The *C. elegans* homolog of *Drosophila* Lethal giant larvae functions redundantly with PAR-2 to maintain polarity in the early embryo

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**SUMMARY**

Polarity is essential for generating cell diversity. The one-cell *C. elegans* embryo serves as a model for studying the establishment and maintenance of polarity. In the early embryo, a myosin II-dependent contraction of the cortical meshwork asymmetrically distributes the highly conserved PDZ proteins PAR-3 and PAR-6, as well as an atypical protein kinase C (PKC-3), to the anterior. The RING-finger protein PAR-2 becomes enriched on the posterior cortex and prevents these three proteins from returning to the posterior. In addition to the PAR proteins, other proteins are required for polarity in many metazoans. One example is the conserved *Drosophila* tumor-suppressor protein Lethal giant larva (Lgl). In *Drosophila* and mammals, Lgl contributes to the maintenance of cell polarity and plays a role in asymmetric cell division. We have found that the *C. elegans* homolog of Lgl, LGL-1, has a role in polarity but is not essential. It localizes asymmetrically to the posterior of the early embryo in a PKC-3-dependent manner, and functions redundantly with PAR-2 to maintain polarity. Furthermore, overexpression of LGL-1 is sufficient to rescue loss of PAR-2 function. LGL-1 negatively regulates the accumulation of myosin (NMY-2) on the posterior cortex, representing a possible mechanism by which LGL-1 might contribute to polarity maintenance.

**KEY WORDS:** PAR proteins, Asymmetric cell division, Polarity, *Caenorhabditis elegans*

**INTRODUCTION**

Polarity is critical for axis specification and for generating cell diversity during development. In metazoans, cell polarity is mediated in part by a conserved set of regulatory proteins, known collectively as the PAR (partitioning-defective) proteins. The one-cell *C. elegans* embryo establishes an anterior-posterior (A-P) axis shortly after fertilization and serves as a model for studying polarity (Goldstein and Macara, 2007; Schneider and Bowerman, 2003). The PAR proteins include the PDZ domain-containing PAR-3 and PAR-6, the atypical protein kinase PKC-3, the serine/threonine kinase PAR-1 and, in nematodes, the putative ubiquitin E3 ligase PAR-2.

In the *C. elegans* embryo, a cue associated with the sperm centrosome signals a local downregulation of contractile forces in the posterior (Motegi and Sugimoto, 2006), triggering a myosin II-dependent contraction towards the future anterior pole (Munro et al., 2004). The contraction generates cortical flows away from the paternal pronucleus, and serves to restrict PAR-3, PAR-6 and PKC-3 to the anterior of the one-cell embryo (Cheeks et al., 2004; Munro et al., 2004). As PAR-3, PAR-6 and PKC-3 become enriched on the anterior cortex, PAR-2 and PAR-1 are recruited to the posterior cortex (Cuenca et al., 2003). The asymmetric distribution of the actomyosin cytoskeleton toward the anterior gives rise to a transient cortical invagination called the pseudocleavage furrow that marks the boundary between the contractile anterior cortex and the smooth posterior cortex (Munro et al., 2004). Additionally, pseudocleavage represents the completion of polarity ‘establishment’ and marks the beginning of the ‘maintenance’ phase (Cuenca et al., 2003). During polarity maintenance, the anterior and posterior PAR proteins act in a mutually antagonistic manner to perpetuate the cortical asymmetries generated by the cortical flows (Cheeks et al., 2004; Cuenca et al., 2003; Munro et al., 2004). PAR-2 prevents the return of the anterior PAR proteins to the posterior cortex by inhibiting a flow of cortical cytoplasm directed towards the posterior (Cheeks et al., 2004; Munro et al., 2004). In turn, phosphorylation by PKC-3 antagonizes the cortical localization of PAR-2, preventing the posterior PAR-2 domain from extending into the anterior (Hao et al., 2006).

Although most of the PAR proteins have been shown to be crucial polarity components in a variety of animal systems (Goldstein and Macara, 2007), PAR-2 is puzzling because it appears to be nematode specific. A possible answer to this puzzle is that PAR-2 has taken on another function in nematodes that is more commonly carried out by another protein, or proteins, in other polarity systems. In many systems, the PAR proteins interact with a number of other polarity modules, one of which includes the conserved tumor-suppressor protein Lethal giant larvae [Lgl; also known as L(2)gl] (Betschinger et al., 2003; Plant et al., 2003; Vasioukhin, 2006; Wirtz-Peitz and Knoblich, 2006; Yamanaka et al., 2003). The overall sequence and domain structure of Lgl are well conserved in metazoans. Lgl family members contain a characteristic C-terminal ‘Lgl domain’ that is not predicted to have a catalytic function. The protein includes a highly conserved series of aPKC phosphorylation consensus sequences (Vasioukhin, 2006). The N-terminal domain of Lgl homologs typically contains a series of WD-40 repeats that are predicted to form consecutive seven-bladed β-propeller structures. β-propeller structures are often involved in scaffolding protein-protein interactions (Vasioukhin, 2006).
Lgl is required in a number of polarized cell types (Vasiosoukhin, 2006; Wirtz-Peitz and Knoblich, 2006). In *Drosophila*, Lgl is involved in the maintenance of polarity in epithelial cells (Bilder et al., 2000; Hutterer et al., 2004). In *Drosophila* embryonic epithelial cells, Lgl is primarily localized to the basolateral membrane, where it contributes to the maintenance of polarity by restricting apical proteins to the appropriate cortical domain (Hutterer et al., 2004). Lgl acts by antagonizing the activity of apical protein complexes that consist of Par-6–Bazooka(Par-3)–aPKC and Crumbs-Stardust-Patj (Hutterer et al., 2004; Tanentzapf and TePas, 2003). Similarly, the apical complexes act to inhibit Lgl function on the apical membrane (Hutterer et al., 2004). This antagonistic relationship results in the maintenance of distinct cortical domains and is reminiscent of the mutual exclusion feedback loop that facilitates polarity maintenance in the *C. elegans* embryo.

