A Kinome RNAi Screen in Drosophila Identifies Novel Genes Interacting with Lgl, aPKC, and Crb Cell Polarity Genes in Epithelial Tissues

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ABSTRACT In both Drosophila melanogaster and mammalian systems, epithelial structure and underlying cell polarity are essential for proper tissue morphogenesis and organ growth. Cell polarity interfaces with multiple cellular processes that are regulated by the phosphorylation status of large protein networks. To gain insight into the molecular mechanisms that coordinate cell polarity with tissue growth, we screened a boutique collection of RNAi stocks targeting the kinome for their capacity to modify Drosophila “cell polarity” eye and wing phenotypes. Initially, we identified kinase or phosphatase genes whose depletion modified adult eye phenotypes associated with the manipulation of cell polarity complexes (via overexpression of Crb or aPKC). We next conducted a secondary screen to test whether these cell polarity modifiers altered tissue overgrowth associated with depletion of Lgl in the wing. These screens identified Hippo, Jun kinase (JNK), and Notch signaling pathways, previously linked to cell polarity regulation of tissue growth. Furthermore, novel pathways not previously connected to cell polarity regulation of tissue growth were identified, including Wingless (Wg/Wnt), Ras, and lipid/Phospho-inositol-3-kinase (PI3K) signaling pathways. Additionally, we demonstrated that the “nutrient sensing” kinases Slat Inducible Kinase 2 and 3 (SIK2 and 3) are potent modifiers of cell polarity phenotypes and regulators of tissue growth. Overall, our screen has revealed novel cell polarity-interacting kinases and phosphatases that affect tissue growth, providing a platform for investigating molecular mechanisms coordinating cell polarity and tissue growth during development.
Drosophila stocks

We used the GAL4/UAS system for tissue-specific expression of transgenes (Brand and Perrimon 1993; glass multimer repeat [GMR - GAL4 (II) and engi galed [en - GAL4 or en - GAL4, UAS - GFP (II)] express GAL4 predominantly in larval eye or wing discs, respectively [obtained from the Bloomington Drosophila Stock Center (BDSG)]. We constructed three cell polarity stocks: GMR - GAL4 was recombined with UAS - apKCCAAXWT (Sotillos et al. 2004) or UAS - crb intra-38.1.2b (Klebes and Knust 2000), and the en > lgl stock was generated by standard genetic techniques. UAS - lgl - RNAi51249 was obtained from the Vienna Drosophila RNAi Center (VDRC) (Dietzl et al. 2007), and we have shown that it efficiently depletes Lgl and can be rescued by the human ortholog Hugl1 (Grzeschik et al. 2010a).

Two independent stocks of each genotype were used to screen for modifiers.

\[
\text{GMR} > aPKCC^A : \text{GMR-GAL4, UAS-aPKCCAAXWT} / \text{CyO.}
\]

\[
\text{GMR} > \text{crb intra} : \text{GMR-GAL4, UAS-crb intra-38.1.2b} / \text{CyO.}
\]

\[
\text{en} > \text{lgl} : \text{en-GAL4, UAS-GFP/CyO; UAS-lgl-RNAi51249} / \text{TM6B.}
\]

UAS - SIK370M and null allele SIK32-2 were obtained from M. Montminy, Salk Institute for Biological Studies, California. UAS - 40D (UASup) was obtained from the VDRC (60101). naked-lacZ (P[ry+7.2] = PZ) nkd[04869a]) and independent SIK3 RNAi stock TRIP.JF03002 were obtained from the BDSG (25111 and 28366 respectively).

