PAR-2, LGL-1 and the CDC-42 GAP CHIN-1 act in distinct pathways to maintain polarity in the C. elegans embryo

Alexander Beatty, Diane G. Morton and Kenneth Kemphues*

SUMMARY
In the one-cell C. elegans embryo, polarity is maintained by mutual antagonism between the anterior cortical proteins PAR-3, PKC-3, PAR-6 and CDC-42, and the posterior cortical proteins PAR-2 and LGL-1 on the posterior cortex. The mechanisms by which these proteins interact to maintain polarity are incompletely understood. In this study, we investigate the interplay among PAR-2, LGL-1, myosin, the anterior PAR proteins and CDC-42. We find that PAR-2 and LGL-1 affect cortical myosin accumulation by different mechanisms. LGL-1 does not directly antagonize the accumulation of cortical myosin and instead plays a role in regulating PAR-6 levels. By contrast, PAR-2 likely has separate roles in regulating cortical myosin accumulation and preventing the expansion of the anterior cortical domain. We also provide evidence that asymmetry of active CDC-42 can be maintained independently of LGL-1 and PAR-2 by a redundant pathway that includes the CDC-42 GAP CHIN-1. Finally, we show that, in addition to its primary role in regulating the size of the anterior cortical domain via its binding to PAR-6, CDC-42 has a secondary role in regulating cortical myosin that is not dependent on PAR-6.

KEY WORDS: Embryo, PAR genes, Polarity

INTRODUCTION
Cell polarity is crucial for embryonic development. In the one-cell C. elegans embryo, anterior-posterior polarity occurs in two temporally distinct phases referred to as establishment and maintenance (Cuenca et al., 2003). Polarity establishment is mediated by an asymmetric contraction of the cortical meshwork that instructs the segregation of two antagonistic sets of conserved polarity regulators known as the partitioning-defective (PAR) proteins (Nance and Zallen, 2011). A cue associated with the sperm centrosome triggers a non-muscle myosin II-dependent asymmetric contraction of the actomyosin cytoskeleton and an associated anteriorly directed cortical flow (Cheeks et al., 2004; Mayer et al., 2010; Munro et al., 2004). This flow requires the small GTPase RhoA (RHO-1) (Motegi and Sugimoto, 2006; Schonegg and Hyman, 2006) and functions to concentrate PAR-3, PAR-6 and the atypical protein kinase C PKC-3 into an anterior cortical domain, likely via advective transport (Goehring et al., 2011b). As the anterior PAR proteins become enriched in the anterior, the posterior proteins, including PAR-1, PAR-2, and LGL-1, become enriched in a reciprocal domain (Nance and Zallen, 2011).

Following the attenuation of cortical flow, polarity maintenance is necessary to perpetuate the cortical asymmetries generated during establishment. Although a number of the key proteins involved in polarity maintenance have been identified, the molecular mechanisms by which the proteins contribute to polarity maintenance, as well as the level of interaction between the components, are not well understood.

The Rho GTPase CDC-42 is an important regulator of polarity maintenance (Aceto et al., 2006; Kumfer et al., 2010; Motegi and Sugimoto, 2006; Schonegg and Hyman, 2006). During maintenance, CDC-42 is enriched on the anterior cortex (Aceto et al., 2006; Motegi and Sugimoto, 2006; Schonegg and Hyman, 2006), and its active form binds PAR-6 (Aceto et al., 2006; Gotta et al., 2001). In cdc-42(RNAi) embryos, PAR-6 and PKC-3 become asymmetrically enriched on the anterior cortex at a reduced level during establishment, and are lost from the cortex around the time of nuclear envelope breakdown. PAR-3 remains cortical but often extends into the posterior and can overlap with PAR-2 (Gotta et al., 2001; Kay and Hunter, 2001). Embryos expressing a PAR-6 mutant unable to bind CDC-42 exhibit defects similar to cdc-42(RNAi) embryos, suggesting that CDC-42 functions in polarity primarily via its physical interaction with PAR-6 (Aceto et al., 2006).

Reduction of CDC-42 function also results in alterations in non-muscle myosin II (NMY-2) localization. In embryos depleted for CDC-42, cortical myosin is largely lost during the transition to the maintenance phase when myosin foci are normally reorganized and replaced by finer fibers (Kumfer et al., 2010; Motegi and Sugimoto, 2006; Schonegg and Hyman, 2006). Because CDC-42 is required for the maintenance of cortical PAR-6/PKC-3 as well as cortical NMY-2, CDC-42 appears to provide a functional link between the anterior PAR proteins and the actomyosin cytoskeleton during polarity maintenance.

In addition to signaling through CDC-42, polarity maintenance is also mediated by mutual exclusion between the anterior and posterior PAR proteins. During the maintenance phase, the sizes of reciprocal PAR domains are stable, but the individual PAR proteins exchange between the cortex and the cytoplasm (Cheeks et al., 2004; Goehring et al., 2011a; Nakayama et al., 2009), and cortical PAR proteins can diffuse freely across the anterior-posterior domain boundary (Goehring et al., 2011a). Mathematical modeling suggests that mutual inhibition between the anterior and posterior PAR proteins occurs via a reaction-diffusion system that does not require the actomyosin cytoskeleton (Dawes and Munro, 2011; Goehring et al., 2011a; Goehring et al., 2011b).