*Drosophila* Lgl also plays a role in asymmetric cell division (Ohshiro et al., 2000; Peng et al., 2000; Wirtz-Peitz et al., 2008). In neuroblasts, Lgl is required for the basal targeting of fate determinants prior to mitotic division and, in this context, plays a role in the formation of polarity early in mitosis (Ohshiro et al., 2000; Peng et al., 2000; Wirtz-Peitz and Knoblich, 2006). Lgl may be involved in spindle positioning in neuroblasts (Albertson and Doe, 2003) and is also involved in the asymmetric cell divisions of sensory organ precursors, where the protein is required for asymmetric localization of the cell-fate determinant Numb (Wirtz-Peitz et al., 2008).

Recent studies have shown that Lgl is involved in the polarization of the A-P axis in the *Drosophila* oocyte (Doerflinger et al., 2010; Fichelson et al., 2010; Li et al., 2008; Tian and Deng, 2008). In the early oocyte, Lgl is required for the proper translocation of cell fate determinants as well as the centrosomes (Fichelson et al., 2010; Tian and Deng, 2008). At mid-oogenesis, phosphorylation by aPKC restricts Lgl to the posterior of the oocyte, along with Par-1 (Tian and Deng, 2008). After the oocyte has been polarized into distinct cortical domains, Lgl is likely to stabilize the cortical localization of Par-1, and these proteins act to reciprocally inhibit the anterior Bazooka complex. As in other contexts, the mutual antagonism between proteins in opposing cortical domains serves to maintain polarity (Doerflinger et al., 2010).

Despite being a fundamental polarity component in a number of polarized cell types, the role of an Lgl homolog in *C. elegans* has not yet been determined. Here, we show that a *C. elegans* homolog of Lgl, LGL-1, functions redundantly with PAR-2 to maintain polarity, and provide evidence that LGL-1 acts by preventing the cortical accumulation of NMY-2 in the posterior cortex of the early embryo.

### MATERIALS AND METHODS

**Nematode strains**

Nematodes were cultured using standard conditions (Brenner, 1974). N2 (Bristol) was used as wild type. Mutations used in this analysis include *par-2* (ec2030), *par-2(it5) (Kemphues et al., 1988), par-2(lw32), *par-2(it87) (Cheng et al., 1995), nmy-2(ne1490), nmy-2(ne3410) (Liu et al., 2010), unc-119(ed4) (Maduro and Pilgrim, 1995), lgl-1(tm2616), provided by the National Bioresource Project at Tokyo Women’s Medical College, and lgl-1(it118) (this study). We confirmed that the tm2616 allele of lgl-1 is a 211 bp deletion with a 9 bp insertion that begins in the sixth intron and ends in the seventh intron. We determined the transcript produced by the tm2616 mutant using RT-PCR (First-Strand cDNA Synthesis Kit, Amersham) followed by sequencing. tm2616 was out-crossed to N2 six times.

We also used the transgene *zuls45[nmy-2::NMY-2::GFP] (Nance et al., 2003). lgl-1 transgenic strains created for this study are listed in Table 1.

**RNA interference (RNAi)**

RNAi was performed by feeding (Timmons and Fire, 1998), with the exception of *lgl-1(RNAi)* in *par-2(it5)*, which was performed either by feeding or by injection (Fire et al., 1998). All RNAi feeding experiments involving *par-2* mutants were performed at 16°C, and the worms were allowed to feed for 48-60 hours. All other RNAi experiments were performed at 25°C, and worms were allowed to feed for 36 hours prior to imaging or immunostaining.

**Imaging**

Images of live embryos using either differential interference contrast (DIC) or wide-field fluorescence microscopy 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). Blastomere cross-sectional areas were measured using Openlab. Fixed embryos were imaged using a Leica TCS SP2 system with a Leica DMRE-7 microscope and an HCX PL APO 63× oil-immersion lens. Images were processed using Leica Confocal Software and Adobe Photoshop CS4. The images in Fig. 8 were captured with a PerkinElmer UltraVIEW LCI confocal scanner with a Nikon Eclipse TE2000-U microscope using UltraView Imaging Suite v5.5. The step size was 1 μm with 10-14 sections per stack. The sections were stacked and processed using ImageJ and Adobe Photoshop CS4.

**LGL-1 polyclonal antibody production**

A C-terminal fragment of LGL-1 (amino acids 490-941) fused to GST was used to generate a polyclonal antibody in guinea pigs. Antibody production was performed by Pocono Rabbit Farm & Laboratory (Canadensis, PA, USA). Crude serum was blot-affinity purified using GST-LGL-1(490-941) prior to use.

**Immunohistochemistry**

Immunostaining of PAR-2, GFP, LGL-1 and PKC-3 in embryos was performed using methanol fixation as described (Guo and Kemphues, 1995). Primary antibodies used include: anti-PAR-2 rabbit polyclonal (Boyd et al., 1996); anti-GFP goat polyclonal (Rockland Immunochemicals); anti-PKC-3 rat polyclonal (Hun and Kemphues, 1999) and anti-LGL-1 guinea pig (this study). Secondary antibodies used

