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

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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), 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 (Boyd et al., 1996; Cuenca et al., 2003; Hao et al., 2006). Recently, LGL-1, the
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 \textit{lgl-1; par-2} double mutants have a stronger phenotype than \textit{par-2} alone. In \textit{par-2} mutants, the anterior PARs expand into the posterior but retain a graded distribution there. In \textit{lgl-1; 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 PAR-2 and LGL-1 act in polarity maintenance, the molecular mechanisms by which the proteins function are not well understood. Both PAR-2 and 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 mediated by the anterior PAR proteins. Hoege and co-workers have suggested that LGL-1 contributes to polarity maintenance via a mutual elimination mechanism (Hoege et al., 2010). This model proposes that PAR-6 and PKC-3 physically interact with LGL-1, likely near the interface of the anterior and posterior cortical domains. The interaction leads to phosphorylation of LGL-1 by PKC-3, which results in the cortical removal of the entire complex. In this way, LGL-1 could contribute to polarity maintenance by facilitating the cortical removal of PAR-6 and PKC-3 that otherwise would diffuse into the posterior cortical domain.

In this study, we investigate the interplay among LGL-1, PAR-2, cortical myosin and the anterior PAR protein PAR-6, and uncover a third redundant polarity maintenance pathway that includes the CDC-42 GTPase-activating protein CHIN-1.

**MATERIALS AND METHODS**

**Nematode strains**

Nematodes were grown using standard conditions (Brenner, 1974) and N2 (Bristol) was wild type. Mutations used in this analysis include \textit{par-2(it5)} (Kemphues et al., 1988), \textit{par-2(bw32)} (Cheng et al., 1995), \textit{par-2(ok1723)}, \textit{par-6(n222)} (Watts et al., 1996), \textit{unc-119(ed4)} (Maduro and Pilgrim, 1995), \textit{lgl-1(tm2616)}, \textit{lgl-1(it31)} (Beatty et al., 2010), \textit{cegf-1(gk261)} (Kumfer et al., 2010) and \textit{mrck-1(or586)} (Gally et al., 2009), \textit{par-2(ok1723)} was out-crossed to N2 five times. We also used the transgenes \textit{zds45 [Pnmy-2::nmy-2::gfp+ unc-119(+)]} (Nance et al., 2003), \textit{olsj40 [Ppie-1::mgfp::wp-1(G-protein binding domain) + unc-119(+)]} (Kumfer et al., 2010), \textit{its2056 [Pglg-1::lgl-1::gfp, unc-119(+)]} (Kumfer et al., 2010), \textit{its279 [Pglg-1::lgl-1::mchr + unc-119(+)]} (Beatty et al., 2010) and \textit{its167 [Ppie-1::mgfp::par-6 + unc-119(+)]} (Li et al., 2010).

**RNA interference**

RNAi was performed by feeding (Timmons et al., 2001). Adults were allowed to lay embryos on RNAi feeding plates, and the progeny were grown to adulthood prior to dissection (60-72 hours). All RNAi feedings were carried out at 25°C with the exception of experiments involving \textit{par-2(bw32)} and \textit{par-2(it5)}, which were carried out at 20°C and 16°C, respectively. In experiments in which \textit{PAR-6} was depleted and NMY-2::GFP was examined, we controlled for the effectiveness of \textit{PAR-6} knockdown by quantifying embryonic lethality prior to dissection and by monitoring loss of asymmetry in NMY-2::GFP during polarity maintenance. Any experiments with less than 100% lethality or residual NMY-2::GFP were excluded from the data sets.

**Imaging**

Differential interference contrast (DIC) and wide-field fluorescence images of live embryos were captured using a Leica DM RA2 microscope with a 63× Leica HCX PL APO oil immersion lens, a Hamamatsu ORCA-ER digital camera and Openlab software (Improvision/PerkinElmer, Waltham MA). Blastomere cross-sectional areas were measured using Openlab. Confocal images were obtained with a PerkinElmer UltraVIEW LCI confocal scanner with a Nikon Eclipse TE2000-U microscope using UltraVIEW Imaging Suite v5.5. The sections were stacked and processed using ImageJ and Adobe Photoshop CS4.

