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CDC-42 and RHO-1 coordinate acto-myosin contractility and PAR protein localization during polarity establishment in *C. elegans* embryos

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In *C. elegans* one-cell embryos, polarity is conventionally defined along the anteroposterior axis by the segregation of partitioning-defective (PAR) proteins into anterior (PAR-3, PAR-6) and posterior (PAR-1, PAR-2) cortical domains. The establishment of PAR asymmetry is coupled with acto-myosin cytoskeleton rearrangements. The small GTPases RHO-1 and CDC-42 are key players in cytoskeletal remodeling and cell polarity in a number of different systems. We investigated the roles of these two GTPases and the RhoGEF ECT-2 in polarity establishment in *C. elegans* embryos. We show that CDC-42 is required to remove PAR-2 from the cortex at the end of meiosis and to localize PAR-6 to the cortex. By contrast, RHO-1 activity is required to facilitate the segregation of CDC-42 and PAR-6 to the anterior. Loss of RHO-1 activity causes defects in the early organization of the myosin cytoskeleton but does not inhibit segregation of myosin to the anterior. We therefore propose that RHO-1 couples the polarization of the acto-myosin cytoskeleton with the proper segregation of CDC-42, which, in turn, localizes PAR-6 to the anterior cortex.

KEY WORDS: Cell polarity, Rho GTPase, PAR, Myosin, Contractility, *C. elegans*

INTRODUCTION

In *C. elegans* one-cell embryos, polarity along the anteroposterior axis is marked by the asymmetric distribution of PAR proteins, which form two distinct domains. The anterior domain is defined by a conserved complex consisting of PAR-3, PAR-6 and atypical protein kinase C (PKC-3) (Cuenca et al., 2003; Etemad-Moghadam et al., 1995; Hung and Kemphues, 1999; Tabuse et al., 1998; Watts et al., 1996). The posterior domain is defined by PAR-1 and PAR-2 (Boyd et al., 1996; Cuenca et al., 2003; Guo and Kemphues, 1995). The establishment of the anterior and posterior PAR domains is a dynamic process. Following fertilization, the anterior and posterior PAR proteins co-localize throughout the entire cortex (Boyd et al., 1996; Cuenca et al., 2003; Etemad-Moghadam et al., 1995; Hung and Kemphues, 1999; Munro et al., 2004). During meiosis II, PAR-2 leaves the cortex, while the anterior PAR proteins remain localized over the whole cortex. The apposition of the sperm-derived centrosome at the posterior cortex triggers regression of the anterior PAR proteins to the anterior cortex of the embryo. PAR-2 returns exclusively to the posterior cortex, the region devoid of the anterior PAR proteins (Cowan and Hyman, 2004b; Cuenca et al., 2003; Munro et al., 2004). Throughout the paper we will refer to this PAR-2 localization cycle as the 'meiotic PAR-2 cycle'.

The initiation of polarity in *C. elegans* induces dramatic cytoskeletal rearrangements that lead to a morphological polarization, which was termed 'contractile polarity' (Cowan and Hyman, 2004a). At the end of meiosis, small transient cortical ruffles can be seen over the entire cortex. Later, the ruffling ceases in the area where the centrosome becomes juxtaposed with the posterior cortex (Cheeks et al., 2004; Cowan and Hyman, 2004b; Cuenca et al., 2003; Munro et al., 2004). This smooth area gradually expands towards the anterior until it is about 50% of the egg-length. A constriction called the pseudocleavage furrow separates the smooth posterior domain from the anterior domain, which remains contractile (Hirsh et al., 1976; Strome, 1986). Fixed sample studies revealed that actin becomes asymmetrically localized in the embryo (Strome, 1986; Strome and Hill, 1988), and suggested that the establishment of contractile polarity is associated with the segregation of the acto-myosin cytoskeleton. More recent studies imaging the non-muscle myosin II heavy chain (NMY-2) fused to GFP revealed that, during contractile polarity establishment, a uniform contractile acto-myosin meshwork disassembles in close vicinity to the posterior nucleus/centrosome complex and segregates towards the anterior pole (Munro et al., 2004). The signal-inhibiting local contractility appears to come from the centrosome (Cowan and Hyman, 2004b; Munro et al., 2004).

Cell polarization depends on communicating a symmetry-breaking event to induce a reorganization of the actin-myosin cytoskeleton, leading to polarized cellular domains and an asymmetric distribution of cytoskeletal functions. The Rho family GTPases Cdc42 and RhoA play important roles in signaling to the downstream cellular machinery that controls actin cytoskeleton organization and, therewith, cell polarity. The activity of GTPases is controlled by regulatory proteins: guanine nucleotide exchange factors (GEFs) activate GTPases by catalyzing the exchange of GDP for GTP (Schmidt and Hall, 2002), whereas GTPase activating proteins (GAPs) inactivate GTPases by stimulating the intrinsic GTPase activity (Bernards, 2003). Cdc42 was identified in *Saccharomyces cerevisae* and shown to be involved in bud site selection (Drubin, 1991; Johnson, 1999). Further analysis in different systems showed that Cdc42 is required for numerous aspects of polarity establishment. For example, in migrating cells, Cdc42 is implicated in the orientation and maintenance of polarized morphology, whereas, in epithelial cells, Cdc42 is implicated in the formation of tight junctions, which separate the apical and the basolateral membranes. Cdc42 plays a further role in the polarized vesicular trafficking required for polarized protein distribution (reviewed by Etienne-Manneville, 2004). Thus, Cdc42 is a component of many cell polarization pathways. RhoA is also essential for many types of cell polarity, as polarized cell shape and

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cell migration depend largely on the acto-myosin cytoskeleton. RhoA is required for the assembly of actin filaments and myosin II into contractile filaments that provide the mechanical force for cortical contractions, motility and cytokinesis (reviewed by Etienne-Manneville and Hall, 2002; Glotzer, 2005). Thus, both RhoA and Cdc42 are essential for many types of cell polarity. However, it remains unclear how their functions are coordinated in cell polarity.

CDC-42 and RHO-1 play essential roles in the *C. elegans* one-cell embryo. Previous studies have shown that after depletion of CDC-42, PAR-2 was found uniformly at the cortex and PAR-6 was either anteriorly enriched, as in wild type, or scattered throughout the entire cortex at the two-cell stage (Gotta et al., 2001; Kay and Hunter et al., 2001). As CDC-42 was shown to interact with PAR-6 in many systems, including *C. elegans* (Gotta et al., 2001; Hutterer et al., 2004; Joberty et al., 2000; Johansson et al., 2000; Lin et al., 2000; Qiu et al., 2000), it seemed likely that CDC-42 would act through PAR-6 to regulate polarity. These studies demonstrated the involvement of CDC-42 in *C. elegans* polarity, although which specific process in polarity establishment is affected by CDC-42 remains unclear.

RHO-1 was shown to function in cytokinesis (Jantsch-Plunger et al., 2000), presumably by regulating acto-myosin activity. It has been demonstrated that polarity establishment in *C. elegans* embryos requires the acto-myosin cytoskeleton. Embryos treated with actin-depolymerizing drugs or depleted of myosin II subunits, or the actin nucleators profilin or formin, do not establish polarity: PAR-2 is unable to localize correctly to the cortex and PAR-3/PAR-6 remain uniformly distributed around the entire cortex (Cuenca et al., 2003; Guo and and Kemphues, 1996; Severson and Bowerman, 2003; Shelton et al., 1999) (S.S. and A.A.H., unpublished). Thus, in *C. elegans* embryos, RHO-1 may be involved in polarity establishment, possibly through regulation of the acto-myosin cytoskeleton, although this remains to be tested.