On the posterior cortex of the one-cell embryo, the putative E3 ubiquitin ligase PAR-2 is required to prevent the anterior cortical domain from expanding during the maintenance phase (Boyed et al., 1996; Cuenca et al., 2003; Hao et al., 2006). Recently, LGL-1, the...
Development 140 (9)

homolog of the Drosophila tumor suppressor protein Lethal Giant Larvae, was found to localize asymmetrically to the posterior cortex and function redundantly with PAR-2 to maintain polarity (Beatty et al., 2010; Hoege et al., 2010). LGL-1 is not required for polarity maintenance, but \( \text{par-2} \) double mutants have a stronger phenotype than \( \text{par-2} \)-alone. In \( \text{par-2} \)-mutant, the anterior PARs expand into the posterior but retain a graded distribution there. In \( \text{lgl-1}; \text{par-2} \) double mutants, the anterior PAR proteins become even more symmetric, although reduced levels of anterior PARs are still apparent in the extreme posterior (Beatty et al., 2010). Although it is clear that \( \text{PAR-2} \) and \( \text{LGL-1} \) act in polarity maintenance, the molecular mechanisms by which the proteins function are not well understood. Both \( \text{PAR-2} \) and \( \text{LGL-1} \) inhibit the cortical accumulation of NMY-2 on the posterior cortex (Beatty et al., 2010; Munro et al., 2004), consistent with the hypothesis that pathways regulated by the two proteins converge on NMY-2. An alternative hypothesis is that the effects of \( \text{LGL-1} \) on NMY-2 are indirect and mediated by the anterior PAR proteins. Hoege and co-workers have suggested that \( \text{LGL-1} \) contributes to polarity maintenance via a mutual elimination mechanism (Hoege et al., 2010). This model proposes that \( \text{PAR-6} \) and PKC-3 physically interact with \( \text{LGL-1} \), likely near the interface of the anterior and posterior cortical domains. The interaction leads to phosphorylation of \( \text{LGL-1} \) by PKC-3, which results in the cortical removal of \( \text{PAR-6} \) and PKC-3 that otherwise would diffuse into the posterior cortical domain.

In this study, we investigate the interplay among \( \text{LGL-1} \), \( \text{PAR-2} \), cortical myosin and the anterior PAR protein \( \text{PAR-6} \), and uncover a third redundant polarity maintenance pathway that includes the cortical myosin and the anterior PAR protein \( \text{PAR-6} \), and uncover

**RESULTS**

**LGL-1 and PAR-2 affect myosin distribution by different mechanisms**

During polarity maintenance in wild-type embryos, myosin is asymmetrically localized to the anterior cortex (Fig. 1A) (Munro et al., 2004). In \( \text{par-2} \)-RNAi embryos, \( \text{LGL-1} \) negatively regulates the posterior cortical accumulation of myosin during polarity
compared with
in the cortical area occupied by myosin in
Materials and methods). Although we can readily detect an increase
par-2
distinguish between these two potential mechanisms, we measured
posterior cortex by the action of LGL-1 (Hoege et al., 2010). To
consequence of the removal of the anterior PAR proteins from the
in cortical myosin in the absence of PAR-2 is a secondary
maintenance (Beatty et al., 2010). However, it is unclear whether
LGL-1 affects asymmetric cortical myosin accumulation
independently of the anterior PAR proteins, or whether the asymmetry
in cortical myosin in the absence of PAR-2 is a secondary
consequence of the removal of the anterior PAR proteins from the
posterior cortex by the action of LGL-1 (Hoege et al., 2010). To
distinguish between these two potential mechanisms, we measured
cortical NMY-2::GFP levels in the presence or absence of LGL-1 in
wild-type and par-2 mutant embryos using two different methods (see
Materials and methods). Although we can readily detect an increase
in the cortical area occupied by myosin in par-2::lgl-1 embryos
compared with par-2 embryos (Beatty et al., 2010) we could detect
no significant difference in total cortical myosin levels between lgl-
1 and wild type or between par-2 and par-2::lgl-1 (Table 1). This
suggested that LGL-1 affected the distribution of cortical myosin
without affecting the overall accumulation of myosin or that the
difference was too small to reliably detect. To test the dependence of
the observed LGL-1-mediated change in myosin distribution, and
possibly levels, on PAR-6, we examined myosin distribution and
measured the cortical myosin levels in par-6(RNAi); par-6(RNAi);
par-2(lw32) and par-6(RNAi); par-2(lw32); lgl-1(tm2616) embryos
during polarity maintenance (see Materials and methods). Consistent
with a previous report of cortical NMY-2 distribution and levels in
par-2; par-3 double mutants (Munro et al., 2004), myosin asymmetry
is lost when the anterior PAR proteins are eliminated from the cortex
and the cortical levels of NMY-2 in par-6(RNAi); par-2(lw32)
embryos were substantially higher than in par-6(RNAi) embryos
(Fig. 1B,C; Table 1). Furthermore, we found no significant difference
in the distribution or amount of cortical myosin between par-6(RNAi);
par-2(lw32) and par-6(RNAi); par-2(lw32); lgl-1(tm2616)
(Fig. 1B,C; Table 1). Therefore, we conclude that PAR-2 negatively
regulates the cortical accumulation of NMY-2 on the posterior cortex
in a manner that is independent of the asymmetry generated by the
anterior PAR proteins, whereas LGL-1 reduces cortical myosin in the
posterior via its effect on the anterior PAR proteins.

