LGL Can Partition the Cortex of One-Cell Caenorhabditis elegans Embryos into Two Domains

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Summary

Many metazoan cell types are polarized by asymmetric partitioning of the conserved PAR (PAR-3/PAR-6/PKC-3) complex [1–5]. Cortical domains containing this PAR complex are counterbalanced by opposing domains of varying composition [6–10]. The tumor-suppressor protein LGL [11, 12] facilitates asymmetric localization of cell fate determinants, in part through modulating the activity of the PAR complex [13, 14]. However, the mechanisms by which LGL acts to maintain a cortical domain remain unclear. Here we identify Caenorhabditis elegans LGL in a biochemical complex with PAR proteins, which localize to the anterior cortex. But LGL itself localizes to the posterior cortex. We show that increasing the amounts of LGL can restrict localization of the PAR complex to an anterior cortical domain, even in the absence of PAR-2. Importantly, LGL must be phosphorylated on conserved residues to exert this function. LGL and the PAR complex can maintain two cortical domains that are sufficient to partition cell fate determinants. Our data suggest a mechanism of “mutual elimination” in which an LGL phosphorylation cycle regulates association of the PAR complex with the cortex: binding of LGL to the PAR complex at the interface of the two domains stimulates its phosphorylation by PKC-3, and the whole complex leaves the cortex.

Results and Discussion

Identification of Caenorhabditis elegans LGL

We purified protein complexes of the conserved protein PAR-6 by immunoprecipitation from synchronized gravid hermaphrodites and identified PAR-6 coprecipitating bands on gels after electrophoresis (Figure 1A). The identity of specifically copurifying species was analyzed by mass spectrometry [15]. The most abundant specific protein band was at 80 kDa and contained the Caenorhabditis elegans atypical protein kinase C (PKC-3); a band at about 120 kDa contained the protein F56F10.4. The closest homologs to F56F10.4 by BLAST are LGL family members, and, indeed, F56F10.4 shows characteristic phosphorylation sites [16, 17] conserved among LGL species (see Figure S1A available online). Therefore, we named f56f10.4 now lgl-1 and refer to it hereafter as lgl. We confirmed that F56F10.4 interacts with PAR-6 and PKC-3 in embryos by expressing GFP-F56F10.4 under the control of the pie-1 promoter and purifying GFP-F56F10.4 protein complexes containing PAR-6 and PKC-3 by immunoprecipitation (Figure 1B).

LGL Localizes Asymmetrically to the Posterior Cortex in C. elegans One-Cell Embryos

We investigated LGL localization by expressing genomic lgl (Figures S1A and S1B) fused to gfp under the control of the pie-1 promoter. After polarity onset, GFP-LGL becomes enriched at the posterior cortex and segregates after division to the posterior P1 cell (Figure 1D). This localization is similar to GFP-PAR-2 [8], and, indeed, both proteins colocalize on the posterior cortex domain in lines expressing both mcherry-PAR-2 and GFP-LGL (Figure 1E). We confirmed the posterior localization by using antibodies raised against LGL (see Experimental Procedures) in immunofluorescence experiments. This showed that LGL localizes to the posterior cortex (Figure 1F), similar to PAR-1 and PAR-2 localization [18, 19]. Therefore, we conclude that LGL is part of the posterior cortex domain.

Genetic Interaction of lgl with par-2 Alleles

We isolated lgl-1 alleles (Figures S1A–S1C and S1E) and found that lgl is not essential for survival and showed no phenotypic abnormalities in the first cell division. The extent of the PAR-6 domain on the cell cortex and nonmuscle myosin organization at the cortex was indistinguishable from wild-type (WT) (Figures S2B, S2D, and S2E). Epithelial integrity at later stages of development also appeared to be unaffected. Adherens junctions and GFP-PAR-6, which mark the apical side of cells in the developing intestine [20, 21], localize normally (Figure S2A). Because lgl is essential for cell polarity in other systems [16, 22–25] and LGL can regulate the subunit composition of the PAR complex [14], we looked for genetic interaction between LGL and the PAR proteins. We fed wild-type N2 or lgl-1(dd21) worms with par-2(RNAi) feeding bacteria for different times and scored the appearance of par-2-specific symmetric first divisions of the embryo. Surprisingly, we found that lgl-1(dd21) mutants are hypersensitive to par-2(RNAi) (Figures 2A and 2B). We further investigated this interaction using par-2 mutants. Although par-2ts(it5) mutants treated with a control RNA (i.e., klp-1(RNAi)) showed about 15% embryonic lethality at the semipermissive temperature (20°C), lethality increased to 87% in combination with lgl(RNAi) (Figures 2C and 2D). Similar lethality was observed when we crossed the lgl-1 alleles into the par-2ts(it5) background. Only about 5% of the par-2ts(it5) embryos failed to hatch at the permissive temperature (16°C), but this fraction increased to 97% in the double mutant, indicating that LGL function is essential in the par-2ts(it5) background (Figures 2E and 2F). Because LGL has been implicated in negative regulation of nonmuscle myosin activity in Drosophila [24, 26–28], we looked for genetic interaction between lgl and nmy-2 mutants. We observed a slight increase in embryo lethality in double mutants, although this was not statistically significant (Figure S2C). In addition, cortex contractility appears to be normal in lgl-1(dd22) mutants (Figure S2B). Therefore, LGL does not seem to directly affect contractility of the cell cortex and instead acts with PAR proteins.