Here, we investigate the roles of CDC-42 and RHO-1 in polarity establishment in *C. elegans* embryos and examine the interaction between these two signaling pathways in cell polarity. Our data suggest that RHO-1 and CDC-42 have separable functions in polarity establishment. We show that RHO-1 activity is required for acto-myosin contractility and organization of the NMY-2 meshwork, which, in turn, is essential for localizing CDC-42 to the anterior half of the embryo. CDC-42, in turn, is required to stabilize the acto-myosin network and for localizing PAR-6 in the anterior. In addition, CDC-42 removes PAR-2 from the cortex during meiosis. We have found that during polarity establishment the roles of RHO-1 and CDC-42 are interdependent, and appear to be coordinated, in part, through the acto-myosin contractile network in *C. elegans* one-cell embryos.

MATERIALS AND METHODS

Worm strains

Worms were handled as described (Brenner, 1974). The following strains were used: N2 (wild type), JH1380 (GFP-PAR-2), TH25 (GFP-PAR-6), JJ1473 (NMY-2-GFP), TH71 (NMY-2-GFP;GFP-PAR-2), TH72 (YFP-CDC-42), KK571 [*par-3(it71)*]. The TH71 strain was constructed by crossing JH1380 to JJ1473 and progeny were selected that expressed both GFPs. TH72 was crossed to KK571 to analyze YFP-CDC-42 localization in a *par-3(it71)* background. The *cdc-42* coding sequence (R07G3.1) was identified using the WormBase web site (release WS155; http://www.wormbase.org) and was amplified by PCR from wild-type strain N2 genomic DNA using the primers 5′-ggcc**actagt**ggaATGCAGACG-ATCAAGTGCGTC-3′ and 5′-ggcc**cccggg**CTAGAGAATATTGCACTT-CTTCT-3′, containing *Spe*I or *Xma*I sites (bold). The N-terminal YFP-fusion was generated in pTH-YFP(N) (a modified version of the pAZ132 plasmid, a gift from Andrei Pozniakovsky, Max Planck Institute of Cell Biology and Genetics, Dresden), expressing YFP under the control of the *pie-1* promoter. Transgenic worms were created by high-pressure ballistic bombardment (BioRad) of DP38 *unc-119(ed3)* homozygotes, as described previously (Praitis et al., 2001).

The YFP-CDC-42 transgene was tested for functionality by using double-stranded RNA against the 3′UTR of *cdc-42* to deplete endogenous CDC-42 in wild-type N2 and YFP-CDC-42 worms. Injected worms were then assayed for embryonic hatching. The YFP-CDC-42 fusion uses the *pie-1* 3′UTR, and therefore was not targeted by the *cdc-42* 3′UTR double-stranded RNA. To test whether RNAi against the 3′UTR of *cdc-42* gives the same phenotype as *cdc-42(RNAi)*, *cdc-42* 3′UTR RNA was injected into GFP-PAR-2. We found that PAR-2 was uniformly localized, as in *cdc-42(RNAi)* embryos (n=7, data not shown). To determine embryonic hatching, injected worms were placed on individual plates for 56 hours at 25°C and allowed to lay eggs for 5 hours at 25°C. These embryos were checked for hatching 48 hours later. The progeny of N2 worms injected with *cdc-42* 3′UTR RNA showed 0% embryonic hatching (21 worms, 193 embryos), whereas the progeny of injected YFP-CDC-42 worms (19 worms, 178 embryos) showed 96.2% hatching, indicating that the YFP-CDC-42 is functional.

RNA-mediated interference

RNAi experiments were performed as described (Oegema et al., 2001). Primers used to amplify regions from N2 genomic DNA are listed in Table 1. Worms were incubated depending on the individual double-stranded RNA for 10-26 hours at 25°C after injection. *Cdc-42(RNAi);rho-1(RNAi)* was performed by co-injection of both RNAs, combined with feeding of *cdc-42(RNAi)* (Timmons and Fire, 1998). *Cdc-42(RNAi);spd-2(RNAi)* was performed by co-injection of both RNAs, combined with feeding of *cdc-42(RNAi)* and *spd-2(RNAi)*. Worms were placed on feeding plates after injection and maintained at 25°C for 22-48 hours.

One general complication of our analyses was that CDC-42, as well as RHO-1 and ECT-2, is essential for oocyte formation in *C. elegans*. Complete depletion by RNAi leads to sterility and therefore we could not analyze polarity under such conditions. We conducted many analyses to determine the maximum depletions that would still yield embryos. In real-time studies, we concentrated our analysis solely on embryos that had cleaved symmetrically after *cdc-42(RNAi)* or failed to undergo cytokinesis after *rho-1(RNAi)* or *ect-2(RNAi)*. For immunofluorescence experiments, we performed RNAi of CDC-42 for 48 hours at 25°C. Under these conditions, in eight out of 10 embryos, PAR-6 did not localize to the cortex. Previous experiments showed some PAR-6 on the cortex after *cdc-42(RNAi)* (Gotta et al., 2001; Kay and Hunter, 2001). The discrepancy between the different results is probably due to a difference in RNAi penetrance, as Gotta et al. (Gotta et al., 2001) used different RNAi

Table 1. Primers used to amplify regions from N2 genomic DNA for the production of double-stranded RNA in vitro

	Gene	Forward	Reverse
rho-1	Y51H4A.3	ATCGTCTGCGTCCACTCTCT	GGCTCCTGTTTCATTTTTGC
rho-1	Y51H4A.3	AAAACTTGCCTGCTCATCGT	TTCCGTCAACTTCAATGTCG
cdc-42	R07G3.1	TCAAAGACCCCATTCTTGTT	ACTTCTCTCCAACATCCGTT
cdc-42 3′ UTR	R07G3.1	GTCTTCCTTGTCTCCATGTTTC	CCTTTATTGTTTTGGATCGCA
ect-2	T19E10.1	TGGATCCGATTCTCGAACTT	ACATTTGGCTTTGTGCTTCC
spd-2	F32H2.3	AATGGTGGTCGCTTCAAAAC	TGACGGTAGAGACGGATGTG
par-3	F54E7.3	GTGACCGGACGTGAAACTG	TTTTCCTTCCGAGACCTTCC
par-6	T26E3.3	ATGTCCTACAACGGCTCCTA	TCAGTCCTCTCCACTGTCCG

conditions. After RNAi of CDC-42 for 26 hours at 25°C, we also found some PAR-6 on the anterior cortex, but at very reduced levels (data not shown).

Time-lapse microscopy

Worms were shifted to 25°C before recording. Embryos were dissected and mounted in a solution containing 0.1 M NaCl and 4% sucrose, with and without 2% agarose. GFP, YFP and differential interference contrast (DIC) recordings were acquired at 10-15 second intervals (exposure time 400 mseconds, 2×2 binning) with a Hamamatsu Orca ER 12 bit digital camera mounted on a spinning disk confocal microscope (Zeiss Axioplan using a 63× 1.4 NA PlanApochromat objective and Yokogawa disk head). Illumination was via a 488 nm Argon ion laser (Melles Griot). Movies acquired for Fig. 2 were done on a wide-field microscope (Zeiss Axioplan II using a 63× 1.4 NA PlanApochromat objective equipped with a Hamamatsu Orca ER 12 bit digital camera). Image processing was done with MetaView Software (Universal Imaging Corporation).