### Table 1. Transgenic strains used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
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<tbody>
<tr>
<td>KK1019</td>
<td>unc-119(ed3); <em>zuls45[nmy-2::NMY-2::GFP + unc-119(+)]</em>; lgl-1(tm2616)</td>
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<tr>
<td>KK1063</td>
<td>itls256[plg1-1::lgl-1::gfp + unc-119(+)]; unc-119(ed4); lgl-1(tm2616)</td>
</tr>
<tr>
<td>KK1075</td>
<td>itls279[plg1-1::lgl-1::mCherry + unc-119(+)]; unc-119(ed4); lgl-1(tm2616)</td>
</tr>
<tr>
<td>KK1077</td>
<td>itls281[plg1-1::lgl-1::S661E;S665E;T669E::mCherry + unc-119(+)]; unc-119(ed4); lgl-1(tm2616)</td>
</tr>
<tr>
<td>KK1080</td>
<td>itls282[plg1-1::lgl-1::S661E;S665E;T669E::mCherry + unc-119(+)]; unc-119(ed4); lgl-1(tm2616)</td>
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<tr>
<td>KK1089</td>
<td>itls256[plg1-1::lgl-1::gfp + unc-119(+)]; unc-119(ed4); par-2(it5); lgl-1(tm2616)</td>
</tr>
<tr>
<td>KK1090</td>
<td>itls285[plg1-1::lgl-1::S677N::gfp + unc-119(+)]; unc-119(ed4); par-2(it5);sc1 [dpy-1(s2171)]; lgl-1(tm2616)</td>
</tr>
<tr>
<td>KK1115</td>
<td>itls285[plg1-1::lgl-1::S677N + unc-119(+)]; unc-119(ed4); lgl-1(tm2616)</td>
</tr>
<tr>
<td>KK1116</td>
<td>itls281[plg1-1::lgl-1::mCherry::S661E;S665E;T669E + unc-119(+)]; unc-119(ed4); par-2(it5);sc1 [dpy-1(s2171)]; lgl-1(tm2616)</td>
</tr>
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<td>KK1117</td>
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</tr>
<tr>
<td>KK1118</td>
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</tr>
<tr>
<td>KK1119</td>
<td>par-2(lw32), unc-45(ce286ts), itls256[plg1-1::lgl-1::gfp + unc-119(+)]; unc-119(ed4)</td>
</tr>
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</table>
include: donkey anti-rat Cy3, donkey anti-rabbit Cy3, donkey anti-goat Cy3, goat anti-guinea pig Cy3 (Jackson Laboratories, West Grove, PA, USA) and donkey anti-goat Alexa 488 (Invitrogen). Samples were mounted using VectaShield with DAPI (Vector Laboratories).

Transgenic strains

lgl-1 transgenic constructs were generated by fosmid recombinocuring using a galK-positive/counterselection cassette (Warming et al., 2005). To begin, the galK cassette (Warming et al., 2005) was amplified using primers that include 75 bp arms on the 5’ ends that are homologous to the regions flanking the region of genomic DNA to be modified. The purified PCR product was then transformed into SW102 cells containing a fosmid that included lgl-1 (WRM065B311). Homologous recombination was induced and recombinants were selected as described previously (Warming et al., 2005). Next, another cassette containing the region to be inserted flanked by the 75 bp homology arms was generated either using PCR (as was the case for fluorophores) or by annealing two homologous single-stranded primers (as was the case for mutations). The purified cassette was transformed, homologous recombination was induced, and recombinants were selected as previously (Warming et al., 2005). After making the desired modification to lgl-1 in the fosmid, the gene as well as the upstream and downstream intergenic regions was recombinocered into pJKL702 (unc-119 in pBSIIK+), kindly provided by Kelly Liu, Cornell University. Approximately 500 bp homology regions, corresponding to the regions directly downstream of the gene upstream of lgl-1 (X:872011-872531) and the region directly upstream of the gene downstream of lgl-1 (X:863852-864392), were cloned into pJKL702 adjacent to one another in the same orientation. The vector was then linearized by cutting between the homology regions and used as a cassette for recombinocering. Constructs were transformed using microapticle bombardment (Praitis et al., 2001). At least two independent integrated transgenic lines were examined for each construct. For the KK1080 strain (see Table 1), we sequenced the transgene expressed in the line after bombardment to ensure that the correct construct was transformed. One line for each construct was used to test for rescue.

RESULTS

Loss of lgl-1 function enhances the maternal-effect embryonic lethality of weak par-2 mutants

The C. elegans gene F56F10.4 (lgl-1) is predicted to encode a protein homologous to Lgl (Vasioukhin, 2006). Although clearly a member of the Lgl family, lgl-1 is more diverged at the primary sequence level than other family members [13.7% identical to Drosophila lgl and 14.1% identical to mouse Lgl1 (Mgl1)]. To determine whether lgl-1 is required for polarity in the early embryo, we examined nematodes homozygous for a deletion/insertion allele of lgl-1, tm2616. tm2616 is predicted to result in a frameshift after codon 342, causing a premature stop codon after amino acid 350 (Fig. 1). Embryos from lgl-1(tm2616) mutants were 99.2±0.4% embryonic viable (Fig. 2A, n = 15). Furthermore, as in wild type, the second cleavages in lgl-1(tm2616) embryos were asynchronous, and the mitotic spindle was oriented transversely in the AB cell and longitudinally in the P1 cell (Fig. 2C, n = 15). Thus, in contrast to its essential role in other polarity systems (Vasioukhin, 2006; Wirtz-Peitz and Knoblich, 2006), LGL-1 function is dispensable for polarity in the C. elegans embryo.

In C. elegans, the putative ubiquitin E3 ligase PAR-2 is required to maintain polarity in the early embryo by restricting the distribution of the anterior polarity proteins PAR-3, PAR-6 and PKC-3 (Cuenca et al., 2003; Hao et al., 2006; Kemphues et al., 1988). Of the PAR proteins, only PAR-2 is not conserved outside of nematodes (Goldstein and Macara, 2007). Because the role of Lgl proteins in polarity in other animals is analogous to that of PAR-2 in C. elegans, we hypothesized that C. elegans LGL-1 functions redundanty with PAR-2. To test this hypothesis, we used RNAi to deplete LGL-1 in par-2(it5) at the permissive temperature of 16°C. We noted a dramatic enhancement of embryonic lethality. Embryos from homozygous par-2(it5) are predominantly viable at the permissive temperature, but the mutants exhibit strong temperature sensitivity (Cheng et al., 1995). At permissive temperature, RNAi control par-2(it5) worms exhibited 15.4±4% embryonic lethality (n = 878). By contrast, the embryonic lethality of par-2(it5) treated with lgl-1(RNAi) was 97.0±0.5% (Fig. 2A, n = 1252). Furthermore, par-2(it5): lgl-1(tm2616) double mutants were 100% maternal-effect embryonic lethal at the permissive temperature (Fig. 2A, n = 1000). This enhancement was not allele specific: lgl-1(tm2616) in double mutant combination with the maternal-effect sterile par-2 alleles e2030 or i887 (Cheng et al., 1995; Kemphues et al., 1988) gave over 99% maternal-effect embryonic lethality (Fig. 2B, n = 765 or 857, respectively).

