Aurora A Triggers Lgl Cortical Release during Symmetric Division to Control Planar Spindle Orientation

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Summary

Mitotic spindle orientation is essential to control cell-fate specification and epithelial architecture [1]. The tumor suppressor Lgl localizes to the basolateral cortex of epithelial cells, where it acts together with Dlg and Scrib to organize apicobasal polarity [2]. Dlg and Scrib also control planar spindle orientation [3, 4], but how the organization of polarity complexes is adjusted to control symmetric division is largely unknown. Here, we show that the Dlg complex is remodeled during Drosophila follicular epithelium cell division, when Lgl is released to the cytoplasm. Lgl redistribution during epithelial mitosis is reminiscent of asymmetric cell division, where it is proposed that Aurora A promotes aPKC activation to control the localization of Lgl and cell-fate determinants [5]. We show that Aurora A controls Lgl localization directly, triggering its cortical release at early prophase in both epithelial and S2 cells. This relies on double phosphorylation within the putative aPKC phosphorylation site, which is required and sufficient for Lgl cortical release during mitosis and can be achieved by a combination of aPKC and Aurora A activities. Cortical retention of Lgl disrupts planar spindle orientation, but only when Lgl mutants that can bind Dlg are expressed. Hence, our work reveals that Lgl mitotic cortical release is not specifically linked to the asymmetric segregation of fate determinants, and we propose that Aurora A activation breaks the Dlg/Lgl interaction to allow planar spindle orientation during symmetric division via the Pins (LGN)/Dlg pathway.

Results and Discussion

Remodeling of Polarity Proteins during Follicle Cell Division

How evolutionarily conserved polarity complexes establish distinct membrane domains and the polarized assembly of junctions along the apicobasal axis has been extensively characterized [6]. One general feature is that it relies on mutual antagonism between apical atypical protein kinase C (aPKC) and Crumbs complexes and a basolateral complex formed by Scribble (Scrib), Lethal giant larvae (Lgl), and Discs large (Dlg) [7–9]. We used the Drosophila follicular epithelium as an epithelial polarity model to address how polarity is coordinated during symmetric division. Dlg and Scrib have been shown to provide a lateral cue for planar spindle orientation [3, 4]. Accordingly, Scrib and Dlg remain at the cortex during follicle cell division (Figures 1A and 1B and Movie S1 available online) [3]. In contrast, Lgl is released from the lateral cortex to the cytoplasm during mitosis (Figure 1A and Movie S1). This subcellular reallocation begins during early prophase, since Lgl starts to be excluded from the cortex prior to cell rounding, one of the earliest mitotic events [10], and is completely cytoplasmic before nuclear envelope breakdown (NEB). Thus, the Dlg complex is remodeled at mitosis onset in epithelia.

The subcellular localization of Lgl is controlled by aPKCmediated phosphorylation of a conserved motif, which blocks Lgl interaction with the apical cortex [11–14]. To address the mechanism of cortical release during mitosis, we expressed the nonphosphorytable form Lgl^{3A}-GFP in the follicular epithelium. Lgl^{3A}-GFP remains at the cortex throughout mitosis (Figure 1C and Movie S1), indicating that Lgl dynamics during epithelial mitosis also rely on the aPKC phosphorylation motif. Although the apical aPKC complex depolarizes during follicle cell division [3, 15], Lgl cortical release precedes aPKC depolarization (Figure S1A). Using Par-6-GFP as a marker for the aPKC complex and the Lgl cytoplasmic accumulation as readout of its cortical release, we found that maximum cytoplasmic accumulation of Lgl occurs when most Par-6 is still apically localized (w70% relative to interphase levels; Figures 1D and 1E). Thus, Lgl cortical release is the first event of the depolarization that characterizes follicle cell division, indicating that Lgl reallocation does not require extension of aPKC along the lateral cortex.

Aurora A Triggers Lgl Cortical Exclusion during Mitosis

Although the major pools of Lgl and aPKC are segregated during interphase, Lgl has a dynamic cytoplasmic pool that rapidly exchanges with the cortex [5, 16]. Thus, further activation of aPKC at mitosis onset would be expected to shift the equilibrium toward cytoplasmic localization. Lgl dynamic redistribution in epithelia is similar to the neuroblast, where activation of Aurora A (AurA) leads to Par-6 phosphorylation and subsequent aPKC activation [5]. To test whether a similar mechanism induced Lgl cortical release during epithelial mitosis, we analyzed Lgl subcellular localization in *aPKC* mutants and in *par-6* mutants unphosphorylatable by AurA. Lgl cytoplasmic accumulation is unaffected in *par-6*; $par\text{-}6^{S34A}$ mutant cells (Figures 1F and 1G). Temperature-sensitive $aPKC^{ts}/aPKC^{k06403}$ mutants display strong cytoplasmic accumulation of Lgl during prophase, with a minor delay relatively to the wild-type (Figures 1F, 1G, and S1B). Moreover, homozygous mutant clones for null ($aPKC^{k06403}$) and kinase-defective ($aPKC^{psu141}$) alleles also display Lgl cortical release during mitosis (Figures 1H and 1I). These results implicate that although aPKC activity may contribute for Lgl mitotic dynamics, the putative aPKC phosphorylation motif is under the control of a different kinase, which triggers Lgl cortical release in the absence of aPKC.