Immunofluorescence

Immunofluorescence was performed as described (Gönczy et al., 1999). For the PAR-2 immunostaining, the GFP-PAR-2 strain (JH1380) was used. A sheep polyclonal antibody to GFP (1:1000; a gift from Francis Barr, Max-Planck-Institute of Biochemistry, Martinsried, Germany) was used to visualize PAR-2. DM1α (1:300, Sigma) and SPD-2, 1:5000, (Pelletier et al., 2004) were used to detect microtubules and centrosomes. PAR-6 was stained with a C-terminal peptide (amino acid 291-308) anti-PAR-6 antibody. The antibodies were visualized with TR- and Cy5-conjugated antibodies (Jackson Immunochemicals), and with a donkey anti-sheep antibody coupled to Alexa 488 (Molecular Probes). Imaging was performed on a DeltaVision microscope and stacks were deconvolved as described (Oegema et al., 2001). SPD-2 and DNA images are projections of *z*-sections representing the entire cell. PAR-2, PAR-6 and Tubulin images are projections of four to 10 *z*-sections.

Contractility tracking

The ruffle kymographs were performed as described (Cowan and Hyman, 2004b). Briefly, the ruffles were tracked starting around the time of the beginning of pronuclear appearance for an interval of 1000 seconds. The position of cortical ruffles was manually tracked and projected onto a calculated ellipse. One half of the ellipse was straightened to generate the *x*-axis, the anteroposterior axis. This procedure was done for each time point and laid down sequentially along the *y*-axis (time). Lines connect ruffles within nearest neighbor groups.

Tracking of PAR-2 and PAR-6 domain extent

The extent of the GFP-PAR-2 domain was manually tracked after the domain reached its maximal size. The extent of the GFP-PAR-6 domain was tracked after pseudocleavage regression. The domain size was calculated as a fraction of the respective embryo circumference. Manual tracking was performed using a custom-written macro (Stephan Grill) for NIH-Image (NIH). Further analysis was done with Mathematica 4.1 (Wolfram Research).

Kymograph analysis

Kymographs were done with Metamorph Software (Universal Imaging Corporation) from cortical YFP-CDC-42 time-lapse recordings (7-12 minutes total). Kymographs were made from a straight line along the long axis of the embryo.

Measurement of the position of the posterior boundary of YFP-CDC-42

Position was measured with Metamorph Software (Universal Imaging Corporation) as a distance along the long axis from the anterior pole. The distance was standardized to total embryo length (100%). 0% indicates the anterior (ANT) pole.

Tracking of NMY-2 foci

GFP-NMY-2 foci were manually tracked with Metamorph Software (Universal Imaging Corporation).

RESULTS

CDC-42 is required to remove PAR-2 from the cortex during meiosis

In wild-type embryos, after completion of meiosis, a polarizing signal from the centrosome leads to cortical PAR-2 localization at the posterior pole and the subsequent spreading of cortical PAR-2 over half of the embryo (Fig. 1, see Movie 1 in the supplementary material) (Cowan and Hyman, 2004b; O'Connell et al., 2000). Previous work has shown that in CDC-42-depleted embryos, PAR-2 was uniformly distributed throughout the cortex (Gotta et al., 2001; Kay and Hunter, 2001); however, the reason for this uniform distribution remained unclear. To investigate whether the uniform PAR-2 localization in *cdc-42(RNAi)* embryos is dependent on the centrosomal signal, CDC-42 was depleted together with SPD-2, a centrosomal protein essential for polarity establishment (Cowan and Hyman, 2004b; O'Connell et al., 2000). In *spd-2(RNAi);cdc-42(RNAi)* embryos, GFP-PAR-2 localized uniformly at the cortex (Fig. 2), showing that the uniform PAR-2 distribution in *cdc-42(RNAi)* embryos is independent of the centrosome-dependent polarity signal. These data suggest that the aberrant PAR-2 distribution could be caused by an earlier defect in the PAR-2 localization mechanism. We next examined the meiotic cycle of GFP-PAR-2 in *cdc-42(RNAi)* embryos. We found that PAR-2 did not leave the cortex during meiosis II, but instead remained uniformly distributed throughout the cortex during the entire cell cycle (n=10, Fig. 1; data not shown). This data suggests that the defect in PAR-2 localization in *cdc-42(RNAi)* embryos results from a failure to remove PAR-2 from the cortex during the meiotic cycle.

CDC-42 is required to localize PAR-6 to the cortex

The uniform distribution of PAR-2 in *cdc-42(RNAi)* embryos is similar to the PAR-2 distribution seen in *par-6* and *par-3* mutant embryos, as the localization of the anterior and posterior PAR proteins is interdependent (Etemad-Moghadam et al., 1995; Hung

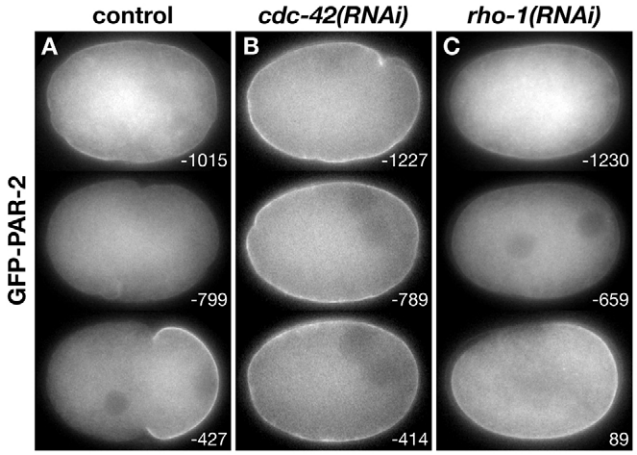

Fig. 1. CDC-42 is required for the PAR-2 localization cycle. (**A-C**) Time-lapse images of GFP-PAR-2 polarity establishment in (A) control, (B) *cdc-42(RNAi)* and (C) *rho-1(RNAi)* embryos. Times (seconds) are relative to nuclear envelope breakdown. In this and subsequent figures, the embryos are approximately 50 μm in length; the embryo posterior is to the right. (A) In control embryos, GFP-PAR-2 localizes uniformly along the cortex around the time of meiosis (top). After meiosis, GFP-PAR-2 disappears from the cortex (middle) and becomes confined to the posterior pole (bottom). (B) In *cdc-42(RNAi)* embryos, GFP-PAR-2 localized uniformly at the cortex. (C) *Rho-1(RNAi)* did not affect the PAR-2 localization cycle.

