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

Noah Jenkins,* Jennifer R. Saam,* Susan E. Mango‡

The Caenorhabditis elegans anteroposterior axis is established in response to fertilization by sperm. Here we present evidence that RHoA, the guanine nucleotide–exchange factor ECT-2, and the Rho guanosine triphosphatase–activating protein CYK-4 modulate myosin light-chain activity to create a gradient of actomyosin, which establishes the anterior domain. CYK-4 is enriched within sperm, and paternally donated CYK-4 is required for polarity. These data suggest that CYK-4 provides a molecular link between sperm entry and anterior PAR localization.

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Materials and Methods

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during polarization, we examined the fertilized embryo, a well-characterized model for polarity (2). Antibody staining revealed that CYK-4 was dramatically enriched in sperm (64 out of 64 embryos examined) (Fig. 1, A and B). Inactivation of cyk-4 by RNA interference (RNAi) indicated that staining was specific and RNAi effective (fig. S1) (7). Upon fertilization, CYK-4 could be detected at the posterior cortex of the one-cell embryo of both wild-type embryos and embryos lacking paternal CYK-4 (Fig. 1, C to K, and figs. S2 and S3) (7). We observed paternal CYK-4 in punctate structures, derived from sperm membranous organelles (MOs) and often associated with the sperm pronucleus (8). Based on nuclear morphology, paternal CYK-4 remained associated with the cortex and MOs during meiosis and the onset of polarity, a period of about 30 min (Fig. 1, C to K) (2).

To determine whether cyk-4 was important for polarity, we examined anterior PAR proteins using green fluorescent protein (GFP) reporters in cyk-4(RNAi) embryos (Fig. 2, A and B) (7). In wild-type embryos, PAR-6::GFP was confined to 47% of egg length at the time of pronuclear meeting. In cyk-4(RNAi) embryos, PAR-6::GFP expanded to 87% of egg length, and endogenous PAR-3 was observed throughout the cortex (table S1 and fig. S4). These data suggest that cyk-4 is required to establish anterior polarity.

In other organisms, cyk-4 orthologs function with the guanine nucleotide exchange factor (GEF) ect-2 during cytokinesis (9), which prompted us to examine ect-2. Antibody staining revealed that ECT-2 was enriched with nonmuscle myosin NMY-2::GFP at the cell cortex (n > 20) (Fig. 3). Colocalization of these two proteins in multiple images suggested that ECT-2 moves anteriorly coincident with NMY-2::GFP. Reduction of ect-2 by RNAi indicated that staining was specific and RNAi effective (13 out of 16) (fig. S1). Inactivation of ect-2 led to distribution of PAR-6::GFP and PAR-3 throughout the cortex at the time of pronuclear meeting, in addition to pronounced cytokinesis defects (Fig. 2C, and table S1 and fig. S4) (7). Because ect-2 and cyk-4 polarity phenotypes were visible before the first cell division, mislocalization of anterior PAR proteins was not a secondary consequence of failed mitosis. We conclude that cyk-4 and ect-2 are critical to establish the anterior PAR domain.

ect-2 and cyk-4 are predicted to control Rho family GTPases, which suggested a possible mechanism for controlling polarity. In vitro, cyk-4 can function as a GAP for rho-1, cdc-42, or rac, and in vivo, it likely controls RhoA during cytokinesis (10). We found that in embryos with reduced RhoA [rho-1(RNAi)], PAR-6::GFP was dispersed throughout the cortex at pronuclear meeting (Fig. 2D and table S1). This phenotype resembled that of cdc-2(RNAi), which suggested that ECT-2 and RHO-1 function in a common pathway. The similarity of phenotypes contrasts with those associated with other C. elegans GTPases. For example, cdc-42 is required for posterior PAR localization and spindle positioning, but not for initial anterior PAR localization (11). No early polarity defects have been noted for the three C. elegans rac genes ced-10, mig-2, and rac-2, even when they were inactivated together (12). These observations suggest that RHO-1/RhoA is a good candidate effector for ECT-2 and, by extension, CYK-4 during the initial stages of polarization. We propose that the regulatory casette of rho-1, cyk-4, and ect-2 that is used during cytokinesis is also deployed for polarity.

Normally, anterior PAR localization depends on a gradient of actomyosin toward the anterior pole (3, 4). Because Rho proteins control the actomyosin cytoskeleton in many contexts, we examined the actomyosin cytoskeleton with nonmuscle myosin NMY-2::GFP. In wild-type embryos, NMY-2::GFP was present at the egg cortex, where it formed coalescing foci that advanced anteriorly (Fig. 4, A to C) (7). In 7 out of 10 ect-2(RNAi) and 6 out of 8 rho-1(RNAi) embryos, a lower proportion of NMY-2::GFP localized cortically, and this remaining protein failed to coalesce into large foci (Fig. 4, D to I) (7). These data suggest that ect-2 and rho-1 are critical to generate a contractile actomyosin network.