Genetic screens

Genetic screens are described in Figure 1 and Figure 3. Two virgin females from GMR > aPKCCA or GMR > crb intra stocks were crossed to two males carrying UAS - RNAi transgenes. Two virgin females from en > lgl stocks were crossed to two males from UAS - RNAi lines that showed a modification of the GMR > aPKCCA and/or GMR > crb intra phenotype. RNAi fly stocks were obtained from the National Institutes of Genetics (NIG-Fly, Japan) or the VDRC (Supplemental Material, Table S1 in File S1). At least 30 adult F1 flies were scored for each cross, representative images are shown. All flies were raised on standard cornmeal agar food at 25°C unless stated otherwise. RNAi lines that modified GMR > aPKCCA adult eyes or en > lgl adult wings were rescanned at 18°C or room temperature, respectively, as both phenotypes were weaker at lower temperatures.

Analysis of adult eye phenotypes

F1 progeny from crosses of GMR > aPKCCA or GMR > crb intra to UAS - RNAi were scored for modification of parental eye phenotypes. Adult eyes showing modification were imaged with a Scitec Infinity1 camera. Images were processed through Adobe Photoshop CS2 and Adobe Illustrator CS2.

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Analysis of wing size
F1 progeny from crosses of en > lgi to UAS - RNAi were scored for modification of parental wing phenotype. Adult wings were mounted in Canada Balsam/Xylene (Sigma) and imaged with an Olympus Stereomicroscope connected to a Scitec Infinity1 camera. Total wing area was measured with Adobe Photoshop CS2. Wing images were processed using Adobe Photoshop and Illustrator CS6.

Statistical analysis
All statistical tests were performed separately for each data set of wing sizes. Probability values were calculated using an unpaired t-test with Welch’s correction to reject the null hypothesis (variation of wing size through random, independent actions of UAS - RNAi transgenes) in Graph Pad Prism. \( P < 0.05 \) was considered statistically significant.

Gene ontology (GO) term (biological function) analysis
To functionally annotate gene lists and identify enriched GO term classes from genetic modifiers, the Princeton University web based tool, generic GO term finder, was used.

Signaling pathway analysis
The online pathway annotation tools DAVID (Database for Annotation, Visualization and Integrated Discovery, https://david.ncifcrf.gov), PANTHER (Protein ANalysis THrough Evolutionary Relationships, http://www.pantherdb.org), and KEGG (Kyoto Encyclopedia of Genes and Genomes, http://www.genome.jp/kegg) were used to place modifier genes into functional groups.

Immunohistochemistry
For analysis of third-instar larval wing discs, discs were dissected in phosphate-buffered saline (PBS), fixed in 4% paraformaldehyde, washed in PBS + 0.1% Triton X-100 (PBT), and blocked in PBT + 2% normal goat serum. Antibodies used were mouse anti-β-galactosidase (1:500; Rockland), rabbit anti-Lgl (1:500; Dennis Strand, Johannes Gutenberg University, Germany), and Alexa Fluor-conjugated 561, (1:500; Abcam). Confocal images were taken with an Olympus FV 1000, processed through Fiji and Adobe Photoshop CS6, and assembled in Adobe Illustrator CS6.

Data availability
Drosophila stocks and antibodies are available upon request or from stock centers as listed in the Materials and Methods. File S1 contains four supplemental figures and seven supplemental tables.

RESULTS AND DISCUSSION
Primary adult eye screen
The primary RNAi screen for modifiers of cell polarity phenotypes was conducted using the adult Drosophila eye, which has a regular, lattice-like structure due to the repetitive organization of group of epithelial retinal cells (Figure 1A). The organized structure of the Drosophila retina makes it sensitive to defects in epithelial structure and tissue

