 µm intervals every 10s.

NMY-2::GFP (Myosin II heavy chain) was imaged by acquiring 4 z-planes at 1 µm intervals at the cell cortex, followed by a single central plane image every 10s. Analysis of the post-anaphase accumulation of cortical NMY-2::GFP as a function of embryo length was performed on maximum-intensity projections of the cortical z-series. A line bisecting the embryo was drawn from the anterior to the posterior tip of the embryo, and MetaMorph software (Molecular Devices, Downingtown, PA) was used to generate an average intensity line scan (50 pixels wide ~1/2 of the width of the embryo) for each time point. Embryos were divided into 20 equal length segments from anterior (0% embryo length) to posterior (100% embryo length), and the mean NMY-2::GFP in each segment was calculated for each time point, after subtraction of a background measurement for that segment made just prior to anaphase onset. The values for each data set were normalized by dividing all intensity values by the average maximum value for controls (55-65% embryo width). The amount of NMY-2::GFP in the contractile ring at the leading edge of the ingressing furrow was quantified using the central plane images as outlined in Fig. S3A.
**Immunofluorescence and imaging fixed embryos:**

Methanol fixation was performed as described (S6) on worms that were pre-shifted to restrictive temperature for 1-2 hours, and dissection was done in a 26°C room. Images were acquired as described (S7) using a Deltavision microscope equipped with a 100x Plan Apo 1.35 N.A. objective. All antibodies were used at a concentration of 1µg/ml.

**RNAi:**

dsRNA was made as described (S6). After injection, worms were allowed to recover for 44-50 hrs at 16°C prior to filming unless the dsRNA led to sterility in which case the worms were allowed to recover for 12-15 hrs (ect-2), 22-24 hrs (cyk-4), and 36-38 hrs (rho-1), respectively.
**Supplemental Figure Legends:**

**Figure S1: Genetic interactions and rescue of cyk-4 GAP alleles.** Genetic data demonstrating that *cyk-4*(or749ts) and *cyk-4*(or570ts) (*CYK-4*\(^{GAP(E448K)}\) and *CYK-4*\(^{GAP(T546I)}\), respectively) are strictly recessive (orange), fail to complement each other (brown), and also fail to fully complement *cyk-4*(t1689ts) (*CYK-4*\(^{CSA(S15L)}\), an allele that affects centralspindlin assembly, CSA). Expression of a GFP fusion with CYK-4 from a transgene rescues the embryonic lethality of both *cyk-4*(or749ts) and *cyk-4*(or570ts) (green).

**Figure S2: Centralspindlin mutations do not affect the accumulation of myosin II on the equatorial cortex prior to furrow ingression.** A) Schematic summarizing the temporal progression of cytokinesis at 25°C. Immediately following anaphase onset, myosin II becomes enriched on the equatorial cortex, forming a band that encircles the cell equator. The cortex subsequently buckles inwards to form a furrow that ingresses inwards towards the spindle center, closing the hole between the daughter cells. B,C) During the 80s immediately following anaphase onset, the amount and distribution of cortical myosin II in the GAP domain and centralspindlin assembly mutants is similar to that in controls. B) Images of the cortex from time-lapse series of embryos expressing a GFP-fusion to the *C. elegans* myosin II heavy chain (NMY-2::GFP). Scale bar, 10 \(\mu\)m. C) The mean post-anaphase accumulation of cortical NMY-2::GFP fluorescence is plotted as a function of embryo length for control and centralspindlin mutant embryos at the indicated time points. Values were normalized by dividing by the average maximum value for controls (between 55-65% embryo length). Error bars=SEM. NOTE: Prior to anaphase onset, cortical NMY-2::GFP is present in an anterior cap that contributes to cellular polarity maintenance. In the early post-anaphase interval, this localization is super-imposed with its accumulation at the cell equator. In the quantification shown in C, the post-anaphase accumulation is measured by subtracting the distribution at anaphase onset from the distribution at subsequent time points to eliminate the contribution from the anterior cap.
Figure S3: Myosin II levels at the furrow tip and the rate of furrow ingression are reduced in centralspindlin mutants.  
A) Schematic illustrating the quantification method used to measure the amount of myosin II heavy chain (NMY-2::GFP) at the furrow tip.  
B) Mean NMY-2::GFP fluorescence at the furrow tip, measured ~180s after anaphase onset, is reduced in centralspindlin mutants relative to controls.  Error bars=SEM.  
C) The mean rate of furrow ingression in the CYK-4 GAP mutants is nearly identical to that in mutants disrupting centralspindlin assembly and following RNAi-mediated depletion of CYK-4, and is reduced ~3-fold relative to controls.  Error bars=SEM.