In addition, we determined that depleting another C. elegans homolog of Lgl, tom-1 (Vasioukhin, 2006), could not enhance par-2(it5) lethality. RNAi control par-2(it5) worms gave 10.3±12% embryonic lethality (n = 715), whereas par-2(it5) worms treated with tom-1(RNAi) showed 14.9±10.3% lethality (P = 0.50, n = 839).

Mutation of lgl-1 enhances par-2 polarity defects in the early embryo

Although loss of lgl-1 function enhanced the maternal-effect embryonic lethality of hypomorphic par-2 mutants, it was unclear whether the embryonic lethality was a result of enhanced early embryonic polarity defects or whether it revealed a cryptic role for the proteins in later embryogenesis. We used DIC microscopy to examine the first two mitotic divisions of embryos from par-2(it5); lgl-1(tm2616) mutants at permissive temperature. At this temperature, most embryos from par-2(it5) mothers divided asymmetrically (n = 18/19) and exhibited an asynchronous second mitotic division with spindles oriented transversely in AB and longitudinally in P1 (Fig. 2C, n = 10/13). By contrast, all embryos from par-2(it5); lgl-1(tm2616) exhibited a strong par-2 mutant phenotype (Fig. 2C) (Cheng et al., 1995; Hao et al., 2006; Kemphues et al., 1988); the double mutant embryos exhibited a symmetrical first cleavage (the AB blastomere accounted for 49.9±1.9% of the total area of the two-cell embryo) and a synchronous second cleavage (Fig. 2C, n = 12/12). Additionally, the mitotic spindles in both the AB and P1 cells were of transverse orientation with respect to the longitudinal axis of the embryo (Fig. 1C, n = 12/12). Therefore, compromising lgl-1 function enhances the polarity defects associated with par-2 in the early embryo.

Fig. 1. The C. elegans Lgl protein (LGL-1). The conserved Lgl domain [Pfam LGL2 (Kato, 2004)] spans amino acids 273-368. Conserved serines 661 and 665 and threonine 669 (asterisks) are the sites of the LGL-1 maternal-effect sterile (Mgl1) phenotype (Fig. 2C) (Cheng et al., 1995; Hao et al., 2006; Kemphues et al., 1988). The mutant and the frameshift mutants were 765 or 857, respectively).

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LGL-1 is asymmetrically localized to the posterior of the one-cell embryo and to the basolateral cortex in epithelial cells

To determine the subcellular localization of LGL-1 in the early embryo, we generated transgenic lines that express gfp- and mCherry-tagged lgl-1 under the control of its endogenous promoter (see Materials and methods). Similar to PAR-2 (Boyd et al., 1996), LGL-1::GFP and LGL-1::mCherry localized asymmetrically to the posterior cortex of the one-cell embryo (Fig. 3A; see Movie 1 in the supplementary material; see below). Unlike PAR-2, however, low levels of LGL-1::GFP were present throughout the cortex just prior to polarization, and the anterior localization of the protein persisted even as it became enriched at the posterior (see Movie 1 in the supplementary material).

The fluorescently tagged transgenes rescued the enhanced maternal-effect embryonic lethality of par-2(it5) by lgl-1(tm2616) at the permissive temperature. For example, expression of LGL-1::GFP in par-2(it5); lgl-1(tm2616) mutants resulted in 98% viable embryos (Fig. 3B, n=927). Immunostaining fixed embryos with a polyclonal antibody raised against the N-terminus of LGL-1 confirmed the localization pattern of LGL-1 (see Fig. S1 in the supplementary material). The subcellular localization of LGL-1 to the posterior cortex in the early embryo is consistent with the hypothesis that LGL-1 acts redundantly with PAR-2 to maintain polarity.

We also observed that LGL-1::GFP localized asymmetrically in differentiated epithelial cells. In the elongating embryo, LGL-1::GFP localized to the basolateral cortex of gut and epidermal cells (Fig. 4). This subcellular distribution suggests that LGL-1 has a role in polarity in differentiated epithelial cells in addition to its role in the early embryo. Because the embryos from lgl-1(tm2616) are viable, the function of lgl-1 in these epithelial tissues, if any, is likely to be redundant.

PKC-3 is required for the asymmetric cortical localization of LGL-1

In asymmetrically dividing Drosophila neuroblasts, migrating fibroblasts and polarized mammalian epithelial cells, Lgl is a substrate for aPKC (Betschinger et al., 2003; Plant et al., 2003; Yamanaka et al., 2003). In Drosophila, phosphorylation of Lgl on a series of conserved aPKC consensus sites results in an intramolecular association between the N-terminal and C-terminal domains of the protein, resulting in cortical disassociation and, presumably, inactivation (Betschinger et al., 2005).

C. elegans LGL-1 includes a highly conserved motif that contains three putative aPKC phosphorylation sites (S661, S665 and T669), suggesting that LGL-1 might be similarly regulated by the aPKC homolog PKC-3 in the early embryo (Fig. 1) (Vasioukhin, 2006). To test this hypothesis, we used RNAi to deplete PKC-3 and monitored the localization of LGL-1::GFP. After depleting PKC-3, LGL-1::GFP was no longer restricted to the posterior cortex following polarity establishment (Fig. 5A; see Movie 2 in the supplementary material). Thus, PKC-3 is required for the asymmetric localization of LGL-1::GFP.