LGL Activity Compensates for PAR-2 Depletion

To understand how LGL could act together with PAR proteins, we looked at whether LGL localization depends on PAR...
proteins. After depletion by RNA interference (RNAi) of either par-3 or pck-3, GFP-LGL behaved like PAR-2 [18], localizing throughout the cortex, and the embryos divided symmetrically (Figure 3A). In par-1(RNAi) embryos, GFP-LGL localization is not notably affected, but these embryos showed a par phenotype and divided symmetrically. However, in par-2(RNAi) embryos, GFP-LGL localization is also not notably affected, but embryos divide asymmetrically like in WT embryos (Figure 3B). This is surprising because par-2(RNAi) leads to a loss of endogenous LGL localization and a uniform localization of anterior PAR proteins to the cell cortex in otherwise WT embryos (Figures S3A and S3A'). Furthermore, GFP-LGL localizes to the cell periphery of cells of the P lineage, and asymmetric divisions of the P lineage and GFP-LGL localization are not changed by par-2(RNAi) (Figure 3B). We eliminated the possibility that GFP-LGL expression is preventing depletion of PAR-2 by par-2(RNAi) using immunoblots and obtained high embryonic lethality for wild-type N2 worms with both RNAi conditions as expected, we observed rescue of par-2/control(RNAi) for GFP-LGL-expressing worms (Figure 3D). However, when we silenced both par-2 and the gfp::lgl transgene by par-2/gfp(RNAi), we observed high embryonic lethality (Figure 3D), strongly arguing for suppression of the par-2 phenotype by GFP-LGL expression. A clue to explain how GFP-LGL expression suppresses the par-2 (RNAi) phenotypes came from immunofluorescence experiments. They showed that LGL fluorescence intensities on the posterior cortex of the one-cell embryo. (F) Immunofluorescence of LGL in embryos treated with control(RNAi), lgl(RNAi), or GFP-LGL-expressing embryos, or GFP-LGL embryos silenced only for the transgene by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry [15]. PAR-6 (36 kDa) stains only weakly by Coomassie. The 80 kDa band contained PKC-3, the 120 kDa band contained the protein of F56F10.4/lgl, and a third band at 180 kDa contained the protein of W07E11.1. * denotes IgG bands.

(B) GFP-LGL interacts with PKC-3 and PAR-6 in C. elegans embryos. Immunoblots were made from anti-GFP immunoprecipitation from GFP-LGL-expressing embryos.

(C) The LGL complex is sensitive to phosphatase treatment. Anti-GFP immunoprecipitations from GFP-LGL-expressing embryos were left untreated (−) or treated (+) with calf intestine phosphatase (CIP). Proteins were detected by immunoblot. (D) GFP-LGL localizes to the posterior cortex after polarity onset. Images are from time-lapse recording. Times are relative to nuclear envelope breakdown (0 s). Top row: differential interference contrast images; bottom row: GFP-LGL fluorescence. Note the blow-up of the anterior and posterior cortex region. (E) LGL colocalizes with PAR-2, mcherry-PAR-2 and GFP-LGL from a double fluorescent cell line colocalize on the posterior cortex of the one-cell embryo. (F) Immunofluorescence of LGL in embryos treated with control(RNAi), lgl(RNAi), or GFP-LGL-expressing embryos, or GFP-LGL embryos silenced only for the transgene by gfp(RNAi) (DNA, blue; microtubules, green; LGL, red). All images were taken with identical exposure conditions and processed identically. For quantifications, see Figure S1F.

immunofluorescence (Figure 3C; Figures S3B and S3C). Therefore, we conclude that GFP-LGL expression is suppressing the phenotype of par-2 (RNAi).