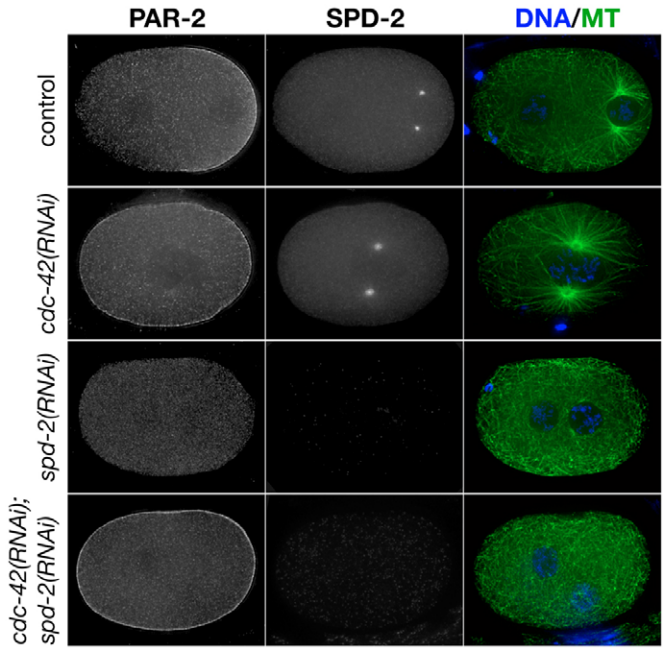

Fig. 2. Meiotic GFP-PAR-2 localization is independent of the centrosomal signal. Embryos expressing GFP-PAR-2 were stained for GFP, SPD-2, microtubules (MT, green) and DNA (blue). In *spd-2(RNAi)* embryos, GFP-PAR-2 did not localize to the cortex. However, in *cdc-42(RNAi);spd-2(RNAi)* embryos, GFP-PAR-2 was found uniformly on the cortex as observed for *cdc-42(RNAi)* alone.

and Kemphues, 1999; Tabuse et al., 1998; Watts et al., 1996). PAR-6 and CDC-42 physically interact in *C. elegans* and other systems (Gotta et al., 2001; Hutterer et al., 2004; Joberty et al., 2000; Johansson et al., 2000; Lin et al., 2000; Qiu et al., 2000), and studies in *C. elegans* have suggested that CDC-42 is required to maintain PAR-6 in the anterior half (Gotta et al., 2001; Kay and Hunter, 2001). We re-examined the requirement of CDC-42 for PAR-6 localization (Fig. 3A). Because complete depletion of CDC-42 leads to sterility (data not shown), we determined the maximum depletion that would still yield embryos, and fixed and stained for PAR-6. Polarity formation can be divided into two phases: an establishment phase in which symmetry is broken and a PAR-6 domain is formed independently of PAR-2; and a maintenance phase, in which the maintenance of the PAR-6 domain in the anterior requires PAR-2 (Cuenca et al., 2003). We found that in four out of four embryos examined during polarity establishment, PAR-6 was absent from the cortex. In four out of six embryos examined during the polarity maintenance phase, we also could not detect PAR-6 at the cortex (in two out of these six embryos, PAR-6 was weakly present in the anterior). This indicated that CDC-42 is required for localization of PAR-6 to the cortex during all stages of the mitotic cell cycle.

The lack of cortical PAR-6 in *cdc-42(RNAi)* embryos could be directly due to CDC-42 depletion. This would be consistent with a previous result showing that PAR-6 binds to CDC-42 (Gotta et al., 2001); in addition the phenotypes of CDC-42 and PAR-6 depletion are similar. Alternatively, the aberrant localization of PAR-2 may prevent PAR-6 from localizing to the cortex. We attempted to distinguish between the two possibilities by performing double RNAi of *cdc-42* and *par-2* (Fig. 3A). We found that PAR-6 can localize to the cortex after the reduction of both proteins, although at reduced levels relative to wild-type embryos (n=16/16). These data suggest that the aberrant PAR-2 localization may prevent PAR-6 from localizing to the cortex in *cdc-42(RNAi)* embryos, similar to recent work showing that mutant PAR-2 expressed ectopically in embryonic somatic blastomeres can displace PAR-3 from the cortex (Hao et al., 2006). However, because we are not working under loss-of-function conditions for RNAi of CDC-42 (stronger RNAi conditions produce sterile worms), we cannot rule out the possibility that residual CDC-42 activity could directly recruit PAR-6 to the cortex after the removal of PAR-2. In support of this idea, the residual PAR-6 in the double RNAi embryos still localizes to the anterior cortex. Therefore, we conclude that a CDC-42-dependent activity is required to remove PAR-2 from the cortex and a CDC-42-dependent activity is necessary to localize PAR-6 to the cortex.

CDC-42 localizes to the anterior cortex

Because CDC-42 and PAR-6 form a complex (Gotta et al., 2001), we hypothesized that the requirement of CDC-42 for PAR-6 localization may be reciprocal. To examine whether the anterior PAR proteins are required for CDC-42 localization, we generated a YFP-labeled CDC-42. The YFP-CDC-42 transgene rescues the loss of endogenous CDC-42 (see Materials and methods), suggesting that the fusion protein complements the function of endogenous CDC-42. YFP-CDC-42 formed dynamic structures at the cortex (Fig. 4; see Movie 2 in the supplementary material) that segregated to the anterior cortex during polarity establishment (n=8), recapitulating the behavior of anterior PAR proteins (Cuenca et al., 2003; Munro et al., 2004). Cortical YFP-CDC-42 disappeared around the time of pronuclear rotation (data not shown). To test whether the anterior PAR proteins are required for CDC-42 localization, we examined YFP-CDC-42 dynamics in the *par-3(it71)* loss-of-function mutant and in *par-6(RNAi)* embryos (Fig. 4; Movie 3 in the supplementary material). We made kymographs from time-lapse recordings in control, mutant and RNAi embryos. In *par-3(it71)* (n=8) and *par-6(RNAi)* (n=7) embryos, CDC-42 segregated to the anterior, although segregation was slower than in control embryos (Fig. 4B). Importantly, YFP-CDC-42 eventually localized in the anterior half as it did in control embryos.

Acto-myosin contractility is required to form an anterior cortical domain of CDC-42

CDC-42 segregation to the embryo anterior occurred coincident with the segregation of contractility. Therefore, we speculated that CDC-42 segregation might by regulated by the acto-myosin cytoskeleton. To test this idea, we followed the dynamics of CDC-42 distribution in embryos depleted of myosin II (NMY-2). We found that in *nmy-2(RNAi)* embryos, YFP-CDC-42 localized to the cortex, but did not segregate into an anterior domain (Fig. 4, n=5). Thus, similar to the establishment of PAR polarity, the asymmetric distribution of CDC-42 requires acto-myosin activity.

RHO-1 is required for organization of the cortical myosin II network

During polarity establishment, acto-myosin contractility has to be temporally and spatially regulated such that contractile polarity is coordinated with other events in cell polarization. RhoA is a conserved regulator of acto-myosin contractility. Therefore, we investigated whether *C. elegans* RHO-1 and the putative RhoGEF ECT-2 regulate contractility during polarization. In all our experiments (see below), RNAi of *ect-2* phenocopied the defects observed in *rho-1(RNAi)* embryos, but did not yield defects

