

Regulation of Notch signaling and endocytosis by the Lgl neoplastic tumor suppressor

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The evolutionarily conserved neoplastic tumor suppressor protein, Lethal (2) giant larvae (Lgl), plays roles in cell polarity and tissue growth via regulation of the Hippo pathway. In our recent study, we showed that in the developing *Drosophila* eye epithelium, depletion of Lgl leads to increased ligand-dependent Notch signaling. *lgl* mutant tissue also exhibits an accumulation of early endosomes, recycling endosomes, early-multi-vesicular body markers and acidic vesicles. We showed that elevated Notch signaling in *lgl*⁻ tissue can be rescued by feeding larvae the vesicle de-acidifying drug chloroquine, revealing that Lgl attenuates Notch signaling by limiting vesicle acidification. Strikingly, chloroquine also rescued the *lgl*⁻ overgrowth phenotype, suggesting that the Hippo pathway defects were also rescued. In this extraview, we provide additional data on the regulation of Notch signaling and endocytosis by Lgl, and discuss possible mechanisms by which Lgl depletion contributes to signaling pathway defects and tumorigenesis.

Introduction

Decades of fundamental research utilizing the vinegar fly, *Drosophila melanogaster*, have revealed many important genes and signaling networks that have subsequently proven to be important in human cancer (reviewed by¹⁻⁴). Of

particular interest for epithelial and neural cancers are the junctional neoplastic tumor suppressors, Lgl, Discs large (Dlg) and Scribbled (Scrib) (reviewed by⁵). These genes function in a common genetic pathway to control epithelial apical-basal cell polarity and tissue growth during *Drosophila* development.⁶⁻⁸ Lgl, Dlg and Scrib antagonize the activity of the Par apical polarity complex (consisting of Par3 (Bazooka (Baz) in *Drosophila*), atypical protein kinase C (aPKC), Par6 and Cdc42), which is important in polarity regulation and tissue growth control (reviewed by^{11,12}). Conversely, aPKC can phosphorylate Lgl and thereby restrict its access to the apical membrane. aPKC also phosphorylates and activates Crumbs (Crb), a component of the Crb-Patj-Pals (Stardust) apical polarity complex, which is required for Crb-Crb extracellular domain interactions and the establishment of the apical membrane domain.¹³ However, our recent studies have revealed that Lgl acts distinctly from Dlg and Scrib in epithelial tissue growth control, and that this function can be separated from Lgl's role in apical-basal cell polarity.^{9,10} In addition to their cell polarity function, *Drosophila* Lgl/aPKC and Crb also regulate tissue growth via the Hippo signaling pathway, a conserved pathway that acts to phosphorylate and inhibit the activity of the Yki/Yap co-transcriptional activator (reviewed by¹⁴⁻¹⁶). Lgl/aPKC and Crb regulate the Hippo pathway by distinct mechanisms; Lgl/aPKC affects the

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Abbreviations: Lgl, Lethal (2) giant larvae; aPKC, atypical Protein Kinase C.

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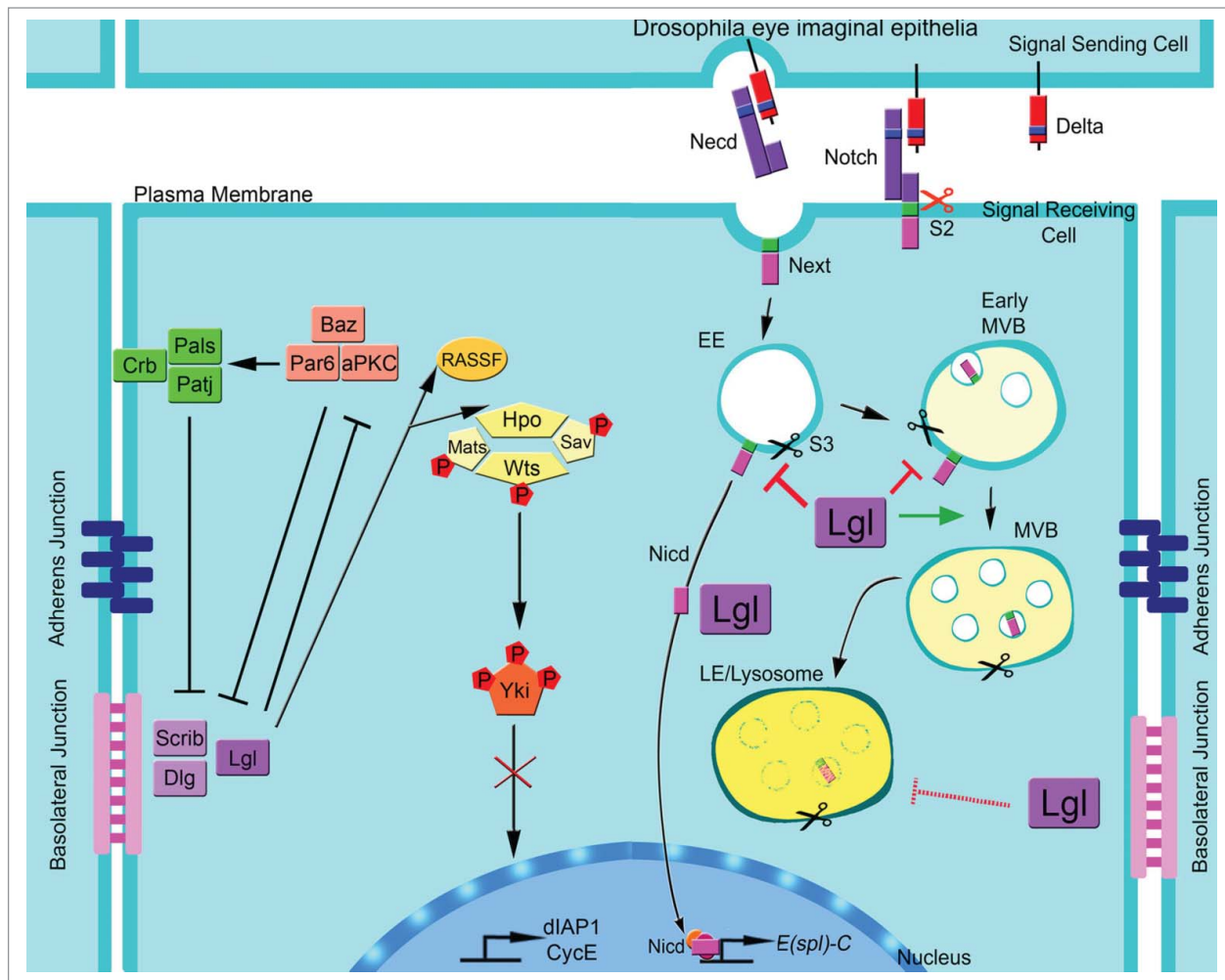