To further verify that par-2 (RNAi) rescue is indeed caused by transgenic GFP-LGL expression, we developed an assay in which we could specifically silence the transgene (for further details, see Experimental Procedures). We fed either wild-type or transgenic gfp::lgl worms an equal mix of par-2/control(RNAi) bacteria or par-2/gfp(RNAi) bacteria. gfp(RNAi) will deplete gfp::lgl but not endogenous lgl. Although we
LGL Activity Compensates for PAR-2 Depletion by Restoring Cell Polarity

We wanted to investigate whether embryo viability in GFP-LGL-expressing worms is restored by rescuing the asymmetric localization of the PAR complex on the cortex but also cell fate determinants in the cytosol. Indeed, mcherry-PAR-6 was restricted to the cortex (Figures S3D–S3I). We conclude that LGL partitions not only the PAR complex but also cell fate determinants in the cytosol.

P granules are cytosolic cell fate determinants. Their formation and localization depends on the activity of PAR proteins that localize to the posterior cortex [18, 19, 29, 30]. To test whether LGL expression could compensate for P granule defects seen in par-2-depleted embryos, we analyzed the P granule component PGL-1. GFP-LGL expression restored the normal size and shape of P granules, which accumulated in the posterior half of the embryo in par-2/control(RNAi) embryos (Figure 3I). However, when gfp::lgl is silenced by RNAi, P granules mostly disintegrate (Figure 3J). Because formation of P granules also requires PAR-1 activity [19, 30], we reasoned that PAR-1 localization to the cortex might be restored by GFP-LGL expression. However, this is not the case, and only residual PAR-1 remains on the cortex (Figures S3J–S3I). We conclude that LGL partitions not only the PAR complex on the cortex but also cell fate determinants in the cytosol.

LGL Activity Is Regulated by PKC-3-Dependent Phosphorylation

We have shown so far that when we silenced par-3 or pkc-3 by RNAi, GFP-LGL localization extended over the whole embryo cortex (Figure 3A). This means that a functional anterior PAR...
regulated by PKC-3-dependent phosphorylation, we expressed a version of GFP-LGL in which we mutated all three conserved sites to alanines. The localization of the GFP-LGL AAA mutant protein is not restricted to the posterior cortex anymore but localizes uniformly to both domains throughout the first cell division (Figure 4A) and is not restricted to the P lineage (data not shown). We also looked at phosphomimetic LGL [10] by exchanging the three conserved serine/threonines with glutamates (GFP-LGL EEE). Strikingly, GFP-LGL EEE was unable to associate with the cortex (Figure 4A), even in the absence of a functional PAR complex (Figures S4F–S4H). Therefore, the association of LGL with the cortex appears to take place only if the protein is not phosphorylated on the conserved sites.

We next asked whether the uniform localization of GFP-LGL AAA is sufficient to remove the anterior PAR complex from the cortex by looking at the distribution of PAR-3 and PAR-6 in par-2(RNAi) embryos expressing GFP-LGL AAA. Interestingly, although GFP-LGL AAA localized uniformly to the cortex in these embryos (Figures S4A and S4B), PAR-3 and PAR-6 still localize to the cortex, and their localization is not restricted to the anterior (Figures S4C and S4D). Confirming this result, GFP-LGL AAA expression could not rescue the lethality of par-2(RNAi) (Figure 4B; Figure S4E). We conclude that LGL must be phosphorylated to

Figure 3. GFP-LGL Expression Compensates for Depletion of PAR-2 by Restoring Cell Polarity

(A) GFP-LGL localization depends on anterior PAR proteins. GFP-LGL localizes uniformly to the cortex in par-3(RNAi) and pck-3(RNAi) embryos. Note that there is still asymmetric division in the par-2(RNAi) embryo.

(B) GFP-LGL localizes to the cell perimeter of P lineage cells. Depletion of par-2 by RNA interference (RNAi) does not lead to symmetric division of P lineage cells in GFP-LGL-expressing embryos, nor to loss of LGL localization (in 14 of 14 embryos).

(C) PAR-2 is efficiently depleted by RNAi in GFP-LGL-expressing embryos. Anti-PAR-2 immunofluorescence was made from par-2/control(RNAi)-treated embryos (DNA, blue; microtubules, green; PAR-2, red). For quantification of PAR-2 cortex fluorescence, see Figure S3C.

(D) Embryonic lethality of par-2(RNAi) is rescued by GFP-LGL expression (par-2/control(RNAi)). Silencing of par-2 and the transgene gfp::lgl by par-2/gfp(RNAi) leads to high embryo lethality. Mean ± SEM, n indicates total number of counted embryos.