Figure 1 Overexpression of cell polarity components in the developing Drosophila eye alters adult eye structure. (A–C) Adult female eye images, side and dorsal views, respectively. Posterior to the left in (A–C). White bar, 250 \( \mu \text{M} \). (A and A') Control (GMR-GAL4) adult eyes displaying highly organized, regular, lattice-like organization of ommatidia to form the adult retina. (B and B') GMR > crb\(^{intra}\) adult eyes are slightly larger and rougher than control eyes. (C and C') GMR > aPKC\(^{CA}\) adult eyes are smaller, areas of the retina show disruptions in retinal architecture and necrosis (arrowheads). (D) Genetic scheme of F1 cell polarity modifier screen. Virgin females expressing components
growth, and therefore an ideal system for genetic modifier screens. When compared with control (GMR - GAL4/+), Figure 1, A and A’), overexpression of the transmembrane-intracellular domain of crb (UAS - crb\textsuperscript{mut}u) in the posterior region of the developing larval and pupal eye (using GMR - GAL4) results in weak overgrowth and lens defects in the adult eye (Figure 1, B and B’) (Grzeschik and Knust 2005; Grzeschik et al. 2010a; Johnson et al. 2002; Robinson et al. 2010). Expression of membrane-tethered constitutively-active aPKC (aPKCCA\textsubscript{AXXWT}, hereafter referred to as aPKCCA) via GMR - GAL4 resulted in a small and rough adult eye with necrosis (arrowhead Figure 1, C and C’). We and others have previously demonstrated the utility of GMR > crb\textsuperscript{mut}u (crb\textsuperscript{mut}u) and GMR > aPKCCA (aPKCCA) to detect genes capable of modifying cell polarity phenotypes (Grzeschik et al. 2010a; Ogawa et al. 2009; Parsons et al. 2010, 2014a,b; Robinson et al. 2010). Thus, we conducted an F1 modifier screen to detect genes capable of altering the morphology and/or growth of the crb\textsuperscript{mut}u and/or aPKCCA adult eye phenotypes to identify novel factors connecting cell polarity to tissue architecture and growth.

Since epithelial structure and cell polarity are integrated with cellular networks controlled by phosphorylation, we focused on genes predicted to encode kinases, phosphatases, and associated factors (Tables S1 and S2 in File S1, respectively). To identify those factors capable of genetically interacting with crb and/or aPKC, we conducted an F1 screen for modifiers of the crb\textsuperscript{mut}u and/or aPKCCA adult eye phenotypes using transgenic UAS - RNAi hairpin lines targeting 365 kinases or phosphatases (Figure 1D). From this screen, we identified 185 genes that modified the adult Drosophila cell polarity eye phenotypes. The 185 genes identified were grouped into four classes based on their interaction with crb\textsuperscript{mut}u and/or aPKCCA and/or GMR – GAL4 alone. Class 1 genes only modified aPKCCA [e.g., Dp110 (P33K92b); Figure 2, A–A’]. Class 2 genes only modified crb\textsuperscript{mut}u [e.g., calcium calmodulin regulated kinase Phk\gamma; Figure 2, B–B’]. Class 3 genes modified both aPKCCA and crb\textsuperscript{mut}u but did not generate a phenotype with GMR - GAL4 alone (e.g., membrane-associated guanylate kinase CASK; Figure 2, C–C’). Class 4 hits not only interacted with crb\textsuperscript{mut}u, aPKCCA, but their knockdown alone with GMR - GAL4 resulted in a visible phenotype [e.g., JNK pathway member misshapen (msn); Figure 2, D–D’] because these RNAi lines crossed to GMR - GAL4 generate an eye phenotype, it is possible that some of these genes may be false positive Crb and aPKC modifiers; however, so as to not miss any Crb and aPKC interactors, we proceeded with the analysis of this class of interactors. Of the 185 genes identified, 43 belonged to Class 1, 18 belonged to Class 2, 72 belonged to Class 3, and 52 were in Class 4 (Figure 2E). For a full list of genes identified in the screen and a brief description of their phenotype alone and/or phenotypic modification of crb\textsuperscript{mut}u and/or aPKCCA see Table S3 in File S1. The high proportion of genetic interactions (51% of genes screened) suggests that phosphoprotein networks play in important role in epithelial organization and/or tissue growth control together with aPKC and/or Crb in Drosophila eye development. Interestingly, the CMGC family of kinases (CDK, MAPK, GSK3, and CLK), comprising protein kinases involved in the MAPK cascade and mitotic tissue cell cycle (Table 1), is the most highly enriched, suggesting that these kinases might play important roles in aPKC and/or Crb function.