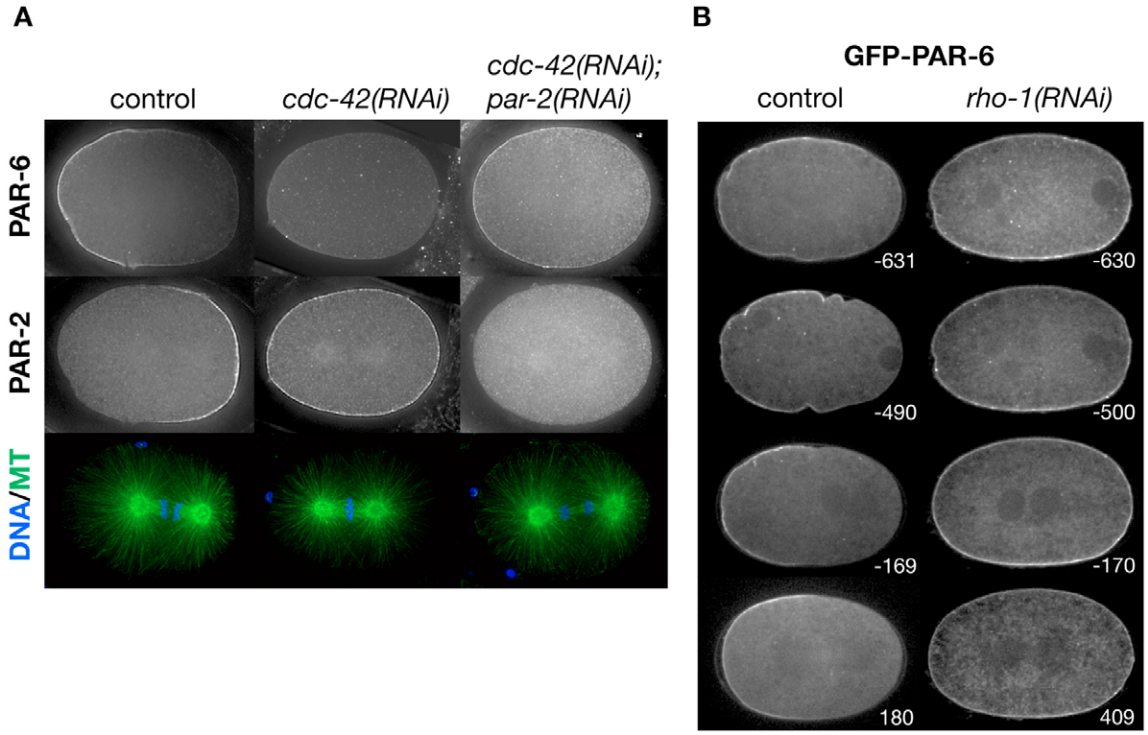

Fig. 3. CDC-42 and RHO-1 are required for PAR-6 localization. (**A**) Embryos expressing GFP-PAR-2 were stained for GFP, PAR-6, microtubules (MT, green) and DNA (blue). In control embryos, PAR-6 localizes to the anterior pole, whereas PAR-2 localizes to the posterior pole. In *cdc-42(RNAi)* embryos, PAR-6 is not detectable at the cortex and PAR-2 is found at the entire cortex. In *cdc-42(RNAi);par-2(RNAi)* embryos, PAR-6 relocalizes anteriorly, but only at reduced levels. (**B**) Time-lapse images of GFP-PAR-6 polarity establishment in control and *rho-1(RNAi)* embryos. Times (seconds) are relative to nuclear envelope breakdown.

characteristic of CDC-42 depletion. This suggests that ECT-2 acts primarily on RHO-1 and not on CDC-42 during polarity establishment.

Depletion of RHO-1 or ECT-2 abolished actin-dependent processes such as cortex ruffling and pseudocleavage furrow formation. Thus, contractile polarity was not established (Fig. 5). The embryos also failed to extrude the polar bodies (data not shown) and cytokinesis failed, as has been previously shown (Jantsch-Plunger et al., 2000). These results suggest that depletion of RHO-1/ECT-2 disrupts the dynamics of the acto-myosin cytoskeleton.

To investigate the relationship between RHO-1 activity and cortical dynamics in more detail, we used a strain expressing NMY-2-GFP (Munro et al., 2004) to monitor myosin organization and dynamics in control and RNAi embryos (Fig. 6A). In control embryos, NMY-2-GFP first forms a dynamic network throughout the entire cortex consisting of clustered foci interconnected by small filaments. At the onset of polarity, concomitant with the apposition of the centrosome at the posterior cortex, this network begins to disassemble in the vicinity of the nucleus/centrosome complex, and the remaining network segregates towards the anterior half (Munro et al., 2004) (see Movie 4 in the supplementary material). Reducing the function of either RHO-1 (n=10, data not shown) or ECT-2 (n=21) by RNAi altered the NMY-2-GFP organization (Fig. 6A; Movie 5 in the supplementary material). Specifically, the early network of interconnected foci clusters did not form. Instead, small foci were uniformly distributed throughout the cortex, reminiscent of the small foci that appear after polarity establishment in control embryos (Fig. 6A, t=750 seconds, 914 seconds). Despite these defects in myosin organization, we noticed that in *ect-2(RNAi)* (Fig. 6A, t=742 seconds, t=918 seconds) and *rho-1(RNAi)* (data not shown) embryos the small myosin foci collectively segregated into an anterior cap in a concerted direction at similar speeds (average velocity 0.17 μm/second, n=10 foci in one embryo; Fig. 6C). However, this cap was not stable (data not shown). In some *ect-2(RNAi)* embryos (six out of 21), the segregation occurred off the long embryo axis, as shown in Fig. 6A. This might reflect a failure in posteriorization (reviewed by Cowan and Hyman 2004a), but as little is known about the molecular details of posteriorization, we did not analyze this further. In four out of 21 *ect-2(RNAi)* embryos, NMY-2 segregation did not take place at any time. Similar results were found for embryos depleted of RHO-1 (data not shown).

The *cdc-42(RNAi)* embryos did not display any obvious structural alterations during the initial assembly of the NMY-2-GFP network (n=9; Fig. 6A, see also Movie 6 in the supplementary material). The contractile network formed and retracted towards the anterior to form a cap as in control embryos. However, the NMY-2-GFP cap was unstable. While the pseudocleavage furrow was regressing, small bright foci appeared and moved back towards the posterior (Fig. 6A, t=763 seconds, t=913 seconds), implicating CDC-42 in stabilizing the acto-myosin network in the anterior half. Ruffle kymographs of *cdc-42(RNAi)* embryos revealed that the establishment of contractile polarity occurred, but the ruffles were more pronounced and less dynamic (Fig. 5).

RHO-1 is required to form an anterior cortical domain of CDC-42

We have demonstrated above that the anterior cortical localization of CDC-42 depends on acto-myosin activity and that RHO-1 regulates the organization of the acto-myosin network. The