(E and F) mcherry-PAR-6 localization is restricted to the anterior by GFP-LGL expression in par-2-depleted embryos. Of par-2/control(RNAi) embryos, 5 of 5 showed par-2 phenotype rescue; 7 of 7 par-2/gfp(RNAi) embryos showed a par-2 phenotype.

(F) Quantification of anterior-to-posterior cortex fluorescence of mcherry-PAR-6 and GFP-LGL (from images in E). (G and H) PAR-6 localization is restricted to the anterior AB cell when gfp::lgl is expressed (in 10 of 10 embryos; par-2/control(RNAi)) but is not restricted if gfp::lgl is silenced (in 6 of 7 embryos; par-2/gfp(RNAi)).

(I and J) GFP-LGL expression restores posterior localization of P granules in par-2-depleted embryos (8 of 8 embryos), but depletion of gfp::lgl leads to loss of P granules (7 of 7 embryos).
deplete PAR-3 and PAR-6 from the posterior cortex and to restore embryo viability. However, because the GFP-LGL EEE phosphomimetic protein localizes to the cytosol and cannot rescue lethality of par-2(RNAi) embryos (Figure 4B), phosphorylated LGL is also not sufficient to restore embryo viability. Therefore, we conclude that cortex partitioning activity of LGL requires phosphorylation changes in LGL.

LGL Phosphorylation Modifies the Association with PAR Complex Proteins
To investigate whether phosphorylation of LGL affects the binding of complex partners, we purified either GFP-LGL WT, GFP-LGL AAA, or GFP-LGL EEE protein complexes from early embryos by immunoprecipitations. Importantly, the WT and the unphosphorylated AAA form both bind well to PAR-6 and PKC-3 (Figure 4C). However, the association of the phosphomimetic EEE form with PKC-3 and PAR-6 is much weaker (Figure 4C). We also noted in our initial immunoprecipitation experiments that treatment of the immunopurified LGL complex with phosphatase increased association of PKC-3 with the complex (Figure 1C). Therefore, we conclude that phosphorylation not only changes the localization of LGL but also affects the composition of the LGL complex.

Model and Conclusions
Although genetic results have indicated that the formation of cortical domains of PAR proteins requires mutual inhibition [6, 9, 18, 31–33], we do not have a good understanding of the enzymology of the interactions between proteins of the two cortex domains. In this work we have now identified the LGL homolog in C. elegans, shown that LGL is sufficient to partition the cortex, and demonstrated that this activity is directly linked to its phosphorylation. An explanation of how LGL activity is involved in polarity of the first-cell C. elegans embryo must explain the seemingly contradictory results that LGL is found associated with the anterior PAR complex proteins PAR-6 and PKC-3 by immunoprecipitation, but that, by light microscopy, LGL localizes to the opposite posterior cortex. To reconcile these observations with the activities of the GFP-LGL AAA and EEE mutants, we propose the following model (see Figure 4D): LGL on the cortex binds the PAR complex most likely at the boundary between the anterior PAR domain and the posterior LGL domain, forming the PAR-6/PKC-3/LGL complex. PKC-3 will then phosphorylate LGL, and the whole complex will drop off the cortex. This model can be thought of as “mutual elimination,” in which interaction between LGL and the anterior PAR complex at the boundary between the two complexes causes both to leave the cortex. PAR proteins
appear to move on the cortex by lateral diffusion [34] (Goehring et al., personal communication). LGL might show similar diffusion, and mutual elimination between LGL and the anterior PAR complex at domain boundaries could, in part, explain maintenance of two domains on the cell cortex.

Different strategies seem to have evolved in cells to maintain two cortex domains on the cell membrane, but all might include direct inhibition of the PAR complex by an opposing activity. Because LGL is conserved in metazoans [35], future work needs to address the contribution of LGL and other opposing activities on the PAR complex in different cell types.

Previous work in Drosophila has shown that Aurora A phosphorylation of PAR-6 stimulates phosphorylation of LGL by aPKC, which activates the PAR complex [14]. This work suggested that LGL acts as a buffer, possibly in the cytoplasm where it holds the PAR complex inactive until phosphorylated by Aurora A. In C. elegans, Aurora A activity is likely high throughout the first cell cycle [36] and could maintain an active PAR complex by releasing sufficient LGL. More generally, the rate of LGL dissociation from the LGL/PAR-6/aPKC complex may determine the available pool of PAR complexes for cell polarity in the one-cell embryo. Therefore, the combination of these two models can explain the function of LGL. On the one hand, it acts as a buffer [14]. On the other hand, LGL acts through mutual elimination to remove the conserved PAR complex from the cortex.