To determine whether the putative PKC-3 phosphorylation sites are required for LGL-1 asymmetry, we generated transgenic lines expressing a mutant form of LGL-1::mCherry (LGL-13A::mCherry) in which the three putative PKC-3 phosphorylation sites are mutated to alanine. Consistent with the expected role of these conserved serines, LGL-13A::mCherry was strongly cortical prior to, and failed to become asymmetric during, polarity establishment. Instead, the mutant protein remained uniformly distributed throughout the cortex (Fig. 5B). Additionally, the cortical signal of LGL-13A::mCherry appeared more intense relative to the cytoplasmic signal when compared with wild type (Fig. 5B). LGL-13A::mCherry failed to rescue the enhancement of par-2(it5) by lgl-1(tm2616) at the permissive temperature: both par-2(it5); lgl-1(tm2616) and par-2(it5); lgl-1(tm2616) expressing LGL-13A::mCherry were 100% maternal-effect embryonic lethal (n=1000 for each genotype). Furthermore, par-2(it5); dpy-1(s2171); lgl-1(tm2616) worms expressing LGL-13A::mCherry were 91±4.0% viable at the permissive temperature, suggesting that ectopic localization of LGL-13A::mCherry at the anterior of the one-cell embryo did not substantially affect embryonic viability in a dominant-negative manner (n=587).

Fig. 2. Loss of lgl-1 function enhances weak par-2 mutants. (A) The percentage of C. elegans embryos of the indicated genotypes that failed to complete embryogenesis. Error bars indicate s.d. (B) Percentage embryonic lethality of weak par-2 alleles compared with the respective par-2; lgl-1 double mutant. The lgl-1 mutation used is tm2616. (C) DIC images of two-cell embryos during interphase (left column) and prior to the second mitotic division (right column). The black lines mark the orientation of the mitotic spindles. Anterior is to the left in all figures.
We also generated a phosphomimetic mutant (LGL-1S661E;S665E;T669E, or LGL-1S3E) by mutating the potential PKC-3 phosphorylation sites to glutamic acid. We expected this mutant to be cytoplasmic. However, although cytoplasmic levels were clearly higher than for wild-type LGL-1::mCherry, LGL-1S3E::mCherry was still detectable at the cortex (Fig. 5B). Expression of LGL-1S3E::mCherry failed to rescue the enhancement of par-2(it5) by lgl-1(tm2616); embryos from par-2(it5); lgl-1(tm2616); lgl-1S3E::mCherry exhibited 100% lethality (n=1000).

We conclude that one or more of the three putative PKC-3 phosphorylation sites is required for LGL-1 asymmetry, consistent with the hypothesis that phosphorylation of LGL-1 by PKC-3 negatively regulates the cortical accumulation of LGL-1 in the anterior.

**Overexpression of LGL-1 is sufficient to rescue PAR-2 loss of function**

par-2(lw32) is a strong allele that produces a truncated PAR-2 protein of a predicted 233 amino acids (Levitan et al., 1994) that lacks the domain required for cortical localization (Hao et al., 2006). If LGL-1 and PAR-2 function redundantly, we hypothesized that overexpression of LGL-1 might be sufficient to rescue the lethality of par-2(lw32). To test this, we crossed the lgl-1::gfp transgene into par-2(lw32) and quantified embryonic lethality of par-2(lw32) expressing LGL-1::GFP. Embryos from par-2(lw32) exhibited 98.0±0.4% lethality (n=2216). By contrast, the embryonic lethality of par-2(lw32); lgl-1::gfp was 5.7±0.6%, suggesting that overexpression of LGL-1 robustly rescued par-2 loss of function (Fig. 6A, n=1128).

par-2(lw32) produces a truncated protein and might not be a true functional null. To confirm that expression of the lgl-1::gfp transgene bypassed the need for PAR-2, rather than acting through residual PAR-2, we treated par-2(lw32); lgl-1::gfp worms with par-2(RNAi). As expected, if par-2(lw32) were a functional null, par-2(RNAi) did not enhance the embryonic lethality of par-2(lw32) (Fig. 6A, n=2473). Furthermore, par-2(lw32); lgl-1::gfp treated with par-2(RNAi) had similar levels of embryonic lethality to those fed bacteria containing empty vector (Fig. 6A, n=2091). Assuming that par-2 RNAi removes any residual active PAR-2 in the lw32 background, we conclude that expression of the lgl-1::gfp transgene can bypass the need for PAR-2.

Finally, to confirm that the viability of par-2(lw32); lgl-1::gfp resulted from LGL-1 overexpression, we depleted LGL-1 in par-2(lw32); lgl-1::gfp using RNAi and scored embryonic lethality. Embryos from par-2(lw32); lgl-1::gfp; lgl-1(RNAi) worms exhibited 99.4±0.8% lethality, indicating that the rescue of par-2(lw32) was the result of LGL-1 overexpression (Fig. 6A, n=696). Embryos from par-2(lw32) worms treated with lgl-1(RNAi) exhibited 100% lethality, suggesting that the small percentage of viable embryos produced by the par-2(lw32) mutant can be attributed to LGL-1 function (Fig. 6A, n=917).

We also compared the cortical polarization of early embryos from par-2(lw32), par-2(lw32); lgl-1(tm2616) and par-2(lw32); lgl-1::gfp by immunostaining endogenous PKC-3. In one-cell wild-type embryos during anaphase, PKC-3 occupied 63.3±3.8% of the cortex (Fig. 6B, n=10). In par-2(lw32), PKC-3 extended significantly further into the posterior (84.2±9.8%, Fig. 6B; P=1.7×10^-4, n=10). Furthermore, PKC-3 was found throughout the entire cortex in par-2(lw32); lgl-1(tm2616) embryos (Fig. 6B; P=6.7×10^-4, n=8), consistent with the hypothesis that both PAR-2 and LGL-1 contribute to polarity maintenance. By contrast, the extent of cortex occupied by PKC-3 in par-2(lw32); lgl-1::gfp embryos was not significantly different than in wild type (65.5±7.0%, Fig. 6B; P=0.40, n=10), indicating that overexpression of LGL-1 can rescue the loss of par-2 function.

