Supporting Online Material for

CYK-4/GAP Provides a Localized Cue to Initiate Anteroposterior Polarity upon Fertilization
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EXPERIMENTAL PROCEDURES

Strains and Alleles. We maintained worms as described (1). Wildtype worms were N2 Bristol. Other strains were: SM1052: JJ1473: *unc-119(ed3)* III; *zuls45[nmy-2::GFP unc-119(+)]* (2), JJ1579: *unc-119(ed3)* III; *zuls77[par-6::PAR-6::GFP; unc-119(+)]* (2). CB1489: *him-8(e1489)* (3), CB3844: *fem-3(e2006ts)* (4).

Microscopy. Fluorescent microscopy: JJ1579 or JJ1473 gravid worms were dissected in PBS(137 mM NaCl, 2.7 mM KCl, 4.3 mM Na2HPO4·7H2O, 1.4 mM KH2PO4 [pH 7.3]) in a watch glass and transferred by mouth pipet onto 4% agarose pads. The embryos were compressed slightly by 18x18mm glass coverslips to optimize viewing. Embryos were imaged at 22-24°C using a 60x1.4 NA oil immersion lens using an Applied Precision DeltaVision RT deconvolution microscope. Images were recorded and an exposure time of 0.80 seconds.

Nomarski Microscopy: Embryos from gravid mothers were dissected and mounted on agar pads as described above. Embryos were imaged at 22-23°C using a 63x1.4 NA oil immersion lens and Zeiss Axioskop microscope. For time-lapse, images were captured every 15 seconds with Improvision Openlab. Time-lapse movies were then encoded as Quicktime movies.

RNA-Mediated Interference T19E10.1(ect-2) and K08E3.6 (cyk-4) were obtained from the RNAi feeding library of Ahringer and colleagues and used according to described methods (5, 6). In brief, a 3ml LB culture containing 30ug/ml carbenicillin was inoculated and incubated for 16-18 hours at 37°C. We added 50 uL of this solution to agar worm plates + 5uM IPTG and 6ug/mL carbenicillin. The bacteria were allowed to grow for 48-72 hours at room temperature (22°C). L3 worms were placed on these plates and allowed to feed for 24-30 hours, after which embryos were removed from gravid adults for analysis. Larval stages were determined by size and extent of vulval development.

For *cyk-4* RNAi of males, *him-8(e1489) ~*L3 worms were placed on *cyk-4* RNAi feeding plates and allowed to feed for 24 hours. The appropriate age for performing RNAi was determined by the presence of a ventral clearing, an indication of gonad development. Adult males were subsequently identified by morphological criteria and used in experiments that called for paternal absence of CYK-4. For *cyk-4(RNAi)* of females, NMY-
2::GFP mothers were fed bacteria expressing fem-1 dsRNA. Progeny larvae were removed from fem-1 as L4 larvae and moved to bacteria expressing cyk-4 dsRNA. Females were identified subsequently by morphological criteria and used in matings. Only those embryos with meiotic and mitotic cytokinesis defects were scored for polarity.

For rho-1 RNAi, we cloned a 389bp fragment from wildtype genomic DNA that included exon 3 of the C. elegans rho-1 gene using the primers: 5'-GGT CGA ACT TGC TCT ATG GGA TAC-3’ and 5'-TCC CAT CTC TCA ATT CGG TGC TAC TGC-3’ into pDONRdT7 (7). C56G7.1 (mlc-4) was obtained from the Orfeome library of Vidal (8). The inserts were amplified by PCR and in vitro transcription was performed to produce double-stranded RNA, which was injected into NMY-2::GFP or PAR-6::GFP adults at a concentration of 800ng/ul. Progeny of injected adults were analyzed 24hrs. later.

**Immunostaining:** Immunostaining was performed according to (9). Hermaphrodites were dissected in 25ul of PBS on a subbed slide. A cover slip was placed on top of the embryos and the liquid was wicked out using a Kimwipe. After freeze/crack on dry ice, the slides were immersed in -20MeOH for 10 minutes. Slides were placed in PBST (1xPBS, 0.1%Tween) for 15 minutes followed by 30 minutes blocking in TNB (100mM Tris-HCl pH7.5, 200mM NaCl, 1% BSA) +10%Normal Goat Serum. Primary antibodies were diluted in TNB and placed on slide and incubated overnight at 15°C except for CYK-4, which was performed at 4°C. Slides were washed in TBS (150mM NaCl, 50mM Tris-HCl pH7.5, 1.5mM NaOH), treated with 2° antibodies and mounted. Images were analyzed on a Delta Vision microscope. PAR-3 monoclonal P4A1, developed by Jim Priess, was obtained from the Developmental Studies Hybridoma Bank, developed under the auspices of the NICHD and maintained by the University of Iowa Department of Biological Sciences, Iowa City, IA 52242. Antibodies were diluted as follows: PAR-3 was used at 1:10, αGFP (Invitrogen/Molecular Probes, cat. # A11122) at 1:200, αCYK-4 (10) at 1:200, 1CB4 (11) at 1:100 and αECT-2 at 1:100. Polyclonal antibodies that recognized ECT-2 were made by Harlan BioProducts from rabbits immunized with the peptide DSEKELPRSKTSSYRVD.