Figure S4: Partial depletion of the ECT-2 GEF enhances the CYK-4\(^{GAP(E448K)}\) cytokinesis defect, and Rac\(^{CED-10}\) depletion rescues furrow ingression in ZEN-4\(^{CSA(D520N)}\) but not in AuroraB\(^{AIR-2(P265L)}\) embryos.  
A) Partial depletion of the ECT-2 GEF by RNAi enhances the CYK-4\(^{GAP(E448K)}\) cytokinesis defect.  
B) Rac\(^{CED-10}\) depletion rescues the furrow ingression defect in the centralspindlin assembly mutant ZEN-4\(^{CSA(D520N)}\), allowing 85% of the furrows to ingress to completion.  However, in 71% of these embryos cytokinesis fails to complete and the furrow ultimately regresses.  
C) Rac\(^{CED-10}\) depletion does not suppress the cytokinesis phenotype in AuroraB\(^{AIR-2(P265L)}\) mutants which also fail to form a central spindle and have a partial furrow ingression defect.  
D) Co-depletion of Rac\(^{CED-10}\) and Rac\(^{RAC-2}\) by RNAi did not increase the efficiency of rescue over that observed following depletion of Rac\(^{CED-10}\) alone (Fig. 3D).  However, due to two (30-40 bp) stretches of identity at the nucleotide level we cannot rule out that RNAi depletion of Rac\(^{CED-10}\) also targets Rac\(^{RAC-2}\) (albeit less-efficiently) and vice versa.  Scale bar, 20 \(\mu\)m.

Figure S5: Strains used in this study.  
Genotype and strain names used in this study.  
Note: CYK-4\(^{GAP(E448K)}\) is cyk-4(or749ts), CYK-4\(^{GAP(T546I)}\) is cyk-4(or570ts), CYK-4\(^{CSA(S15L)}\) is cyk-4(t1689ts), and ZEN-4\(^{CSA(D520N)}\) is zen-4(or153ts) in this manuscript.

Figure S6: dsRNAs used in this study.  
Primers, template, and concentrations of the dsRNAs used in this study.
Figure S7: Negative regulation of Rac and its effectors, WASp/WAVE and the Arp2/3 complex, by CYK-4 GAP activity is essential for cytokinesis. Model for signaling during cytokinesis.
Supplemental Movie Legends:

Movie S1: Movie montage of cytokinesis in cyk-4 mutant and control embryos. The GAP domain mutants CYK-4\textsuperscript{GAP(E448K)} and CYK-4\textsuperscript{GAP(T546I)} show a partial ingression cytokinesis defect that resembles the cytokinesis defect in the centralspindlin assembly (CSA) mutant CYK-4\textsuperscript{CSA(S15L)}. Embryos are co-expressing a GFP fusion with a PH domain that binds to a phospholipid specifically produced on the plasma membrane and a RFP\textsuperscript{mCherry} fusion with histone H2B, which labels the chromosomes. Images were collected every 15s and are played back at 150x real time.