In wild-type two-cell embryos, PKC-3 is enriched on the anterior cortex of the AB blastomere and in the most anterior portion of P1 (Fig. 6C) (Tabuse et al., 1998). By contrast, PKC-3 was cortically...
localized in both AB and P1 in par-2(lw32) embryos, although the intensity of the PKC-3 signal was notably weaker in the posterior than in the anterior (Fig. 6C). Furthermore, in embryos from par-2(lw32); lgl-1(tm2616), PKC-3 was also localized to the cortex of both the anterior and posterior blastomeres during the two-cell stage, but the difference in signal intensity was less substantial (Fig. 6C). We quantified the difference and found that in par-2(lw32) two-cell embryos, the posterior to anterior signal ratio was 0.65±0.22 (n=8), whereas the ratio in par-2(lw32); lgl-1(tm2616) embryos was 0.97±0.32 (n=8), indicating that the distribution of PKC-3 is significantly more symmetrical in par-2(lw32); lgl-1(tm2616) than in par-2(lw32) (P=0.03). These data are consistent with the idea that PAR-2 and LGL-1 function redundantly to maintain polarity. Finally, in embryos from par-2(lw32); lgl-1::gfp, the asymmetrical cortical localization of PKC-3 was restored and PKC-3 was again enriched in the AB blastomere and in the most anterior portion of P1 (Fig. 6C). Embryos from par-2(lw32); lgl-1::gfp worms exhibited asymmetric first cell divisions (n=17/19), and the mitotic spindles of the second cell division were oriented in transverse to the A-P axis in the AB cell and along the A-P axis in the P1 cell (n=16/18). LGL-1::GFP was also asymmetrically localized in these embryos (see Movie 3 in the supplementary material).

We conclude that overexpression of LGL-1 is sufficient to restore viability in the absence of functional PAR-2 by rescuing the early embryonic failure in polarity maintenance of par-2 mutants.

it31 is a hypomorphic allele of lgl-1

par-2(e2030) was initially isolated in a strain with a maternal-effect embryonic lethal phenotype; however, the embryonic lethality of the strain was dependent on an additional locus linked to the X chromosome (K.K., unpublished). When separated from the X-linked locus, par-2(e2030) exhibits a maternal-effect sterile phenotype (Kemphues et al., 1988). The X-linked mutation it31 also enhanced the maternal-effect embryonic lethality of par-2(it5). At the permissive temperature, par-2(it5) single mutants and par-2(it5); (it31) double mutants displayed 4.9±2.8% (n=509) and 61.6±14.9% (n=1077) maternal-effect embryonic lethality, respectively (Fig. 7A). As a single mutant, it31 was viable (2.5±1.9% embryonic lethality, Fig. 7A, n=440) and did not display
any detectable mutant phenotype. Genetic mapping placed it31 at the left end of linkage group X at approximately –20 cM, very near lgl-1 at –19.5 cM, raising the possibility that it31 is a mutation in lgl-1. Sequencing revealed a missense mutation, S877N. Furthermore, it31 failed to complement lgl-1(tm2616) for the ability to enhance par-2(it5). An average of 99.8±0.3% of embryos from six par-2(it5); lgl-1(tm2616)/it31 mothers failed to hatch (n=636). Thus, it31 is an allele of lgl-1.

To determine the effect of the it31 S877N mutation on the subcellular localization of LGL-1 we generated transgenic lines expressing LGL-1S877N::GFP. In early embryos, LGL-1S877N::GFP localized very weakly to the posterior cortex when compared with LGL-1::GFP (Fig. 7B). Additionally, we stained endogenous LGL-1 in it31 embryos and observed that the cortical signal of LGL-1 was notably weaker than in the wild type (see Fig. S1 in the supplementary material). Taken together, these data suggest that the serine at position 877 is required for the normal cortical localization of LGL-1 or for stability of the protein. As expected for a hypomorphic mutation, LGL-1S877N::GFP suppressed the lethality of lgl-1(tm2616);par-2(it5ts) embryos less well than did LGL-1::GFP (Fig. 7A, n=970; compare with Fig. 3B).

Mutation of lgl-1 affects the cortical accumulation of NMY-2 during polarity maintenance

During the maintenance phase of polarity, PAR-2 is required to prevent the recruitment of NMY-2 to the posterior cortex (Cuenca et al., 2003; Munro et al., 2004). Because LGL-1 functions redundantly with PAR-2 during polarity maintenance, we hypothesized that LGL-1 might also affect the posterior cortical accumulation of NMY-2. To test this, we compared the localization of NMY-2::GFP in par-2(RNAi) and par-2(RNAi); lgl-1(tm2616) embryos during the first mitotic division. In wild-type embryos, NMY-2::GFP foci became asymmetrically distributed to the anterior half of the embryo during the establishment phase (Fig. 8A; see Movie 4 in the supplementary material). Around the time of pronuclear meeting, the NMY-2 foci were reorganized into finer filaments, which remained enriched in the anterior of the embryo (Fig. 8B) (Munro et al., 2004). During metaphase, NMY-2::GFP was mostly restricted to the anterior half of the embryo, although there was a patch of NMY-2::GFP that appeared at the posterior pole (Fig. 8C). In lgl-1(tm2616), the dynamics of NMY-2::GFP were similar to wild type (Fig. 8E,F; see Movie 5 in the supplementary material). In par-2(RNAi) embryos, establishment of NMY-2 asymmetry occurred relatively normally, although the cap of NMY-2::GFP foci extended further into the posterior than in wild type (Fig. 8G; see Movie 6 in the supplementary material) (Munro et al., 2004). The asymmetry failed to be maintained, and shortly after pseudocleavage NMY-2::GFP asymmetry was lost (Fig. 8H) (Munro et al., 2004). Around the time of nuclear envelope breakdown, however, NMY-2::GFP partially cleared from the posterior (Fig. 8I, n=10/12). NMY-2::GFP filaments extended into the posterior, but little NMY-2::GFP was observable on the posterior pole. In par-2(RNAi); lgl-1(tm2616) embryos, the dynamics of NMY-2::GFP were similar to that of par-2(RNAi) embryos until nuclear envelope breakdown (Fig. 8J,K; see Movie 7 in the supplementary material); thereafter, in contrast to par-2(RNAi)
embryos, the NMY-2::GFP filaments remained almost uniformly distributed around the cortex in par-2(RNAi); lgl-1(tm2616) embryos and, in most cases, failed to clear from the posterior (Fig. 8L, n=11/12). We quantified the extent of NMY-2::GFP clearing based on total embryo length in par-2(RNAi) and par-2(RNAi); lgl-1(tm2616). Although NMY-2 failed to clear in most par-2(RNAi); lgl-1(tm2616) embryos, if we included the few embryos that showed some clearing, the average clearing was 5.3±7.1%, compared with 21.8±11.1% in par-2(RNAi) (n=10, P=0.001). These data suggest that LGL-1 functions redundantly with PAR-2 to negatively regulate the accumulation of NMY-2 in the posterior of the one-cell embryo.