**Immunohistochemistry**

PAR-6 immunostaining was carried out according to Hung and Kemphues (Hung and Kemphues, 1999).

**Measuring cortical NMY-2 levels**

The fraction of the cortex with NMY-2::GFP signal was measured using ImageJ. Twelve confocal sections from the top of the cortex to the midsection and spaced at 1.0 μm were stacked. Threshold levels were selected such that most of the NMY-2::GFP fibers in each of the stacks were recognized as particles by ImageJ. Then the area of the particles relative to the total area of the embryo cross-section was calculated using ImageJ, and the ratio was referred to as the ‘fraction of the cortex with signal’. The same threshold was applied to all the data sets being analyzed, and the means were compared. As an additional method for quantifying cortical NMY-2::GFP signal, the average signal intensity across the area of two to three stacked cortical sections was measured using ImageJ, and these values were compared after subtracting the background. Means were compared using Welch’s \textit{t}-test. The threshold for statistical significance corresponded to a \textit{P}-value of 0.05. Both methods yielded similar results.

**Measuring PAR-6 levels**

Cortical and cytoplasmic PAR-6 levels were measured using ImageJ. For each embryo, three confocal sections (0.5 μm spacing) centered on the midsection were stacked. Signal for the entire embryo was assumed to be proportional to the signal measured for these stacked cross-sections. To determine the average cortical PAR-6 signal, nine one-pixel-wide lines of roughly equal length were traced along the cortical domain (three near the anterior pole and six laterally), and measured the average signal intensity for each. The average signal intensities were averaged after subtracting the background signal to yield an average cortical signal for the embryo. The average cytoplasmic signal was determined similarly using three lines per embryo. The area of the embryo cross-section occupied by the cortical domain was determined by selecting a signal threshold so that the cortical PAR-6::GFP signal, and not the cytoplasmic signal, was recognized as a particle by ImageJ, and by measuring the corresponding area (see supplementary material Fig. S1). The cortical signal was determined by multiplying the area of the cortical domain by the average cortical signal. To validate this approach, we also calculated the average cortical signal by multiplying the mean signal intensity for the cortical domain by the area of the cortical domain for some of the data sets (see supplementary material Table S1). The cytoplasmic signal was determined by multiplying the total area of the cross-section minus the area of the anterior cortical domain by the average cytoplasmic domain signal. Three independent sets of 5 to 10 embryos each were examined for each genotype, except for \textit{par-2(RNAi)} and \textit{lgl-1(tm2616); cgef-1(RNAi)}, in which two sets were used. Means for individual experiments were compared using Student’s \textit{t}-test. Results for independent trials were combined using Fisher’s method.

**Measuring cortical enrichment of GFP::GDBwsp-1**

Cortical enrichment of GFP::GDBwsp-1 was measured as above for PAR-6. The anterior and posterior signal intensities were determined by drawing three lines on the anterior or posterior cortex, respectively, and measuring the average signal intensity for each. These values were averaged and the background subtracted to yield the cortical signal. The cytoplasmic signal was determined similarly.

**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 \textit{par-2(RNAi)} embryos, LGL-1 negatively regulates the posterior cortical accumulation of myosin during polarity...
compared with par-2 in the cortical area occupied by myosin in Materials and methods). Although we can readily detect an increase in wild-type and par-2 cortical NMY-2::GFP levels in the presence or absence of LGL-1 in consequence of the removal of the anterior PAR proteins from the cortex with NMY-2::GFP signal. Anterior is towards the left in all figures.