**GO analysis of adult Drosophila eye cell polarity modifier genes**

As the starting population of genes for this boutique screen was highly enriched for GO terms, such as phosphorous metabolic process genes (323/365, 88.7%), it was not possible to detect GO term enrichment for highly represented terms from Class 1–4 gene sets. Nevertheless, examination of GO terms with lower levels of enrichment between Classes 1–4, compared with the starting pool of genes screened, revealed modest enrichment of GO terms between the starting pool and modifier genes (Table 1). Classes 1–4 all showed different distributions for the 10 GO terms analyzed (Table 1). Genes associated with the GO term "establishment or maintenance of cell polarity” were only found in Class 3 (Table 1), highlighting the sensitivity of both the aPKCCA and crb\textsuperscript{mut}u adult eye phenotypes to changes in cell polarity network activity. Although all classes showed association with the GO term "signal transduction,” only classes with aPKCCA modifiers showed enrichment for MAPK signaling pathway genes. Furthermore, depletion of genes associated with "regulation of cellular response to stress” was only observed in Class 1 (aPKCCA). Taken together, these genetic screens demonstrated that cell polarity network activity was sensitive to several cellular processes, including proliferation, stress, and signaling pathways; however, distinct cell polarity modules may have different sensitivities and responses to these inputs.

### Secondary adult wing screen

Examination of gene lists corresponding to RNAi lines that modified aPKCCA and/or crb\textsuperscript{mut}u (Classes 1–4) revealed numerous genetic interactors that might be new genes involved in cell polarity regulation of tissue growth (Table S3 in File S1). To confirm these genetic interactions, as well as to reveal genes involved in linking cell polarity regulation to tissue growth, we conducted a secondary genetic screen where we upregulated aPKC and Crb in the developing wing by knocking down Lgl. Due to the antagonistic interaction between Lgl and aPKC, knockdown of Lgl results in increased aPKC activity (Betschinger et al. 2005), which in turn phosphorylates and activates the Crb complex (Fletcher et al. 2012; Sotillos et al. 2004). We used the wing epithelium, rather than the eye, since it is easier to quantify effects on tissue growth in the adult wing than in the eye, as well as to reveal genes that interact with deregulated Lgl/aPKC and Crb in another epithelial tissue. To knockdown Lgl, we used the engrailed (en)-GAL4 driven expression of a UAS-lgl-RNAi line, which is expressed in the posterior compartment of the developing wing from embryogenesis (Figure 3A). In this wing model, we quantified altered tissue growth by measuring adult wing size. Depletion of Lgl in the posterior half of the developing wing disc [en - GAL4 driven UAS - lgl-RNAi (en > lgl)] (Figure S1 in File S1) resulted in a 10% increase in total adult wing area (Figure 3, B and C, overlayed in Figure 3D, and quantified in Figure 3E). Thus, we conducted a secondary screen of the 185 cell polarity modifier hits for those able to modify the wing overgrowth due to Lgl depletion.

Of the 185 positive hits identified in the adult eye screen, 93 also interacted with en > lgl or en-GAL4 alone (Figure 4A). en > lgl interactors were broadly classified into three groups (Table S4 in File S1). Class 1 comprised 41 genes that resulted in pupal lethality with en > lgl and/or en – GAL4 alone, which precluded the specific effect of the gene knockdown on the wing growth being analyzed. Class 2 included 10 genes that produced adults following coknockdown of lgl, but the cramped wing phenotype precluded measurement. The most informative class, Class 3, comprised 42 genes, where either codepletion with lgl and/or depletion alone altered adult wing size. Essentially all Class 3 genes, except Tao-1 and SIK3, suppressed adult wing growth associated with lgl depletion.