PAR-6 levels in the early embryo are increased in lgl-1(RNAi)
If LGL-1 functions solely by removing PAR-6 from the cortex in the
one-cell embryo (Hoege et al., 2010), we hypothesized that cortical
levels of PAR-6 should be increased and cytoplasmic levels should be
reciprocally decreased after depleting LGL-1. To test this, we
compared the relative amount of cortical and cytoplasmic PAR-6::GFP
in control and lgl-1(RNAi) during polarity maintenance at the
time of nuclear envelope breakdown (see Materials and methods). In
contrast to our predictions, we found that both cortical and
cytoplasmic PAR-6::GFP levels were reproducibly higher after
depleting LGL-1 (Fig. 2). The cortical and cytoplasmic amounts of
PAR-6::GFP were increased by 15.1±4.4% (P=0.009) and 26.0±6.1%
(P<0.0001), respectively, in lgl-1(RNAi) embryos compared with the
controls (n=24 for each genotype). Despite this increase in PAR-6
levels, the area of the PAR-6 cortical domain with respect to the total
area of the embryo cross-section was similar in lgl-1(RNAi) and
control embryos; the measured change in the PAR-6 domain in lgl-
1(RNAi) embryos with respect to wild type was −4.2±2.9% (Fig. 2;
P=0.61). Thus, LGL-1 normally acts to reduce levels of PAR-6 protein,
but our experiment does not address whether LGL-1 might
also remove PAR-6 from the cortex (see Discussion).

We performed a similar experiment comparing PAR-6 levels with
and without depleting PAR-2. In par-2(RNAi) embryos, consistent
with expectations, the area of the cortical PAR-6::GFP domain was
expanded by 24.2±4.8% (P=2×10−5); however, neither the cortical
nor cytoplasmic PAR-6 levels changed compared with the control
(Fig. 3; P=0.36, 0.22, respectively; n=18). Consistent with the
previously described role for PAR-2 in polarity maintenance
(Cuenca et al., 2003; Hao et al., 2006), we conclude that PAR-2
negatively regulates the size of the cortical PAR-6 domain, but not
the steady state level of PAR-6. By contrast, in the presence of PAR-
2, LGL-1 has no effect on anterior domain size, but instead regulates
the overall amount of PAR-6.

Depletion of CHIN-1 blocks the ability of LGL-1 overexpression to rescue par-2
Overexpression of LGL-1 is sufficient to maintain cortical
asymmetry in par-2 mutants (Beatty et al., 2010; Hoege et al.,

Fig. 1. The effect of LGL-1 on myosin accumulation in par-2 is
epistatic to par-6(RNAi). (A) Confocal projections of cortical NMY-2::GFP
prior to symmetry breaking (left), at pseudocleavage (center) and at
nuclear envelope breakdown (NEB) of the first mitotic division (right).
(B) The left column shows confocal projections of cortical NMY-2::GFP in
embryos at NEB. The column on the right depicts the threshold mask (red
overlay) applied to the images on the left to quantify the fraction of the
cortex with NMY-2::GFP signal. Anterior is towards the left in all figures.
(C) The average fraction of the cortex with NMY-2::GFP signal±s.d. based
on the threshold mask exemplified in B. The values for the average
fraction of the cortex with signal are provided in Table 1 in the ‘Area
method’ column. Asterisks indicate significant differences relative to par-
6(RNAi). P<0.002.
2010). In an attempt to understand how LGL-1 influences polarity maintenance, we screened a small set of known cytoskeletal regulators by RNAi depletion to identify any that prevented LGL-1::GFP overexpression from rescuing par-2(lw32), but were not required in wild type (Beatty et al., 2010). One protein that emerged from the screen was the putative CDC-42 GAP, CHIN-1, which has been shown to inhibit the accumulation of NMY-2 fibers on the posterior cortex during polarity maintenance (Kumfer et al., 2010). Embryos from chn-1(RNAi) were mostly viable (0.6±0.9% lethality, n=872), as were embryos from lgl-1::gfp; par-2(lw32) (10.3±8.0% lethality, n=436). However, lgl-1::gfp; par-2(lw32); chn-1(RNAi) embryos were 83.9±10.7% lethal (Fig. 4A; n=1286). We obtained a similar result using a different lgl-1 transgene and different par-2 allele. Embryos from par-2(ok1723); lgl-1::mCherry were 48.5±22.6% lethal (n=912), while embryos from par-2(ok1723); lgl-1::mCherry; chn-1(RNAi) were 98.1±1.4% lethal (n=1312). Thus, depletion of CHIN-1 severely impairs the ability of LGL-1 overexpression to rescue par-2.

We determined that the increased lethality in the CHIN-1 knockdown was due to a loss of polarity maintenance. Early embryos from lgl-1::gfp; par-2(lw32); chn-1(RNAi) exhibited a characteristic Par-2 phenotype. In contrast to chn-1(RNAi) and lgl-1::gfp; par-2(lw32) embryos, lgl-1::gfp; par-2(lw32); chn-1(RNAi) embryos exhibited similarly sized blastomeres and transverse spindle orientations in the P1 cell, like par-2(lw32) embryos (Fig. 4B) (Kumfer et al., 2010). The AB blastomere accounted for 53.9±1.5% (n=11) of the total embryo area in lgl-1::gfp; par-2(lw32) as compared to 51.1±2.4% (n=17) in lgl-1::gfp; par-2(lw32); chn1-1(RNAi) (P=0.0004). We examined the subcellular localization of LGL-1::GFP in one-cell lgl-1::gfp; par-2(lw32); chn1-1(RNAi) embryos. In these embryos, LGL-1::GFP became asymmetrically localized to the posterior cortex during polarity establishment, but the cortical domain was not maintained (n=8/8, see Movies 1 and 2 in the supplementary material), suggesting CHIN-1 is required to maintain the posterior cortical domain in lgl-1::gfp; par-2(lw32).

Consistent with a role for CHIN-1 in maintenance, we found that depleting CHIN-1 enhanced the lethality and early polarity defects of par-2(it5s) at the permissive temperature of 16°C, similar to lgl-1 (Beatty et al., 2010). Embryos from par-2(it5); chn1-1(RNAi) were 82.3±15.6% (n=1428) embryonic lethal compared with chn1-1(RNAi) and par-2(it5) worms at 16°C, which yielded 1.6±1.2% (n=377) and 11.4±3.8% (n=1071) dead embryos, respectively (Fig. 4).