AurA is a good candidate to induce Lgl cortical release as it controls polarity during asymmetric division [5, 17, 18]. Furthermore, *Drosophila* AurA is activated at the beginning of prophase, which coincides with the timing of Lgl cytoplasmic reallocation [19]. To examine whether AurA controls Lgl dynamics in the follicular epithelium, we generated homozygous mutant clones for

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the kinase-defective allele *aurA*^{37} [20]. In contrast to wild-type cells, we could only detect low amounts of cytoplasmic Lgl during prophase in *aurA*^{37} mutants, which display a pronounced delay in the cytoplasmic reallocation of Lgl during mitosis (100%, n = 16; Figure 2A and Movie S2). This delayed cortical release of Lgl has been previously reported during asymmetric cell division in *aurA*^{37} mutants [5], possibly resulting from residual kinase activity. Thus, AurA is essential to trigger Lgl cortical exclusion at epithelial mitosis onset.

The idea that Lgl mitotic reallocation is directly controlled by a mitotic kinase implies that Lgl should display similar dynamics regardless of the polarized status of the cell. Consistently, Lgl-GFP is also released from the cortex before NEB in nonpolarized *Drosophila* S2 cells (Figure 2B). Furthermore, Lgl^{3A}-GFP is retained in the cortex during mitosis, revealing that Lgl cortical release is also phosphorylation dependent in S2 cells (Figure 2C). Treatment with a specific AurA inhibitor (MLN8237), or with aurA RNAi, strongly impairs Lgl cortical release during prophase, as Lgl is present in the cortex at NEB (Figures 2D, 2E, S2A, and S2C). However, inhibition of AurA still allows later cortical exclusion, which could result from the activity of another kinase. Despite their distinct roles, AurA and Aurora B (AurB) phosphorylate common substrates in vitro [21]. We therefore analyzed whether AurB could act redundantly with AurA. Inactivation of AurB with a specific inhibitor, Binucleine 2, enables normal Lgl cytoplasmic accumulation before NEB and still allows later cortical exclusion in cells treated simultaneously with the AurA inhibitor (Figure S2B). As AurB does not seem to participate on Lgl mitotic dynamics, we used RNAi directed against aPKC to examine whether it could act redundantly with AurA. aPKC depletion did not block Lgl cortical exclusion, but it was slightly delayed (Figures 2D, 2E, and S2D). However, simultaneous AurA inhibition and *aPKC* RNAi produced almost complete cortical retention of Lgl during mitosis (Figure 2E). Thus, AurA induces Lgl release during early prophase, but aPKC retains its ability to phosphorylate Lgl during mitosis.

Double Phosphorylation of Lgl Controls Cortical Release

To address which serine(s) within the phosphorylation motif of Lgl control its dynamics during mitosis, we generated individual and double mutants. As complete cortical release occurs before NEB, we quantified the ratio of cytoplasmic to cortical mean intensity of Lgl-GFP at NEB to compare each different mutant. All the single mutants displayed similar dynamics to Lgl^{WT}, exiting to the cytoplasm prior to NEB (Figures 3A and 3C and Movie S3). In contrast, all double mutants were cortically retained during mitosis (Figures 3B and 3C and Movie S3), indicating that double phosphorylation is both sufficient and required to efficiently block Lgl cortical localization.

The ability to doubly phosphorylate Lgl would explain how AurA drives Lgl cortical release. Accordingly, the sequence surrounding S656 perfectly matches AurA phosphorylation consensus ((R/K/N)RX(Sp/Tp)Φ; Φ denotes any hydrophobic amino acid with exception of proline, and X denotes any residue [22]), whereas the S664 surrounding sequence shows an exception in the -3 position (Figure 3D). In contrast, the sequence surrounding S660 does not resemble AurA phosphorylation consensus, and AurA does not directly phosphorylate S660 in vitro as detected by phosphospecific antibodies against S660 [5]. We confirmed that S656 is directly phosphorylated by recombinant AurA in vitro using a phosphospecific antibody for S656 (Figure 3E). Moreover, AurA inhibition or *aurA* RNAi results in a similar cortical retention at NEB to Lgl^{S656A,S664A} (Figure 3C [green] and Movie S3), suggesting that AurA also controls S664 phosphorylation during mitosis, whereas aPKC would be the only kinase active on S660 (Figure 3D). Consistent with this, *aPKC* RNAi increases the cortical retention of Lgl^{S656A,S664A}, mimicking the localization of Lgl^{3A} (Figure 3C [red] and Figure 3F). Furthermore, whereas S660A mutation does not significantly affect the cytoplasmic accumulation of Lgl in *aPKC* RNAi, S656A and S664A

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mutations disrupt Lgl cortical release in *aPKC*-depleted cells, leading to the degree of cortical retention of Lgl^{S656A,S660A} and Lgl^{S660A,S664A}, respectively (Figure 3C [blue], Figure 3G, and Movie S3). Altogether, these results support that AurA controls S656 and S664 and that these phosphorylations are partially redundant with aPKC phosphorylation to produce doubly phosphorylated Lgl, which is released from the cortex.