After we had completed our screens, we were alerted to a recent report suggesting that ~25% of the VDRC KK RNAi collection can generate false positive enhancement of impaired Hippo pathway signaling, due to ectopic expression of the tiopip (tio) transcription factor insertion.
GMR showed no interaction with Adult eyes expressing a normal phenotype with 33054) showed no interaction with phenotypes. (D) Adult eyes expressing reduce necrosis. (D also resulted in an aberrant eye phenotype. Classes of interactors (Classes 3.1, 3.3, and 3.4) (Figure S3 and Tables S6 and S7), we have identified 18 kinases and phosphatases where knockdown of but not with GMR – GAL4 or GMR – aPKCCA, the gene from the 40D insertion site (Vissers et al. 2016). To determine if aberrant tio expression might be influencing our screen results, we tested the polarity phenotypes with the tio tester stock (40D:GALA). We observed modification of the GMR > cb*tra, en – GAL4, and en > lgl, but not GMR – GAL4 or GMR > aPKCCA, with the tio tester stock (40D:GALA) (Figure S2 in File S1). We note that in some instances where two or more RNAi lines for a given gene were tested for modification of cb*tra and aPKCCA, the genetic interaction produced opposite results [e.g., walter (wan), Table S3 in File S1] this may be due to a false positive interaction of the KK line with cb*tra, off-target effects, or the ability of different RNAs to efficiently suppress target genes. As KK lines interacting with both cb*tra and lgl may represent false positives, we intersected cb*tra and lgl modifiers, which revealed four interactors: CG1830 (PhKyi), CG10417, CG8866, and CG32484 (Sk2) (Figure 4A and Table S5 in File S1). Three of these interactors, CG10417, CG8866, and CG32484 (Sk2), were identified with KK lines and may represent false positives that need further verification by testing with independent RNAi lines.

Systematic analysis of wing sizes of en > lgl compared with en – GAL4 interactors in Class 3 revealed four subclasses: Subclass 3.1 interactors only modified en > lgl; Subclass 3.2 modifiers affected en – GAL4 and en > lgl wing size equivalently; Subclass 3.3 en > lgl wings were smaller than en – GAL4 modified wings; and Subclass 3.4 en > lgl wings were larger than en – GAL4 wings. Since genes in Subclass 3.2 modified en – GAL4 and en > lgl wing size equivalently, these genes were ruled out as being specific en > lgl interactors, leaving 18 genes in the remaining classes as Lgl modifiers. Additionally, as both en – GAL4 and en > lgl crossed to 40D:GALA wings displayed an ~10% decrease in wing size (Figure S2 in File S1), 15 Subclass 3.2 wing modifiers (where equivalent reduction in en > lgl and en wing growth was observed) may also be false positives (indicated with *; Table S6 in File S1). In summary, the en > lgl screen identified 18 kinases and phosphatases where knockdown of the modifier gene only showed modification of wing growth with lgl but not with en, or modified en > lgl wing size more than en alone (Table S6 in File S1). These genes include CG9784 (IPP, lipid phosphatase) and PpV (predicted Wnt pathway regulator; Sarup et al. 2015), which dramatically modified lgl-depleted wings, but had little effect or ~10% reduced growth following knockdown alone (Figure 4, B–E, respectively, quantified in Figure 4F). Thus, although screening the VDRC KK RNAi collection can generate false positive genetic interactions and some modifier genes require further validation (see Table S6 in File S1), we have identified 18 kinase/phosphatase genes that might coordinate cell polarity cues and tissue growth signals during organ development.