Figure 1. Model for the regulation of Hippo and Notch by Lgl. In epithelial cells, Lgl/aPKC regulate the Hippo pathway by promoting the correct localization of Hpo/Rassf, therefore allowing the phosphorylation of Yki and preventing its translocation into the nucleus and the activation of Hippo pathway targets, dIAP1 and CycE. Lgl also regulates the ligand-dependent activation of Notch, in an aPKC-independent manner. In ligand-dependent Notch activation, the ligand (Delta) binds to the extracellular domain (N-ecd), it is cleaved by S2 protease and the Delta/Necd complex is internalized in the signal sending cell. S2 cleaved Notch (N-ext) is endocytosed and cleaved by γ -secretase (S3) in early endosomes (EE), or in early-multivesicular body (early-MVBs), releasing the active cytoplasmic N-icd fragment, which translocates into the nucleus, thereby inducing transcription of target genes, such as Enhancer of split complex (*E(spl)-C*). Unprocessed Notch can traffic through to the late endosome (LE)/lysosome where it undergoes proteasomal degradation. Extrapolating from our data, we propose that Lgl plays a role in controlling the activity of Notch by regulating its trafficking from the EE, early-MVB, to MVB-LE stage. Lgl also directly or indirectly limits the acidification process of the endosomes, thereby attenuating Notch signaling by preventing its cleavage by γ -secretase.

localization of the Hippo core protein kinase and the negative regulator Rassf (Fig. 1), while Crb acts to control the stability of an upstream Hippo pathway activator, Expanded.^{15,17-21} However, *Drosophila* Dlg and Scrib do not directly regulate the Hippo pathway, although once cell polarity is lost the Hippo pathway is impaired, most likely due to the aberrant aPKC activity.^{9,16,22,23}

In our recent study,²⁴ we have discovered a role for *Drosophila* Lgl in the

regulation of the Notch signaling pathway (Fig. 1). The Notch pathway is an important cell-cell communication pathway required for cell-fate decisions during development, which is commonly deregulated in human cancer (reviewed by²⁵⁻²⁸). Engagement of the ligands (Delta or Serrate in *Drosophila*) in the signal-sending cell with the Notch receptor in the signal-receiving cell leads to cleavage of Notch by an Adam metalloprotease (Kuzbanian in *Drosophila*), to result in the S2 cleavage

to produce the intracellular truncated form of Notch (N-ext). N-ext is then cleaved by γ -secretase (S3 cleavage) to produce the Notch intracellular domain (N-icd) that enters the nucleus and regulates gene transcription in a complex with the transcriptional regulators CSL/Su (Hairless) and Mastermind (Mam). Recently, endocytosis has been shown to play an important role in Notch signaling (reviewed by²⁹⁻³³). The Notch receptor can be internalized by a ligand-dependent

(where it is cleaved and activated) or a ligand-independent manner (where it is downregulated by degradation in the lysosome, or recycled to the plasma membrane). However, specific defects in endocytosis at the multivesicular body (MVB) stage (eg ESCRT II, *vps25* mutants) lead to aberrant Notch cleavage and elevated Notch signaling.^{34,35}

In our analysis, we found *lgl* mutant clones induced in the developing *Drosophila* eye, showed upregulation of Notch targets and accumulation of intracellular Notch (N-ext or N-icd), detected by an antibody to the Notch intracellular domain, but did not affect full-length or N-ecd, revealed by an antibody to the Notch extracellular domain²⁴ (Fig. 1). Consistent with this observation, we found that upregulation of Notch activity is ligand dependent, since *lgl*⁻ tissue that was also deficient for the 2 Notch ligands, Delta and Serrate, did not show ectopic expression of Notch targets. Consistent with the requirement for Notch to be internalized for activation,³⁶ we found that in *lgl*⁻ tissue also deficient in Dynamin (Shibire (Shi)) in *Drosophila*, by expressing a dominant negative transgene, *shi*^{DN} function (required for the initial stages of endosomal budding from the plasma membrane) or in Rab5 early endosome (EE) function (using *Rab5*^{RNAi}), Notch target expression was blocked, similarly to *shi*^{DN} or *Rab5*^{RNAi} clones. Elevated Notch signaling in *lgl*⁻ is correlated with defects in endosomal trafficking (Avl⁺ (Syx7) EEs, Rab11⁺ recycling endosomes (REs) and Hrs⁺ (Escrt 0) early-MVBs accumulated) and with increased acidic vesicles.²⁴ In wild-type cells, we found that Lgl colocalizes with Notch and endocytic markers, consistent with Lgl playing a direct role in the regulation of endocytic trafficking. Based on these findings, we hypothesized that Lgl might regulate a step in endosomal maturation or in vesicle acidification that led to elevated Notch signaling. However, reducing Rab11 or Escrt 0 function did not rescue increased Notch signaling in *lgl*⁻ tissue,²⁴ suggesting that accumulation of these endocytic compartments was not responsible for the increased Notch signaling in Lgl depleted tissue. Instead, we found that reducing vesicle acidity by

feeding the developing larvae the drug chloroquine restored Notch signaling to normal in *lgl*⁻ tissue and dramatically rescued the *lgl*⁻ mosaic adult eye phenotype. This rescue demonstrated that the increased acidity of vesicles in *lgl*⁻ tissue is responsible for ectopic Notch signaling and other defects caused by Lgl depletion. This result is consistent with previous data showing that the activity of γ -secretase, which is required for S3-cleavage activation of Notch, is dependent on vesicle acidification.^{37,38} In contrast to many studies where the primary function of Lgl is to inhibit aPKC, we found that the ectopic Notch signaling observed upon depletion of Lgl is not dependent on aPKC activity for its effect.²⁴ Taken together, our study has revealed a novel aPKC-independent role for Lgl in the regulation of endocytosis and Notch signaling. Here we provide additional data to probe the mechanism by which Lgl regulates endocytosis and Notch signaling. Furthermore, we compare and contrast our findings with other published studies on polarity regulators in endocytosis, and speculate upon possible mechanisms by which Lgl depletion contributes to signaling pathway defects and tumorigenesis.

The Control of Notch Signaling by Lgl

To further explore the interaction of Lgl with the Notch pathway, we sought to examine whether overexpression of Lgl (*lgl*^{WT}) could inhibit Notch signaling. Firstly, we overexpressed Lgl in the developing eye epithelium and examined the effect on endogenous Notch signaling in third instar larval *lgl*^{WT} clones relative to the surrounding wild-type tissue using the *E(Spl)m8-lacZ* reporter as a read out of Notch activity (Fig. 2A, B). No changes were observed relative to the control mosaic eye disc (Fig. 2A, B). Furthermore, no significant changes were observed to Notch-intra levels or localization (Fig. 2C). Since potential effects of *lgl*^{WT} overexpression in reducing endogenous Notch signaling might be difficult to observe, we then tested whether overexpression of Lgl could suppress ectopic Notch signaling. Accordingly, we