Lgl Cortical Release Promotes Planar Spindle Orientation

RNAi-mediated knockdown of Lgl in vertebrate HEK293 cells results in defective chromosome segregation [23]. Furthermore, overexpressed Lgl-GFP shows a slight enrichment on the mitotic spindle (e.g., Figure 2B), suggesting that relocalization of Lgl could be important to control chromosome segregation. However, *lgl* mutant follicle cells assemble normal bipolar spindles, and although we could detect minor defects on chromosome segregation (4% lagging chromosomes in anaphase, n = 25), the mitotic timing (time between NEB and anaphase) is indistinguishable between lgl and wild-type cells (8.8 ± 0.7 min versus 9.3 ± 1 min, p = 0.19; Figures S3A and S3B). Additionally, loss of Lgl activity allows proper chromosome segregation in both *Drosophila* S2 cells and syncytial embryos (Figures S3C–S3E and Movie S4). Thus, Lgl does not seem to have a general role in the control of faithful chromosome segregation in *Drosophila*.

Nevertheless, Lgl cortical release could per se play a mitotic function, as key mitotic events are controlled at the cortex. In fact, the orientation of cell division requires the precise connection between cortical attachment sites and astral microtubules, which relies on the plasma membrane associated protein Pins (vertebrate LGN) [24, 25]. Pins uses its TPR repeat domain to bind Mud (vertebrate NUMA), which recruits the dynein complex to pull on astral microtubules, and its linker domain to interact with Dlg, which participates on the capture of microtubule plus ends [26–28]. Notably, Pins/LGN localizes apically during interphase in *Drosophila* and vertebrate epithelia, being reallocated to the lateral cortex to orient cell division. Pins relocalization relies on aPKC in some epithelial tissues, but not in chick neuroepithelium and in the *Drosophila* follicular epithelium, where Dlg provides a polarity cue to restrict Pins to the lateral cortex [3, 4, 29–33]. Dlg controls Pins localization during both asymmetric and symmetric division, and a recent study has shown that vertebrate Dlg1 recruits LGN to cortex via a direct interaction [33, 34]. However, Dlg uses the same phosphoserine binding region within its guanylate kinase (GUK) domain to interact with Pins/LGN and Lgl [28, 35–37]. Thus, maintenance of a cortical Dlg/Lgl complex during mitosis is expected to impair the ability of Dlg to bind Pins and control spindle orientation.

Interaction between the Dlg's GUK domain and Lgl requires phosphorylation of at least one serine within the aPKC phosphorylation site [36]. Although the phosphorylation-dependent binding of Lgl to Dlg remains to be shown in *Drosophila*, crystallographic studies revealed that all residues directly involved in the interaction with p-Lgl are evolutionarily conserved from *C. elegans* to humans [36]. Thus, whereas Lgl^{3A} does not form a fully functional Dlg/Lgl polarity complex, double mutants should bind Dlg's GUK domain and are significantly retained at the cortex during mitosis due to the inability to be double phosphorylated (Figure 4A). This led us to examine their ability to support epithelial polarization during interphase and to interfere with mitotic spindle orientation. We performed rescue experiments in mosaic egg chambers containing *lgl^{27S3}* null follicle cell clones. *lgl* mutant clones display multilayered cells with delocalization of aPKC (Figure 4B) [38]. This phenotype is rescued by Lgl-GFP, but not by Lgl^{3A}-GFP (Figure 4B). More importantly, in contrast to Lgl^{S660A,S664A}, which extends to the apical domain in wild-type cells (red arrows) and fails to rescue epithelial polarity in lgl mutant cells, Lgl^{S656A,S660A} and Lgl^{S656A,S664A} can rescue epithelial polarity, localizing with Dlg at the lateral

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cortex and below aPKC (Figures 4C and 4D). Hence, aPKC-mediated phosphorylation of S660 or S664 is sufficient on its own to control epithelial polarity and to confine Lgl to the lateral cortex.