For myosin light chain-1P antibody staining, embryos were fixed in 1X PBS, 2% paraformaldehyde for 30 minutes under a coverslip. Embryos were fractured and the slides placed on dry ice for 10 minutes. Coverslips were removed and samples were fixed in fresh, 4° methanol for 3 minutes. Slides were moved to TBS and then blocked in TNB as described above. The MLC-1P antibody is from Cell Signalling (phospho-myosin light chain 2 (Ser19) cat#3671S) diluted 1:100.
Paternal Crosses

We obtained females by either *fem-1(RNAi)* of *nmy-2::GFP* animals or *fem-3(e2006ts); nmy-2::GFP* larvae shifted to 25°. L4 larvae were placed on plates expressing *cyk-4* double-stranded RNA at room temperature (21-23°). Females were identified 18-20 hours later and were mated to *him-8(e1489)* males. Worms were allowed to mate for 1-3 hours. Embryos were dissected from mothers into egg buffer (118mM NaCl, 40mM KCl, 3mM CaCl2, 3mM MgCl2, 5mM HEPES, pH 7.2; (12)) and imaged with Applied Precision DeltaVision RT deconvolution optics.

*par-6::GFP* animals were manipulated similarly to *nmy-2::GFP* except that all females were generated by *fem-1(RNAi)*. L4 mothers were placed on plates expressing *fem-1* double-stranded RNA at room temperature (21-23°) until their progeny reached the L4 stage (which is after the *fem-1* temperature sensitive period (13)). L4 progeny were moved to plates expressing *cyk-4* double-stranded RNA and allowed to mature overnight before the cross was initiated.

To inactivate paternal *cyk-4*, *him-8(e1489)* L3 males were transferred to plates expressing *cyk-4* double-stranded RNA at room temperature (21-23°). Treated males were either crossed to *nmy-2::GFP; fem-1(RNAi)* females for ~2-3 hours of mating at room temperature or processed for antibody staining of CYK-4 in sperm. Embryos from crosses were dissected from mothers into egg buffer (see above) and imaged with Applied Precision DeltaVision RT deconvolution optics.
Table 1.

**Endpoint of Cortical PAR-6::GFP**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>At Pronuclear Meeting (%)</th>
<th>(n)</th>
<th>At Telophase (%)</th>
<th>(n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>47 ± 3</td>
<td>(10)</td>
<td>54.5 ± 5</td>
<td>(7)</td>
</tr>
<tr>
<td><em>cyk-4</em></td>
<td>87 ± 8</td>
<td>(10)</td>
<td>73.1 ± 8</td>
<td>(6)</td>
</tr>
<tr>
<td><em>ect-2</em></td>
<td>98 ± 4</td>
<td>(8)</td>
<td>75.5 ± 19</td>
<td>(12)</td>
</tr>
<tr>
<td><em>rho-1</em></td>
<td>98 ± 3</td>
<td>(5)</td>
<td>84.1 ± 13</td>
<td>(5)</td>
</tr>
<tr>
<td>Paternal *cyk-4(+)</td>
<td>50±5</td>
<td>(8)</td>
<td>72.8± 12</td>
<td>(8)</td>
</tr>
</tbody>
</table>

Endpoints of cortical PAR-6::GFP at pronuclear meeting and at the anaphase-telophase transition. 0% represents the anterior pole and 100% represents the posterior pole. Note that RNAi-treated embryos (*cyk-4, ect-2, rho-1*) are extended around the cortex at pronuclear meeting compared to the wild type, but partially recover by early telophase of the first cell cycle. Wildtype paternal CYK-4 from *him-8* fathers can rescue polarity in embryos lacking maternal *cyk-4* (*cyk-4(RNAi); fem-1(RNAi)* mothers; bottom row). We observe later defects in these embryos, which may reflect secondary effects from cytokinesis.
FIGURE LEGENDS

Supplemental Figure 1. Inactivation of cyk-4 and ect-2 by RNAi
(A,B) Endogenous CYK-4 (red) localizes within sperm, which were co-stained with 1CB4 (green), an antibody that recognizes sperm membranous organelles (11, 14). (C,D) cyk-4(RNAi) generated sperm that lacked CYK-4 protein. (E, H) Endogenous ECT-2 (red) is enriched in puncta at the cell cortex. These puncta co-localize with NMY-2::GFP (green). (I, J) ect-2(RNAi) leads to an absence of ECT-2 staining and a disruption of NMY-2::GFP. Each embryo is 50µM long.