Because LGL-1 inhibits the accumulation of NMY-2::GFP in the posterior in par-2(RNAi) embryos, we hypothesized that decreasing the dose of NMY-2 in the early embryo might be sufficient to partially rescue par-2(it5); lgl-1(tm2616). To test this hypothesis, we used partial RNAi to reduce NMY-2 levels in par-2(it5); lgl-1(tm2616). NMY-2 was depleted such that a low level of lethality was observed in N2 (15.1±13.1%). Similar to par-2(it5); lgl-1(tm2616) controls, par-2(it5); lgl-1(tm2616); nmy-2(RNAi) was 100% embryonic lethal. Results were similar when we compromised NMY-2 function using either of two conditional nmy-2 alleles (Liu et al., 2010) at semi-restrictive temperature, suggesting that reduced NMY-2 function is insufficient to partially rescue par-2(it5); lgl-1(tm2616).

Having determined that LGL-1 influences the cortical accumulation of NMY-2, we screened genes identified in a previous study as affecting the cortical dynamics of the early embryo (Sonnichsen et al., 2005) to determine whether RNAi depletion of any of the genes blocked the ability of LGL-1::GFP to rescue par-2(lw32) mutants. We found several genes that caused higher levels of embryonic lethality in par-2(lw32); lgl-1::gfp relative to wild type. One of these, let-502, a homolog of the Rho-associated kinase ROCK (Piekn and Mains, 2002; Wissmann et al., 1997), also compromised early embryonic polarity in par-2(lw32); lgl-1::gfp (see Fig. S2 in the supplementary material). Specifically, five out of 12 par-2(lw32); lgl-1::gfp; let-502(RNAi) embryos had symmetrical first cleavages, and the mitotic spindles of the second cell division were oriented transversely to the A-P axis in both the AB and P1 cells (see Fig. S2 in the supplementary material, n=13/13). When LET-502 was depleted in N2, similar polarity defects were not observed (see Fig. S2 in the supplementary material; n=15/15) (see also Sonnichsen et al., 2005).

Having identified a requirement for let-502 for rescue of par-2(lw32) by overexpression of lgl-1, we also tested the possible requirement for myotonic dystrophy-related Cdc42-binding kinase homolog (MRCK-1). MRCK-1 is a potential downstream effector of CDC-42, and both LET-502 and MRCK-1 are involved in the cortical recruitment of NMY-2 in the one-cell embryo (Kumfer et al., 2010). In embryos from par-2(lw32); lgl-1::gfp; mrck-1(RNAi) we observed an increased frequency of symmetrical first cleavages (n=6/12) and the mitotic spindles of the second cell division were oriented transverse to the A-P axis in both the AB and P1 cell (n=12/12), but we did not observe similar defects in mrck-1(RNAi) embryos (see Fig. S2 in the supplementary material; n=15/15) (see also Sonnichsen et al., 2005).

**DISCUSSION**

**PAR-2 and LGL-1 function redundantly in polarity maintenance**

We have shown that LGL-1, the *C. elegans* homolog of Lgl, functions redundantly with PAR-2 to maintain polarity in the early embryo. Loss of LGL-1 function robustly enhances both the embryonic lethality and early polarity phenotypes of hypomorphic par-2 mutants. We also found that LGL-1 and PAR-2 colocalize in the early embryo, and overexpression of LGL-1 in a putative par-2 null was sufficient to restore embryonic viability and rescue the early polarity defects associated with par-2 loss of function. These results indicate that LGL-1 and PAR-2 function redundantly and suggest that the respective pathways to which the proteins belong must ultimately converge on a common target or set of targets.

We noted that LGL-1, in addition to posterior cortical localization in the early embryo, is strongly expressed in *C. elegans* epithelial cells and is localized basolaterally. However, lgl-1(tm2616) worms are viable and fertile with no obvious defects in epithelial function. Since we do not detect PAR-2 in epithelial cells and lgl-1(tm2616); par-2(lw32) worms exhibit only maternal-effect lethality, we speculate that a polarity protein other than PAR-2 functions redundantly with LGL-1 in epithelial cells or that LGL-1 has no function in these cells.