We then examined whether exclusion of Lgl from the cortex and the consequent release from Dlg would be functionally relevant for oriented cell division. Expression of Lgl-GFP or Lgl^{3A}-GFP does not affect planar spindle orientation during follicle cell division (Figures 4E and 4G). In contrast, Lgl double mutants display metaphasic cells in which the spindle axis, determined by centrosome position, is nearly perpendicular to the epithelial layer (Figures 4E and 4G). Live imaging revealed that these spindle orientation defects were maintained throughout division as we could follow daughter cells separating along oblique and perpendicular angles to the epithelia (Figure 4F and Movie S5). Moreover, we detected equivalent defects on planar spindle orientation upon expression of $Lgl^{S656A,S664A}$ in the lgl or wild-type background, indicating that cortical retention of Lgl exerts a dominant effect (Figures 4E and 4G). Interestingly, $Lgl^{S656A,S660A}$ and $Lgl^{S656A,S664A}$ induce higher randomization of angles, whereas $Lgl^{S660A,S664A}$, which is less efficiently restricted to the lateral cortex, produces a milder phenotype. Altogether, these results indicate that retention of Lgl at the lateral cortex disrupts planar spindle orientation only if Lgl can interact with Dlg.

Despite the ability of $Lgl^{S656A,S660A}$-GFP to rescue epithelial polarity in *lgl* mutants, strong overexpression of $Lgl^{S656A,S660A}$-GFP, but not of other Lgl double mutants, can dominantly disrupt epithelial polarity during the proliferative stages of oogenesis (Figure 4C, white arrows). One interpretation is that $Lgl^{S656A,S660A}$ forms the most active lateral complex of the mutant transgenes, disrupting the balance between apical and lateral domains. We therefore examined whether the dominant effect of Lgl cortical retention on spindle orientation could solely result from Dlg mislocalization. Dlg is properly localized at the lateral cortex in $Lgl^{S656A,S660A}$-expressing cells presenting misoriented spindles, but this position does not correlate with the orientation of the centrosomes (Figure 4H). Thus, cortical retention of Lgl interferes with Dlg's ability to transmit its lateral cue to instruct spindle orientation, which may result from an impairment of the Dlg/Pins interaction.

In conclusion, our findings outline a mechanism that explains how the lateral domain is remodeled to accomplish oriented epithelial cell division, unveiling that AurA has a central role in controlling the subcellular distribution of Lgl. AurA regulates the activity of aPKC at mitotic entry during asymmetric division [5], and our results are consistent with the ability of aPKC to phosphorylate and collaborate in Lgl cortical release. However, in epithelia, aPKC accumulates in the apical side during interphase, where it induces apical exclusion of Lgl, in part by generating a phosphorylated form that binds Dlg [11, 36]. Consequently, aPKC has a reduced access to the cortical pool of Lgl at mitotic entry and would be unable to rapidly induce Lgl cortical exclusion. Our data show that cell-cycle-dependent activation of AurA removes Lgl from the lateral cortex through AurA's ability to control Lgl phosphorylation on S656 and S664 independently of aPKC. Thus, AurA and aPKC exert the spatiotemporal control of Lgl distribution to achieve unique cell polarity roles in distinct cell types.

We propose that release of Lgl from the cortex allows Dlg interaction with Pins to promote planar cell division in *Drosophila* epithelia. Lgl cortical release requires double phosphorylation, indicating that whereas Lgl-Dlg association involves aPKC phosphorylation, multiple phosphorylations break this interaction, acting as an off switch on Lgl-Dlg binding. Triple phosphomimetic Lgl mutants display weak interactions with Dlg [36], suggesting that multiple phosphorylations could directly block Lgl-Dlg interaction. Alternatively, the negative charge of two phosphate groups may suffice to induce association between the N- and C-terminal domains of Lgl, impairing its ability to interact with the cytoskeleton and plasma membrane as previously proposed [12]. This would reduce the local concentration of Lgl available to interact with Dlg, enabling the interaction of Dlg's GUK domain with the pool of Pins phosphorylated by AurA [28].

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Therefore, AurA converts the Lgl/Dlg polarity complex generated upon aPKC phosphorylation into the Pins/Dlg spindle orientation complex (Figure 4I). This study, together with that of Bell et al. [39], underlines the critical requirement of synchronizing the cell cycle with the reorganization of polarity complexes to achieve precise control of spindle orientation in epithelia.

Author Contributions

E.M. and C.S. conceived the project. C.C., S.M., and E.M. performed all experiments, with the exception of Figure 3E (G.V.). E.M., C.C., S.M., and C.S. analyzed the data. E.M. wrote the paper, and E.M., C.C., S.M., and C.S. revised it.