One possible explanation for these results is that chn-1 and lgl-1 are components of a common genetic pathway, and LGL-1 acts through CHIN-1. An alternative explanation is that chn-1 and lgl-1 are components of parallel genetic pathways, both of which contribute to polarity maintenance. To distinguish between these hypotheses, we depleted CHIN-1 in the presumed null mutation lgl-1(tm2616). If CHIN-1 acts downstream of LGL-1 in a linear pathway, we expected the embryonic lethality of lgl-1(tm2616); chn1-1(RNAi) to be no greater than the sum of the embryonic lethality from lgl-1(tm2616) (1.7±0.3%; n=3787) and chn1-1(RNAi) (0.8±0.1%; n=4100). By contrast, if CHIN-1 and LGL-1 function in parallel, we expected to see synthetic lethality when both proteins were compromised. lgl-1(tm2616); chn1-1(RNAi) yielded 5.0±2.0% lethality (n=3420; P<0.0001, χ^2 test assuming an expected lethality of 2.5% for lgl-1(tm2616); chn1-1(RNAi)). Although the magnitude of the difference is not large, leaving open the possibility that it is due to variation in RNAi effectiveness, the results are consistent with the hypothesis that chn-1 and lgl-1 are components of distinct genetic pathways.

### Loss of CGEF-1 function rescues par-2(lw32)

A putative CDC-42 GEF, CGEF-1, appears to antagonize CHIN-1 (Kumfer et al., 2010). Because CHIN-1 depletion enhanced the phenotype of par-2(it5), we hypothesized that deletion or mutation of CGEF-1 may be sufficient to suppress par-2. Consistent with this hypothesis, embryos from par-2(lw32); cgef-1(RNAi) were 65.0±20.2% viable (n=618), whereas par-2(lw32) worms fed on control bacteria yielded no viable progeny (Fig. 5A; n=653). Similar results were observed using par-2(it5) at the restrictive temperature; viability of embryos from par-2(it5) and par-2(it5); cgef-1(RNAi) were 52.8±7.1% (P=0.21).
were 11.2±2.2% (n=1049) and 42.9±29.0% (n=462), respectively (Fig. 5A). Furthermore, embryos from par-2(lw32); cgef-1(gk261) were 76.4±4.5% viable (Fig. 5; n=505). gk261 is a likely null allele (Kumfer et al., 2010). We conclude that loss of cgef-1 function suppresses the embryonic lethality associated with par-2 mutations.

In an effort to learn the relationship between CGEF-1 function and LGL-1, we also tested whether CGEF-1 depletion could suppress par-2; lgl-1. We made a series of par-2; lgl-1 double mutants by generating all possible pairwise combinations of two likely null alleles (lw32 and tm2616) and two hypomorphic alleles (it5 and it31) of par-2 and lgl-1. We treated each of the par-2; lgl-1 double mutants with cgef-1(RNAi) and quantified embryonic lethality (supplementary material Table S2). We observed that cgef-1(RNAi) can suppress par-2; lgl-1 when a hypomorphic allele of lgl-1 is coupled with a null allele of par-2, but not when a hypomorphic allele of par-2 is coupled with a null allele of lgl-1. Thus, lgl-1 function is required for loss of cgef-1 function to rescue embryonic viability in par-2. Although this result is consistent with a role for regulating active CDC-42 in polarity maintenance, it does not distinguish whether LGL-1 acts in the same pathway as CDC-42 or in a parallel pathway.

### CHIN-1 and CGEF-1 can regulate CDC-42 in par-2(lw32); lgl-1(tm2616)

To determine the functional relationship between CHIN-1/CGEF-1, PAR-2 and LGL-1 with respect to CDC-42 regulation, we used a biosensor, GFP::GBDwsp-1, that reports high concentrations of available active GTP-bound CDC-42 (Kumfer et al., 2010). During polarity maintenance at the time of nuclear envelope breakdown, GFP::GBDwsp-1 is asymmetrically enriched on the anterior cortex of the embryo (Fig. 6A) (Kumfer et al., 2010). In Fig. 6B we show measurements of the signal at the cortex relative to the cytoplasm in the anterior and posterior. Control embryos had cortical to cytoplasmic enrichments of 60.1±0.9% and 5.8±0.9% at the anterior and posterior pole, respectively (n=10). Consistent with published results (Kumfer et al., 2010), both chin-1(RNAi) and cgef-1(RNAi) altered the distribution of the probe during polarity maintenance (Fig. 6A). In chin-1(RNAi) embryos, GFP::GBDwsp-1 was cortically enriched in the anterior (70.9±8.7%), like controls, but...
posterior cortical enrichment increased roughly sixfold relative to the control (35.6±7.6%; n=5). By contrast, cgef-1(RNAi) resulted in a marked reduction of cortical GFP::GBDwsp-1 (6.7±6.6% enrichment on the anterior cortex, −4.0±15.3% on the posterior cortex; n=5).