Experimental Procedures

C. elegans Worm Lines and Cloning

All C. elegans strains were grown on nematode growth medium (NGM) plates and handled as described [37]. Igl was polymerase chain reaction amplified from genomic N2 DNA with primers 5’TATACTAGTGAACTGATGCACTATCTTACAGTT3’ and 5’TATAAGGCCCTATGTTGTGCACTGC3’ and subcloned into pGEM-T (Promega). The sequence was verified, and a SpeI and Stul fragment was cloned into pTH314 (gfp::igu fusion under control of pie-1 promoter and promoter). Transgenic lines expressing GFP-LGL (TH270), GFP-LGL AAA (TH271), or GFP-LGL EEE (TH272) were made from genomic igl by particle bombardment of unc-119(ed3) worms [38] and were maintained at 25°C. Other worm lines were mcherry-PAR-2 (TH29), mcherry-PAR-6 (TH36), and NMV-2-GFP [40]. Double fluorescent lines were made by crossing the relevant single lines. Iggl gene structure was derived from first-strand cDNA made from N2 polyA RNA. Site-directed mutagenesis (QuikChange, Stratagene) and DNA sequencing were used to introduce and verify mutations in igl.

Identification of Iggl Alleles by Deletion Screening

Igl-1(dd21) (TH31) and Igl-1(dd22) (TH32) were generated by ethyl methanesulfonate-mediated mutagenesis [41]. The mutant alleles were isolated with the nested primer pairs 5’GATGTCACCGACTGATGGGCG’ and 5’AC TCGGATGATTGCATCATC’ and 5’ATCAACGAGCTTCTTACCGACA AG3’ and 5’GAAATCTTACACTTGCAGGCGC’ and backcrossed across seven times to N2. The deletions were verified by amplification and sequencing of Igl-1(dd21) and Igl-1(dd22) cDNA and by western blotting with anti-IGL antibody.

Gene Silencing by RNAi and Embryonic Lethality Assays

Gene silencing by RNAi was done by feeding [42, 43]. Briefly, overnight cultures grown in LB Amp Tet were diluted to fresh LB Amp, grown to log phase, and induced with 0.2 mM IPTG at 30°C for 2 hr and plated onto fresh NGM feeding plates for further 1 day growth. L4 or young adults were placed on feeding plates for 24–28 hr at 25°C for RNAi experiments. For feeding assays with two genes, bacterial cultures of similar optical densities were mixed 1:1. Feeding assays for par-2(control)/RNAi and par-2(gfp)/RNAi in gfp::igu-expressing worms were done in parallel on 3–6 plates, using identical conditions, and embryonic lethality was assayed as ratio of dead embryos to total number (n) of embryos (in Figure 3D; Figures S3D–S5J; Figure 4B; Figure S4E). A drop in penetrance of par-2/RNAi) compared to par-2/control(RNAi) or par-2(gfp)/RNAi), respectively, was not observed. For gfp(RNAi), a gfp cDNA fragment was cloned into L4440. control(RNAi) was done with empty L4440 plasmid. Three independent transgenic gfp::igu lines rescued embryonic lethality of par-2(RNAi). For assaying synthetic embryonic lethality by RNAi, igl(RNAi) or skp-1(RNAi) (as a control) was done by feeding for 3 days at 16°C and 1 day at 20°C or 4 days at 16°C in wild-type N2 or par-2ts1(f5) animals. Animals were allowed to lay eggs for 4-6 hr; eggs were counted and embryonic lethality was scored 24 hr later. For assaying synthetic lethality of double mutants, igl-1(dd22) or igl-1(dd22) animals were crossed to the relevant lines and screened for homoyzogous offspring. par-2ts1(f5) worms (KK14) contain an additional deletion (del72) mutation that does not influence lethality with igl. For nmy-2 igl-1(dd21) lethality assays, nmy-2(ne1490)ts (WM180) worms were crossed into the igl background and scored for embryonic lethality at indicated temperatures.

Antibodies

A set of 36 LGL GST-fusion fragments was cloned (USER system, New England Biolabs) and screened for solubility by an enzyme-linked immunosorbent assay. Three soluble fragments with high expression were purified from protein gels, used for injection of rabbits (Charles River Laboratories, France), and tested in worms. Iggl from two sera were further used and affinity purified by corresponding MBP-fusion protein columns (primers of fragments used were forward 5’gagggacacTTAATATGACCAAAATCAGGA TATG3’ and reverse 5’ggggacacCTATGTTAACCCTCCAGTAC3’ and forward 5’gaggagcacCGCACTTCCTAGGATG3’ and reverse 5’ggggacacTGCTT TCCTAGC3’).