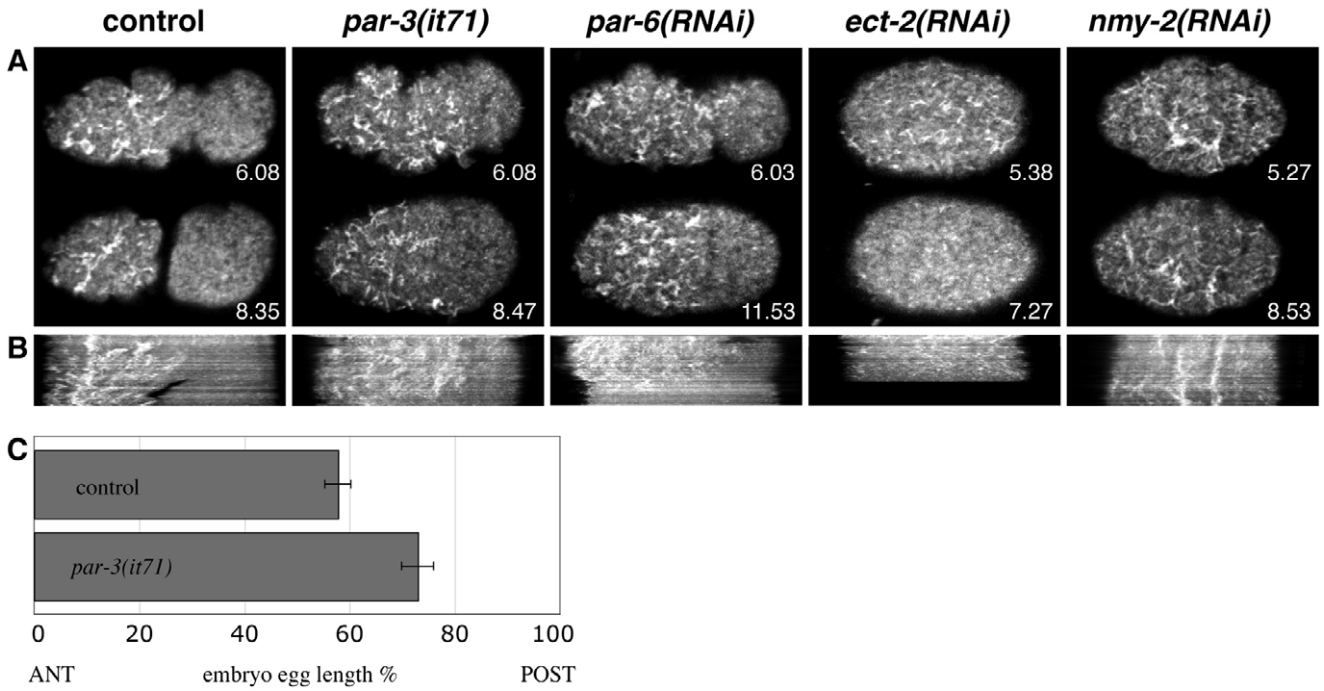

Fig. 4. Cortical YFP-CDC-42 dynamics in mutant and RNAi-depleted embryos. (**A**) Time-lapse images of cortical YFP-CDC-42 recordings after polarity establishment. Times (min.sec) are relative to polarity establishment, as assessed by the proximity of the male pronucleus to the cortex. (**B**) Kymographs of cortical YFP-CDC-42 time-lapse recordings for a period of 7-12 minutes. (**C**) Position of the edge of the cortical YFP-CDC-42 accumulation from the anterior pole 6 minutes after polarity establishment in control and *par-3(it71)* embryos.

segregation of the acto-myosin network, however, could occur independently of RHO-1 activity. We therefore investigated whether CDC-42 segregation could occur in RHO-1- and ECT-2-depleted embryos.

In *ect-2(RNAi)* (n=6) and *rho-1(RNAi)* (n=5) embryos, YFP-CDC-42 remained localized over the whole cortex and did not segregate into an anterior domain (Fig. 4; data not shown; see Movie 7 in the supplementary material). From this, we conclude that RHO-1 activity is essential for CDC-42 segregation to the anterior, but not its localization to the cortex. To test whether PAR-6 localization also requires RHO-1 activity, we made time-lapse movies of GFP-PAR-6 in *rho-1(RNAi)* and *ect-2(RNAi)* embryos. In all *rho-1(RNAi)* (n=6) and *ect-2(RNAi)* (n=11) one-cell embryos studied, GFP-PAR-6 remained localized throughout the cortex during the whole cell cycle and failed to segregate into an anterior domain (Fig. 3B; see Movies 8 and 9 in the supplementary material; data not shown). Thus, RHO-1 activity is also essential for the establishment of an anterior PAR-6 domain. Because CDC-42 distribution appears to dictate cortical PAR-6 localization, it is possible that the symmetric distribution of PAR-6 in *rho-1(RNAi)/ect-2(RNAi)* embryos reflects the defect in CDC-42 segregation. Interestingly, embryos depleted of RHO-1 (n=9/10, data not shown) or ECT-2 (Fig. 6A, n=17/21) sometimes segregated myosin to the anterior, whereas in all embryos studied under the same RNAi conditions, PAR-6 localization remained uniform [*ect-2(RNAi)*, n=63/63; *rho-1(RNAi)*, n=6/6]. RHO-1 activity may therefore couple the anterior movement of myosin II with the anterior segregation of CDC-42 and PAR-6. In support of this idea, co-depletion of RHO-1 and CDC-42 resulted into an additive phenotype (see Fig. S1 in the supplementary material), from which we conclude that RHO-1 and CDC-42 function in separate pathways to localize the PAR proteins.

The coordination of anterior and posterior PAR polarity establishment requires RHO-1

The segregation of myosin II to the embryo anterior in *rho-1(RNAi)* (data not shown) and *ect-2(RNAi)* embryos (Fig. 6A) suggested that some aspects of polarity establishment can occur without the network-like organization of NMY-2, despite the failure to segregate CDC-42 and PAR-6 to the anterior. To determine whether the asymmetric distribution of PAR-2 was established in embryos depleted of RHO-1 activity, we analyzed whether the meiotic PAR-2 cycle is affected after depleting RHO-1. We found that the meiotic cycle is normal (n=6; Fig. 1C); however, we observed two classes of defects with respect to later PAR-2 localization in *rho-1(RNAi)* and *ect-2(RNAi)* embryos. In some embryos, PAR-2 did not localize to the cortex and appeared to remain in the cytoplasm [*ect-2(RNAi)*, n=6/17; *rho-1(RNAi)*, n=4/23; see Fig. S2 and Movie 10 in the supplementary material]. In the remaining embryos, PAR-2 localized to the cortex. However, the onset of the PAR-2 domain was late and its final size was enlarged [*ect-2(RNAi)*, n=11/17; *rho-1(RNAi)*, n=19/23; see Figs S2, S3 and Movie 11 in the supplementary material]. Different amounts of GFP-PAR-2 at the cortex could result from partial RHO-1 depletion. We then determined whether the establishment of PAR-2 polarity in embryos depleted of RHO-1 activity correlated with the segregation of NMY-2. Using a strain expressing both NMY-2-GFP and GFP-PAR-2, we found that NMY-2-GFP migration correlated with formation of the GFP-PAR-2 domain (n=19; Fig. 6B). In embryos in which NMY-2 failed to segregate, we could not detect PAR-2 at the cortex (n=3; data not shown). As we never observed PAR-6 segregating into an anterior domain [Fig. 3B, *rho-1(RNAi)*, n=6 embryos; *ect-2(RNAi)*, n=11 embryos (data not shown)], we concluded that when PAR-2 localizes to the cortex in the absence of RHO-1 activity, it must co-localize with PAR-6. We confirmed this by co-staining for PAR-2 and PAR-6 in *ect-2(RNAi)* embryos (see Fig.