To determine whether PAR-2 and LGL-1 are required for CHIN-1 or CGEF-1 function, or both, we examined and quantified the localization of GFP::GBDwsp-1 in par-2(lw32); lgl-1(tm2616) with and without RNAi-mediated depletion of CHIN-1 or CGEF-1. In contrast to expectations from the hypothesis that levels of active CDC-42 are regulated exclusively by either PAR-2 or LGL-1, or both, GFP::GBDwsp-1 was distributed asymmetrically in par-2(lw32); lgl-1(tm2616) (Fig. 6C). We observed a 38.5±4.0% cortical enrichment of GFP::GBDwsp-1 in the anterior, and no enrichment in the posterior (−2.2±5.3%; n=15). Furthermore, the cortical levels of the biosensor were increased in the posterior in par-2(lw32); lgl-1(tm2616) embryos depleted of CHIN-1 (Fig. 6C; 43.2±14.0% anterior enrichment and 34.3±21.3% posterior enrichment; n=5). By contrast, when CGEF-1 was knocked down in par-2(lw32); lgl-1(tm2616), GFP::GBDwsp-1 levels were reduced throughout the cortex (Fig. 6C, 10.3±4.3% anterior enrichment, −3.5±0.4% posterior enrichment, n=10). We conclude that active CDC-42 asymmetry is maintained, at least in part, by a mechanism that is independent of both PAR-2 and LGL-1. In addition, CHIN-1 and CGEF-1 can function in the absence PAR-2 and LGL-1, consistent with the hypothesis that these proteins are likely components of a genetic pathway that does not include PAR-2 or LGL-1.

Mis-regulation of CDC-42 results in increased amounts of PAR-6 in the early embryo

Next, we asked what role active CDC-42 plays in controlling the amount and cortical distribution of PAR-6 in the early embryo by comparing the cortical and cytoplasmic amounts of PAR-6::GFP in control, chin-1(RNAi) and cgef-1(RNAi) embryos at nuclear envelope breakdown. As in lgl-1(RNAi) embryos, both the cortical and cytoplasmic PAR-6::GFP amounts were increased in chin-1(RNAi) embryos (Fig. 3; 26.9±16.1% increase in cortical amount, $P=0.0005$; 19.3±10.6% increase in cytoplasmic amount, $P=0.006$; n=21). Additionally, the anterior cortical domain extended 14.8±11.6% further into the posterior after depleting CHIN-1 (Fig. 3A; $P=0.0002$).

In embryos depleted of CGEF-1, the area of the PAR-6 cortical domain was 16.5±3.2% smaller than in the controls ($P=0.0006$, n=27) and was sometimes positioned laterally (Fig. 3A; n=5/27). Cortical PAR-6 amounts were similar to controls (−1.2±10.0%; $P=0.18$); but, surprisingly, the cytoplasmic amounts were 34.8±16.6% greater in cgef-1(RNAi) embryos compared with the controls (Fig. 3; $P=6\times10^{-5}$; n=27); indeed, the total PAR-6 (sum of the cortical and cytoplasmic signal) was increased after knocking down CGEF-1 ($P=0.0001$).

Thus, elevating the cortical levels of active CDC-42 during polarity maintenance results in an expansion of the PAR-6 cortical domain, as well as an increase in the amount of cortical PAR-6. By contrast, reducing active CDC-42 at the cortex leads to a reduction in both the size of the PAR-6 domain and amount of cortical PAR-6. However, in both situations, total PAR-6 amounts are elevated.

CHIN-1 and CGEF-1 regulate anterior cortical domain size and PAR-6 levels in lgl-1(tm2616)

If LGL-1 and CHIN-1 are components of parallel pathways that both independently contribute to polarity maintenance, we predicted that depletion of CHIN-1 in lgl-1(tm2616) would result in an expansion of the anterior cortical domain and an increase in the total amount of PAR-6 in the one-cell embryo. For two independent par-6::gfp, lgl-1(tm2616) lines, we found that the area of the cortical PAR-6::GFP domain was increased in chin-1(RNAi) embryos with respect to the controls; the anterior cortical domain was expanded by 25.9±11.3% ($P=6\times10^{-5}$; n=24) in one of the lines and 23.7±6.1% ($P<1\times10^{-8}$; n=26) in the other (Fig. 7A,B). Additionally, depletion of CHIN-1 resulted in an increase in cortical PAR-6::GFP in both lines (25.3±21.0%; $P=0.03$; n=24; 35.1±15.5%; $P=0.003$; n=26) and a statistically significant increase in cytoplasmic PAR-6 signal in one of the two lines (Fig. 7; 9.7±7.0%; $P=0.02$; n=24). The other line showed a similar, but not statistically significant, change in the cytoplasmic signal following CHIN-1 depletion (8.6±13.3%; $P=0.10$; n=26).

Next, we depleted CGEF-1 in lgl-1(tm2616) expressing PAR-6::GFP and observed a 35.6±4.4% reduction in the area of the PAR-6 cortical domain (Fig. 7A,C; $P=1\times10^{-7}$; n=15) along with a 21.7±16.1% reduction in cortical PAR-6::GFP relative to the controls (Fig. 7; $P<0.02$; n=15). In contrast, the cytoplasmic and total amount of PAR-6 were increased in lgl-1(tm2616); cgef-1(RNAi) (Fig. 7; $P=0.0004$ and 0.001, respectively). The cytoplasmic PAR-6::GFP
cgef-1(gk261) (2010), we noted a substantial decrease in cortical NMY-2::GFP in consistent with the data published by Kumfer et al. (Kumfer et al., independent of the anterior PAR proteins. Consistent with an earlier report examining the formation of myosin filaments in the posterior (Munro et al., 2004), we found that PAR-2 does not require the anterior PAR proteins to affect myosin accumulation in the posterior. During polarity maintenance in the one-cell embryo, PAR-2 is required to prevent the expansion of the anterior cortical domain (Cuenca et al., 2003; Goehring et al., 2011b; Hao et al., 2006) and also prevents the formation of NMY-2 filaments on the posterior cortex (Beatty et al., 2010; Munro et al., 2004). How these two pathways maintain C. elegans polarity must be independent of the anterior PAR proteins. Consistent with this possibility, we observed that PAR-2 and LGL-1 act to reduce the overall level of PAR-6 in the embryo. It also reveals a third redundant pathway for polarity maintenance defined by the action of CHIN-1.