Conversely, cyk-4 controlled relaxation or disassembly of the actomyosin network. Of 30 cyk-4(RNAi) embryos, 15 had a dynamic actomyosin network that remained evenly distributed over the cortex (Fig. 4, J to L) (7). In these embryos, initial contractility appeared wild type but sperm-induced asymmetry was lost. In 10 out of 30, asymmetric NMY-2::GFP occurred, but the global transition from foci to puncta was delayed until after pronuclear meeting, which suggested a temporal role for cyk-4 (Fig. 5S) (7). The remaining 5 out of 30 embryos had an intermediate phenotype (fig. S6) (7). The variable cyk-4(RNAi) phenotypes could reflect incomplete inactivation by RNAi or the existence of additional polarity pathways.
Supporting the former hypothesis, we detected CYK-4 protein in 39% of sperm after RNAi treatment (fig. S6) (7). These data suggest that cyk-4 is required to down-regulate the actomyosin cytoskeleton posteriorly and, thereby, to induce asymmetric pulling forces.

One effector of RhoA is RhoA kinase, which phosphorylates myosin light chain (MLC) and MLC phosphatase, which leads to MLC activation and actomyosin contractility (13). In C. elegans, MLC-4 is required for anteroposterior polarity, actomyosin contractility, and anterior PAR localization (14). These observations suggested that RHO-1, ECT-2, and CYK-4 might control MLC-4. To test this idea, we monitored activated MLC using an antibody specific for phospho-MLC (7, 13).

Phospho-MLC was located at the cell cortex of wild-type embryos, where it overlapped with foci of NMY-2::GFP (Fig. 5, A and B, and fig. S7) (7). We detected phospho-MLC associated with the anterior cortex and absent from the posterior after fertilization, indicating loss of active MLC (fig. S8) (7). Loss of immunoreactivity in mcl-4(−) embryos indicated that phospho-MLC staining was specific (fig. S9) (7). Phosphorylation of MLC required ect-2 and rho-1, since neither ect-2(RNAi) (n > 10) nor rho-1(RNAi) (n = 7 out of 9) embryos had detectable phospho-MLC at the cell cortex (Fig. 5, C and G, and fig. S7) (7). As predicted, cyk-4(RNAi) embryos contained phospho-MLC, which colocalized with NMY-2::GFP in an extended domain (n > 10 one-cell embryos with meiotic defects) (Fig. 5E and fig. S8) (7). These findings suggest that ECT-2 and RHO-1 promote, whereas CYK-4 inhibits, activated MLC and, therefore, actomyosin contractility.

To address the importance of sperm-donated CYK-4, we examined embryos from NMY-2::GFP females mated with cyk-4(RNAi) males (7). As monitored by NMY-2::GFP, 29% lacked polarity altogether, whereas 17% had an intermediate phenotype (n = 48) (Fig. 6, D and E); the remainder looked wild type. By antibody staining, 42% of sperm had reduced or absent CYK-4 after RNAi (n = 90) (Fig. 6B). These data indicate that paternally endowed CYK-4 is required to polarize the embryo. Conversely, we showed CYK-4+ from male sperm could rescue polarity, but not meiotic cytokinesis, for fertilized cyk-4(RNAi) eggs (figs. S10 and S11).

We propose that the bolus of CYK-4 donated by sperm down-regulates the actomyosin network in the posterior, thereby generating a gradient of contractility (fig. S12) (7). The gradient of contractility depends on differential activation of MLC. There may be additional effectors, given that RhoA in other organisms influences the actin cytoskeleton in multiple ways. In addition to CYK-4, previous studies have shown that the sperm-donated centrosome is required for anteroposterior polarity (1, 15, 16). Currently, it is unclear whether CYK-4 acts in parallel to the centrosome or whether these two sperm cues function in a common pathway. We note that the requirement for a mature centrosome helps explain why polarity initiates after the completion of meiosis, despite the presence of paternal CYK-4 immediately after fertilization.

Consistent with the model that RhoA, CYK-4, and ECT-2 function during the earliest stage of polarization, we observed the strongest polarity defects during the first half of the first cell cycle (table S1) (7). Subsequently, anterior PAR proteins and CDC-42 contribute to actomyosin dynamics (3, 4, 17). PAR-2 may function even later or in parallel, because anterior PAR are localized normally in par-2 mutant embryos (18). Thus, polarization during the first cell cycle involves multiple stages governed by distinct sets of factors.

Our studies may also have implications for the role of CYK-4 during cytokinesis. Although the spindle midzone is a target of CYK-4 (9), recent studies revealed that a visible spindle midzone is not essential for cytokinesis (19, 20). We suggest that the cortical actomyosin cytoskeleton may be a focus of CYK-4 during cytokinesis as it is during polarization.