activated Notch by expression of N-ext, which is the S2-cleaved derivative that requires γ -secretase to generate the N-icd active version, and therefore serves as a constitutive substrate for γ -secretase.³⁸ We expressed the *UAS-N-ext* transgene via the *equatorial (eq1)-GAL4* driver (*eq > N-ext*), which is expressed in a patch in the center of the developing eye, as well as in the wing disc notum that gives rise to the adult thorax. *eq > N-ext* resulted in a patch of roughness in the center of the adult eye and an overgrown thorax (arrowheads Fig. 2E, E' compared with control flies Fig. 2D, D'). To test that this phenotype was sensitive to modification, we expressed a Mastermind dominant negative transgene (*mam*^{DN}), which inhibits Notch activity at the promoters of its target genes. *mam*^{DN} strongly suppressed the overgrown thorax and rough eye phenotypes of *eq > N-ext* (Fig. 2F, F'). Importantly, co-expression of Lgl with N-ext also dramatically reduced the phenotypic effects of *eq > N-ext* (Fig. 2G, G'). This result shows that increasing Lgl levels is sufficient to inhibit γ -secretase-mediated activation of N-ext. We then tested whether Lgl could also inhibit the phenotypic effects of expression of constitutively active Notch, N-icd (γ -secretase cleaved form) via the *eq* driver. *eq > N-icd* produced a milder phenotype in the eye and the thorax relative to *eq > N-ext*, perhaps due to lower expression levels (Fig. 2H, H'). While expression of *mam*^{DN} could suppress the eye and thoracic defects of *eq > N-icd* (Fig. 2I, I'), overexpression of *Lgl*^{WT} failed to reduce these defects (Fig. 2J, J'). Since *lgl*^{WT} expression can suppress N-ext induced phenotypes but not N-icd phenotypes, this suggests that Lgl is acting at the level of γ -secretase activity rather than on N-icd function. Taken together, these results provide evidence that Lgl directly or indirectly regulates γ -secretase-mediated Notch activation.

Endosomal Trafficking is Affected at the Early-MVB Stage in *lgl* Mutant Tissue

In our recent study, we showed that Avl (Syx7, EE), Hrs (Escrt 0, early-MVB) (Fig. 3A) and Rab11 (RE) were elevated

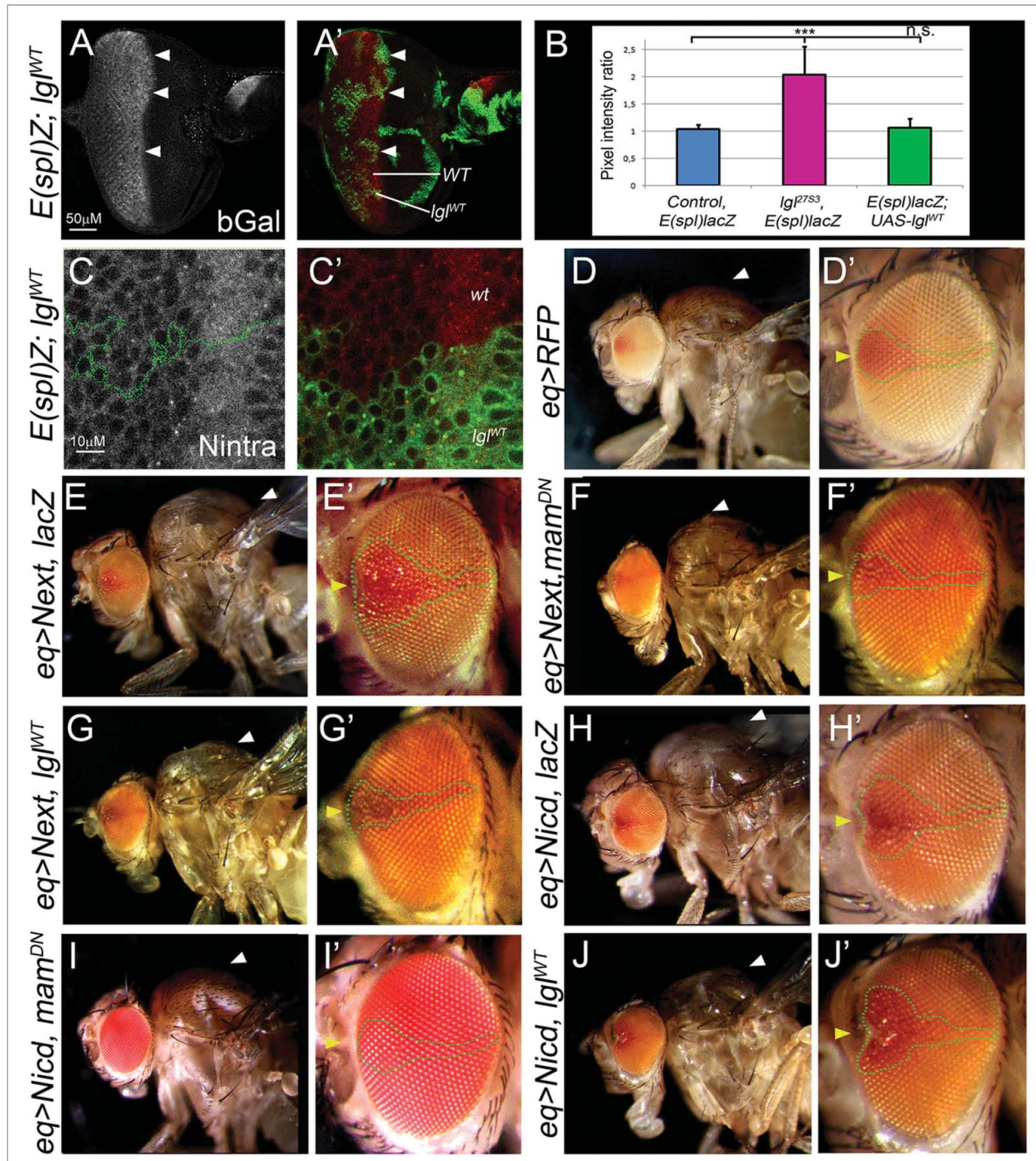


Figure 2. For figure legend, see page 1500.

in *Igl^T* tissue, but that Rab7 (maturing late endosomes, LE) and Car (LE to lysosome fusion) remained unaffected.²⁴ To further investigate the effect of Lgl depletion on LE-lysosomal stage, we examined the localization and levels of Dor (LE³⁹) and Arl8 (lysosome⁴⁰) in *Igl^T* mosaic eye epithelium (Fig. 3B, C). These markers were

not altered in *Igl^T* tissue, confirming that Lgl depleted tissue leads to endosomal trafficking defects at the EE/early-MVB stages. To further explore vesicle trafficking defects in *Igl^T* tissue, we examined SARA endosomes, which are a subclass of EE involved in Notch signaling in *Drosophila* neural and adult gut epithelial

asymmetrically dividing cells^{41,42} and are also present in imaginal disc epithelial cells.⁴³ However, SARA endosomes were not perturbed in *Igl^T* eye disc clones (Fig. 3D), indicating that this specialized endosomal compartment was not involved in the Notch signaling defects in Lgl depleted eye epithelial tissue. Finally, we