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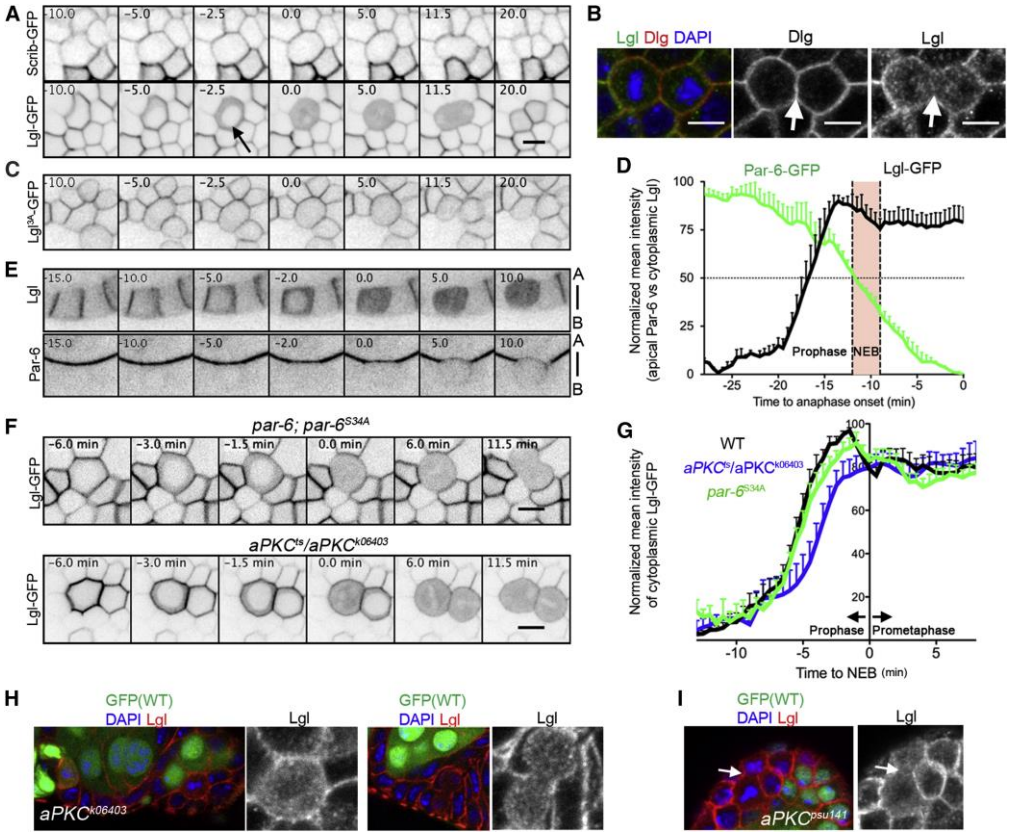

Figure 1. Reorganization of Polarity Determinants during Follicle Cell Division

(A and C) Time-lapse images of follicle cells expressing Scrib-GFP or Lgl-GFP (A) or Lgl^{3A}-GFP (C). The arrow points to the nucleus.

(B) Lgl does not accumulate with Dlg at the interface between dividing cells (arrow).

(D and E) Lgl cortical exclusion precedes apical depolarization. Normalized mean intensity of apical Par-6-GFP and cytoplasmic Lgl-GFP until anaphase onset is shown in (D). The average ± SEM is shown (n = 5). The amplitude of NEB timing is indicated. Longitudinal time-lapse images of follicle cells expressing Lgl-GFP (top) and Par-6-GFP (bottom) are shown in (E). The apical-basal (AB) axis is indicated.

(F) Time-lapse projections of the follicular epithelium in *aPKC^{ts}/aPKC^{k06403}* at 29ºC and *par-6*; *par-6^{S34A}* egg chambers expressing Lgl-GFP.

(G) Normalized mean intensity of cytoplasmic Lgl-GFP relative to NEB. The average ± SEM is shown (n = 7). (H and I) *aPKC^{k06403}* (H) and *aPKC^{psu141}* (I) mutant follicle cells (GFP absence) stained for Lgl. Lgl accumulates in the cytoplasm at metaphase (left) and telophase (right) (H). Lgl is absent from the juxtaposed cortex of neighboring *aPKC^{psu141}* dividing cells (I, arrow). Scale bars, 5 μm.

Time (minutes) is shown relative to NEB. See also Figure S1 and Movie S1.