**Suppression of par-2 by cgef-1 is not mediated by MRCK-1**

Kumfer and colleagues reported that the loss-of-function phenotype of the gene mrck-1/rag-39 is similar to cgef-1(gk261) with respect to cortical NMY-2 accumulation, suggesting that CDC-42 functions through MRCK-1 to influence cortical myosin association (Kumfer et al., 2010). If the observed suppression of par-2 by cgef-1 is a result of a reduction in cortical NMY-2 during polarity maintenance, then loss or depletion of mrck-1 should also suppress the embryonic lethality associated with loss or depletion of par-2. Embryos from par-2(lw32) were 98.8±1.6% lethal (n=562), whereas par-2(lw32); mrck-1(RNAi) worms yielded no viable embryos (n=512). We obtained similar results when we depleted PAR-2 by RNAi in wild type, cgef-1(gk261) and mrck-1(ok586): lethality of 93.9±0.4% (n=1609), 12.6±9.6% (n=1045) and 99±0.1% (n=1242), respectively. Thus, loss or depletion of MRCK-1 is not sufficient to suppress loss or depletion of par-2.

Although loss of cgef-1 affects cortical myosin accumulation independently of PAR-6, it is possible that loss of cortical myosin has an effect on the anterior PARs. If so, our expectation would be that the PAR-6 domain size in mrck-1 mutants, which lack cortical myosin accumulation at maintenance, would be larger than in wild type. Instead we found that the average PAR-6 domain size in pooled mrck-1(ok586) one-cell embryos in late prophase, metaphase and anaphase (56.5±4.7% egg length) was slightly smaller than that of wild type (59.5±5.5% egg length) and the difference was not statistically significant (n=30 for each genotype, P>0.05, two-tailed t-test).

**DISCUSSION**

Our analysis of relationships among PAR-2, LGL-1, cortical myosin accumulation and the anterior PAR proteins in polarity maintenance in the C. elegans zygote provides evidence that PAR-2 and LGL-1 influence myosin distribution via different mechanisms and that LGL-1 acts to reduce the overall level of PAR-6 in the embryo. It also reveals a third redundant pathway for polarity maintenance defined by the action of CHIN-1.

**Mutation of CGEF-1 reduces cortical myosin levels independent of the anterior PAR proteins**

Consistent with the data published by Kumfer et al. (Kumfer et al., 2010), we noted a substantial decrease in cortical NMY-2::GFP in cgef-1(gk261) compared with wild type at nuclear envelope breakdown (Fig. 8; n=21 for each genotype). The residual cortical myosin puncta appeared sparser than in wild type, but were still modestly enriched in the anterior. To determine whether the decrease in cortical myosin fibers was dependent on the anterior PAR proteins, we compared cortical NMY-2::GFP distribution in par-6(zu222) and cgef-1(gk261); par-6(zu222), and found that cortical NMY-2::GFP was reduced in cgef-1(gk261); par-6(zu222) embryos compared with par-6(zu222) embryos (Fig. 8; n=8 for each genotype). Results were similar using par-6(RNAi). Thus, unlike LGL-1, CGEF-1 regulates cortical myosin accumulation in a PAR-6-independent manner.

**Fig. 6. CHIN-1 and CGEF-1 appear to regulate the distribution of active CDC-42 in par-2(lw32); lgl-1(tm2616).** (A,C) Confocal midsection of GFP::GBDwsp-1 at NEB in a (A) wild-type embryo or a (C) par-2(lw32); lgl-1(tm2616) embryo treated with empty vector, chin-1(RNAi) or cgef-1(RNAi). (B,D) The percentage cortical enrichment of GFP::GBDwsp-1 on the anterior and posterior cortex±s.e.m. in (B) wild type and (D) par-2(lw32); lgl-1(tm2616) following the indicated RNAi treatment.

**amount was elevated 31.6±7.4% and the total was 27.2±8.1% greater than the controls (n=15). Furthermore, 40% (6/15) of lgl-1(tm2616); cgef-1(RNAi) had laterally skewed PAR-6 domains (Fig. 7C). Thus, CHIN-1 and CGEF-1 function to regulate the size of the PAR-6 cortical domain and PAR-6 amounts independent of LGL-1.**
2 regulated the size of the anterior domain without significantly affecting the amount of PAR-6 in the one-cell embryo. A role for PAR-2 in regulating PAR-6 through myosin is in apparent contradiction to a report that the actomyosin network is not required for polarity maintenance (Goehring et al., 2011a).

We propose, however, that PAR-2 has separate roles in regulating cortical myosin accumulation and anterior cortical domain size. We found that knockdown of MRCK-1, a likely effector of active CDC-42, fails to suppress par-2. Kumfer and colleagues (Kumfer et al., 2010) reported, and we confirmed, that a loss-of-function mutation in mrck-1 resulted in a robust decrease in cortical myosin during maintenance. If PAR-2 contributes to maintenance only by preventing aberrant cortical flow, we would predict that compromising the actomyosin network during maintenance would suppress par-2 mutants. Because this was not the case, we conclude that PAR-2 affects the size of the anterior PAR domain by another mechanism. However, because depletion of MRCK-1 partially blocks the ability of LGL-1 to compensate for the loss of PAR-2 (Beatty et al., 2010), we conclude that MRCK-1 does have a role in polarity maintenance.