Movie S2: AuroraB\textsuperscript{AIR-2} dynamics during cytokinesis in cyk-4 mutant and control embryos. A GFP-fusion with AuroraB\textsuperscript{AIR-2} localizes to the central spindle normally in CYK-4\textsuperscript{GAP(E448K)} and CYK-4\textsuperscript{GAP(T546I)} embryos, but not in embryos from the centralspindlin assembly mutant CYK-4\textsuperscript{CSA(S15L)}, which do not form a central spindle. Images were collected every 10s and are played back at 100x real time.
Genetic interactions and rescue of cyk-4 alleles at 25°C

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<td>unc-119(ed3) cyk-4(or570ts)/unc-119(ed3) cyk-4(or570ts)</td>
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</table>
Canman et al. Supplemental Figure 2

A

Anaphase Onset

Furrow Ingression
50-250s Post Anaphase Onset

B

NMY-2::GFP (Myosin II Heavy Chain)

Control

CYK-4 GAP(E448K)

CYK-4 GAP(T546I)

CYK-4 CSA(S15L)

ZEN-4 CSA(D520N)

C

Mean GFP Fluorescence Intensity

0% 50% 100% 0% 50% 100% 0% 50% 100%

-1 -0.5 0 0.5 1 1.5

Control n=11
CYK-4 GAP(E448K) n=4
CYK-4 GAP(T546I) n=7
CYK-4 CSA(S15L) n=4
ZEN-4 CSA(D520N) n=5
Canman et al. Supplemental Figure 3

A

NMY-2::GFP (Myosin II Heavy Chain) in contractile ring

Average fluorescence - Average fluorescence

Area

B

NMY-2::GFP (Myosin II Heavy Chain) in Contractile Ring (% Control)

Control n=10
CYK-4 GAP(E448K) n=9
CYK-4 GAP(T546I) n=12
CYK-4 CSA(S15L) n=9
cyk4 (RNAi) n=6
ZEN-4 CSA(D520N) n=9

0% 50% 100%

C

Rate of Ingression 80-120 sec after Furrow Initiation

Rate of ingression (μm/sec)

Control n=10
CYK-4 n=10
cyk4 (RNAi) n=6
ZEN-4 CSA(D520N) n=9

Rate of ingression (μm/sec)

0.25
0.2
0.15
0.1
0.05
0

Rate of ingression (μm/sec)

0.25
0.2
0.15
0.1
0.05
0

Rate of ingression (μm/sec)
Canman et al. Supplemental Figure 4

A. Complete Cytokinesis

- **ect-2(RNAi)**: n=12
  - 0% Complete Cytokinesis
  - 58% Full Ingression Followed by Furrow Regression
  - 42% Partial Ingression Followed by Furrow Regression
  - 0% No Ingression

B. ZEN-4 CSA(D520N)

- n=17
  - 0% Complete Cytokinesis
  - 100% Full Ingression Followed by Furrow Regression
  - 0% Partial Ingression Followed by Furrow Regression
  - 0% No Ingression

C. AuroraB AIR-2(P265L)

- n=12
  - 17% Complete Cytokinesis
  - 17% Full Ingression Followed by Furrow Regression
  - 67% Partial Ingression Followed by Furrow Regression
  - 0% No Ingression

D. ced-10;rac-2(RNAi)

- n=8
  - 100% Complete Cytokinesis

- CYK-4 GAP(E448K); ced-10;rac-2(RNAi)

- n=37
  - 35% Complete Cytokinesis
  - 35% Full Ingression Followed by Furrow Regression
  - 35% Partial Ingression Followed by Furrow Regression
  - 0% No Ingression
## Worm strains used in the study

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<td>cyk-4(or570ts)III.</td>
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### dsRNAs used in the study

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</table>
Canman et al. Supplemental Figure 7

Negative Regulation

CYK-4 (Rac GAP)

↓

Rac

↓

ARP2/3 Complex

Positive Regulation

ECT-2 (Rho GEF)

↓

RhoA

↓

Formin

Actomyosin Constriction

↓

Cytokinesis
Supplemental References: