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Lgl and its phosphorylation by aPKC regulate oocyte polarity formation in *Drosophila*

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Abstract

Specification of the anteroposterior (AP) axis in *Drosophila* oocytes requires proper organization of the microtubule and actin cytoskeleton. The establishment and regulation of cytoskeletal polarity remain poorly understood, however. Here, we show important roles for the tumor suppressor Lethal (2) giant larvae (Lgl) and atypical protein kinase C (aPKC) in regulating microtubule polarity and setting up the AP axis of the oocyte. Lgl in the germline cells regulates the localization of axis-specifying morphogens. aPKC phosphorylation of Lgl restricts Lgl activity to the oocyte posterior, thereby dividing the cortex into different domains along the AP axis. Active Lgl promotes the formation of actin-rich projections at the oocyte cortex and the posterior enrichment of the serine/threonine kinase Par-1, a key step for oocyte polarization. Our studies suggest that Lgl and its phosphorylation by aPKC may form a conserved regulatory circuitry in polarization of various cell types.

Keywords

Lethal (2) giant larvae (Lgl); Atypical protein kinase C (aPKC); Oocyte polarity; Par-1; Microtubule; Oogenesis; *Drosophila*

Introduction

Cell polarity, the asymmetry in distribution of cellular constituents within a single cell, is fundamental to cellular life and essential for generating cell diversity. For example, epithelial cells have a characteristic apicobasal polarity that is necessary for their function as barriers between different extracellular environments; this polarity facilitates directed trafficking of molecules between those environments. The polarity established in neuroblasts is the basis for their asymmetrical division, which produces distinctive cell types. Loss of polarity in cells is frequently related to the disruption of cellular function and can sometimes result in tumorigenesis.

The *Drosophila* oocyte exhibits an anteroposterior (AP) asymmetry with respect to its cytoskeletal organization and the distribution of some mRNAs and proteins (Riechmann and Ephrussi, 2001; van Eeden and St Johnston, 1999). This asymmetry provides the basis for the formation of the major body axes in the subsequent embryo. A key step for oocyte polarity formation is the reorganization of the microtubules in the germline during mid-oogenesis. Previous studies show that the microtubule (MT) nucleating activity is associated with the centrosome-nucleus complex (Januschke et al., 2006). After stage 6, MT reorganization in the

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oocyte occurs at the onset of anterior migration of the nucleus (Januschke et al., 2006), presumably as a consequence of the polarizing signal from the follicle cells (Gonzalez-Reyes and St Johnston, 1998; Roth et al., 1995; Ruohola et al., 1991). Reorganization of the posterior microtubule organizing center is believed to allow formation of a gradient of MT from high density at the anterior to low density at the posterior (Cha et al., 2001; Januschke et al., 2006; Theurkauf et al., 1992). When the reorganization of MT within the oocyte takes place at this stage, the MT plus ends, as visualized by the localization of a protein fusion of the plus-end MT motor kinesin (Kin:β-Gal) (Clark et al., 1994), accumulate in the posterior. This new microtubule orientation is necessary for localization of maternal determinants such as *oskar* (*osk*), *bicoid* (*bcd*), and *gurken* (*grk*) mRNAs, which are the foundation for specification of the AP and dorsoventral axes (Neuman-Silberberg and Schupbach, 1993; Nilson and Schupbach, 1999; van Eeden and St Johnston, 1999).

Lethal (2) giant larvae (Lgl), a WD40 domain-containing protein, is implicated in cellular asymmetry formation in a number of cell types (Vasioukhin, 2006; Wirtz-Peitz and Knoblich, 2006), but its role in the oocyte is unclear. Lgl is evolutionarily conserved across the eukaryotes; orthologs range from budding yeast (Sro7/Sro77) to human (Lgl1 and Lgl2) (Vasioukhin, 2006). In *Drosophila*, *lgl* mutations result in loss of apicobasal polarity in epithelial cells and produce tumors of the brain, the imaginal disc and the follicular epithelium (Bilder et al., 2000; Ohshiro et al., 2000; Peng et al., 2000). In neuroblasts, Lgl is phosphorylated by aPKC in the apical region to direct localization of basal components involved in asymmetric cell divisions (Betschinger et al., 2003). The precise role of Lgl in cell polarity formation in *Drosophila* and other multicellular organisms remains largely unknown. Here, we report that Lgl and its phosphorylation by aPKC are required for oocyte polarity formation. aPKC phosphorylation of Lgl restricts Lgl activity to the oocyte posterior and regulates posterior enrichment of Par-1 and organization of microtubule polarity that is required for morphogen localization and axis specification.

Materials and Methods

Fly strains and genetics

The strains were raised at 25°C on standard media. We used lgl^{4} and lgl^{4w3} as null alleles for *lgl* (Bilder et al., 2000; Manfruelli et al., 1996; Ohshiro et al., 2000). *lgl* and *apkc* germline clones were generated by FLP-FRT-induced mitotic recombination (Xu and Rubin, 1993), and *lgl* germline clones were also generated by the DFS FRT/FLP-ovo^{D1} system (Chou and Perrimon, 1996) from the following strains: lgl^{4}P[neoFRT]40A/Cyo (strong LOF allele), lgl^{4w3}P[neoFRT]40A/Cyo (strong LOF allele), P[ry^{+}, FRT]^{42D} $apkc^{K06403}$/*Cyo* (strong LOF allele), *yw* P[mini-w^{+}, hsFLP]^{1}; P[Ubi-GFP]^{33} P[Ubi-GFP]^{38} P[neoFRT]40A/CyO, *yw* P[mini-w^{+}, hsFLP]^{1}; P[ovoD1-18]^{2La} P[ovoD1-18]^{2Lb} P[neoFRT]40A/CyO, *yw* P[mini-w^{+}, hsFLP]^{1}; P{arm-lacZ.V}36BC P[neoFRT]40A/CyO, w[1118]; P[neoFRT]42D P{w[+mC]=Ubi-GFP(S65T)nls}2R/Cyo. We induced germline clones by administering 2 hour 37°C heat shocks on two consecutive days during the third instar. For aPKC and GFP-Par-1(N1S) overexpression, we obtained *UAS:aPKC* and *UAS:aPKC*$^{\Delta N}$ transgenic fly stocks from C. D. Doe (Lee et al., 2006), transgenic fly stocks with *pUASP: GFP-Par-1(N1S)* on the 2nd chromosome from D. St Johnston (Huynh et al., 2001), and transgenic fly stocks with *pUASP:GFP-Par-1(N1S) K** and *pUASP:GFP-Par-1(N1S)* on the 3rd chromosome from A. Ephrussi (Vaccari et al., 2005). *Matα4-tubulin-Gal4-VP16* (*Mat-Gal4*) was used for the overexpression in the germline.

Molecular biology and biochemical analysis

The cDNA of *lgl* and *lgl-3A* from J. A. Knoblich (Betschinger et al., 2003) and *apkc* from DGRC were amplified, sequenced and subcloned into the pUASP (Rorth, 1998) or

pUASP:GFP vectors for *Drosophila* transgenes. Transgenic lines of (1) *pUASP:Lgl*, (2) *pUASp:Lgl-3A*, *pUASP:Lgl-GFP*, (3) *pUASP-Lgl-3A-GFP*, (4) *pUASP:aPKC-GFP* and (5) *pUASP:aPKC*$^{\Delta N}$*-GFP* constructs were generated by standard methods and overexpressed by means of the germline *Gal4* drivers (*Mat-Gal4*). One of each of these transgenic lines were used for phenotypic and biochemical analyses.

For the co-immunoprecipitation (co-IP) analysis, ovarian extracts were prepared from wild-type flies or transgenic flies expressing pUASP:GFP-Par-1 (N1S) in homogenization buffer [150 mM NaCl, 50 mM HEPES (pH 7.2), 0.5 mM DTT, PMSF, Complete Protease Inhibitor cocktail (Roche)]. Lysate was incubated with primary antibody (rabbit anti-Lgl antibody) (Betschinger et al., 2003) or mouse anti-GFP antibody (Santa Cruz Biotechnology) for 10 hours at 4°C. Protein A/G plus-agarose (Santa Cruz) was then added, and the reaction was allowed to proceed for 4 hours at 4°C. Analysis was conducted with SDS-PAGE followed by western blots. In anti-Lgl immunoprecipitates, controls were only beads (without added antibody) and peptide blocks; in anti-GFP immunoprecipitates, controls were beads (without added antibody) and the ovarian extract from wild-type flies. Anti-Cut antibody was used as a negative control in the co-IP experiment, which did not detect the Cut band in the western blot containing proteins pulled down by anti-Lgl antibody (data not shown).

Antibody staining, imaging and analysis

Antibody staining was performed according to standard procedures (Sun and Deng, 2005). Primary antibodies were diluted as follows: rabbit anti-Lgl and anti-pLgl, 1:200 (Betschinger et al., 2003); rabbit anti-aPKC, 1:1000 (Santa Cruz Biotechnology); rabbit anti-Stau, 1:5000 (St Johnston et al., 1991); mouse anti-Gurken, 1:20 (Developmental Studies Hybridoma Bank (DSHB)); rabbit anti-β-galactosidase, 1:5000 (Sigma); Alexa Fluor 546 Phalloidin, 1:50 (Invitrogen), mouse anti-Cut, 1:50 (DSHB); mouse anti-Hindsight (Hnt), 1:20 (DSHB); mouse anti-Eyes absent (Eya), 1:50 (DSHB). For microtubule staining, samples were fixed for 10 minutes in 8% paraformaldehyde (Doerflinger et al., 2006) and stained with a FITC-conjugated monoclonal anti-α-tubulin antibody DM1A (1:400; Sigma). Secondary antibodies conjugated to Alexa Fluor 546 goat anti-mouse and goat anti-rabbit (Molecular Probes) were used at 1:400. Fluorescently labeled samples were counterstained with DAPI for visualization of DNA. In situ hybridizations were carried out with RNA probes labeled with Digoxigenin-UTP (Roche). Immunohistochemical detection was performed with alkaline phosphatase-conjugated anti-DIG (1:5000; Roche). Images were captured with a Zeiss LSM 510 confocal microscope and assembled in Adobe Photoshop.

Results

Lgl is involved in the germline cells for oocyte polarity formation

In a study designed to determine whether genes regulating epithelial polarity can regulate oocyte polarization, we analyzed the staining patterns of antibodies against Staufen (Stau) and Gurken (Grk) in *lgl*^{4} germline clones using the FLP/FRT system (Xu and Rubin, 1993). Stau, an RNA-binding protein that colocalizes with *osk* mRNA to the oocyte posterior (St Johnston et al., 1991) (Fig. 1A,E), showed abnormal localization in *lgl*^{4} germline clones at stages 9/10 (58%, *n*=81; 17% mislocalized to the center (hereafter called ‘complete mislocalization’), 31% had both ectopic and posterior localization (‘partial mislocalization’) and 10% had diffuse mislocalization (‘diffuse mislocalization’); Fig. 1B and data not shown). The TGF-α homolog Grk, which normally moves with the oocyte nucleus from the posterior to an anterior corner to induce dorsoventral patterning (Neuman-Silberberg and Schupbach, 1993) (Fig. 1C), failed to reach its correct destination in some *lgl*^{4} germline clones (16%, *n*=144; Fig. 1D). In these cases, Grk and the oocyte nucleus were either mislocalized to a lateral position in the oocyte (Fig. 1D) or remained at the posterior (data not shown). To confirm the involvement of Lgl in

localizing oocyte polarity markers, we generated germline clones of a different *lgl* mutant allele, lgl^{4w3}, and similar defects of Stau and Grk localization were found (data not shown). When RNA in situ hybridization was used to stain the lgl^{4} germline clones generated by a dominant female sterile (DFS) technique, the posterior determinant *osk* mRNA (Kim-Ha et al., 1991) (Fig. 1E) was mislocalized in the oocyte at stages 9/10 (68%, *n*=143; 13% complete, 38% partial and 17% diffuse; Fig. 1F and data not shown). *bcd* mRNA, which is normally localized to the extreme anterior cortex of the oocyte in a disc-shaped pattern (Driever and Nusslein-Volhard, 1988) (Fig. 1G), accumulated as dots in the anterior of *lgl* germline clones (38%, *n*=128; Fig. 1H), also indicating a defect in localizing the anterior determinant.

To gain more insight into the oocyte polarity defects of *lgl* germline clones, we analyzed the localization of a microtubule plus-end marker, Kinesin:β-galactosidase (Kin:β-gal) (Clark et al., 1994), which is normally localized at the posterior end of the oocyte during stages 8-9 (Fig. 1I). In *lgl* germline clones, Kin:β-gal was not concentrated at the posterior and instead mislocalized as ectopic dots in the center or partially mislocalized or diffused from the posterior of the oocyte (57%, *n*=97; 14% complete, 36% partial and 7% diffuse; Fig. 1J and data not shown). In oocytes of *lgl* germline mutants, the anterior-to-posterior gradient of microtubules at stages 7-8 (Theurkauf et al., 1992) (Fig. 1K), as visualized by direct staining with a FITC-conjugated antibody against α-Tubulin, was also disrupted. Instead of an AP gradient, a high density of microtubules was detected throughout the oocyte of *lgl* germline clones (Fig. 1L). These defects in microtubule organization could contribute to the defects in Stau/*osk* and Grk mislocalization.

Follicle-cell differentiation is normal in *lgl* germline clones

Because the reorientation of microtubule polarity in the oocyte depends on a polarizing signal from the posterior follicle cells (PFC), we examined whether these follicle cells were specified correctly in egg chambers bearing *lgl* germline clones. First, we stained the *lgl* mosaics with antibodies against Cut and Fasciclin 3 (Fas3), which are both downregulated by Notch after stage 6 (Lopez-Schier and St Johnston, 2001; Sun and Deng, 2005) (Fig. 2A,A′ and data not shown). In the PFC covering the *lgl* germline clones, we found no change in the expression of these two immature follicle cell-fate markers (Fig. 2B,B′ and data not shown). Hindsight (Hnt), a Notch target in follicle cells during mid-oogenesis (Sun and Deng, 2007), maintained its expression correctly in the PFC after stage 6 (Fig. 2C,C′), suggesting Notch signaling is unaffected in *lgl* germline clones (Fig. 2D,D′). We then examined the expression of Eyes absent (Eya), which is normally expressed in both immature follicle cells and the anterior follicle cells after stage 7 (Bai and Montell, 2002) (Fig. 2E,E′), and found no expression in the PFC covering the *lgl* germline clone (Fig. 2F,F′), suggesting that the PFC do not take either the immature or anterior follicle cell fate. In addition, a PFC fate marker, Pointed-lacZ (Pnt-*lacZ*) (Gonzalez-Reyes and St Johnston, 1998; Poulton and Deng, 2006), continued to be expressed in the PFC covering the *lgl* germline clones (Fig. 2H,H′). The correct expression of the follicle cell-fate markers in follicle cells over the *lgl* germline clone suggests that the activation of the Notch and EGFR pathways is unaffected. Therefore, the possibility that Lgl is involved in the germline signaling that induces follicle-cell differentiation can be excluded. Together, these data indicate that Lgl acts downstream of the polarizing signal from the PFC to specify the oocyte AP axis.

Phosphorylation of Lgl by aPKC is critical for oocyte polarity

Lgl can be phosphorylated by aPKC at three conserved serine residues in the central region of the protein, a process required for inactivation of Lgl (Betschinger et al., 2005; Betschinger et al., 2003). Using the co-IP technique, we detected aPKC in the protein complex pulled down by a polyclonal antibody against Lgl from the ovarian lysate (Fig. 6E), suggesting that Lgl and aPKC interact during oogenesis.

To determine whether the phosphorylation of Lgl by aPKC is required for oocyte polarization, we generated UASP:Lgl and UASP:Lgl-3A (a mutated form of Lgl in which all three aPKC phosphorylation sites were mutated to alanine) (Betschinger et al., 2003) transgenes, overexpressed them using a germline Gal4 driver *Matα4-Gal4-VP16* (*Mat-Gal4*), and examined oocyte polarity markers in these egg chambers. To confirm the expression of Lgl and Lgl-3A in the germline, we performed a western blot using the anti-Lgl and anti-phosphorylated-Lgl (pLgl) (Betschinger et al., 2003). We found that levels of pLgl in ovaries with germline overexpression of the wild-type Lgl transgene were more than twice as high as endogenous levels, whereas only endogenous levels of pLgl and a similar level of Lgl were detected in Lgl-3A-overexpressing egg chambers (see Fig. S1A,B in the supplementary material). Therefore, levels of expression of Lgl in Lgl and Lgl-3A overexpressing chambers were similar, as were levels of phosphorylatable Lgl in wild-type and Lgl-3A overexpressing chambers. Stau and *osk* mRNA were mislocalized in the majority of the Lgl-3A-overexpressing egg chambers at stages 9/10 (Stau: 74%, *n*=149; 44% complete, 10% partial and 20% diffuse, Fig. 3C,I; see Fig. S2C in the supplementary material; and data not shown; *osk*: 80%, *n*=130; 46% complete, 15% partial and 19% diffuse; Fig. 3F,I; and data not shown), Grk mRNA in the oocyte nucleus was mislocalized in 8% (*n*=150) of these egg chambers (Fig. 3L), and *bcd* mRNA was mislocalized to the posterior in 5% (*n*=120) of these egg chambers (see Fig. S3B in the supplementary material). In addition, Kin:β-gal was mislocalized in the oocyte (77%, *n*=121; 54% complete, 13% partial and 10% diffuse; Fig. 3O and data not shown), and a high density of microtubules was detected around the cortex (Fig. 3R) or in the central or lateral part of the oocyte (data not shown), indicating severe defects in microtubule organization in the oocyte. By contrast, although wild-type Lgl-overexpression also caused some Stau or *osk* mislocalization, the percentage was much lower (Stau: 31%, *n*=123; 10% complete, 17% partial and 4% diffuse, Fig. 3B,H, and see Fig. S2B in the supplementary material; and data not shown; *osk*: 32%, *n*=149; 9% complete, 20% partial and 3% diffuse; Fig. 3E,H; and data not shown), diffuse localization of *bcd* mRNA was detected at the anterior (3%, *n*=125; data not shown), and no defect was apparent in Grk localization (*n*=123; Fig. 3K). Microtubules appeared to be only mildly affected in these egg chambers (Fig. 3Q), and Kin:β-gal was modestly disrupted at the posterior (36%, *n*=117; 10% complete, 18% partial, and 8% diffuse; Fig. 3N). Because the differences between Lgl-3A and Lgl lie in whether they can be phosphorylated by aPKC, the stronger oocyte polarity defects in Lgl-3A-overexpressing egg chambers suggest that Lgl phosphorylation by aPKC is important for oocyte polarization.

The severe oocyte polarity defects caused by the nonphosphorylatable form of Lgl prompted us to study the requirement for aPKC in oocyte polarization. Previous clonal analysis revealed a requirement for aPKC in the maintenance of the oocyte cell fate (Cox et al., 2001). Because overexpression of Lgl-3A in the germarium using the *nanos-Gal4* drivers (Van Doren et al., 1998; Wang and Lin, 2004) did not cause any oocyte differentiation defect (data not shown), other aPKC targets may be involved in the maintenance of the oocyte fate during early oogenesis. Although 80% (*n*=101) of the $apkc^{k06403}$ germline clones were arrested at stage 5 or 6, we found that some germline clones developed until stages 9 and 10. In oocytes of these clones, the posterior localization of Stau was disrupted (47%, *n*=114; 35% complete, 7% partial and 5% diffuse; Fig. 4B and data not shown), suggesting a previously unidentified role for aPKC in polarizing the AP axis of the oocyte during mid-oogenesis. In these *apkc* germline clones, follicle-cell fate markers (Cut, Eya, and Hnt) showed normal expression patterns (data not shown). These data suggest that aPKC and Lgl may function in the same pathway to regulate the oocyte polarity.

To determine whether *apkc* and *lgl* interact genetically in germline cells, we overexpressed Lgl in a heterozygous *apkc* mutant background. These egg chambers demonstrated a greater penetrance of oocyte polarity defects (Stau: 56%, *n*=128; 16% complete, 38% partial and 2% diffuse; Fig. 4C,D) than did those with Lgl overexpression alone, suggesting that Lgl and aPKC

are functionally related in oocyte polarization. Moreover, co-overexpression of Lgl with aPKC alleviated the oocyte polarity defects (Stau: 10%, *n*=111; 1% complete, 8% partial and 1% diffuse; Fig. 4F,H) caused by Lgl overexpression alone (Fig. 4G and Fig. 3B), whereas overexpression of aPKC alone did not result in any obvious oocyte polarity defects (Stau: 96%, *n*=117; Fig. 4E). By contrast, co-overexpression of Lgl-3A with aPKC produced oocyte polarity defects similar to those of Lgl-3A overexpression alone (data not shown), suggesting that aPKC cannot modify the phenotypes caused by overexpression of the nonphosphorylatable form of Lgl. Together, these results strongly suggest that the phosphorylation of Lgl by aPKC plays a crucial role in regulating oocyte polarity formation.

aPKC restricts Lgl localization to the posterior of the oocyte

To examine the localization of Lgl in the oocyte, we stained egg chambers with anti-Lgl antibody, but endogenous Lgl was only detected at a minimal level in the germline cells (data not shown). We therefore used the UASP:Lgl transgene and stained for Lgl in egg chambers with Lgl overexpression driven by *Mat-Gal4*. Lgl was enriched at the posterior oocyte cortex in the majority of these egg chambers starting at stage 7 (stage 7, posterior: 79%; the entire cortex: 21%, *n*=115; Fig. 5A and data not shown; stage 9/10, posterior: 35%; posterior and half of the lateral: 42%; the entire cortex: 23%, *n*=106; Fig. 5B and data not shown). To confirm these localization patterns, we generated GFP-tagged Lgl (UASP:Lgl-GFP) and expressed it in the germline using the same driver, *Mat-Gal4*. Consistent with the Lgl pattern, Lgl-GFP was enriched at the posterior cortex in the majority of the egg chambers also starting at stage 7 (Fig. 5C,D).

To determine the effect of phosphorylation of Lgl on its localization, we stained for Lgl protein in Lgl-3A-overexpressing egg chambers and found Lgl localization throughout the oocyte cortex (100%, *n*=153; Fig. 5E,F). This localization pattern was confirmed in another transgenic line with overexpression of GFP-tagged Lgl-3A (Lgl-3A-GFP) in the germline (Fig. 5G,H). The expansion of nonphosphorylatable and active Lgl along the oocyte cortex suggests that Lgl phosphorylation by aPKC plays an important role in restricting Lgl to the posterior of the oocyte.

To analyze further the relationship between Lgl phosphorylation and the subcellular localization of Lgl in the oocyte, we monitored the distribution of Lgl in egg chambers with co-overexpression of Lgl (or Lgl-GFP) and aPKC$^{\Delta N}$-GFP (or aPKC$^{\Delta N}$) (a dominant active form of aPKC) (Betschinger et al., 2003). Most of these egg chambers failed to show Lgl association with the oocyte cortex, and the rest showed only weak posterior or weak entire cortex Lgl localization (no cortex localization: 59%; weak posterior: 23%; weak entire cortex: 18%, *n*=87; Fig. 5I,J and data not shown), indicating Lgl is excluded from the cellular cortex by aPKC phosphorylation. By contrast, the localization of Lgl-3A along the oocyte cortex was not changed in egg chambers with co-expression of Lgl-3A and aPKC$^{\Delta N}$-GFP when compared with Lgl-3A overexpression alone (see Fig. S4 in the supplementary material). These results suggest that phosphorylation of Lgl by aPKC is crucial for Lgl localization in the oocyte.

Active Lgl is involved in posterior enrichment of Par-1

The GFP tagged Par-1 (N1S) showed enrichment of the serine/threonine kinase Par-1 in the posterior (Fig. 6A), starting at stage 7, which was the earliest step of oocyte polarization after the PFC-oocyte signaling (Doerflinger et al., 2006;Vaccari et al., 2005). Because Lgl is also localized at the posterior cortex beginning at stage 7, we performed co-IP experiments to determine whether Par-1 (N1S) and Lgl interact biochemically in the germline cells. In the lysate from GFP-Par-1(N1S)-overexpressing egg chambers (Benton and St Johnston, 2003;Huynh et al., 2001), Lgl and GFP-Par-1 (N1S) immunoprecipitated together (Fig. 6E), suggesting that Lgl and Par-1 could form a complex in the germline cells during oogenesis.

To determine the relationship of Lgl and Par-1 localization in the oocyte, we used both UASP:GFP-Par-1(N1S) and UASP:GFP-Par-1 (N1S) K*, a kinase-dead form of the Par-1 (N1S) isoform that has exactly the same localization pattern as the normal Par-1 (N1S) protein (Vaccari et al., 2005) (Fig. 6B), to monitor any change in Par-1 localization in *lgl* germline clones. We found that both GFP tagged forms of Par-1 [(N1S) K* and (N1S)] were no longer enriched at the posterior but became uniformly distributed in the cytoplasm and cortical region of the oocyte (55%, *n*=31; Fig. 6C,C′ and data not shown), suggesting that Lgl is required for Par-1 (N1S) enrichment at the oocyte posterior. In egg chambers with Lgl-3A overexpression, active Lgl expanded towards the lateral domains of the oocyte cortex. We asked whether mislocalized active Lgl would disrupt Par-1 localization as well. In egg chambers with co-expression of Lgl-3A and GFP-Par-1 (N1S) in the germline, we found that GFP-Par-1 (N1S) expanded throughout the oocyte cortex, colocalizing with Lgl-3A (Fig. 6D-D″), indicating that active Lgl may play a role in directing Par-1 (N1S) to the oocyte cortex.

Lgl regulates actin organization in the oocyte

Par-1 (N1S) localization depends on actin but not microtubule organization in the oocyte (Doerflinger et al., 2006), and GFP-Par-1 (N1S) showed tight association with the cortical actin at the posterior cortex (Fig. 7A). In egg chambers with Lgl-GFP overexpression, Lgl-GFP also colocalized with the cortical actin cytoskeleton (Fig. 7B). Interestingly, under high magnification, the cortical actin in Lgl-GFP-overexpressing egg chambers exhibited projections mainly at the oocyte posterior (Fig. 7B). Careful observation of wild-type oocytes also identified such actin-rich structures at the posterior (Vanzo et al., 2007) (84%, *n*=45; Fig. 7C), albeit weaker than those in the Lgl-overexpressing oocytes, indicating that cortically localized Lgl may promote the formation of these actin projections. To test this hypothesis, we monitored actin organization in egg chambers with Lgl-3A overexpression or containing lgl^4 germline clones. If Lgl function is involved in the formation of the actin projections, the lateral oocyte cortex should have actin projections in egg chambers with Lgl-3A overexpression, which have active Lgl localized along the entire cortex. Indeed, in these egg chambers we observed long and thick actin projections throughout the cortex of the oocyte (91%, *n*=55; Fig. 7D). When treated with an actin depolymerizing drug, latrunculin A, the actin projections were dramatically compromised and mislocalized into the cytoplasm (data not shown). By contrast, actin projections were undetectable in lgl^4 germline clones (74%, *n*=35; Fig. 7E). These data suggest that active Lgl in the oocyte regulates the organization of the cortical actin cytoskeleton.

Discussion

The reorganization of the microtubule cytoskeleton during mid-oogenesis that leads to the formation of an AP gradient of microtubule density in the oocyte is essential for body-axis formation in *Drosophila* (Cha et al., 2001; Januschke et al., 2006; Theurkauf et al., 1992). Our findings show that genes such as *aPKC* and *lgl* that are important in polarizing other cell types play important roles in regulating the microtubule polarity in the oocyte. As in the neuroblast, aPKC regulates Lgl function by affecting its localization along the cellular cortex (Betschinger et al., 2003). Lgl-3A, the nonphosphorylatable and active form of Lgl, was localized along the oocyte cortex, whereas wild-type Lgl overexpression is mainly enriched at the posterior. Lgl was less localized to the posterior cortex when it was co-expressed with $aPKC^{\Delta N}$. For establishment of the posterior-to-anterior gradient of cortical Lgl, anterior aPKC activity is probably stronger than at the posterior. Although preferential localization of aPKC to the anterior cortex of the oocyte is plausible, similar to the apical localization of aPKC in the cortex of the neuroblast or epithelial cells, we did not observe such a strict anterior localization of aPKC in oocytes with GFP-tagged aPKC overexpression or UAS-aPKC overexpression using anti-aPKC antibody (data not shown). Although the transgene may not localize exactly the same as the endogenous form, other mechanisms may also be involved in restricting aPKC

activity in the anterior so that Lgl is enriched at the posterior oocyte cortex. For example, the mysterious signaling that is sent from the posterior follicle cells might be involved in inactivating aPKC in the posterior.

A previous report indicated that aPKC germline clones did not adversely affect oocyte polarity in stage 9 egg chambers (Doerflinger et al., 2006), but in our experiments we did observe defects in oocyte polarity in some *apkc* germline clones. Because no information was provided on the allele used or the number of clones analyzed in the report (Doerflinger et al., 2006), we do not know why the polarity defect we observed was not detected in this previous study. A possible reason is that not all *apkc* (*apkc*^{K06403}) germline clones that develop to stage 9/10 possess oocyte polarity defects, so a significant number of escapers have to be analyzed before the phenotype can be observed.

The level or dose of Lgl and its phosphorylation by aPKC appear to be crucial for oocyte polarity formation. Both excessive and insufficient amounts of unphosphorylated Lgl cause oocyte polarity defects, probably explaining why overexpression of the wild-type Lgl also caused some defects in oocyte polarity, perhaps because of an upper limit on the amount of Lgl that can be phosphorylated by endogenous aPKC. When overexpression of Lgl is massive, only some of the overexpressed Lgl can be phosphorylated by aPKC, and the rest remains active and cortically localized. The alleviation of Lgl-overexpression-induced oocyte polarity defects by co-overexpression of Lgl and aPKC supports this hypothesis. A fine balance appears to be necessary between the amount of phosphorylated and unphosphorylated Lgl in the oocyte. Alteration of the levels of Lgl or aPKC can therefore cause varying severities of defects in oocyte polarity. Interestingly, *lgl* loss of function and Lgl-3A overexpression caused similar Stau/*osk* localization defects, which are probably results of disrupted actin and microtubule organization in these mutant backgrounds. The cytoskeletal defects could have been translated into defects in the Par-1 posterior enrichment that is crucial for Stau and *osk* localization.

Indeed, our data suggest that posterior enrichment of Par-1 is regulated by Lgl. Par-1 has also been shown to be a phosphorylation target of aPKC in HEK293 cells (Hurov et al., 2004), and Par-1 localization could be regulated by aPKC phosphorylation in the oocyte (Doerflinger et al., 2006). aPKC therefore probably has dual function in restricting Par-1 kinase activity to the oocyte posterior. First, it prevents Par-1 from localizing to the anterior cortex through direct phosphorylation, and second, it promotes Par-1 localization to the posterior by restricting active Lgl to the posterior. To explain the function of Lgl in localizing molecules in cell-polarity formation, two different, but not mutually exclusive, models have been proposed (Vasioukhin, 2006). The first model is based on findings in yeast and vertebrate cells, demonstrating that Lgl homologs regulate docking and fusion of post-Golgi vesicles with the plasma membrane through the SNARE complex (Lehman et al., 1999). In this scenario, Lgl could be involved in the exocytosis pathway and membrane trafficking. A *sec5* mutant has been reported (Murthy et al., 2005) to show impaired membrane trafficking of Grk, but the cytoskeleton was correctly oriented. These phenotypes differ from the defects we found in *lgl* loss- and gain-of-function mutants. The potential involvement of Lgl in the exocytosis pathway in the oocyte is therefore unlikely to be the sole explanation of the serious polarity defects observed in *lgl*^{4}- and Lgl-3A-overexpressing egg chambers. The second model is related to the role of Lgl in actomyosin cytoskeletal regulation, which is based on genetic interactions between Lgl and actin-binding proteins such as Myosin II [encoded by *zipper* (*zip*)] and Myosin VI (Ohshiro et al., 2000; Petritsch et al., 2003; Strand et al., 1994). Our finding that the cortical actin morphology was dramatically changed when the active Lgl was overexpressed supports this model. In neuroblasts, Lgl can promote the cortical localization of Miranda by restricting Myosin II localization to the apical cortex. Germline clones of a *zip* loss-of-function allele, however, caused arrest of oogenesis at an early stage, before the reorganization of the microtubules takes place (data not shown). By contrast, *miranda* loss-of-function germline clones showed no

defects in oocyte AP polarity (Irion et al., 2006). A recent study demonstrated that actin projections at the oocyte cortex require Osk (Vanzo et al., 2007). Whether Lgl localization is normal in *osk* mutants is unknown. Because *osk* is mislocalized in the Lgl-3A overexpressing egg chambers, which still have long actin projections, the impact of Osk in actin organization is probably indirect. Lgl and Osk may affect some common targets that regulate cortical actin in the oocyte.

Our data indicate that genetic manipulations of either *lgl* or *apkc* produced incomplete penetrance of oocyte polarity defects. In fact, a number of previously reported mutations also had partial penetrance of Stau mislocalization (*14-3-3*, 62%; $BAZ^{S151A,S1085A}$ misexpression, 49%; *rab6*, 64.47%), and the penetrance of mislocation of Grk/oocyte nucleus has not been reported in these mutants (Benton and St Johnston, 2003; Benton et al., 2002; Coutelis and Ephrussi, 2007). Because many genes and pathways are involved in the regulation of cytoskeletal organization in the oocyte, functional redundancy and crosstalk between different pathways are probably unavoidable in this complicated polarization process. Other genes may act in parallel with Lgl and aPKC to regulate actin organization and Par-1 localization, which in turn controls posterior localization of morphogens.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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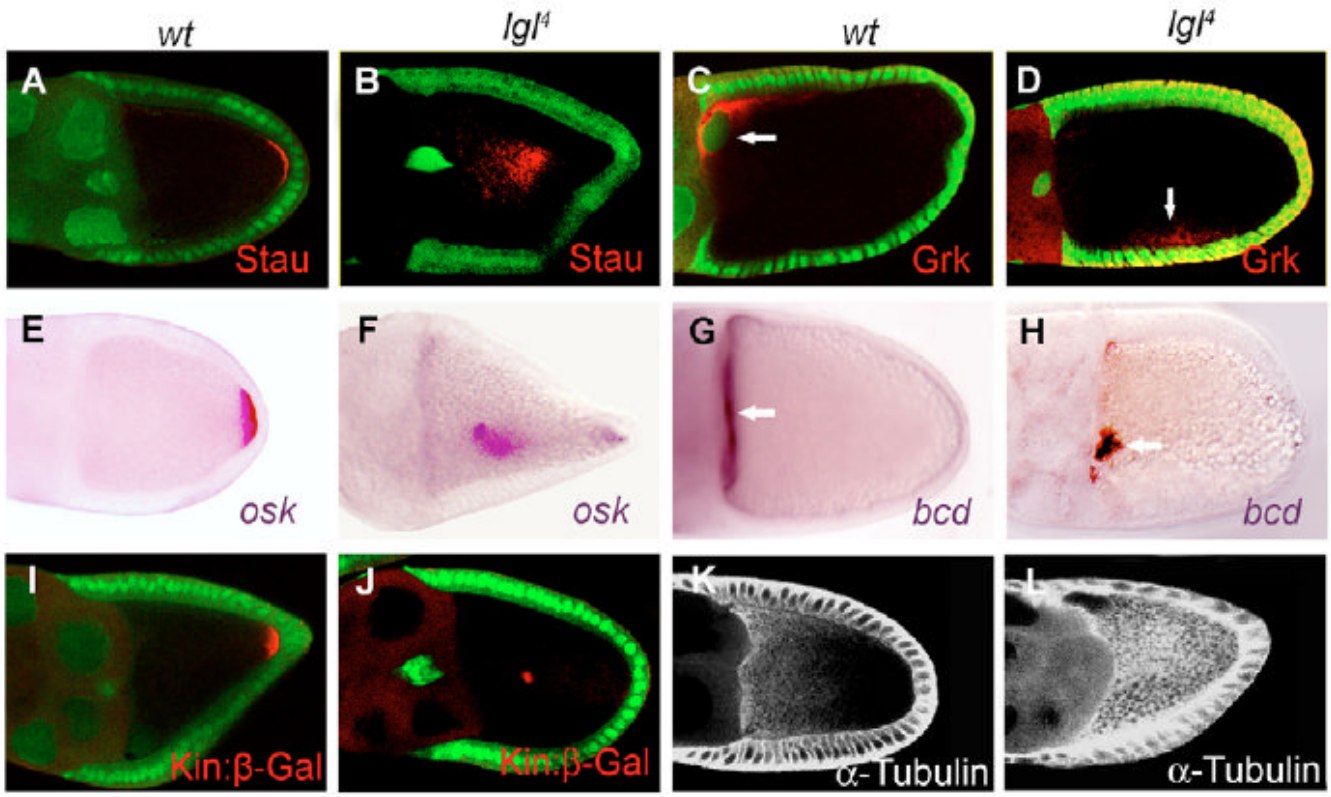

Fig. 1. *lgl* germline clones affect oocyte polarity

(**A,B**) Stau was localized at the posterior in the wild type (A) but is mislocalized in *lgl*^{4} mutant germline clones (B). (**C,D**) Grk and the oocyte nucleus (arrow) were localized to the anterior corner (C) but were mislocalized to a lateral position in some *lgl*^{4} mutant germline clones (D). (**E,F**) *osk* mRNA in wild-type egg chambers showed a posterior localization at stage 10 (E), whereas *lgl*^{4} germline clones displayed clear mislocalization of *osk* mRNA to the center of the oocyte (F). (**G,H**) *bcd* mRNA in wild-type egg chambers showed disc-shaped localization in the oocyte anterior (G, arrow), but in *lgl*^{4} germline clones *bcd* sometimes accumulated as dots at the anterior of the oocyte (H, arrow). (**I,J**) Kin:β-Gal showed posterior localization in the wild type (I) but was mislocalized to the center of the oocyte in *lgl*^{4} germline clones (J). (**K,L**) α-Tubulin showed an anterior-to-posterior gradient in the wild-type oocyte (K) but was uniformly distributed in *lgl*^{4} germline clones (l). Mutant germline clones were marked by the absence of GFP (green) in the nuclei of the nurse cells (B,D,J) or were generated by the dominant female sterile method (F,H,L). Anterior is towards the left and posterior is towards the right in all panels.

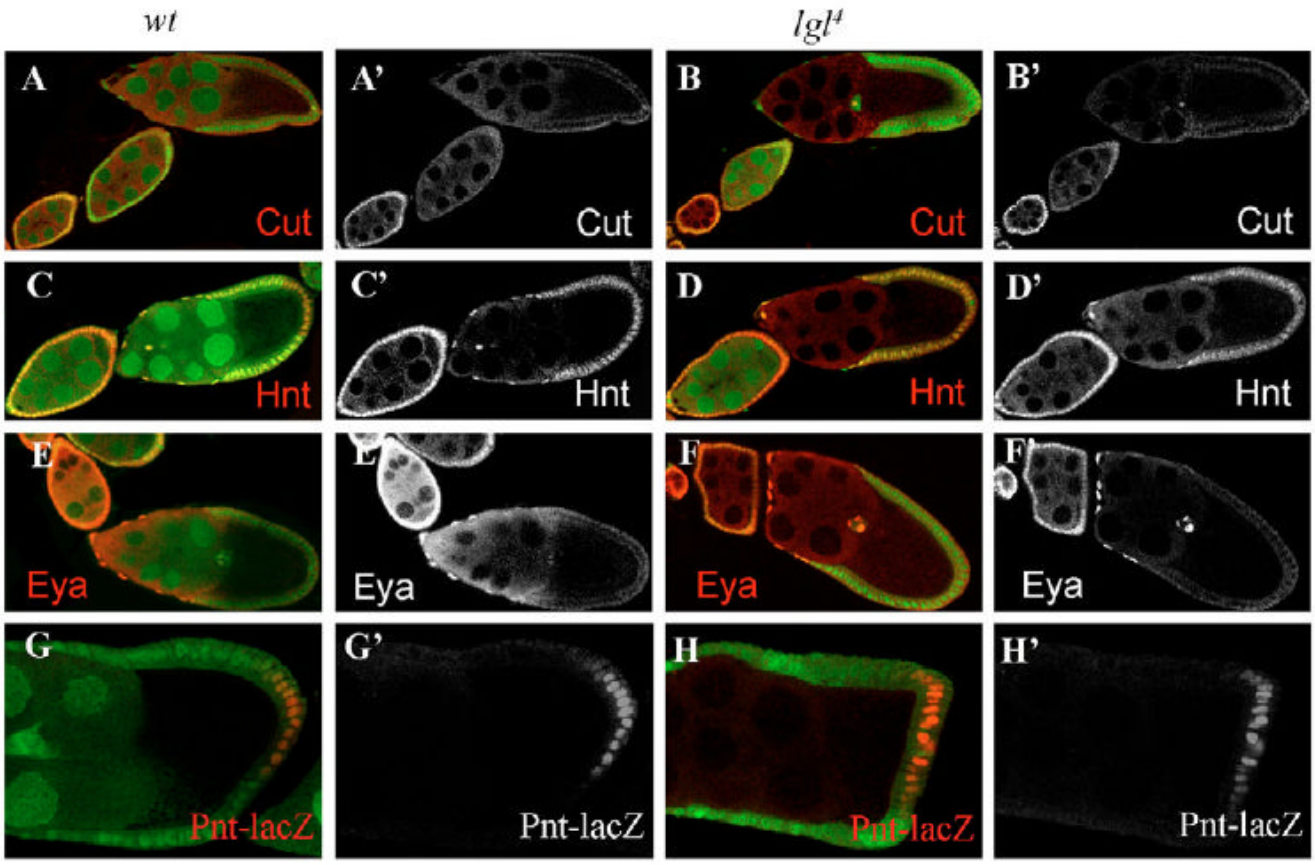

Fig. 2. Follicle-cell fate markers are unaffected in *lgl* germline clones

(**A-H′**) Cut is an immature cell fate marker that is downregulated by Notch signaling at stage 6. Hindsight (Hnt) is expressed after stage 6 and Eyes absent (Eya) is normally expressed in anterior follicle cells and immature follicle cells. Pointed-*lacZ* is a posterior follicle-cell fate marker. No differences in expression of these markers between lgl^4 germline clones (B,D,F,H) and the wild type (A,C,E,G) were detected. Mutant germline clones were marked by the absence of GFP (green) in the nuclei of the nurse cells.

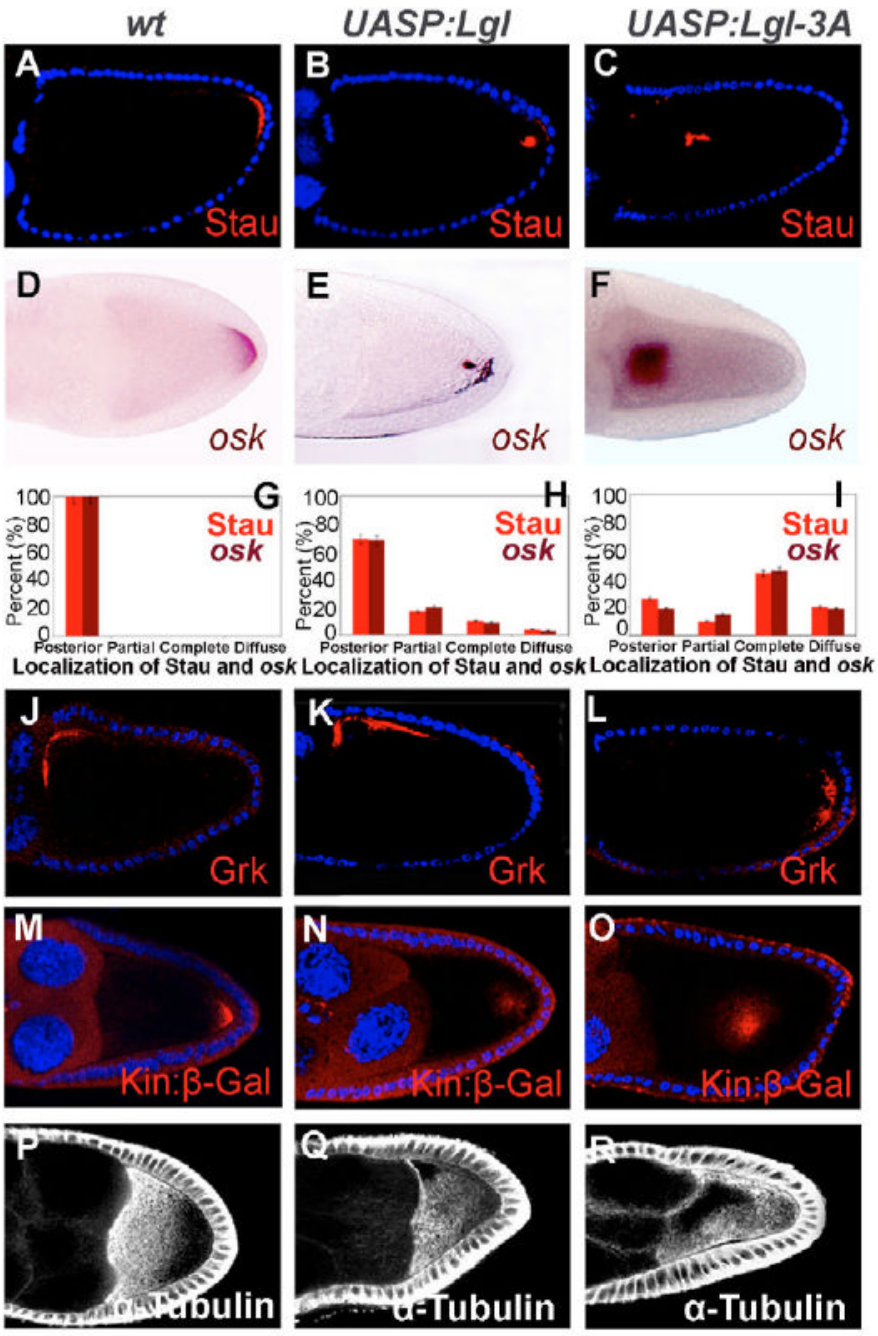

Fig. 3. Lgl phosphorylation can regulate oocyte polarity

Stau (**A-C**), *osk* (**D-F**), the quantification of Stau and *osk* mRNA localization (**G-I**), Grk (**J-L**), Kin:β-Gal (**M-O**), α-Tubulin (**P-R**) in the wild type (driver only) (A,D,G,J,M,P), Lgl-overexpressing (B,E,H,K,N,Q) and Lgl-3A-overexpressing (C,F,I,L,O,R) egg chambers are shown. In egg chambers with Lgl overexpression, Stau (B,H), *osk* (E,H) and Kin:β-gal (N) were partially mislocalized, but Grk (K) showed normal localization, and α-Tubulin (Q) displayed a weaker anterior-posterior gradient than in the wild type (P). In egg chambers with Lgl-3A overexpression, Stau (C,I), *osk* (F,I) and Kin:β-gal (O) were frequently mislocalized to the center of the oocyte, Grk and the oocyte nucleus (L) were mislocalized in some egg

chambers, and α-Tubulin showed higher concentrations along the cortex and lower concentration at the center of the oocyte (R).

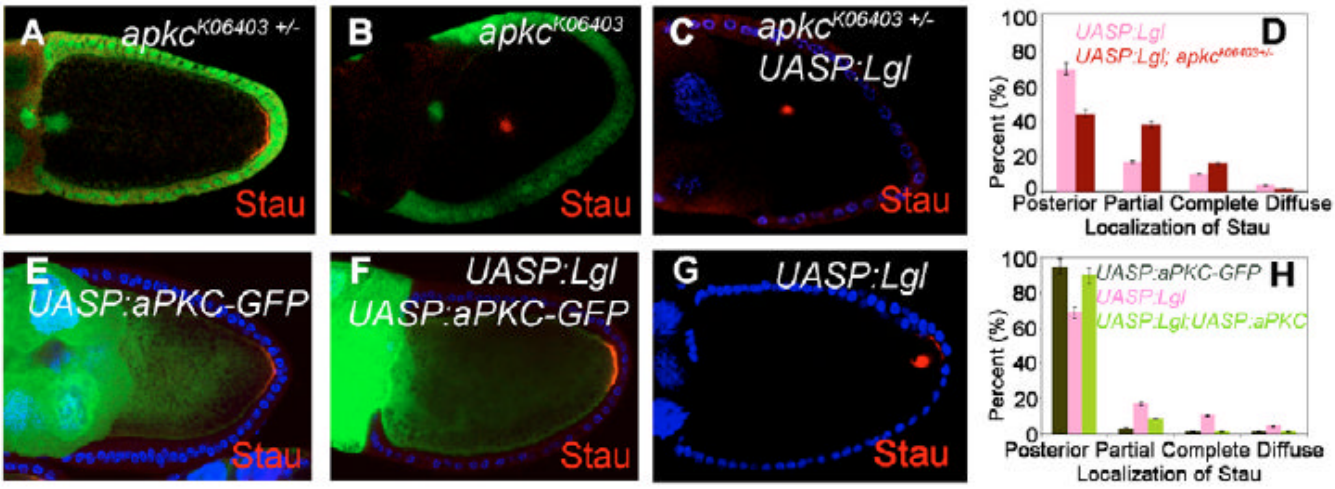

Fig. 4. The genetic interaction between Lgl and aPKC shows that Lgl phosphorylation by aPKC can regulate oocyte polarity

(**A, B**) Stau was normally localized in the *apkc*^{k06403} heterozygous mutant (A), but mislocalized in *apkc*^{k06403} mutant germline clones (B); mutant clones were marked by the absence of nuclear GFP (green). (**C, D**) Loss of one copy of *apkc* exaggerated the Stau mislocalization phenotype stemming from Lgl overexpression. Stau localization in these backgrounds was quantified (D). (**E**) Stau is normally localized in aPKC-GFP overexpression. (**F**) Overexpression of aPKC-GFP can rescue the Stau mislocalization phenotype caused by the Lgl overexpression. (**G**) Stau is mislocalized in the Lgl-overexpressing egg chambers. (**H**) Stau localization in these backgrounds was quantified.

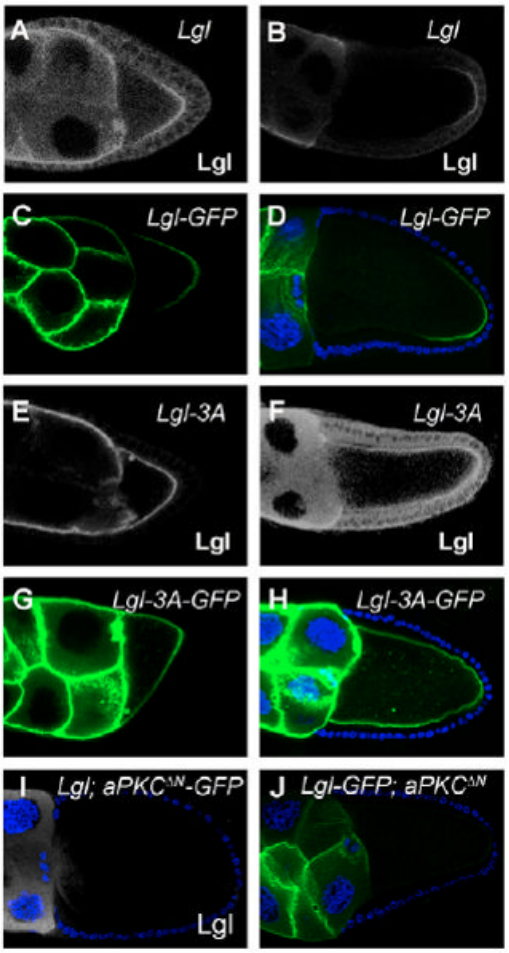

Fig. 5. aPKC phosphorylation of Lgl restricts Lgl localization to the oocyte posterior

(**A,B**) Lgl was localized at the oocyte posterior in Lgl-overexpressing egg chambers at stage 7 (A) and stage 9/10 (B). (**C,D**) Lgl was localized at the oocyte posterior in Lgl-GFP-overexpressing egg chambers at stage 7 (C) and stage 9/10 (D). (**E,F**) Lgl was localized throughout the cortex in Lgl-3A-overexpressing egg chambers at stage 7 (E) and stage 9/10 (F). (**G,H**) Lgl was localized throughout the cortex in Lgl-3A-GFP-overexpressing egg chambers at stage 7 (G) and stage 9/10 (H). (**I,J**) When Lgl or Lgl-GFP was co-overexpressed with aPKC$^{\Delta N}$-GFP (GFP is not shown here) or aPKC$^{\Delta N}$, Lgl or Lgl-GFP was excluded from the cortex.

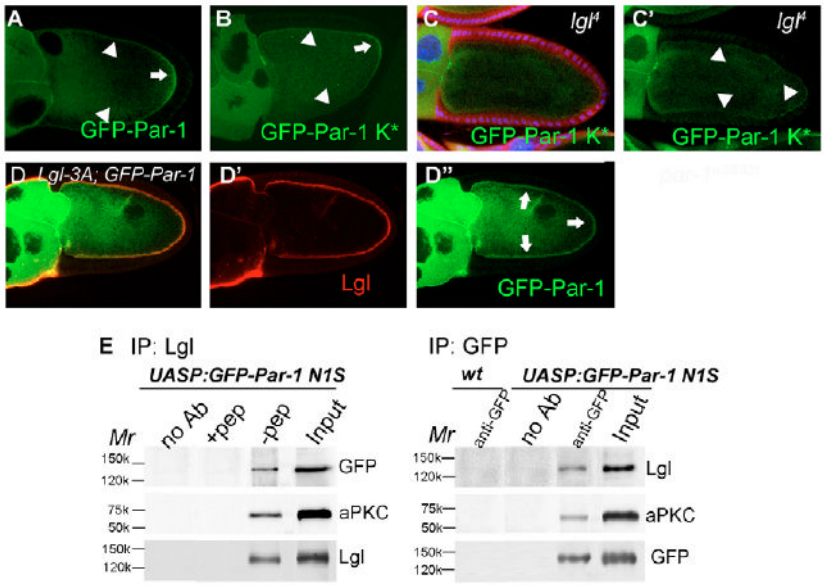

Fig. 6. Lgl interacts with Par-1 and is required for Par-1 localization

(**A,B**) Both the GFP-Par-1(N1S) (A) and GFP-Par-1(N1S) K* (B) were enriched at the posterior at stage 10. (**C,C′**) The GFP-Par-1(N1S) K* lost the posterior enrichment in *lgl*^{4} germline cells. Mutant germline clones were marked by the absence of β-gal staining (red) in the nuclei of the nurse cells. (**D-D″**) GFP-Par-1(N1S) colocalized with Lgl and was detected throughout the oocyte cortex in egg chambers with co-overexpression of Lgl-3A and GFP-Par-1(N1S). Arrows indicate the strong GFP-Par-1 and arrowheads indicate the weak GFP-Par-1. (**E**) Immunoblots of anti-Lgl or anti-GFP immunoprecipitation from ovaries with GFP-Par-1 (N1S) overexpression (*mat-Gal4* driver) or the wild-type control (*mat-Gal4* driver only). aPKC and GFP-Par-1 (N1S) proteins were precipitated by the anti-Lgl antibody from GFP-Par-1 (N1S) overexpression, and aPKC and Lgl proteins were precipitated by an anti-GFP antibody from GFP-Par-1 (N1S) overexpression. Input was one-fifth of the total ovarian extract. In anti-Lgl immunoprecipitates, controls were beads (without added antibody) and peptide blocks (+pep); in anti-GFP immunoprecipitates, controls were beads (without added antibody) and ovarian extract from wild-type flies.

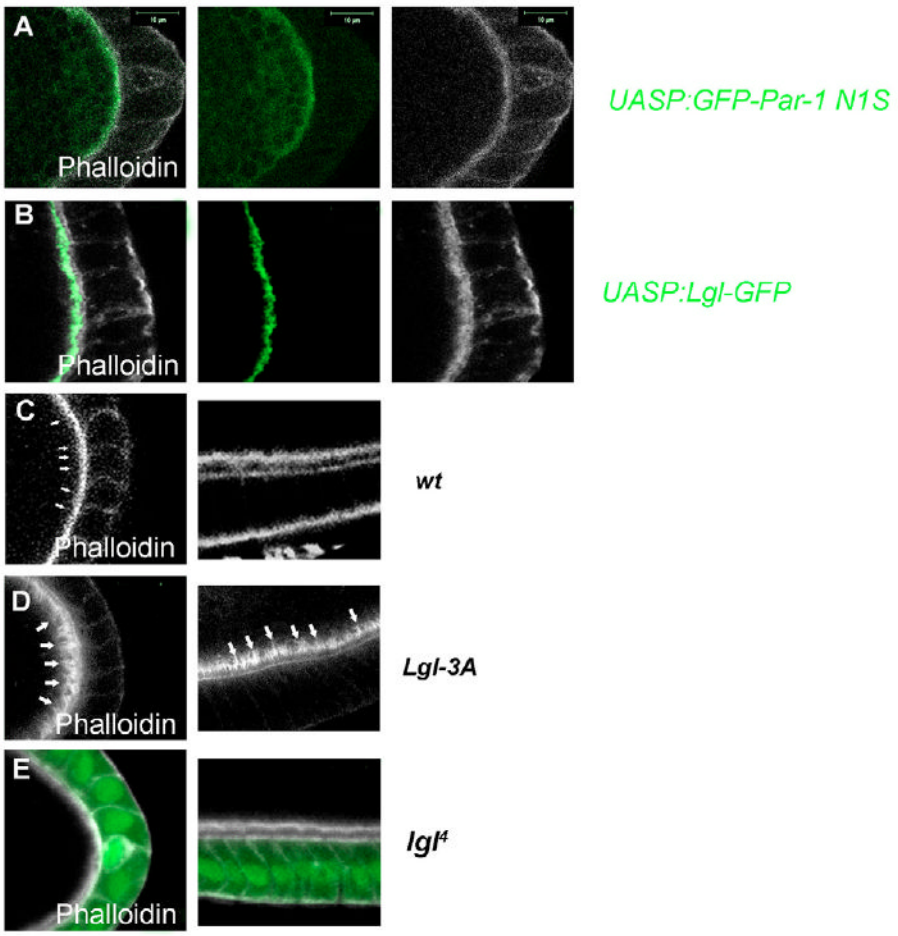

Fig. 7. Lgl regulates actin organization in the oocyte

(**A**) GFP-Par-1 (N1S) was tightly associated with the cortical actin at the oocyte posterior. (**B**) In Lgl-GFP-overexpressing egg chambers, Lgl-GFP was enriched at the oocyte posterior and showed tight association with cortical actin, which had projections at the posterior of the oocyte. (**C**) Actin staining in the wild-type egg chamber. The weak long actin projections were indicated by arrows. (**D**) The long actin-rich projections (arrows) were very obvious along the oocyte cortex in Lgl-3A-overexpressing egg chambers. (**E**) In *lgl*^{4} germline clones, no cortical actin projections were detected. All of them are stage 10 egg chambers.