Supplemental Figure 2. CYK-4 localization
Panel A shows a sperm (asterisk) and an egg. After fertilization, CYK-4 (red) is detected between foci of NMY-2::GFP (green, B). Panel B is a cortical view of a fertilized NMY-2::GFP embryo whereas all others are cross sections. CYK-4 (C) and sperm contents (D, 1CB4), are localized at the posterior cortex and cytoplasm (arrowheads) after fertilization. CYK-4 is also associated with meiotic cytokinesis during oocyte maturation (C, left). Male pronucleus is demarked with an asterisk (D).

Supplemental Figure 3. Complementary expression of CYK-4 and NMY-2::GFP at the cell cortex. Endogenous CYK-4 (red, middle and right panels) in a fertilized egg is detected at the posterior cortex (arrowheads), complementary to NMY-2::GFP (green, left and right panels). Merge is yellow (C). We also observe CYK-4 associated with the meiotic divisions that occur during oocyte maturation. All panels are cross sections, anterior is left, embryos are ~50 µm long.

Supplemental Figure 4. Anterior localization of PAR-3 depends on cyk-4/GAP and ect-2/GEF. Endogenous PAR-3 is localized throughout the cortex of cyk-4(RNAi) (B, 19/26) and ect-2(RNAi) (C, 13/14) embryos compared to anterior localization in the wild type (WT, A). Embryos were scored at or before pronuclear meeting. PAR-3 is red, DNA is blue.

Supplemental Figure 5. Actomyosin is regulated by cyk-4/GAP. In wildtype (WT) embryos, there is a meshwork of non-muscle myosin NMY-2::GFP upon fertilization (A,
early), which moves towards the anterior (B, wave) and subsequently disperses into
puncta upon pronuclear meeting (C). In a third of cyk-4 embryos, NMY-2::GFP moves
anteriorly, but fails to relax at pronuclear meeting (F).

Supplemental Figure 6. Quantitation of cyk-4(RNAi)
NMY-2::GFP hermaphrodites were treated with cyk-4 double-stranded RNA beginning at
the L3 larval stage. Mothers were cut open and examined, either for staining with αCYK-4
antibody (10) or for NMY-2::GFP clearing in living embryos. For staining, we scored CYK-4
associated with sperm (A, n=119) and with the meiotic spindle during oocyte maturation
(B, n=9). These two features were chosen because both gave strong signals and because
they may reflect the paternal and maternal CYK-4 contributions, respectively. NMY-2::GFP
was scored for no clearing, partial clearing or wildtype clearing (C, n=30). Note that the
penetrance of the NMY-2::GFP polarity phenotype after cyk-4(RNAi) resembles the
penetrance of CYK-4 expression in sperm compared to that of the meiotic spindle, after
RNAi.

We relied on RNAi against cyk-4 because the available mutants were not helpful.
The temperature-sensitive allele of cyk-4 (cyk-4(t1689)) is a missense mutation within the
ZEN-4 binding site, which inactivates ZEN-4 binding (15). We have no evidence that zen-4
is part of the paternal cue: ZEN-4 protein is absent from sperm and zen-4(RNAi) has minor
effects on polarity, at best. Therefore, it’s not surprising that cyk-4(t1689) does not have a
polarity phenotype.

Supplemental Figure 7. Phosphorylated myosin light chain at the cortex during
polarization. Antibodies that recognize phospho-serine-myosin light chain (mlc-1P) detect
activated endogenous myosin light chain (red) at the cell cortex, co-localized with NMY-
2::GFP (green) in wildtype (WT) embryos (top panels). The merge is yellow. In ect-
2(RNAi) embryos, phospho-MLC is rarely detected at the cortex (ect-2). cyk-4(RNAi)
embryos exhibit phospho-MLC patches that co-localize with NMY-2::GFP (cyk-4). In rho-
1(RNAi) embryos, phospho-MLC is rarely detected at the cortex (rho-1). All panels are at
the cell surface.

Supplemental Figure 8. Distribution of MLC-1P in wildtype and cyk-4(RNAi)
embryos. Top panels: a wildtype (WT) embryo during the pronuclear migration stage
exhibits phospho-MLC (MLC-1P) at the anterior cortex whereas a cyk-4(RNAi) embryo
(bottom panels) has MLC-1P distributed all over the cortex at pronuclear meeting. Only one pronucleus is visible; the second one is out of the plane of focus. Note that we also observe immunoreactivity at the pronuclear surface in both wildtype and cyk-4(RNAi) embryos, but we do not know if this staining is specific.