Organisms such as tunicates and teleosts undergo asymmetric actomyosin contraction upon fertilization, which contributes to polarization of the fertilized egg (21). In P. mammillata, contraction depends on an actomyosin basket with its opening located at the site of sperm entry. Thus, asymmetry in these embryos may

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**Fig. 4. ect-2/GEF, rho-1/rhoA, and cyk-4/GAP regulate the actomyosin wave.** In wild-type (WT) embryos, a meshwork of nonmuscle myosin NMY-2::GFP (A, early) is enriched anteriorly during pronuclear migration (B, wave) and subsequently disperses into puncta at pronuclear meeting (C). In ect-2(RNAi) embryos (D to F) and rho-1(RNAi) embryos (G to I), contractile foci are rarely observed at any stage. In cyk-4(RNAi) embryos, 50% of embryos remain contractile over the entire embryo at all stages (J to L).

**Fig. 5. Phospho-MLC localized at the cortex during polarization.** (A and B) In wild-type (WT) embryos, antibodies that recognize phospho-MLC (mlc-1P, red) detect activated, endogenous MLC at the cell cortex, colocalized with NMY-2::GFP (green). In ect-2(RNAi) (C and D) and rho-1(RNAi) (G and H) embryos, phospho-MLC is rarely detected. (E and F) cyk-4(RNAi) embryos exhibit phospho-MLC throughout the cortex, with NMY-2::GFP. DNA is blue.

**Fig. 6. Paternal cyk-4 is required for polarity.** Sperm from cyk-4(RNAi); him-8 males exhibit reduced CYK-4 (B), while others appear unaffected (A). Sperm were counterstained for membranous organelles (1CB4) (C). Embryos from NMY-2::GFP; fem-1 females and cyk-4(RNAi); him-8 males exhibit loss of polarized NMY-2::GFP (D), or have a partial defect (E).
Heart formation and suggest the involvement of the mevalonate pathway in congenital heart disease.

from mutations in the genes encoding HMG-CoA reductase, downstream enzymes in the mevalonate pathway, and G protein Ga1, which is geranylgeranylated, thus representing an end point of isoprenoid biosynthesis. Our findings reveal a cardiac cell-autonomous requirement of Ga1 geranylgeranylation for heart formation and suggest the involvement of the mevalonate pathway in congenital heart disease.

References and Notes
7. Materials and methods and additional data are available as supporting material on Science Online.
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The Mevalonate Pathway Controls Heart Formation in Drosophila by Isoprenylation of Gγ1

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The early morphogenetic mechanisms involved in heart formation are evolutionarily conserved. A screen for genes that control Drosophila heart development revealed a cardiac defect in which pericardial and cardiac cells dissociate, which causes loss of cardiac function and embryonic lethality. This phenotype resulted from mutations in the genes encoding HMG-CoA reductase, downstream enzymes in the mevalonate pathway, and G protein Ga1, which is geranylgeranylated, thus representing an end point of isoprenoid biosynthesis. Our findings reveal a cardiac cell-autonomous requirement of Ga1 geranylgeranylation for heart formation and suggest the involvement of the mevalonate pathway in congenital heart disease.

Mutations in genes controlling heart development frequently cause fatal cardiac malformations, the most common type of birth defect in humans. Because many of the mechanisms involved in heart development are evolutionarily conserved, the fruit fly Drosophila melanogaster represents a powerful model for genetically dissecting this complex developmental process. The Drosophila heart, or dorsal vessel, which pumps bloodlike cells through an open circulatory system, is composed of parallel rows of contractile cardiac cells (cardioblasts) tightly attached to pericardial cells; the latter perform supportive and secretory functions (Fig. 1A) (1).

We performed a P-element genetic screen (2) for Drosophila mutants with heart defects using transgenic flies harboring a green fluorescent protein (GFP) transgene under control of the Hand enhancer (3), which is specific for cardiac cells, pericardial cells, and the lymph gland—a hematopoietic organ in fruit flies (Fig. 1B). The Hand-GFP transgene allows visualization of the developing heart at single-cell resolution. Among a collection of mutants with cardiac abnormalities, we observed a heart defect in which pericardial cells dissociated from cardioblasts in the dorsal vessel at the end of embryogenesis. We termed this phenotype “broken hearted” (bro). Here, we describe five such mutants of different genetic loci (Fig. 1, C to G). In contrast to the wild-type dorsal vessel in which the pericardial cells are intimately associated with cardioblasts, in each of these mutants, the relative positions of pericardial cells and cardioblasts changed with each heartbeat.

The P element in the bro1 locus [(3)01152] is located in the first exon of the hydroxymethyl-

Fig. 1. Mutants in different genetic loci gave rise to a common broken hearted (bro) cardiac defect. (A) Schematic drawing of a late stage 17 embryonic heart (dorsal view, anterior to the left). (B) To (G) Stage 17 embryonic heart labeled by Hand-GFP (3) in wild-type embryo (B) or five bro homoyzygous mutants (C to (G) (pericardial cells are indicated by arrows) (O) HMGCR, bro1, (I)bro1(301152); (D) GGPPS/qmL14, bro2; (E) jggf2, bro3; (F) Ga1, bro4, (I)bro2(08017); (G) Sar1, bro5, (I)bro2(07408).

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