Overlap between modifier genes that specifically affected adult eye cell polarity phenotypes (Classes 1–3) and the en > lgl wing size (Classes 3.1, 3.3, and 3.4) (Figure S3 and Tables S6 and S7 in File S1) revealed that 15 of the Lgl wing size modifier genes interacted with both aPKC and Crb, suggesting that these genes were general cell polarity

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**Figure 2** Classification of adult Drosophila eye cell polarity modifiers. (A–D*) Side view, adult female eyes, posterior to the left. Classification of genetic modifiers was based on genetic interaction with GMR > cb*tra and/or GMR > aPKCCA and if RNAi expression alone via GMR-GAL4 also resulted in an aberrant eye phenotype. Classes of interactors are indicated. (A–A*) Class 1: modifier genes only interacted with GMR > aPKCCA. (A) Adult eyes expressing UAS-Pi3Ki (VDRC 38985) showed no interaction with GMR-GAL4 or (A*) GMR > cb*tra but modified (A*) GMR > aPKCCA to generate a small, glassy eye with decreased necrotic areas. (B–B*) Class 2: modifier genes only interacted with GMR > cb*tra. (B) Adult eyes expressing UAS-PhKyi (VDRC 33054) showed no interaction with GMR-GAL4 or (B*) GMR > aPKCCA but modified (B*) GMR > cb*tra to generate a larger eye with slight ruffling at the posterior edge (arrowhead). (C–C*) Class 3: modifier genes interacted with both GMR > cb*tra and GMR > aPKCCA. (C) Adult eyes expressing UAS-CASKi (VDRC 104793 and 34184) showed a normal phenotype with GMR – GAL4 alone but modified (C*) GMR > cb*tra to generate slightly larger eyes and (C*) GMR > aPKCCA to reduce necrosis. (D–D*) Class 4: genes modified all three parental phenotypes. (D) Adult eyes expressing UAS-msni (NIG 1697R-1) interact with GMR-GAL4 to generate glassy eyes. (D*) UAS-msni also modified GMR > cb*tra to produce elongated rough adult eyes and (D*)
Table 1 Frequency analysis of GO terms Class 1–4 modifiers of GMR > crb\textsuperscript{intra} and GMR > aPKC\textsuperscript{CA}

<table>
<thead>
<tr>
<th>GO TERM</th>
<th>GO ID</th>
<th>% Genes in Screen</th>
<th>% Genes in Class 1</th>
<th>% Genes in Class 2</th>
<th>% Genes in Class 3</th>
<th>% Genes in Class 4</th>
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The frequency of GO terms in Classes 1–4 is shown as a percentage of genes within the individual class that associate with the GO term. Fold enrichment was calculated by dividing the percentage of genes associated in individual classes by the percentage of genes in the total number of genes screened. Fold enrichment range reflects lowest to highest enrichment from all four Classes. Similarities and differences in GO term frequency between Classes are depicted graphically in pie charts (see Legend for color coding of GO terms analyzed). Individual pie charts for Classes 1–4 lack the range of GO terms observed in the total pool of genes screened, indicating that the cell polarity phenotypes selectively interacted with modifiers. Moreover, each Class has different GO term association patterns suggesting that the cell polarity phenotypes are not equivalent and therefore may impact different signaling networks.
tissue growth regulators. Three genes, Btk29A, Ror, and PpY-55A, did not interact with Crb, suggesting that these genes might be specific for the Lgl–aPKC axis of the tissue growth regulatory pathway. For simplicity, we will henceforth refer to these 18 genes as cell polarity–tissue growth interactors.

**Cell polarity–tissue growth-interacting genes are associated with many signaling pathways**

Analysis of the 18 cell polarity–tissue growth interactors (Classes 3.1, 3.3, and 3.4) for their links to signaling pathways (Figure 5 and Table S7 in File S1), revealed that many were associated with signaling pathways that Lgl, aPKC, or Crb have previously been shown to regulate in tissue growth control; the Hippo, Notch, and JNK pathways (Grzeschik et al. 2010a; Parsons et al. 2014a,b; Portela et al. 2015; Sun and Irvine 2011; Zhu et al. 2010). Notably, other Lgl-interacting genes were associated with signaling pathways not previously linked to the negative regulation of tissue growth by Lgl: Wg, Decapentaplegic (Dpp), Hedgehog (Hh), Src, Ras, and lipid signaling/PI3K (Figure 5 and Table S7 in File S1). However, three Lgl-interacting genes, the protein kinase genes Ror and CAMKIIB and the phosphatase gene PpY-55A, have been poorly studied and have not yet been associated with known signaling pathways.