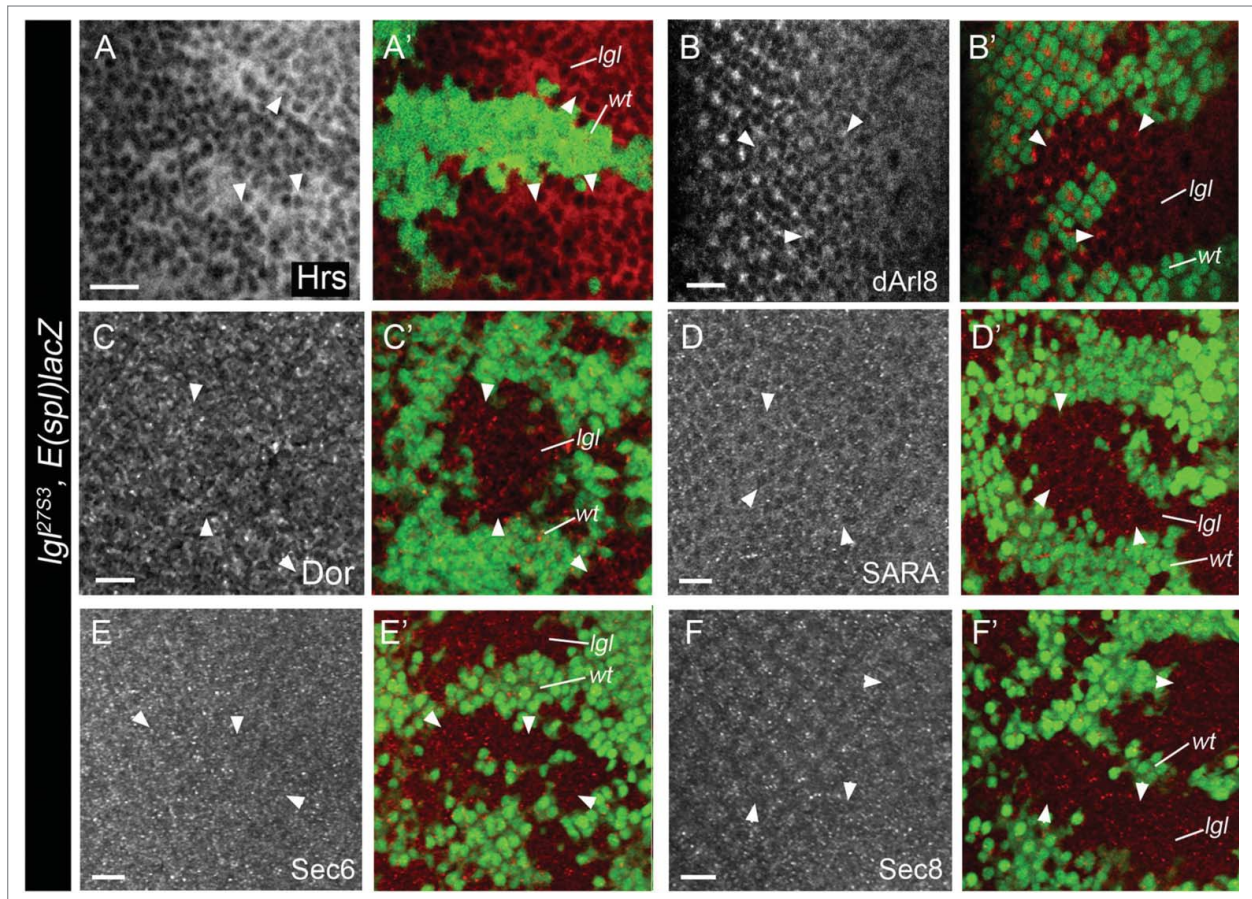


Figure 3. Lgl depletion affects vesicle trafficking at the early-MVB stage. *lgl*⁻ third instar larval eye disc clones stained for Hrs (Early-MVB) (A), Arl8 (lysosome) (B), Dor (LE/lysosome) (C), SARA endosomes (D), Sec6 (exocyst) (E) and Sec8 (exocyst) (F) (gray or red in merges). Note that *lgl*⁻ clones accumulate Hrs, but show normal levels and localization of other endosome or exocyst markers, compared with surrounding wild-type clones. *lgl*⁻ clones are marked by the absence of GFP (green, arrowheads). Scale bar = 10 μM. Genotype for all panels: *eyFLP; lgl*^{27S3}, *FRT40, E(spl)m8-lacZ / Ubi-GFP, FRT40*. Antibodies: Guinea Pig Hrs (1:500, H. Bellen), Rabbit dArl8 (1:500, I. Hofmann), Guinea pig Dor (H. Kramer, 1:100), Rabbit SARA (1:500, M. Gonzales Gaitan), Guinea pig Sec6 (1:1000, U. Tepass) and Guinea pig Sec8 (1:2000, U. Tepass).

examined the exocyst, which is involved in trafficking of vesicles from REs to the plasma membrane, and is important in Notch signaling.⁴⁴⁻⁴⁷ We found that the localization and levels of Sec6 and Sec8

exocyst components were not altered in *lgl* mutant eye disc clones (Fig. 3E, F). Thus, although Rab11 RE accumulate in *lgl*⁻ tissue,²⁴ there is no effect on the levels or localization of Sec6 or Sec8 exocyst

components. Furthermore, since the exocyst is required for E-cadherin (E-cad) trafficking,⁴⁸ we investigated E-cad localization in *lgl*⁻ clones and did not find any visible alterations. It is thus unlikely that

Figure 2 (See previous page). Overexpression of Lgl can suppress N-ext, but not N-icd, phenotypes, and does not affect endogenous Notch signaling. (A) Confocal images of β-gal staining (gray, red in merge) of *lgl*^{WT} mosaic third instar larval eye discs in the *E(spl)m8-lacZ* background. Clones are marked by the expression of GFP (green, arrowheads). (B) Quantification of *E(spl)m8-lacZ* expression in *lgl*^{WT} clones compared with wild-type clones, showing that *lgl*^{WT} does not significantly affect the expression of β-gal relative to wild-type tissue. The data is shown relative to the elevated expression of *E(spl)m8-lacZ* in *lgl*⁻ clones and control mosaic eye discs. (C) Confocal images of Notch-intra staining (gray, red in merge) of *lgl*^{WT} mosaic third instar larval eye discs in the *E(spl)m8-lacZ* background. Clones are marked by the expression of GFP (green, outlined area). *lgl*^{WT} does not affect the levels or localization of Notch-intra. (D) Control *eq-GAL4* adult thorax (D) and eye (D') (E-G) *eq > N-ext* crossed to *UAS-lacZ* (E), *UAS-mam*^{DN} (F) or *UAS-lgl*^{WT} (G) adult thorax and eyes. Note that expression of *mam*^{DN} or *lgl*^{WT} can suppress the adult eye roughing (outlined areas) and thoracic overgrowth phenotypes (arrowheads) of *eq > N-ext*, indicating that Lgl can suppress ectopic Notch signaling at the γ-secretase step. (H-J) *eq > N-icd* crossed to *UAS-lacZ* (H), *UAS-mam*^{DN} (I) or *UAS-lgl*^{WT} (J) adult thorax and eyes. Only *mam*^{DN} expression is able to rescue the adult eye roughing (outlined areas) and thoracic overgrowth phenotype (arrowheads). Genotypes: (A, C) *eyFLP, UAS-GFP; FRT40, E(spl)m8-lacZ; UAS-lgl*^{WT} / *tubGAL80 FRT40; tubGAL4* (D) *w*; *UAS-myrRFP, eq1-GAL4/TM6B* (E) *w*; *UAS-myrRFP, eq1-GAL4, UAS-N-ext/UAS-lacZ* (F) *w*; *UAS-myrRFP, eq1-GAL4, UAS-N-ext/UAS-mam*^{DN} (G) *w*; *UAS-myrRFP, eq1-GAL4, UAS-N-ext/UAS-lgl*^{WT} (H) *w*; *+UAS-N-icd; UAS-myrRFP, eq1-GAL4, UAS-lacZ* (I) *w*; *+UAS-N-icd; UAS-myrRFP, eq1-GAL4, UAS-mam*^{DN} (J) *w*; *+UAS-N-icd; UAS-myrRFP, eq1-GAL4, UAS-lgl*^{WT} Flystocks: *Eq1-Gal4* (H Sun), *E(spl)m8-lacZ* (A. Bergmann), *UAS-N-ext* (III) (T. Vaccari), *UAS-lacZ-nls* (III) (G. Baeg), *UAS-mam*^{DN} (BL26672) (Bloomington stock center), *UAS-lgl*^{WT} (III) (J. Knoblich), *UAS-N-icd* (II) (S. Artavanis-Tsakonas). Antibodies: mouse β-galactosidase (Sigma, 1:500), mouse Notch-intra (Developmental Studies Hybridoma Bank, 1:50).