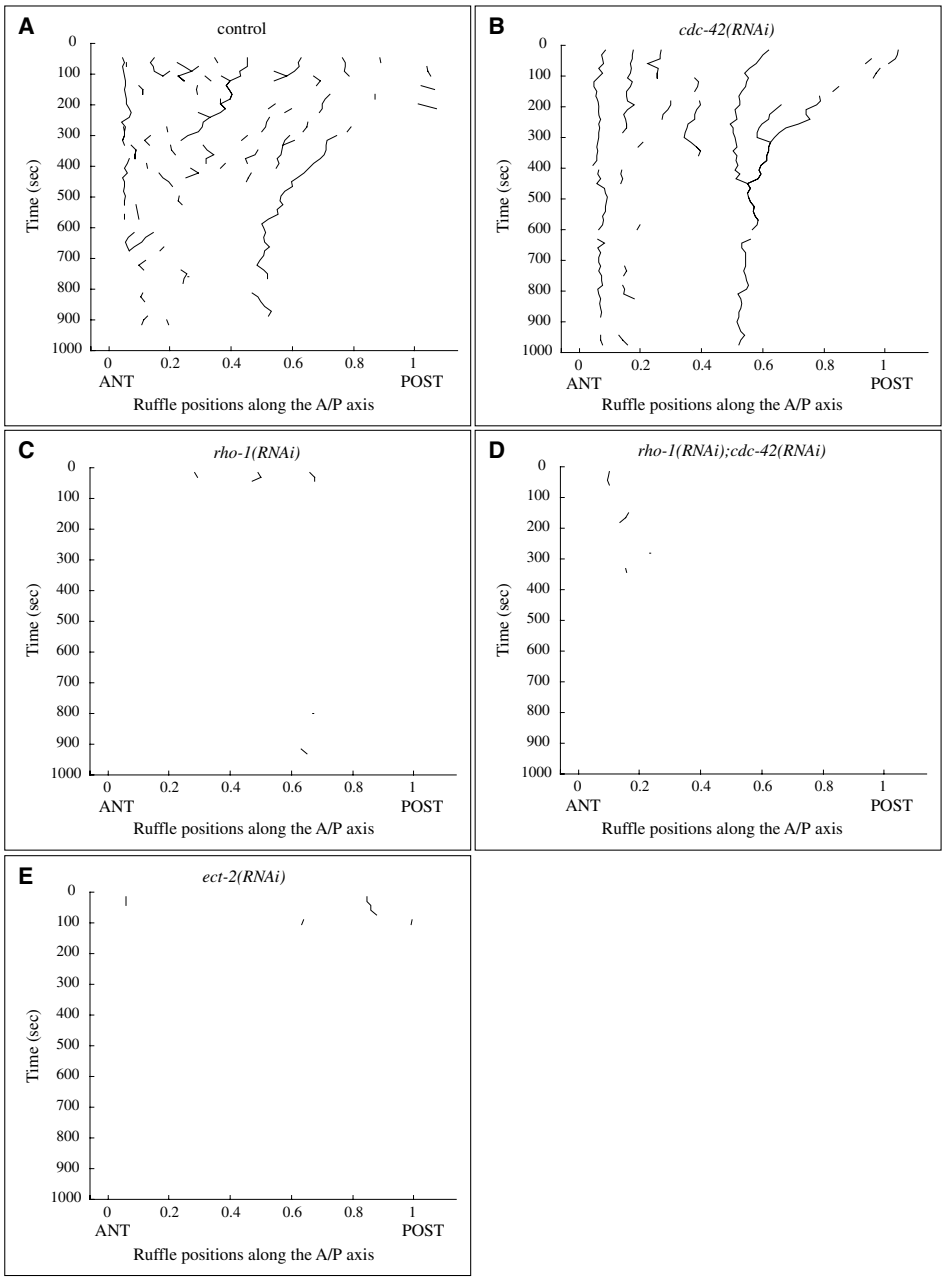

Fig. 5. Ruffle kymographs monitoring the establishment of contractile polarity over time. The position of cortical ruffles along the anterior (ANT)-posterior (POST) axis is projected onto a calculated ellipse. One half of the ellipse was straightened to generate the *x*-axis (see Materials and methods). (**A**) In control embryos, the cortex contracts uniformly after the completion of meiosis. During anteroposterior polarity establishment, the posterior cortex becomes cleared from contractions, whereas the anterior cortex continuous to ruffle. (**B**) *Cdc-42(RNAi)* did not prevent the establishment of the contractile polarity. Ruffles were deeper and persisted longer than in control embryos. (**C-E**) *Rho-1(RNAi)* (C), *cdc-42(RNAi);rho-1(RNAi)* (D) and *ect-2(RNAi)* (E) abolished contractile polarity establishment.

S4 in the supplementary material). In all *ect-2(RNAi)* embryos analyzed, PAR-6 was uniformly localized (*n*=22/22). PAR-2 co-localized with PAR-6 in 17 out of 22 *ect-2(RNAi)* embryos. In five out of 22 *ect-2(RNAi)* embryos, PAR-2 did not localize to the cortex (data not shown). Therefore, we conclude that the formation of a PAR-2 domain is uncoupled from the establishment of the PAR-6 domain.

DISCUSSION

Our data show that RHO-1 and CDC-42 have distinct but coordinated functions in cell polarity in the first cell division of *C. elegans*. RHO-1 is required for the asymmetric distribution of CDC-42 during polarity establishment. CDC-42 is essential for localizing PAR-6 to the cortex during polarity establishment and for stabilizing the acto-myosin network.

After depleting RHO-1 activity, NMY-2 segregation is uncoupled from the anterior segregation of CDC-42 and PAR-6. Thus, the function of RHO-1 may be to couple the segregation of the acto-myosin cytoskeleton to the segregation of the anterior PAR complex. Evidence for how RHO-1 might function in this regard comes from our analysis of the NMY-2 cytoskeleton. In control embryos, NMY-2 forms foci clusters interconnected by small filaments. In *rho-1(RNAi)* and *ect-2(RNAi)* embryos, the myosin foci are smaller and interconnections are not formed, but they can still segregate to the anterior (Fig. 6A). We propose that RHO-1 links CDC-42 and PAR-6 with the segregation of the acto-myosin cortex by organizing the NMY-2 meshwork.

One interesting aspect of the *rho-1(RNAi)* phenotype is that the PAR-2 domain was often expanded, and, in extreme cases, uniformly distributed along the cortex (see Fig. S2 in the supplementary material). By contrast, PAR-6 was always uniformly localized (Fig. 3B). Depletion of PAR-5 or proteins implicated in the regulation or formation of the cytoskeleton showed overlapping anterior and posterior PAR domains (Cuenca et al., 2003; Guo and Kemphues, 1996; Hill and Strome, 1990; Severson et al., 2002;

Severson and Bowerman, 2003; Shelton et al., 1999). However, an aberrant spreading of PAR-2 along the cortex leading, in some cases, to almost uniform PAR-2 distribution has not been observed previously. This implicates RHO-1 activity in the regulation of PAR-2 domain size and suggests that RHO-1-dependent acto-myosin contractility may also help to define the boundaries between anterior and posterior cortical domains in the embryo. One model of cortical polarity establishment suggests that the cortical acto-myosin network is under tension. A local break in the meshwork causes the meshwork to collapse away from the break point (Hird and White, 1993), leaving the voided region of the cortex available for PAR-2 localization. RHO-1 activity might modulate the contractile forces within the network, resulting in an alteration of the boundary between the cytoskeleton network and the PAR-2 domain.