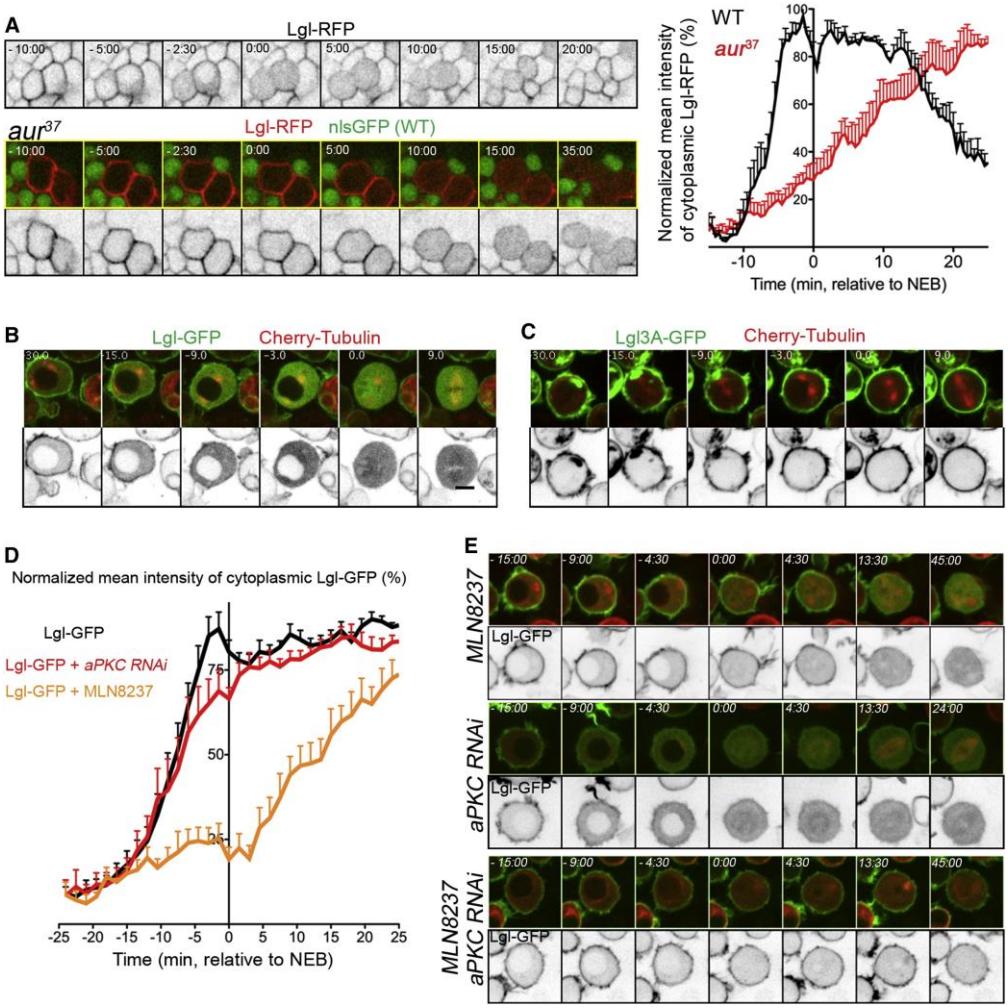

Figure 2. Aurora A Triggers Lgl Cortical Exclusion at Mitosis Onset In Epithelial and S2 Cells

(A) Time-lapse projections of wild-type follicle cells (top) and homozygous *aurA*^{37} mutant cells (bottom, nls-GFP absence) expressing Lgl-RFP. *aurA*^{37} mutant clones only show strong cytoplasmic accumulation of Lgl-RFP after NEB. Quantification of the normalized mean intensity of cytoplasmic LglRFP in wild-type (n = 3) and *aur*^{37} (n = 5) cells is shown. The mean average ± SEM is shown.

(B and C) Time-lapse images of S2 cells expressing Lgl-GFP (B) or Lgl3A-GFP (C) and Cherry-Tubulin. Scale bar, 5 μm.

(D) Normalized mean intensity of Lgl-GFP in the cytoplasm during mitosis in control cells and upon inactivation of AurA or *aPKC* RNAi. aPKC depletion induces a minor delay, whereas a strong delay in Lgl-GFP cytoplasmic accumulation is observed upon MLN8237 treatment. The mean average ± SEM (n = 9) is shown for each time point.

(E) Time-lapse images showing S2 cells expressing Lgl-GFP and treated as indicated. Simultaneous treatment with MLN8237 and *aPKC* RNAi prevents LglGFP cytoplasmic accumulation (bottom).

Time (minutes:seconds) is shown relative to NEB. See also Figure S2 and Movie S2.