exocyst function is altered in Lgl depleted eye epithelial tissue. These results, together with our recent study,²⁴ support a specific role for Lgl in endosomal trafficking at the EE/early-MVB or RE stages.

Does Lgl Control N-Ext Cleavage Directly Via Regulation of V-ATPase Activity or Indirectly Through Endosomal Maturation Defects?

In analyzing the functional consequences of the endosomal trafficking defects upon Lgl depletion, we found that Dynamin (Shibire, initial step of vesicle budding from the plasma membrane) and Rab5 (EE) functions are required, but not Hrs/Stam (Escr 0) or Rab11 (RE) functions, for elevated Notch signaling in *lgl*⁻ tissue.²⁴ While markers for other Escr components in multivesicular body function are not available to determine whether they are altered in *lgl*⁻ tissue, it remains possible that Lgl might regulate their activity. Since defective Escr I, II and III activity is associated with ectopic ligand-independent Notch signaling,^{33-35,49} we envisioned that upregulation of Escr I-III components might rescue elevated Notch signaling in *lgl*⁻ tissue. Accordingly, we tested whether overexpression of Vps25 (Escr II)⁵⁰ could restore the Notch target reporter, *E(Spl)lacZ*, expression to normal in *lgl*⁻ clones, however no rescue was observed (arrowheads, Fig. 4C, D compared with Fig. A, B). Although the LE protein, Rab7, was not altered in Lgl depleted tissue,²⁴ we hypothesized that its function may be defective. Consistent with a possible role for Rab7, a recent study revealed that a Rab7 deficiency leads to accumulation of Hrs⁺ vesicles (early-MVBs) in the *Drosophila* developing eye,⁵¹ similar to Lgl depletion. We therefore tested whether overexpression of wild-type or constitutively active *Rab7* (*Rab7*^{Q67L}) might rescue elevated *E(Spl)lacZ* gene expression in *lgl*⁻ clones. However, neither wild-type *Rab7* nor *Rab7*^{Q67L} were capable of rescuing elevated Notch signaling in Lgl depleted tissue (arrowheads, Fig. 4E-H, compared with Fig. A, B). Thus, reduced Vps25 or Rab7 function is not responsible

for elevated Notch activation in *lgl*⁻ tissue. However, it remains to be determined whether other Escr I-III components are affected in *lgl* mutant tissue and if they are required for the effect on Notch signaling in Lgl depleted tissue. Furthermore, Lgl might regulate the activity of an unknown Rab protein, given recent evidence for a role for rat Lgl1 in the regulation of the exocytic Rab10 in neural outgrowth.⁵² In this study, Lgl1 was shown to act by releasing the GDP dissociation inhibitor (GDI) from Rab10, and it is therefore possible that Lgl might regulate an endocytic Rab via a similar mechanism. Since we have shown that Rab5 (EE) activity is required for elevated Notch signaling in *lgl*⁻ tissue,²⁴ Rab5 is a potential candidate for a direct target of Lgl.

Alternatively, Lgl might function in Notch signaling via regulation of the vacuolar ATPase (V-ATPase), which is required for vesicular acidification and γ -secretase activity.^{37,38,53} Lgl could negatively regulate V-ATPase activity by affecting the localization of V-ATPase in the membrane, the composition of V-ATPase isoforms or the assembly of the V0 and V1 V-ATPase complexes. Since increased Vha44 (V-ATPase subunit C) expression results in increased Notch signaling,³⁷ it is also possible that Lgl regulates the level of this V-ATPase component to modulate in vesicle acidification. Thus, further research is required to determine whether V-ATPase function is increased or required for elevated Notch signaling in *lgl*⁻ tissue.

Vesicle Acidification is Important for the Overgrowth Phenotype of the *lgl* Mutant Mosaics

Our recent study revealed that *lgl*⁻ clones accumulated acidic vesicles, and vesicle acidification was necessary for elevated Notch signaling and overgrowth of Lgl deficient tissue.²⁴ Specifically, we found the deacidifying compound, chloroquine,⁵⁴ rescued ectopic Notch signaling and the *lgl*⁻ adult eye phenotype.²⁴ This is not due to the elimination of *lgl*⁻ tissue, since large *lgl*⁻ clones (GFP-negative) were present in larval eye discs²⁴ and in adult eyes (dark

areas, arrowheads, Fig. 5A, B). Thus, *lgl*⁻ tissue overgrowth, as well as morphological defects that occur during the pupal stage and contribute to the adult eye phenotype,¹⁰ are strongly rescued by feeding larvae chloroquine.²⁴ Blocking upregulation of Notch in *lgl*⁻ tissue by *mam*^{DN} expression did not rescue adult eye defects to the same extent as chloroquine treatment,²⁴ therefore we predict that chloroquine is impacting other signaling pathways or processes that are defective in the *lgl*⁻ tissue. Vesicle acidification, and its inhibition by chloroquine, has been reported to affect various signaling pathways in addition to Notch, including mTorc1, Wnt/Wingless, PI3K-Akt and hypoxia pathways.⁵⁵⁻⁶³ Furthermore, chloroquine is being trialed as an anti-cancer agent where its mode of action has been proposed to act by blocking autophagy, which is a catabolic process where cellular proteins and organelles are targeted for degradation in the lysosome in order to generate energy (reviewed by⁶⁴⁻⁶⁷). This raised the possibility that Lgl deficient tissue might exhibit increased autophagy and chloroquine may act to suppress this. To determine whether Lgl depletion promoted autophagy, we stained *lgl*⁻ mosaic eye discs for p62, a marker of autophagic flux.⁶⁸ However, *lgl*⁻ tissue did not exhibit changes in p62 levels (arrowheads, Fig. 5C), indicating that autophagy is not affected. This result suggests that it is unlikely that autophagy induction is contributing to the *lgl*⁻ phenotype and that chloroquine rescues the Lgl deficient phenotype by blocking autophagy. Since we know that the Hippo negative growth signaling pathway is impaired in *lgl*⁻ tissue and contributes to its tissue growth defects⁹ (see Fig. 1), this raises the question of whether the Hippo pathway might also be regulated by endocytosis or vesicle acidification and be a target of chloroquine in *lgl*⁻ tissue. Interestingly, recent proteomics analysis of the Hippo pathway uncovered many endocytosis proteins as interactors with core Hippo pathway components.⁶⁹ Furthermore, downregulation of endocytic regulators impairs Hippo signaling,⁷⁰ although whether this occurs without affecting cell polarity is unclear. Clearly, further analysis is needed to dissect the molecular mechanism by which chloroquine acts to rescue the *lgl*⁻ defects and