Several of the cell polarity–tissue growth interactors affected the Hippo signaling pathway; depletion of Tao-1 (Class 3.4) (Boggiano et al. 2011; Poon et al. 2011), Hipk (Class 3.3) (Chen and Verheyen 2012), dco (Class 3.1) (Milton et al. 2010; Sopko et al. 2009), SIK2 (Class 3.3), and SIK3 (Class 3.4) (Wehr et al. 2013) modified the Lgl, aPKC, and Crb cell polarity phenotypes. Interestingly, the founding members of the Hippo pathway, hpo and wts (Class 3.1) (Harvey and Tapon 2007), also interacted with en, resulting in pupal lethality, although at lower temperatures hpo-RNAi produced adults with wing size defects (Table S4 in File S1). Since Lgl, aPKC, and Crb are known to regulate the Hippo pathway (Chen et al. 2010; Grzeschik et al. 2010a; Ling et al. 2010; Parsons et al. 2010; Robinson et al. 2010), the identification of Hippo pathway regulators in our en screen demonstrates that this phenotype is sensitive to modifier genes that regulate tissue growth.

We have previously demonstrated that Notch signaling is impaired in lgl mutant eye epithelial tissue and contributes to the tissue growth effects (Parsons et al. 2014a; Portela et al. 2015), and Crb has also been shown to regulate Notch signaling in the eye (Richardson and Pichaud 2010). HIPK, identified as an lgl, aPKC and crb interactor, in addition to its regulation of Hippo pathway signaling, also acts a positive
regulator of Notch signaling in eye development (Lee et al. 2009). HIPK also regulates JNK signaling in the Drosophila wing epithelium (Huang et al. 2011), and in this tissue Lgl depletion-mediated cell polarity and tissue growth effects are JNK-dependent (Sun and Irvine 2011; Zhu et al. 2010). Thus, HIPK’s role in regulating JNK signaling in the wing tissue and in Notch signaling in the eye tissue might also underlie its genetic interactions with lgl, aPKC, and crb. However, although core members of the JNK pathway, Bsk, Tak1, Takl2, and Misshapen (Msn), were identified as modifiers of the GMR > aPKC or GMR > aPKC and GMR > crb eye phenotypes (Table S3 in File S1), knockdown of Tak1 or Msn were lethal with en > lgl and en > crb, precluding analysis of their specific interaction with lgl, while Bsk or Takl2 knockdown did not noticeably modify the en > lgl phenotype (Table S4 in File S1).

Our analysis of the Lgl-interacting genes revealed several novel signaling pathways (Dpp, Wg, Hh, Src, Ras, and lipid-P13K) not previously implicated in the negative regulation of tissue growth by Lgl, aPKC, or Crb cell polarity regulators, which we will detail below.

Lgl regulates Dpp [Bone Morphogenetic Protein (BMP)] signaling in the wing epithelium by promoting the secretion of the Dpp morphogen (Arquier et al. 2001). Pka-C1, which genetically interacts with lgl,
aPKC, and crb phenotypes, negatively regulates Dpp signaling (Li et al. 1995), so may genetically interact with Lgl, by affecting Dpp signaling. However, since Dpp positively regulates wing tissue growth (Brumby and Richardson 2005) and Lgl depletion would be expected to decrease Dpp signaling, this is unlikely to directly account for the wing size increase. Furthermore, knockdown of Pka-C1 should lead to increased Dpp signaling, as well as Hh and Wg signaling in the wing epithelium (Li et al. 1995), and therefore it is difficult to understand how Pka-C1 leads to a reduction in en > lgl wing growth. Interestingly, Pka-C1 promotes Ras-induced stem cell proliferation in the malpighian tubules (Zeng et al. 2010), and therefore reduced Pka-C1 might also reduce Ras signaling in the wing leading to the reduced tissue growth of en > lgl wings; however, further investigation is required to investigate this possibility. Interestingly, there is cross talk between the Dpp and Hippo pathways that might also impact on this interaction (Oh and Irvine 2011; Rogulja et al. 2008).