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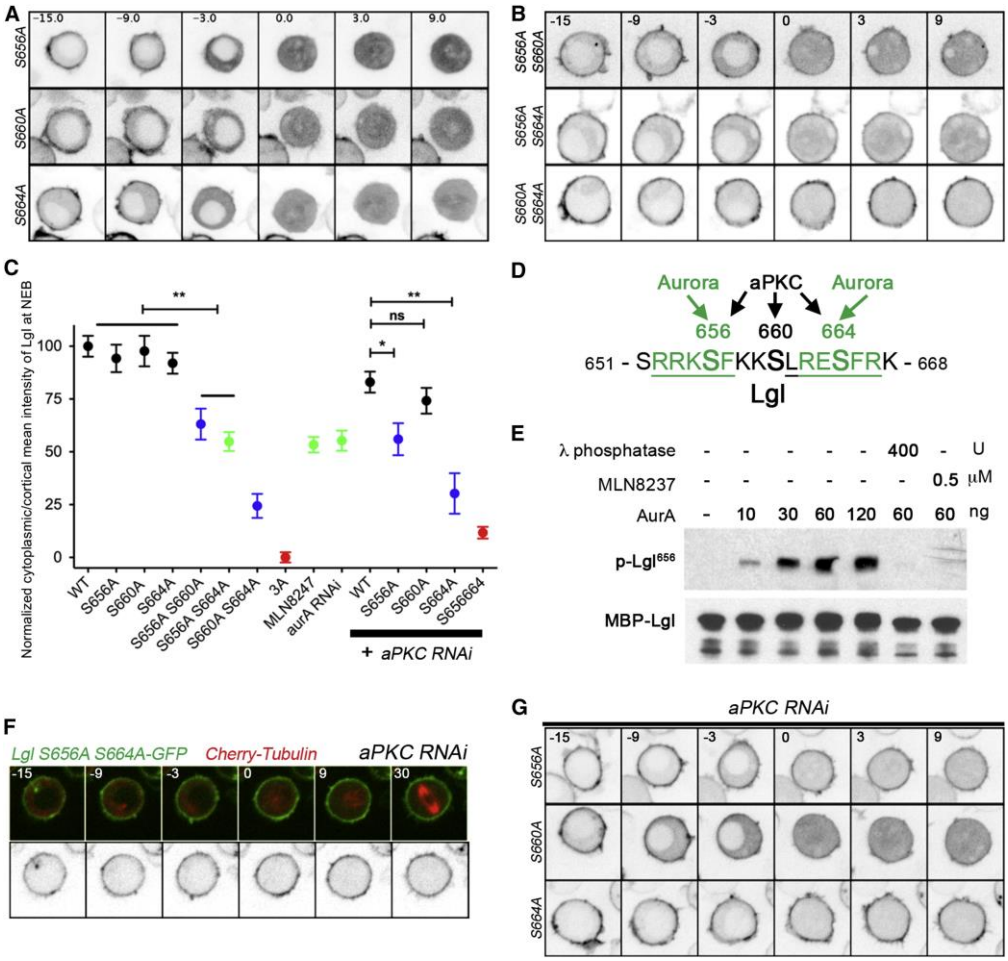

Figure 3. Aurora A Controls S656 and S664 Phosphorylation

(A and B) Time-lapse images of S2 cells expressing the indicated Lgl mutants show that any combination of double phosphorylation is sufficient and required for efficient Lgl cortical release.

(C) Quantification of the cytoplasmic enrichment at NEB (ratio of the mean intensity of cytoplasmic Lgl to the mean intensity of cortical Lgl, normalized to the wild-type ratio [100%] and corrected for the ratio of Lgl^{3A} [0%]). The average ± SEM is shown from quantification of at least five movies (*$p < 0.05$, **$p < 0.01$; Student's t test with two-tailed distribution).

(D) Representation of the Lgl sequences that match the AurA phosphorylation consensus (green). The serines phosphorylatable by AurA and aPKC are shown.

(E) In vitro kinase assay showing that increasing amounts of AurA lead to increased phosphorylation of S656, monitored by western blot using a phosphospecific antibody. anti-MBP was used to control for the amount of MBP-LGL.

(F and G) Time-lapse images of S2 cells expressing $Lgl^{S656A,S664A}$ (F) or single mutants (G) with *aPKC* RNAi. Mutations on S656 or S664 induce further cortical retention of Lgl upon aPKC depletion, whereas double mutations fully prevent Lgl cortical release.

Time (minutes) is shown relative to NEB. See also Movie S3.