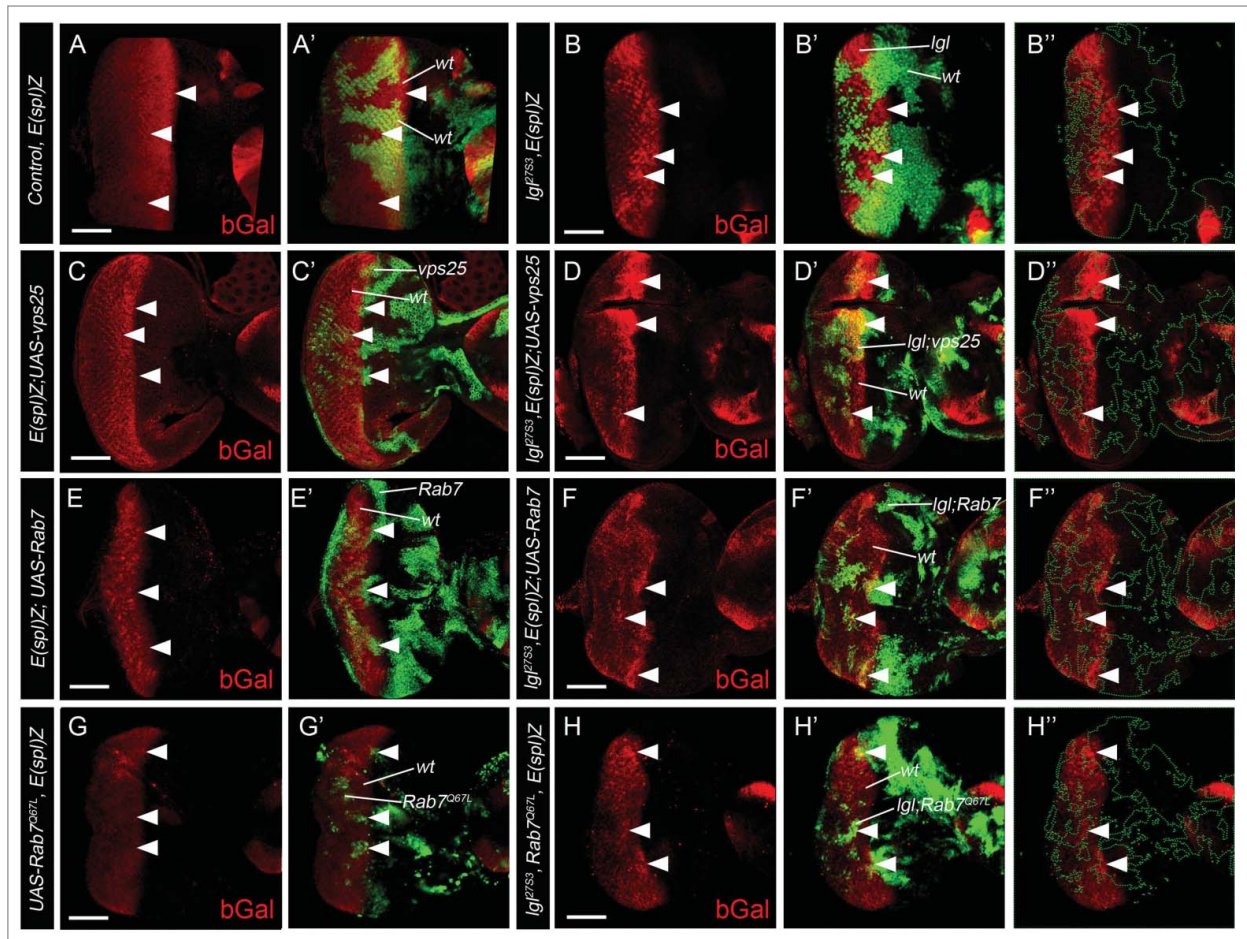


Figure 4. Vps25 or Rab7 functions are not responsible for ectopic Notch signaling in Lgl depleted tissue. **(A, B)** β -gal staining (red) of wild-type **(A)**, lgl^{-} **(B)** $E(spl)m8-lacZ$ third instar larval mosaic eye discs, showing upregulation of the Notch target in lgl^{-} clones just posterior to the Morphogenetic Furrow where ligand-dependent Notch signaling occurs (arrowheads). lgl^{-} mutant tissue is GFP (green)-negative (arrowheads). **(C, D)** β -gal staining (red) of $vps25$ **(C)** and $lgl^{-} vps25$ **(D)** $E(spl)m8-lacZ$ third instar larval mosaic eye discs, showing no substantial rescue of the elevated β -gal expression in lgl^{-} clones. Mutant tissue is marked by GFP (green, arrowheads). **(E-H)** β -gal staining (red) of $Rab7^{WT}$ **(E)** $Rab7^{Q67L}$ **(F)** $lgl^{-} Rab7^{WT}$ **(G)** and $lgl^{-} Rab7^{Q67L}$ **(H)** $E(spl)m8-lacZ$ third instar larval mosaic eye discs, showing no substantial effect on the elevated β -gal expression in lgl^{-} clones. Mutant tissue is GFP-positive (green, arrowheads). Scale bar = 50 μ M. Green-dashed line in B, D, F and H outline the clones. Genotypes: **(A)** $eyFLP; FRT40, E(spl)m8-lacZ / Ubi-GFP, FRT40$ **(B)** $eyFLP; lgl^{2753}, FRT40, E(spl)m8-lacZ / Ubi-GFP, FRT40$ **(C)** $eyFLP, UAS-GFP; FRT40, E(spl)m8-lacZ; UAS-vps25/ tubGAL80; tubGAL4$ **(D)** $eyFLP, UAS-GFP; lgl^{2753}, FRT40, E(spl)m8-lacZ; UAS-vps25/ tubGAL80, FRT40; tubGAL4$ **(E)** $eyFLP, UAS-GFP; FRT40, E(spl)m8-lacZ; UAS-Rab7-GFP/ tubGAL80, FRT40; tubGAL4$ **(F)** $eyFLP, UAS-GFP; lgl^{2753}, FRT40, E(spl)m8-lacZ; UAS-Rab7-GFP/ tubGAL80, FRT40; tubGAL4$ **(G)** $eyFLP, UAS-GFP; UAS-YFP-Rab7^{Q67L}, FRT40, E(spl)m8-lacZ/ tubGAL80, FRT40; tubGAL4$ **(H)** $eyFLP, UAS-GFP; lgl^{2753}, UAS-YFP-Rab7^{Q67L}, FRT40, E(spl)m8-lacZ/ tubGAL80, FRT40; tubGAL4$ Flystocks: $UAS-vps25-7$ (T. Vaccari), $UAS-Rab7-GFP$ (BL42706), $UAS-YFP-Rab7^{Q67L}$ (BL 24103, Bloomington Stock Center). Antibody: mouse β -galactosidase (Sigma, 1:500).

whether this is associated with Hippo pathway restoration.