**LGL-1 contributes to regulating the overall amount of PAR-6 in the one-cell embryo**

LGL-1 functions redundantly with PAR-2 to maintain polarity in the early embryo, and negatively regulates cortical myosin accumulation in the absence of PAR-2 (Beatty et al., 2010; Hoege et al., 2010). In contrast to our results with PAR-2, we found that the effect of LGL-1 on cortical myosin in embryos lacking PAR-2 was dependent on the anterior PAR proteins, precluding a direct role for LGL-1 in regulating cortical myosin, but consistent with a mutual elimination mechanism proposed by Hoege and colleagues (Hoege et al., 2010). However, our measurements of PAR-6 levels are not consistent with the mutual elimination model. If LGL-1 acts solely by removing the anterior PAR proteins at the boundary between anterior and posterior cortical domains, we expected that removing LGL-1 would lead to an increase in PAR-6 at the cortex and a reciprocal decrease in cytoplasmic PAR-6. Instead we observed an increase in both cortical and cytoplasmic levels of PAR-6 in the embryo, suggesting LGL-1 is involved in regulating cortical myosin, but consistent with a mutual elimination mechanism proposed by Hoege and colleagues (Hoege et al., 2010). Notably, whereas loss of LGL-1 results in anterior domain expansion in the absence of PAR-2 (Beatty et al., 2010), lgl-1(RNAi) in the presence of PAR-2 does not, despite increasing both cortical and cytoplasmic PAR-6 levels. Thus, limiting cytoplasmic pools of PAR proteins is likely not the only constraint on domain expansion (Goehring et al., 2011b).

Taken together, our results suggest that LGL-1 negatively regulates the overall amount of PAR-6 in the early embryo and thus may buffer against expansion of the anterior cortical domain (Dawes and Munro, 2011). Although the molecular mechanism by which LGL-1 acts remains elusive, we suggest that polarity maintenance in the one-cell *C. elegans* embryo may represent another instance where LGL functions as a molecular buffer, but in a method that is distinct from that reported for the peripheral nervous system of *Drosophila* (Wirtz-Peitz et al., 2008).
Three pathways maintain *C. elegans* polarity

**CHIN-1 and CGEF-1 are involved in controlling anterior domain size and PAR-6 levels**

CHIN-1 and CGEF-1 regulate cortical myosin localization during polarity maintenance, presumably by modulating the activity of CDC-42 (Kumfer et al., 2010). Additionally, these CDC-42 regulatory proteins appear to contribute to controlling the size of the PAR-6 cortical domain and the overall levels of PAR-6 in the early embryo. In agreement with their antagonistic roles in regulating CDC-42, we found that *cgef-1(RNAi)* caused a reduction in the PAR-6 cortical domain, whereas *chin-1(RNAi)* resulted in an expansion of the PAR-6 domain. However, depletion of either protein led to an increase in the overall amount of PAR-6 in the early embryo, suggesting there must be a regulatory mechanism curtailing anterior domain growth in *cgef-1(RNAi)* embryos, despite an excess of cytoplasmic PAR-6 relative to wild type. One likely explanation for this observation is that depletion of CGEF-1, through its effect on levels of activated CDC-42, reduces the ability of PAR-6 to localize to the cortex. It has previously been reported that PAR-6 must interact with CDC-42 in order to be robustly maintained at the cortex (Aceto et al., 2006). Modulating the affinity of the cortex for PAR-6 may provide an additional layer of regulation to a reaction diffusion mechanism of polarity maintenance. The reason for the increase in the overall amount of PAR-6 in both *chin-1(RNAi)* and *cgef-1(RNAi)* embryos is unclear, but may be evidence of a feedback loop between CDC-42 and the anterior PAR proteins.

We found that reducing the levels of active CDC-42 via *cgef-1* mutation during polarity maintenance results in a substantial decrease in cortical myosin, even in *par-6* mutants, suggesting that CDC-42 has two separable functions in polarity maintenance. In contrast to the role of CDC-42 in binding to PAR-6, the role of CDC-42 in cortical myosin regulation via MRCK-1 during polarity maintenance appears to be dispensable, consistent with the observation that the actomyosin cytoskeleton is not required for the maintenance of distinct cortical domains (Dawes and Munro, 2011; Goehring et al., 2011a; Goehring et al., 2011b).

**A third pathway for polarity maintenance**

Our results lead us to propose that there are at least three pathways contributing to polarity maintenance in the early *C. elegans* embryo: a primary pathway that requires PAR-2, a second redundant pathway that requires LGL-1 and a third independent pathway that requires CHIN-1, the putative CDC-42 GAP. This third pathway is either consistent with or supported by the following evidence: (1) *chin-1(RNAi)* blocks the ability of LGL-1 overexpression to rescue *par-2* loss-of-function mutations; (2) depletion of CHIN-1 in an *lgl-1-null* mutant resulted in modest but statistically significant synthetic lethality; (3) embryos lacking functional PAR-2 and LGL-1 exhibited persistent asymmetry of active CDC-42; (4) the distribution of active CDC-42 in the double *par-2; lgl-1* mutant is responsive to knockdown of CHIN-1 or CGEF-1; (5) loss of LGL-1 and depletion of CHIN-1 have an additive effect on the level of PAR-6::GFP and synergistic effect on the size of the PAR-6::GFP domain; (6) LGL-1 affects myosin levels through the anterior PAR proteins whereas CGEF-1 does not.

**Putting it all together**

In Fig. 9 we present a summary of the proposed relationships among LGL-1, PAR-2, myosin and regulation of CDC-42 during polarity maintenance. Negative regulatory activities of LGL-1, PAR-2 and CHIN-1 independently contribute to polarity maintenance, but each acts differently. LGL-1 acts by regulating PAR-6 cortical accumulation and overall levels. PAR-2 appears to have two independent roles, restricting the anterior PAR domain and preventing myosin accumulation in the posterior. Because eliminating myosin accumulation during maintenance by mutating MRCK-1 fails to suppress the absence of PAR-2, the main activity of PAR-2 must not require its blocking cortical myosin accumulation; unfortunately, the nature of that activity remains elusive. The role of CHIN-1, presumably acting through CDC-42, is minor compared with that of PAR-2.