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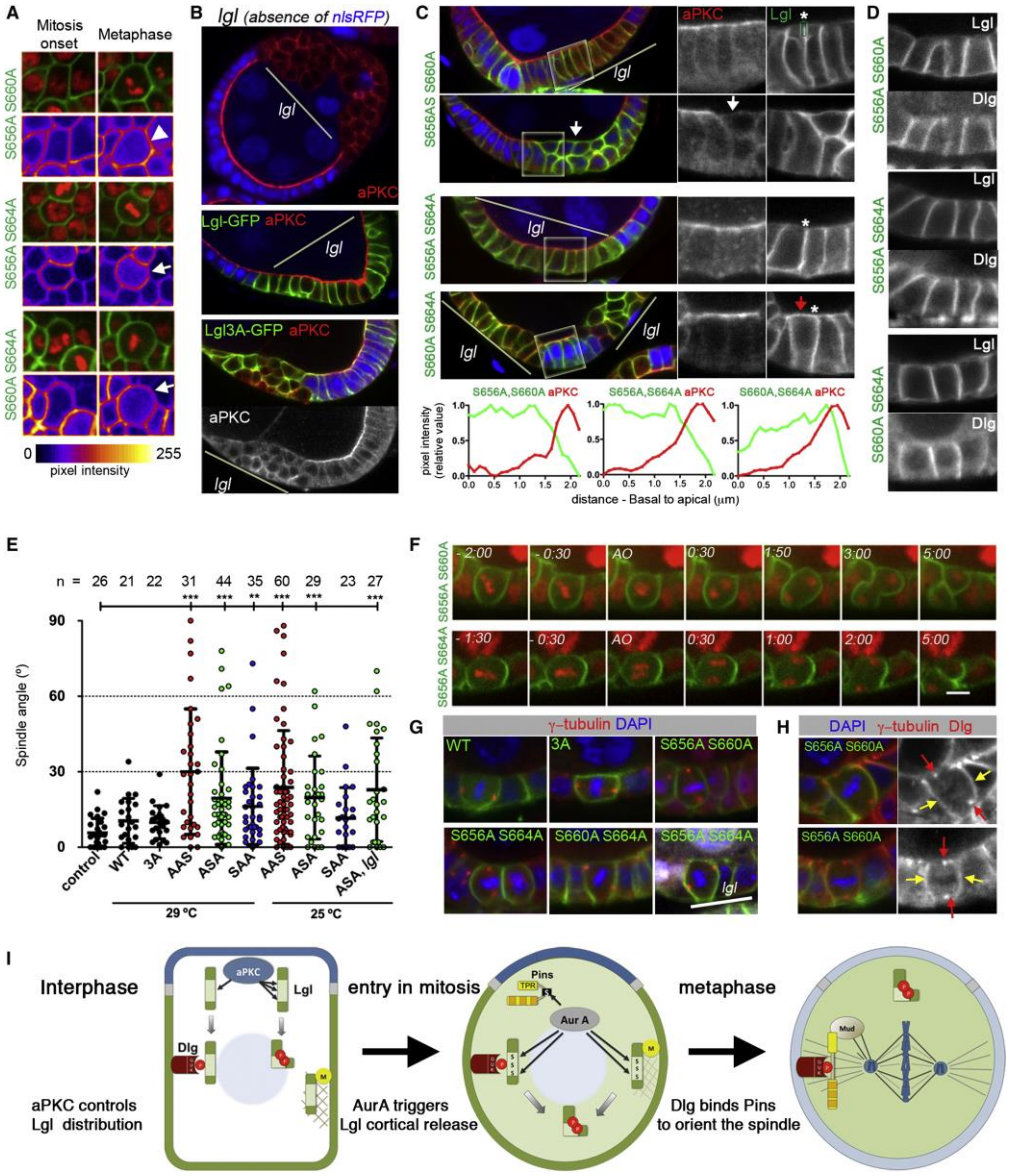

Figure 4. Lgl Cortical Release Promotes Mitotic Spindle Orientation

(A) Pseudocolored frames from surface time-lapse projections show that Lgl double mutants are strongly retained at the cortex during mitosis as high pixel intensity values are detected at the interface between dividing cells (arrowhead) or at the interface of dividing cells with neighbors with lower expression of Lgl (arrows).

(B and C) Mosaic egg chambers of *lgl*^{27S3} null mutant follicle cell clones (absence of nlsRFP, which is colored in blue) expressing the indicated Lgl variants (green) and stained for aPKC (red). Enlarged areas of the depicted regions are shown as separate channels in (C). Plots show mean cortical pixel intensity along a 2.16 μm region crossing the apicolateral border until the apical domain (bottom). The measured cortices are marked with asterisks.

(D) Separate channels showing follicle cells expressing the indicated GFP-tagged Lgl double mutants and stained for Dlg.

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(E) Quantification of the angle between the mitotic spindle and the plane of the epithelium in control and follicle cells expressing the indicated Lgl transgenes at 29ºC and 25ºC. The mean ± SD and n are shown (***p < 0.001, **p < 0.01; Kolmogorov-Smirnov test).

(F) Time-lapse images of cells expressing $Lgl^{S656A,S660A}$ and $Lgl^{S656A,S664A}$, which fail to orient cell division. His-RFP labels chromosomes. Scale bar, 5 μm. Time (minutes:seconds) is shown relative to anaphase onset (AO).

(G) Confocal images of follicle cells expressing the indicated Lgl forms and stained with γ-tubulin (red) to label centrosomes and DAPI to identify metaphasic plates. *lgl* mutant cells labeled by absence of RFP (gray) and expressing $Lgl^{S656A,S664A}$-GFP are shown (bottom right).

(H) Confocal images of $Lgl^{S656A,S660A}$-GFP-expressing cells showing that centrosome position (red arrows) during metaphase does not follow the position of Dlg (yellow arrows).

(I) Model showing the subcellular localization of Lgl at different cell-cycle stages. The scheme depicts how Lgl release from the cortex converts the Dlg/Lgl complex into the Dlg/Pins complex, which mediates spindle positioning.

See also Figure S3 and Movie S5.

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