The Regulation of Endocytosis and Notch Signaling by Apical-Basal Cell Polarity proteins during development

While our study has revealed a role for Lgl in endocytic regulation of Notch signaling, independent of aPKC, in the

developing eye neuroepithelium,²⁴ alternative regulatory mechanisms for Lgl in Notch signaling have been described in other *Drosophila* neural tissues.⁷¹⁻⁷⁴ In the *Drosophila* neuroblasts within the central and peripheral nervous systems, Lgl is required for the basal localization of Numb (a negative regulator of Notch), which segregates into daughter cells upon cell division and inhibits Notch signaling, thereby enabling neural cell differentiation.⁷² In these cells, Lgl acts by

sequestering aPKC, which when free phosphorylates Numb and prevents its localization to the cell cortex on one side of the cell.^{75,76} Lgl also prevents Sanpodo (a transmembrane protein that binds Notch and promotes its endocytosis) from localizing to the plasma membrane and thereby inhibits Notch signaling. In contrast, in another type of neural stem cell in *Drosophila*, the sensory organ precursors (SOPs), Lgl acts in parallel to Numb to block Notch function.⁷¹ This regulation

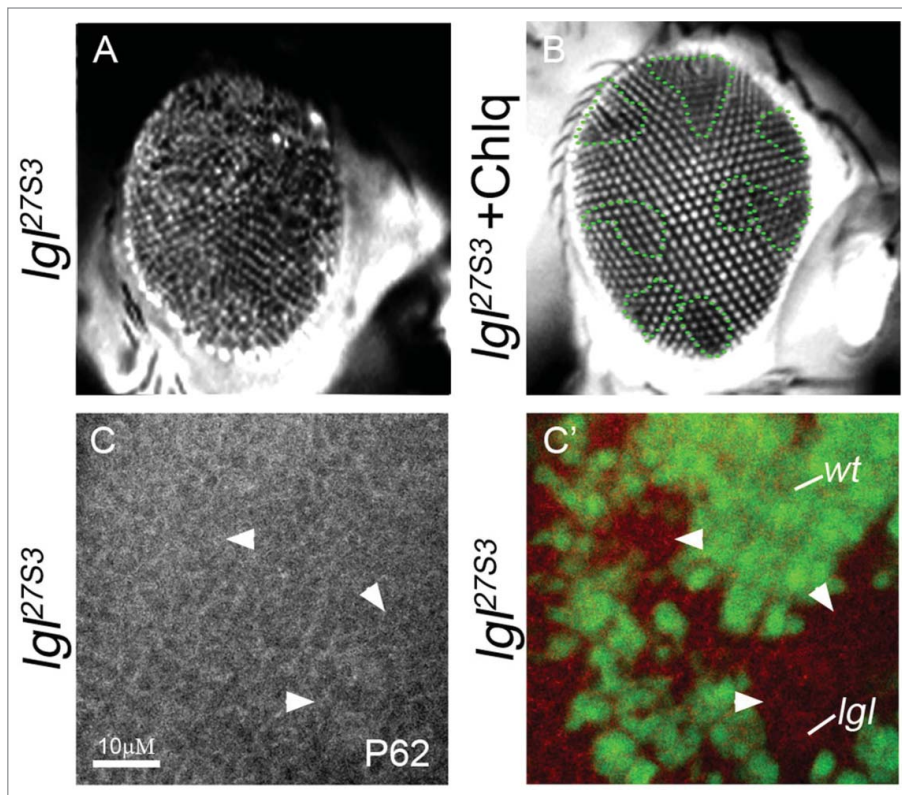


Figure 5. Chloroquine rescues the *Igf*⁻ adult eye phenotype without affecting autophagy. **(A, B)** Adult eyes viewed under the fluorescent-microscope; **(A)** GFP-negatively marked *Igf* mosaic eyes from mock-treated larvae, and **(B)** *Igf* mosaic eyes from larvae fed chloroquine (at 1 mg/mL). GFP-negatively marked *Igf*⁻ clones (dark) are still detected in the adult eyes when treated with chloroquine. Note, the *Igf*⁻ eye phenotype is dramatically rescued by chloroquine treatment. **(C)** p62 staining (gray or red in merge) of *Igf*⁻ mosaic third instar larval eye discs showing no obvious change between *Igf*⁻ and wild-type tissue. *Igf*⁻ clones are marked by the absence of GFP (green). Genotypes: **(A–C)** *eyFLP; Igf^{27S3}, FRT40, E(spl)m8-lacZ / Ubi-GFP, FRT40*. Antibody: Rabbit p62 (1:2000, G. Juhasz).

most likely occurs via Lgl's effect on the plasma membrane localization of Sanpodo,⁷³ by controlling the endocytosis of Sanpodo.⁷⁴ The precise mechanism by which Lgl acts to control endocytosis in these cells is unknown, however this endocytic role for Lgl may be fundamental in its *modus operandi* in controlling Notch signaling in all neural tissues. In non-neural *Drosophila* tissues, whether Lgl plays a role in regulating Notch signaling and the dependency on aPKC requires further investigation. Our analysis (this study) has revealed that overexpression of Lgl represses ectopic Notch signaling in the epithelial cells of the wing notum (that generates the adult thorax), although we presently do not know whether this is aPKC-dependent. Moreover, further studies are required to determine if Lgl plays a physiological role in Notch signaling in

the wing epithelial tissue. However, in another non-neural tissue, the ovarian follicle cells, functional analysis has shown that Lgl is not involved in regulating Notch signaling.^{36,77} Thus, there may be developmental and tissue-type requirements for *Drosophila* Lgl in Notch signaling and different mechanisms involved in each context.

Similarly in vertebrates, from limited information currently available, Lgl and aPKC have been shown to regulate Notch signaling via different mechanisms in different systems.^{78,79} During mouse brain development, the Lgl ortholog, Lgl1, is involved in the differentiation of neuro-progenitor cells.⁷⁸ These Lgl1 mutant neuro-progenitor cells fail to exit the cell cycle and differentiate, which is associated with the failure to segregate Numb and inhibit Notch signaling in the progenitor

cells. Whether aPKC is involved here was not investigated, however, in the chick central nervous system and myogenic precursors, an aPKC-dependent mechanism controls Notch signaling.⁷⁹ In these cells, aPKC ζ regulates ligand-dependent Notch1 signaling via phosphorylation of the Notch1 receptor on Ser-1791, which promotes trafficking of ligand-activated Notch1 to the nucleus, where it activates target gene expression. In the absence of ligand, aPKC instead promotes Notch1 internalization from the cell surface and from the secretory golgi-ER pathway to intracellular vesicles, where it is inactive. However, this mechanism is unlikely to occur in *Drosophila*, since the *Drosophila* Notch protein lacks the conserved Ser-1791 residue. In order to reconcile these studies, it will be important to determine whether Numb or Sanpodo are also regulated by aPKC in the chick central nervous system and myogenic precursors to contribute to Notch signaling, and in the mouse whether aPKC is involved and regulates Notch1 directly, as well as via Numb.