Antibodies against PAR-1, PAR-2, and PAR-3 were made from insoluble 6×Histidine-tagged fusions, purified under denaturing conditions (according to QIAGEN protocols), and used for the identification of PAR proteins (rubs of rabbits (Charles River Laboratories, France). PAR-6 antibodies were made from an N-terminal GST-PAR-6 fragment in rabbits and were affinity purified against PAR-6. Iggl from PAR-1 and PAR-2 serum was affinity purified on corresponding GST-fusion columns and stored in phosphate-buffered saline (PBS) with 50% glycerol at −20°C. Anti-PGL-1 antibody (guinea pig) was a gift from Christian Eckmann (MPI-CBG, Dresden) and anti-PKC-3 antibody for biochemistry was a gift from Monica Gotta (Geneva); for PKC-3 immunofluorescence, we used PKCzeta (C20, Santa Cruz Biotechnology); and MH27 (Jam-1) was received from the Developmental Studies Hybridoma Bank (DSHB).

Immunoprecipitations and Western Blots

For large-scale anti-PAR-6 immunoprecipitation (Figure 1A), 1 × 106 dauer larva from wild-type N2 were released on 15 egg plates and grown for 35 hr at 24.5°C. Adult worms containing 1 to 8 cell embryos were harvested and washed twice with H100 buffer (50 mM HEPES, pH 7.5, 100 mM KCl, 1 mM MgCl2, 1 mM EGTA, 10% glycerol) and frozen in liquid nitrogen. An equal volume of H100 buffer (with 0.05% NP-40 and protease inhibitors) (Dulbecco) was added to pellets, and worm lysates were lysed by sonicating six cycles (15 s). Lysates were clarified by 100,000 × g centrifugation for 10 min and incubated with 12 μg anti-PAR-6 IgGs for 45 min at 4°C. Immunocomplexes were collected by protein A agarose (General Electric), washed five times with lysis buffer, and eluted with 1 M glycine (pH 2.3), neutralized and supplemented with Laemmli buffer. Proteins were separated on SDS-PAGE gels (Hoefer). Coomassie-stained bands were in gel digested with trypsin, and proteins were identified by matrix-assisted laser desorption/ionization time-of-flight mass spectrometric peptide mapping [15]. Worms for immunoprecipitation from early embryos (Figures 1B and 1C; Figure 4C) were grown on peptone plates made with 3.6% agar and seeded with C600 bacteria. Embryos were purified by bleaching and hatched overnight in S-Base Complete, and the first larva were seeded with a density of about 1 × 105 per 15 cm peptone (seeded with C600 bacteria) plate and grown at 25°C. Synchronized, adult worms were harvested before embryos were laid, and embryos were purified by bleaching, frozen in liquid N2, and stored at −80°C. Embryo pellets (500 μg) were taken up in 500 μl volume 2 × H100 buffer (100 mM HEPES at pH 7.5, 200 mM KCl, 2 mM MgCl2, 2 mM EGTA, 20% glycerol, protease and phosphatase inhibitors), lysed on ice by 0.5 s sonications pulses for a total of 20 s, supplemented with 0.2% final Triton X-100 for 20 min on ice, and centrifuged for 1 hr at 230,000 × g in a TLA120.2 rotor. Supernatants were filtered (Ultrafree MC Millipore 0.45 μm) and incubated with goat-anti-GFP IgG (protein expression facility, MPI-CBG) coupled to magnetic protein G beads (Invitrogen). Beads were washed four times with 1 × H100 with 0.2% Triton X-100, once with 1 × H100, 300 mM KCl with 0.2% Triton X-100, and once with H100 buffer and eluted with 50 μl 1% SDS. For phosphatase treatment (Figure 1C), the washed immunocomplexes were incubated for 20 min at 37°C with or without phosphatase (40 units calci intestinal phosphatase, New England Biolabs). For immunoblots, protein extracts were diluted with sample buffer, run on gels as above,
blotted, and detected on polyvinyldiene fluoride membranes (Millipore) by enhanced chemiluminescence (ECL, General Electric).

**Microscopy**

Live imaging of fluorescent worm lines was either on a Zeiss Axioi Plan 2 Widefield microscope with a Zeiss Axioi Plan 2 1.4 Apochromat and a Hamamatsu Orca ER 12-bit color as described [39] or on a Zeiss Axioi Imager Z1 microscope body (with an Apochromat, 63× and 1.4 lens), equipped with a Yokagawa spinning disc head, a Melles Griot 488 nm Argon ion laser, and a Hamamatsu Orca ER 12-bit camera, and images were processed with ImageJ (http://rsb.info.nih.gov/ij/). For immunofluorescence, embryos were freeze cracked in liquid N$_2$ fixed for 20 min in −20 °C methanol, rehydrated in PBS with 0.05% Tween-20 (PBS-T) and stained with antibodies overnight, washed with PBS-T, and probed with secondary antibodies (Alexa Dyes, Molecular Probes) and fluorescein isothiocyanate-conjugated anti-tubulin dm1α (Sigma). Images were taken on a DeltaVision RT imaging system (Applied Precision, LLC; IX70 Olympus) equipped with a charge-coupled device camera (CoolSNAP HQ; Roper Scientific) in 0.2 μm serial Z sections using an Olympus 100× NA 1.40 NA PlanApo. Image stacks were deconvolved using Softworx (Applied Precision, LLC) and processed in ImageJ. Images were maximum-intensity projected (all planes for microtubules and DNA; 10 midplane images for cortex proteins). All images of embryos show anterior on the left and posterior on the right.

**Image Quantifications**

Average fluorescence intensities from immunofluorescence pictures were measured from deconvolved and projected images (10 planes of 0.2 μm distance) by cortical 5-pixel-wide segmented lines spanning 0%–25% (anterior) or 75%–100% (posterior) of embryo length. Average cortex fluorescence intensities were normalized to background. Fluorescence intensities from live embryos were measured from a cortical 5-pixel-wide segmented line spanning 0%–100% of embryo length (cortex position anterior to posterior in pixel). All images for quantification were exposed and processed identically and made from slides prepared in parallel experiments. PAR-6 domain and boundary shape (Figures S2D and S2E) was extracted from 20-pixel-wide lines (spinning disk images from 6 frames, 20 s intervals). The sum of the three brightest pixels corresponding to an approximately 600 nm thick region spanning the membrane was taken at each point to generate a fluorescence profile around the entire embryo, and the absolute intensity was normalized to the mean fluorescence of the entire profile. The edges of the domain were fit using error functions (erf), with the center of each erf taken as the domain edge. Domain size is taken as the distance between these two edges and was normalized to the total length of the profile. To generate the average shape of the boundary profiles, we aligned individual boundary gradients by the domain edge, as defined by the center of the corresponding erf, and averaged them. Distance is absolute.

**Supplemental Information**

Supplemental Information includes four figures and can be found with this article online at doi:10.1016/j.cub.2010.05.061.

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2 functions in hemidesmosome formation, maintenance of cellular morphology and growth regulation in the developing basal epithelium. Development 122, 3255–3265.


Supplemental Information

LGL Can Partition the Cortex of One-Cell Caenorhabditis elegans

Embryos into Two Domains

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Figure S1. Cloning of the \textit{lgl} Gene and Isolation of \textit{lgl} Mutants

(A) \textit{lgl} gene structure. X chromosome location is based on wormbase version WS206. Gene model and location of isolated \textit{lgl} deletions is based on cloned \textit{lgl}. Numbers in parentheses indicate base pairs. Green numbered boxes indicate exons.

(B) Open reading frames (ORF) of \textit{lgl-1} and the alleles \textit{lgl-1(dd21)} and \textit{lgl-1(dd22)}. \textit{lgl-1} transcripts are spliced from 13 exons to a length of 2826 bases. cDNA from \textit{lgl-1(dd21)} and \textit{lgl-1(dd22)} confirmed the ORFs of the deletion alleles. Numbers in parentheses indicate basepairs. The electropherograms indicate the region of \textit{lgl-1} deletion before the Stop codon.

(C) LGL wildtype and mutant proteins. Numbers indicate amino acids, numbers in brackets indicate additional amino acids which are not present in wildtype LGL.
(D) LGL phosphorylation sites are highly conserved among nematodes and other species. Alignment was done with ClustalX, 2.0.10. (*), identical residues; (:), similar residues; (.) related residues.

(E) *lgl-1(dd21)* protein does not show posterior cortex localization using anti LGL immunofluorescence. Samples were processed in parallel with identical conditions. (DNA blue; microtubules green; LGL red). Anterior of the embryo is on the left, posterior is on the right. Grey box: blow-up of posterior cortex region.