**The cortical asymmetry of LGL-1 is regulated by PKC-3**

Lgl proteins in flies and mammals are regulated via phosphorylation by aPKC (Betschinger et al., 2003; Plant et al., 2003; Tian and Deng, 2008; Yamanaka et al., 2003). Our results are consistent with conservation of this regulation in *C. elegans*. The asymmetric localization of LGL-1 depends on the aPKC homolog PKC-3, and mutating three conserved putative PKC-3 target sites in LGL-1 to alanines blocks asymmetry. However, mutating these three phosphorylation sites to glutamic acid yielded unexpected results. We hypothesized that the phosphomimetic mutant would fail to localize to the cortex. Instead, LGL-1*E3::mCherry localized cortically and symmetrically, in addition to being distributed to the cytoplasm. There are several possible explanations for this unexpected result. Perhaps glutamic acid insufficiently mimics a phosphate group to completely block the cortical localization of LGL-1. Another possibility is that residual cortical LGL-1 is the result of overexpression of the transgene relative to wild type lgl-1. Alternatively, regulation of the cortical localization of LGL-1 might depend on additional sites in the protein. Finally, it is possible that the conserved sites do not serve as phosphorylation sites in *C. elegans*.

**Two potential modes of LGL-1 action in C. elegans**

Currently, the molecular mechanism by which Lgl participates in polarity is not well understood. Results from *Drosophila* and mammalian cells suggest three non-mutually exclusive hypotheses to explain how LGL-1 could function: (Vasioukhin, 2006; Wirtz-Peitz and Knoblich, 2006). One hypothesis, based initially on work on the LGL-1 homologs Sro7/77 in yeast (Hattendorf et al., 2007; Vasioukhin, 2006; Wirtz-Peitz and Knoblich, 2006), is that LGL could regulate polarized vesicular trafficking. Additional evidence from metazoans supports this role: MGl binds a component of the exocytic machinery, syntaxin 4, in mammalian epithelial cells (Musch et al., 2002). The second hypothesis proposes that Lgl could negatively regulate the activity of non-muscle myosin II. *Drosophila* and human Lgl proteins bind non-muscle myosin II (Strand et al., 1994; Strand et al., 1995). Additionally, in *Drosophila* neuroblasts, reducing the dosage of the myosin II gene zipper suppresses the loss of basal protein targeting associated with the lgl mutation (Oshiro et al., 2000; Peng et al., 2000). Furthermore, Lgl may function in neuroblasts to restrict myosin to the apical cortex (Barros et al., 2003), although this may be...
facilitated indirectly by inhibition of aPKC activity at the basal cortex (Atwood and Prehoda, 2009). Finally, in asymmetrically dividing cells in the *Drosophila* nervous system (Atwood and Prehoda, 2009; Betschinger et al., 2003; Wirtz-Peitz et al., 2008), Lgl appears to function by regulating the activity of aPKC, either by inhibiting its activity (Atwood and Prehoda, 2009) or by altering its target specificity (Wirtz-Peitz et al., 2008). It accomplishes this, at least in part, by exchanging with PAR-3 in the PAR-6–aPKC complex (Wirtz-Peitz et al., 2008). A similar exchange with PAR3 also occurs in mammalian epithelial cells (Plant et al., 2003; Yamanaka et al., 2003).

The mode of action of LGL-1 in the early *C. elegans* embryo is unclear. Our evidence argues strongly that the major role of LGL-1 in the early embryo is in the maintenance, rather than establishment, of polarity. Thus, at the time that LGL-1 acts, it is not in a complex with PKC-3 and PAR-6 but acts to exclude these proteins from the posterior cortex. It is possible that LGL-1 prevents binding of PAR-6 and PKC-3 at the cortex by forming PAR-6–LGL-1–PKC-3 complexes that, in contrast to the situation in *Drosophila* and mammalian cells, can no longer bind cortically. In this model, the observed accumulation of myosin in the posterior of *lgl-1(tm2616); par-2(RNAi)* embryos is a consequence, rather than a cause, of the abnormal presence of the PAR-6–PKC-3–PAR-3 complex. However, if LGL-1 acted by promoting dissociation of PAR complexes from the cortex, we would expect to see dominant effects of mislocalizing LGL-1 to the anterior and we do not.

Alternatively, LGL-1 might have an activity that is independent of complex formation with PAR-6 and PKC-3, such as regulating membrane trafficking or myosin activity. Of these, regulating the recruitment of myosin or its activity at the cortex is most consistent with our data. Inhibition of myosin activity by Lgl was previously proposed in *Drosophila* embryonic neuroblasts (Peng et al., 2000). In *Drosophila* neuroblasts, lgl mutations can be suppressed by compromising myosin activity (Peng et al., 2000). We carried out similar experiments in *C. elegans* to no effect, however: reducing myosin activity using either temperature-sensitive *myo-2* mutations or weak *myo-2(RNAi)* failed to suppress the enhancing effects of loss of LGL-1 on par-2 mutants. Evidence that LGL-1 does act at least indirectly through myosin comes from our discovery that rescue of *par-2* mutants via LGL-1 overexpression is dependent upon the activities of Rho kinase (*let-502*) and *mrck-1*, a downstream effector of CDC-42. However, in contrast to the proposed role as an inhibitor of myosin contractility, the requirement for LET-502 and MRCK-1 argues that LGL-1 promotes myosin contractility. Perhaps, by blocking myosin accumulation in the posterior, LGL-1 indirectly promotes increased myosin accumulation and hence contractility in the anterior.

The discovery of a role for Lgl in polarity in *C. elegans* underscores the degree to which cell polarity mechanisms are conserved. The creation of a *C. elegans* strain that is completely dependent upon LGL-1 provides a new opportunity to explore the precise mode of action of this key protein.

Note added in proof

While this paper was under review, a similar analysis was published by Hoege and colleagues (Hoege et al., 2010).

Acknowledgements

We thank Wendy Hoose and Mona Hassab for technical support; Sylvia Lee for helpful comments on the manuscript; the National Bioresource Project for the experimental animal *C. elegans* and for *lgl-1(tm2716)*; the Biological Resources Branch at NCI for recombining reagents; the Caenorhabditis Genetics Center for providing worm strains; and Jun Kelly Liu for providing pJKL702. This research was supported by National Institutes of Health grants HD27689 and GM079112 to K.K. Deposited in PMC for release after 12 months.

Competing interests statement

The authors declare no competing financial interests.

Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.056028/-/DC1

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Development 21, 266-277.