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) 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.,
Table 1. Comparison of cortical myosin levels

<table>
<thead>
<tr>
<th>Genotype</th>
<th>n</th>
<th>Area method</th>
<th>Signal intensity method</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>7</td>
<td>0.27±0.09</td>
<td>42.0±4.8</td>
</tr>
<tr>
<td>lgl-1(tm2616)</td>
<td>8</td>
<td>0.24±0.07 (P=0.70)</td>
<td>41.0±5.3 (P=0.69)</td>
</tr>
<tr>
<td>par-2(RNAi)</td>
<td>10</td>
<td>0.25±0.10</td>
<td>41.5±5.7</td>
</tr>
<tr>
<td>par-2(tm2616); lgl-1(tm2616)</td>
<td>10</td>
<td>0.20±0.07 (P=0.24)</td>
<td>37.9±6.4 (P=0.20)</td>
</tr>
<tr>
<td>par-6(RNAi)</td>
<td>15</td>
<td>0.21±0.08</td>
<td>42.1±5.6</td>
</tr>
<tr>
<td>par-6(RNAi); par-2(lw32)</td>
<td>8</td>
<td>0.49±0.11 (P=2.8±10^-5)</td>
<td>54.9±5.2 (P=3.7±10^-5)</td>
</tr>
<tr>
<td>par-6(RNAi); par-2(lw32); lgl-1(tm2616)</td>
<td>10</td>
<td>0.43±0.14 (P=0.42)</td>
<td>52.5±7.1 (P=0.21)</td>
</tr>
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</table>

The values provided by the area method represent the average fraction of the embryo with NMY-2::GFP signal. P values shown compare wild type with lgl-1(tm2616) in row 2, par-2(RNAi) to par-2(RNAi); lgl-1(tm2616) in row 4, par-6(RNAi) with par-6(RNAi); par-2(lw32) in row 7 and par-6(RNAi); par-2(lw32) with par-6(RNAi); par-2(lw32); lgl-1(tm2616) in row 8.

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 chin-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); chin-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; chin-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); chin-1(RNAi) exhibited a characteristic Par-2 phenotype. In contrast to chin-1(RNAi) and lgl-1::gfp; par-2(lw32) embryos, lgl-1::gfp; par-2(lw32); chin-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); chin1-1(RNAi) (P=0.0004). We examined the subcellular localization of LGL-1::GFP in one-cell lgl-1::gfp; par-2(lw32); chin-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(it5ts) at the permissive temperature of 16°C, similar to lgl-1 (Beatty et al., 2010). Embryos from par-2(it5); chin1-1(RNAi) were 82.3±15.6% (n=1428) embryonic lethal compared with chin-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 chin-1 and lgl-1 are components of a common genetic pathway, and LGL-1 acts through CHIN-1. An alternative explanation is that chin-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 genetic pathway, we expected the embryonic lethality of lgl-1(tm2616); chin-1(RNAi) to be no greater than the sum of the embryonic lethality from lgl-1(tm2616) (1.7±0.3%; n=3787) and chin1-1(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); chin1-1(RNAi) yielded 5.0±2.0% lethality (n=3420; P<0.0001, χ² test assuming an expected lethality of 2.5% for lgl-1(tm2616); chin1-1(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 chin-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 depletion 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 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. 3C). 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=6×10^{-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=6×10^{-5}; n=24) in one of the lines and 23.7±6.1% (P<1×10^{-3}; 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±1.3%; 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=1×10^{-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...
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.

**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(tag-59) 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.

**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 functions relate to one another is not clear. However, our results rule out one possibility — that the accumulation of posterior myosin is a secondary consequence of the effect of PAR-2 on the anterior PAR proteins. Consistent with an earlier report examining par-2; par-6(RNAi) embryos (Munro et al., 2004), we found that par-2; par-6(RNAi) embryos have increased cortical myosin during polarity maintenance compared with par-6(RNAi). This observation is consistent with the previously proposed hypothesis that PAR-2 influences the size of the anterior domain indirectly by preventing the formation of myosin filaments in the posterior (Munro et al., 2004). Also consistent with this possibility, we observed that PAR-
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 \textit{par-2}. Kumfer and colleagues (Kumfer et al., 2010) reported, and we confirmed, that a loss-of-function mutation in \textit{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 \textit{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. However, because this regulation could affect cortical and cytoplasmic PAR-6 differentially, our experiment does not test the mutual elimination model. Notably, whereas loss of LGL-1 results in anterior domain expansion in the absence of PAR-2 (Beatty et al., 2010), \textit{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 \textit{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 \textit{Drosophila} (Wirtz-Peitz et al., 2008).
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; indeed, 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 mcrk-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.

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The authors declare no competing financial interests.

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