A key finding is that CDC-42 has a dual role: a major role in regulating the size of the anterior cortical domain via its binding to PAR-6 and a minor role in regulating cortical myosin during maintenance. The basis for this conclusion is that *cgef-1* can suppress *par-2* mutation or depletion but lowering the level of MRCK-1, a downstream effector of CDC-42, fails to suppress *par-2* depletion; instead, it appears to enhance it. It is well established that lowering the steady state level of PAR-6 can suppress *par-2* mutations (Hyenne et al., 2008; Watts et al., 1996). Lowering active CDC-42 levels by mutation of *cgef-1* will affect both cortical PAR-6 accumulation (Aceto et al., 2006) and MRCK-1 activity (Kumfer et al., 2010). We propose that the two effects exert unequal influence on polarity maintenance and that suppression of PAR-2 by CGEF-1 occurs because reduced active CDC-42 will restore the balance between the antagonistic forces of the anterior and posterior domains. Lowering MRCK-1 levels lowers the levels of cortical myosin in the anterior (Kumfer et al., 2010) but appears to have no effect on the size of the PAR-6 domain, consistent with the observation that the actomyosin cytoskeleton is not required for the maintenance of distinct cortical domains (Dawes and Munro, 2011; Goehring et al., 2011a; Goehring et al., 2011b). As mentioned above, however, the ability of *mrck-1(RNAi)* to partially block LGL-1 overexpression rescue of *par-2* argues that MRCK-1 normally contributes to maintaining the anterior cortical domain, although this contribution is non-essential in a normal genetic background.

An unexpected finding of our analysis is that the embryo has a mechanism to maintain an asymmetric distribution of active CDC-42 that is independent of PAR-2 and LGL-1. Whether this asymmetric CDC-42 activity is dependent on the centrosome-
mediated polarity signals remains to be tested. The relationship between CDC-42 activity and steady state PAR-6 levels is also unclear. Increasing posterior CDC-42 activity by depleting CHIN-1 results in expansion of the PAR-6 domain and increased amounts of cytoplasmic PAR-6. Although this could be explained if cortical PAR-6 was protected from turnover, this explanation would predict that lowering levels of active CDC-42 by depleting CGEF-1 should lower steady-state amounts of cytoplasmic PAR-6. Because the latter is not the case, we clearly have more to learn.

Overall, our results reveal an additional level of redundancy that ensures faithful polarization of the early embryo, clarify the role of CDC-42, and add to a growing body of evidence indicating that numerous regulatory processes function in concert to consistently establish and maintain cell polarity.

Acknowledgements
We thank Ed Munro for helpful suggestions on the manuscript, Rich McCloskey for valuable discussions and the Caenorhabditis elegans Genetics Center for strains.

Funding
This work was supported by grants from the National Institutes of Health (HD27689 and GM79112). Deposited in PMC for release after 12 months.

Competing interests statement
The authors declare no competing financial interests.

Supplementary material
Supplementary material available online at http://dev.biologists.org/lookup/suppl;doi:10.1242/dev.088310;+/-DC1

References
Fig. S1. Procedure to determine the domain size of PAR-6::GFP. (A-C) Confocal midsection of a representative PAR-6::GFP embryo at nuclear envelope breakdown. The red overlay on the embryo in B represents the threshold mask applied in order to measure the area of the PAR-6::GFP cortical domain. The red overlay in C shows the threshold mask used to measure the area of the embryo cross-section. (D,E) The area within the outline in D was designated as area of the cortical PAR-6::GFP domain and the area defined by the outline in E was designated as the area of the embryo.
Movie 1. Control showing distribution of LGL-1::GFP in lgl-1::gfp; par-2(lw32). Time-lapse florescence micrographs of lgl-1::gfp; par-2(lw32) embryos.

Movie 2. CHIN-1 is required for the cortical maintenance of LGL-1::GFP in lgl-1::gfp; par-2(lw32). Time-lapse florescence micrographs of lgl-1::gfp; par-2(lw32); chin-1(RNAi) embryos.
Table S1. Comparison of measured changes in cortical PAR-6::GFP levels using alternative methods for quantifying cortical PAR-6::GFP. Two alternative methods for calculating the cortical PAR-6::GFP signal were applied to the cgef-1(RNAi) and the par-2(RNAi) data sets. The column on the left shows the percentage change in cortical PAR-6::GFP and the associated s.e.m. obtained using the method of averaging a set of lines drawn on the cortex that is described in the Materials and methods. The column on the right shows the results obtained by using the average signal associated with the full area of the cortical signal. Both methods led to the same conclusions; the measured changes in PAR-6::GFP relative to the negative control were not significant using either method. The $P$ value associated with each comparison is shown in parentheses next to the measured change.

<table>
<thead>
<tr>
<th>Change in cortical signal (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>cgef-1(RNAi)</td>
</tr>
<tr>
<td>par-2(RNAi)</td>
</tr>
</tbody>
</table>

Table S2. Embryonic lethality in par-2; lgl-1 after depleting CGEF-1.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Embryonic lethality</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Genotype</strong></td>
<td><strong>Embryonic lethality</strong></td>
</tr>
<tr>
<td><em>par-2</em> allele</td>
<td><em>lgl-1</em> allele</td>
</tr>
<tr>
<td>lw32</td>
<td>tm2616</td>
</tr>
<tr>
<td>lw32</td>
<td>it31</td>
</tr>
<tr>
<td>it5</td>
<td>tm2616</td>
</tr>
<tr>
<td>it5</td>
<td>it31</td>
</tr>
</tbody>
</table>