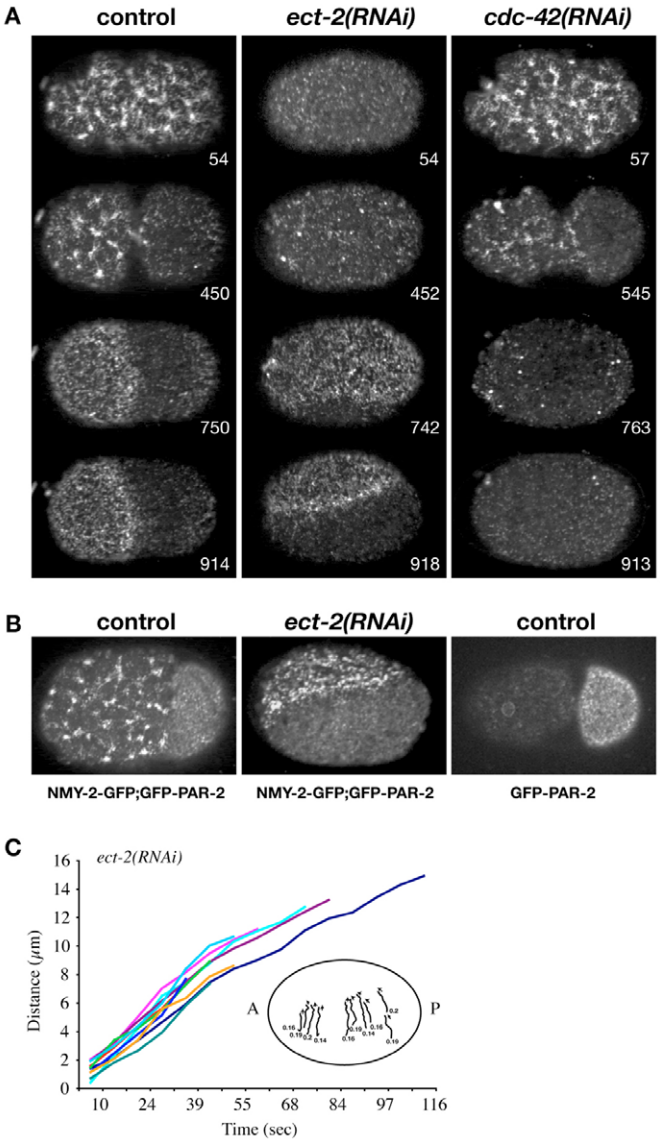

Fig. 6. RHO-1 activity organizes NMY-2 into foci clusters. (**A**) Time-lapse images (surface view) of GFP-NMY-2 during polarity establishment of control, *ect-2(RNAi* and *cdc-42(RNAi)* embryos. Times (seconds) are relative to pronuclei appearance. (**B**) Images of the combined NMY-2-GFP;GFP-PAR-2 line (surface view) of a control (left) and an *ect-2(RNAi)* embryo (middle); cortical view of GFP-PAR-2 (right). GFP-PAR-2 labels the posterior cortex. (**C**) Tracking of GFP-foci in an *ect-2(RNAi)* embryo. The small foci moved concomitantly at the same time and with similar velocities (average velocity=0.17 μm/second).

We have shown that PAR-3 and PAR-6 are neither required to localize CDC-42 to the cortex nor essential for the segregation of CDC-42 to the anterior cortex. However, both proteins appear to be involved in regulating the velocity of the segregation of CDC-42 (Fig. 4B). The segregation of CDC-42 depends on RHO-1 and NMY-2 activity (Fig. 4), which correlates with previous findings that anterior PAR proteins have some regulatory function on the cytoskeleton (Munro et al., 2004). Recent studies in MDCK II epithelial cells have also shown that PAR-3 modulates actin dynamics by regulating the Rac activity through the interaction with a Rac-specific GEF, Tiam1 (Chen and Macara, 2005). So far, no evidence exists that Rac activity or a *C. elegans* homolog of Tiam1 is required for polarity establishment in one-cell embryos. However, it is possible that CDC-42 might regulate acto-myosin dynamics as part of a PAR-3/PAR-6/aPKC/CDC-42 complex. This idea is supported by studies that have shown that ECT-2 interacts with PAR-6 in a two hybrid assay (Liu et al., 2004), and that PAR-3 inactivates cofilin by LIM kinase 2 (LIMK2), a downstream effector of the RHO-1 and CDC-42, which alters contractility in MDCK II cells (Chen and Macara, 2006).

Our data indicate that a key function of CDC-42 during polarity establishment is to facilitate the localization of PAR-6 to the cortex. In the absence of CDC-42, PAR-6 is unable to localize to the cortex and PAR-2 is uniformly distributed along the cortex (Fig. 1, Fig. 3A). This differs from previously published data, which suggested that CDC-42 was required to maintain PAR-6 in an anterior domain. However, in our study we obtained no evidence of PAR-6 localization to the anterior cortex after RNAi of CDC-42, either during the establishment or during the maintenance phase of polarity (Cuenca et al., 2003). The most likely reason for this difference is that we are working under stronger RNAi conditions (see Materials and methods).

How might CDC-42 act to localize PAR-6? In other systems, CDC-42 binds to PAR-6 and activates its PDZ domain, enabling it to bind other partners. Thus, one likely possibility is that CDC-42 localizes to the cortex, where it in turn recruits PAR-6, triggering the assembly of the anterior PAR complex. In *cdc-42(RNAi)* embryos, PAR-2 stays localized uniformly over the cortex as the embryo enters mitosis. This is similar to the phenotype of *par-6(RNAi)*, providing additional support for the idea that CDC-42 operates in concert with the anterior PAR complex. We show that this aberrant PAR-2 localization in *cdc-42(RNAi)* embryos excludes PAR-6 from localizing to the cortex (Fig. 3A). In support of this idea, recent work has shown that ectopic mutant PAR-2 excludes PAR-3 from the apical cortex of embryonic somatic blastomeres (Hao et al., 2006). Therefore, our data, and the findings of Hao et al. (Hao et al., 2006), would support a model in which ectopic PAR-2 localization, in *cdc-42(RNAi)* embryos, is sufficient to displace PAR-6 from the cortex. However, it is unclear whether these two experimental situations rely on the same molecular machinery. Hao et al. (Hao et al., 2006) examined the ability of PAR-2 to displace PAR-3 from the cortex in cells that exhibit epithelial (apical-basal) polarity, and it has not been investigated whether localization of the anterior PAR complex in somatic blastomeres has the same molecular requirements, such as for CDC-42, as in one-cell embryos. Additionally, one important caveat in our experiments is that we are not working under full loss-of-function conditions for *cdc-42(RNAi)* embryos (see Materials and methods) and, thus, we cannot rule out the possibility that residual CDC-42 in *cdc-42(RNAi);par-2(RNAi)* embryos can localize PAR-

6 after removal of PAR-2. Interestingly, PAR-6 is enriched in the anterior cortex in *cdc-42(RNAi);par-2(RNAi)* embryos. Because PAR-2 has been depleted, one might expect that PAR-6 would spread back to the posterior during the maintenance phase. This anterior enrichment of PAR-6 in *cdc-42(RNAi);par-2(RNAi)* embryos could suggest some partially counterbalancing antagonistic activities, as proposed by Gotta et al. (Gotta et al., 2001).

In wild-type embryos, PAR-2 does not prevent the localization of PAR-6 at the cortex during meiosis. PAR protein distribution changes from co-localization at meiosis to mutually exclusive domains at mitosis, which suggests that the distribution of PAR proteins is controlled differently during meiosis and mitosis. Thus, it is possible that the mechanism of PAR-6 localization to the cortex differs between the meiotic and mitotic cell cycle, and that cortical PAR-6 localization is CDC-42 independent during meiosis but CDC-42 dependent during mitosis. The cortical PAR-6 localization we observed in *par-2(RNAi);cdc-42(RNAi)* embryos would reflect the meiotic localization pathway. Ultimately, our experiments cannot distinguish between the varieties of models at this time. We conclude that CDC-42 has two activities: to remove PAR-2 from the cortex at the end of meiosis, and to localize PAR-6 throughout the cell cycle.

Taken together, our data suggest the following model: prior to polarity establishment, active RHO-1, perhaps regulated by ECT-2, organizes the acto-myosin into a contractile network. Both actin polymerization and NMY-2 activity contribute to the structure of the network and could be regulated by RHO-1. RHO-1-independent localization and/or activation of CDC-42 at the cortex triggers assembly of the anterior PAR complex. Upon perception of the centrosomal polarization signal, the myosin network, together with CDC-42 and the anterior PAR complex, segregates to the embryo anterior. This segregation is dependent on RHO-1 activity. At the same time, PAR-2 responds to the altered cortical structure resulting from the anterior segregation of myosin and establishes a posterior cortical domain.

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Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/133/18/3507/DC1

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