In our studies in the *Drosophila* eye epithelium, we have separated Lgl's role in tissue growth and in Hippo and Notch signaling from its cell polarity role.^{9,10,24} A polarity independent role has also been discovered for Lgl in the zebrafish retinal neuroepithelial cells in regulating Notch signaling.⁸⁰ Here depletion of zebrafish Lgl, Lgl1, results in elevated Notch signaling and neurogenesis defects. Furthermore, blocking Notch activity in the Lgl1 depleted retina rescues the neurogenesis defects, showing that aberrant Notch signaling is critical for this occurrence. The mechanism of Notch activation by Lgl depletion in this tissue is thought to be due to the expansion of the apical cellular domain and Notch receptor accumulation, although whether endocytosis defects also occur requires further investigation. Also whether the Hippo pathway is impaired in the zebrafish Lgl1 depleted retinal neuroepithelial cells would be interesting to determine.

In our previous study, we found that Lgl acts distinctly from Scrib and Dlg in the regulation of Hippo signaling.¹⁵ However, a recent study in *Drosophila* epithelial cells has revealed a novel trafficking

role for Lgl, together with Scrib and Dlg.⁸¹ This study showed that *lgl*, *scrib* and *dlg* mutants show defective trafficking of retromer cargo, such as Crb and Wntless (GPR177), to the apical membrane via aPKC-dependent and aPKC-independent mechanisms. Scrib was also found to have a role in retromer cargo trafficking in mammalian cells,⁸² although there are differences in the mechanism involved compared with *Drosophila*.⁸¹ In contrast to our system, where Lgl depleted cells do not lose polarity^{9,10,24} in the de Vreede study cell polarity was lost, and since Lgl regulates Dlg and Scrib localization and function,⁷ *lgl*, *scrib* and *dlg* mutant cells would be expected to behave similarly. Thus, it remains to be clarified whether Lgl is a direct regulator of the retromer trafficking pathway. However, the localization, levels or function of apical Crb complex components are not affected in *lgl*⁻ mosaic larval eye epithelia.^{9,10} This is important in regard to Notch signaling, since Crb also regulates Notch signaling/endocytosis separate to its role in cell polarity.⁸³ The extracellular Crb domain negatively regulates Notch signaling possibly by affecting S2 cleavage of Notch, as well as Notch and Delta endocytosis, whereas cell polarity function is coordinated by the Crb intracellular domain. Furthermore, Crb regulation of Notch signaling is distinct to that of Lgl, since Crb loss-of-function affects trafficking of full-length Notch,⁸³ which is not observed in Lgl depleted tissue.²⁴ Thus, Crb and Lgl affect Notch signaling by distinct mechanisms, and both have separable roles in the regulation of endocytosis/Notch signaling versus their roles in apical-basal cell polarity control.

Implications for Human Cancer

Improved cancer diagnosis relies on the establishment of tools that detect the earliest stages of cellular transformation. Our discovery that Lgl depletion in *Drosophila* epithelial cells results in sustained Notch activation indicates that the deregulated activity of neoplastic tumor suppressors can impact on cell signaling pathways prior to changes in cell polarity. In *lgl*⁻ tissue, the effect of Notch signaling on

expression of its targets, Cyclin A and Rst, is expected to contribute to the cell proliferative and survival effects observed in Lgl depleted tissue,²⁴ together with impairment of Hippo signaling and Yki activation.⁹ Indeed the incomplete suppression of the *lgl*⁻ adult eye phenotype by *mam*^{DN}, *aPKC*^{CAAX-DN} or *baz-RNAi* individually, suggests that aPKC-dependent (Yki activation) and Notch-dependent mechanisms contribute to the eye developmental defects of *lgl*⁻ mosaic flies.²⁴ Lgl acts as a tumor suppressor in human cancers, showing downregulation or mislocalization in many epithelial tumor types (reviewed by⁵). Deregulation of Lgl function might occur directly through mutation or indirectly via transcriptional regulation or polarity changes due to cells undergoing an epithelial to mesenchymal transition. Elevated Notch signaling is also associated with leukemia, breast and brain cancer (reviewed by^{25,84}), although how this is related to Lgl function is presently unknown. Given our findings it will be important to investigate the connection between Lgl and Notch and Hippo signaling in tumorigenesis in cell lines or mouse models.

Furthermore, our discovery of a novel function for *Drosophila* Lgl in the regulation of endosomal acidification, and the striking suppressing effect of chloroquine treatment on the adult *lgl*⁻ mosaic phenotype, reveals the importance of acidification in tumor growth. Indeed, higher acidity appears to be important for cancer progression and metastasis (reviewed by⁸⁵). V-ATPase subunits are upregulated in several human cancers, and are associated with increased tumor growth and metastasis,⁸⁶⁻⁸⁸ and reducing acidity can abrogate tumorigenesis.^{89,90} It is therefore important to investigate whether Lgl might be linked to the regulation of acidification in human cancers.

Chloroquine is a widely-used and well-tolerated FDA-approved drug for the treatment of malaria and inflammatory conditions, such as rheumatoid arthritis and systemic lupus erythematosus (reviewed by⁵⁶). More recently, chloroquine is being trialled as an anti-cancer agent with varying success (reviewed by^{64,67,91-93}). The rationale for using chloroquine as an anti-cancer therapy has been

to block autophagy, and since autophagy has context-dependent effects on tumor growth and metastasis (reviewed by^{67,94}), chloroquine's therapeutic success may depend on the type and grade of the cancer. Moreover, given chloroquine's effect on specific signaling pathways,⁵⁵⁻⁶³ the molecular profile of each patient's cancer needs to be considered to triage patients to select those who would most benefit from chloroquine therapy. Based on the dramatic rescue of the *lgl*⁻ phenotype that we have observed,²⁴ our studies suggest a novel use for this compound as an anti-cancer therapy against Notch and polarity defective cancers.

In summary, our studies in *Drosophila* have revealed that Lgl depletion leads to defects in endocytosis and key signaling pathways that promote tissue overgrowth. Based on our findings and given the importance of Lgl and Notch in human cancers, further investigation of the relationship between Lgl, cell polarity, endocytosis, regulation of acidification and Notch signaling in normal and malignant tissues is warranted. Moreover, the possible restoration of the Hippo pathway defects exhibited in the *lgl*⁻ tissue by chloroquine highlights the need to further explore possible connections between the Hippo pathway, endocytosis and vesicle acidification. Such studies have the potential to reveal novel therapeutic avenues to treat human cancers.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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