**Supplemental Figure 9. Specificity of MLC-1P antibody.** Upper panels: Embryos from heterozygous mlc-4(or253)/+ mothers were co-stained with phospho-MLC antibody (MLC-1P, red) and αAJM-1 MH27 antibody (green; (16)) and seam cells examined (17). 76% (25/33) of embryos were MLC-1P+ and had normal morphology. 24% (8/33) lacked MLC-1P immunoreactivity and most of these were morphologically abnormal, suggesting they were mlc-4(or253) homozygotes (17). AJM-1+ ≥2 fold embryos were chosen for analysis since both MLC-1P and AJM-1 stain at this time, and since we could detect mutant embryos morphologically. Bottom panels: one-cell mlc-4(RNAi) embryos stained with phospho-MLC antibody (red) and α-GFP antibody (green) for NMY-2::GFP. Loss of NMY-2::GFP foci was our control for the efficacy of RNAi in treated embryos. Lack of cortical phospho-MLC was seen in 5/5 embryos.

**Supplemental Figure 10. Rescue of cyk-4(RNAi) polarity defects by wildtype sperm.** Progeny from cyk-4(RNAi); NMY-2::GFP; fem-1(RNAi) females and cyk-4(+); him-8(e1489) males exhibit wildtype NMY-2::GFP clearing (left panels) but fail to complete meiosis (right panels). We scored embryos that failed to complete meiotic and mitotic cytokinesis, our positive control for cyk-4(RNAi). 21/21 embryos were rescued for posterior NMY-2::GFP clearing in response to fertilization. Similar results were obtained for PAR-6::GFP (8/8). Five different examples are shown. Note that NMY-2::GFP localization is disrupted after cytokinesis failure (right panels).

**Supplemental Figure 11. Rescue of cyk-4(RNAi) polarity defects by wildtype sperm.** Progeny from cyk-4(RNAi); PAR-6::GFP; fem-1(RNAi) females and cyk-4(+); him-8(e1489) males exhibit anterior PAR-6::GFP localization, like the wild type (left panels), but fail to undergo mitotic cytokinesis (right panels). Five different examples are shown. Note that PAR-6::GFP localization is disrupted after cytokinesis failure (right panels).

**Supplemental Figure 12. Model for initiation of anterior PAR asymmetry.** Anterior PAR proteins (red) are localized throughout the cortex prior to fertilization (left embryo).
Upon sperm entry (S), CYK-4 (blue) is donated to the embryo (middle embryo), where it inhibits actomyosin contractility (right embryo). ECT-2/GEF activates RHO-1/RhoA, leading to MLC phosphorylation and actomyosin contraction. The asymmetry in forces, regulated by these factors, favors movement to the nascent anterior (arrows, right embryo), thereby localizing anterior PAR proteins. In cases of lateral sperm entry, lateral flows are initiated, and these ultimately re-orient along the longitudinal axis (1, 3).

SUPPLEMENTAL MOVIES:
Embryos were either NMY-2::GFP (2) or PAR-6::GFP (2). Frames were taken every ten seconds for a duration of fifteen minutes. Either wildtype or RNAi-treated embryos were examined, as described in the text and Supplemental Data.

- **Movie S1.** NMY-2::GFP in a wild-type embryo
- **Movie S2.** NMY-2::GFP in a cyk-4(RNAi) embryo
- **Movie S3.** NMY-2::GFP in an ect-2(RNAi) embryo
- **Movie S4.** NMY-2::GFP in a rho-1(RNAi) embryo
- **Movie S5.** NMY-2::GFP in an embryo from a cyk-4(RNAi) mother mated to a cyk-4(+) father (paternal rescue)

REFERENCES
Jenkins, Saam and Mango, Figure 2
Jenkins Supplemental Figure 3
Jenkins Supplemental Figure 4

PAR-3

WT

A

cyk-4

B

ect-2

C
Jenkins, Saam and Mango, SF5
Jenkins Supplemental Figure 7

**mlc-1P**

**NMY-2::GFP**

**merge**

**WT**

**ect-2**

**cyk-4**

**rho-1**
Jenkins Supplemental Figure 8
Jenkins Supplemental Figure 9

WT

MUT

MLC-4P stain:

+  25
-  8

MLC-4P stain:

+  25
-  8

MERGE

MLC-4

NMY-2::GFP

wild type

mcl-4 RNAi
Jenkins, Saam and Mango, Supplemental Figure 12

anterior PAR  \[\text{CYK-4/GAP} \uparrow \text{sperm}\]

actomyosin contraction

posterior relaxation

\[\text{CYK-4/GAP} \uparrow \text{sperm}\]