(F) LGL on the posterior cortex is increased in embryos expressing GFP-LGL. Silencing of transgenic GFP-LGL by *gfp(RNAi)* reduces fluorescence to wildtype intensities. Quantification from experiment shown in (Fig. 1F) (Mean ± SEM; n=5). (See methods for quantification), anterior of the embryo is on the left, posterior is on the right.
Figure S2. *lgl* Is Redundant for Cell Polarity and Cortex Contractility

(A) *lgl-1(dd21)* embryos do not show adherens junctions or apical GFP-PAR-6 localization defects in the intestine. Immunofluorescence with anti JAM-1 (MH27) antibody (DAPI staining for DNA) and live images from GFP-PAR-6 embryos.

(B) NMY-2-GFP dynamic localization appears unaffected in *lgl-1(dd22)* embryos. Stills of embryo cortex plane during polarity establishment (top). Kymograph (central horizontal 30pixel line) of embryo cortex plane till end of polarity establishment phase (-550s to 0s).

(C) *lgl-1(dd21)* slightly increases lethality of *nmy-2(ne1490)ts* embryos. Mean ± SEM, n indicates number of total counted embryos.

(D) GFP-PAR-6 profile at the domain boundary appears unchanged in *lgl-1(dd21)* embryos. Mean ± SD.
(E) GFP-PAR-6 fluorescence intensity on the cortex is unaltered in \textit{lgl-1(dd21)} embryos (distance 0 represents the center of the PAR-6 domain).
Figure S3. GFP-LGL Expression Restores Cell Polarity in PAR-2 Depleted Embryos

(A-A') Localization of endogenous LGL depends on the anterior PAR complex. (A) par-2(RNAi) leads to uniform PKC-3 localization (4 of 4 embryos). (A') LGL localization to the posterior cortex is lost in par-2(RNAi) embryos (in 5 of 5 embryos). Note blow up of cortical region.

(B) PAR-2 depletion is efficient in wt and GFP-LGL expressing worms after par-2(RNAi). Embryo lethality in wt worms was 100%, in GFP-LGL expressing worms only 8%. Anti PAR-2 western blot and anti tubulin as loading control.

(C) PAR-2 is efficiently depleted by RNAi in GFP-LGL expressing embryos. Quantification of anti PAR-2 immunofluorescence of par-2/control(RNAi) treated embryos as shown in Fig. 3C. Mean ± SEM (n=5 embryos).

(D-E) PKC-3 localization is restricted to the AB cell when gfp::lgl is expressed (in 6 of 6 embryos par-2/control(RNAi)), but not restricted if gfp::lgl is silenced (10 of 10 embryos; par-2/gfp(RNAi)).

(F-G) PAR-3 localization is restricted to the anterior when gfp::lgl is expressed (in 4 of 4 embryos) and uniform if gfp::lgl is silenced (in 5 of 7 embryos).

(H-I) PAR-1 localization to the posterior cortex is not restored by gfp::lgl expression (in 7 of 11 embryos; par-2/control(RNAi)), 4 of 11 show only very faint cortex signal). PAR-1 is cytosolic if gfp::lgl is silenced (in 9 of 9 embryos; par-2/gfp(RNAi)). Compare to PAR-1 stainings in wildtype N2 (I').

(J) PAR-6 fluorescence ratio are restored. Ratio of PAR-6 anterior to posterior cortex fluorescence from wildtype N2 or GFP-LGL expressing worms treated by RNAi as indicated. Mean ± SEM.

(A-I') Anterior of the embryo is on the left, posterior is on the right. (DNA blue; microtubules green; PAR / PKC-3 / LGL red).
Figure S4. LGL Phosphomutant and Phosphomimetic Proteins

(A-D) Unphosphorylated LGL can not remove anterior PAR proteins from the cortex. (A-B) GFP-LGL AAA expression does not rescue par-2 phenotypes. Note the symmetric division in the par-2/control(RNAi) embryo. (C-D) PAR-6 and PAR-3 are not depleted from the cortex by GFP-LGL AAA expression. Anti PAR-6 and anti PAR-3 immunofluorescence. Worms from same experiment were tested for embryo lethality (see E). (DNA blue; microtubules green; PAR red). (E) GFP-LGL wt, but not GFP-LGL AAA rescues embryonic lethality of par-2/control(RNAi). Mean ± SEM. (F-H) Mimicking LGL phosphorylation also inhibits cortex association in absence of a functional PAR complex. GFP-LGL EEE does not associate with the cortex (F) nor in par-3(RNAi) (G), nor in pkc-3(RNAi) (H) embryos. (A-H) Anterior of the embryo is on the left, posterior is on the right.