The Wg (Wnt) signaling pathway was associated with many of the Lgl interactors. The Wg/Wnt pathway regulates many biological processes, including proliferation and differentiation, to coordinate organ growth and planar cell polarity (Clevers and Nusse 2012). Interestingly, as measured by increased expression of the naked (nkd)-lacZ Wg signaling reporter (Tyler and Baker 2007; Zeng et al. 2000), we found that Wg signaling was upregulated in lgl mutant clones relative to the surrounding wild-type cells in larval eye discs (Figure S4 in File S1). Consistent with Lgl-modulating Wg signaling, two cell polarity tissue growth interactor genes are implicated in Wg signaling [discs overgrown (dco) (Class 3.1) (Klein et al. 2006), and cAMP-dependent protein kinase 1 (Pka-C1) (Class 3.3) (Li et al. 1995)]. Furthermore, comparison between the cell polarity tissue growth interactors, and kinase and phosphatase genes recently predicted to regulate Wg signaling in Drosophila (Swarup et al. 2015), revealed dco and three other kinase genes [VRK (Class 3.3), Hipk (Class 3.3), and Eip63E (Class 3.1)], as well as five phosphatases genes [wun2, Pp2c1, PpV, wdb (all Class 3.3), and PP2C (Class 3.1)] (Figure 5 and Tables S6 and S7 in File S1). Therefore, these genes might regulate Wg signaling to modify the Lgl, aPKC, or Crb phenotypes. Consistent with the association of these interactors with the Wg pathway, previous studies have revealed genetic interactions between the Wg signaling pathway and Lgl/aPKC in Drosophila embryo epithelial morphogenesis (Dollar et al. 2005; Kaplan et al. 2009, 2011; Kaplan and Tölovinski 2010) and in Xenopus (Choi and Sokol 2009). Thus, cell polarity regulation of Wg signaling might be important in coordinating epithelial structure and organ growth.

However, it should also be noted that the Wg signaling pathway can cross talk to the Hippo pathway in tissue growth control during wing development (Zecca and Struhl 2010), and therefore the effect of these cell polarity tissue growth interactors on the Wg pathway might indirectly affect the Hippo pathway to modulate the Lgl, aPKC, or Crb phenotypes.

Other signaling pathways associated with the cell polarity–tissue growth interactors were also associated with lipid-PI3K signaling (Tables S6 and S7 in File S1): PI3K, phospho-inositol-3-kinase.

Cell polarity tissue growth interactors were also associated with lipid-PI3K signaling (Tables S6 and S7 in File S1): CG9784 (IPP), encoding a Inositol phosphate phosphatase, which is involved in membrane trafficking and lipid signaling, Class 3.1; (De Craene et al. 2017; Erneux...
Overexpression of kinase dead transgene (SIK3K70M), RNAi treatment of UAS-SIK3 RNAi, and GMR-GAL4 drivers with GMR>GAL4 were used to study SIK3 activity in Drosophila. (A) Expression of UAS-SIK3-RNAi with GMR-SIK3i (B) Expression of UAS-SIK3 RNAi in conjunction with GMR>SIK3K70M; (C) GMR>aPKCC42; (D) Reduction of SIK3 activity by RNAi depletion en > GFP; (E) Overexpression of kinase dead transgene en > GFP; UAS-SIK3K70M; or (H) null allele en > GFP/SIK372 has no effect on adult wing size (< 1%). (F) In conjunction with reduced lgl activity, en > lgl decreased SIK3 activity by RNAi depletion with UAS-SIK3-RNAi. (G) Overexpression of kinase dead transgene (UAS-SIK3K70M) or (I) null allele SIK3722 significantly increased wing size. (J) Quantification of total wing area (D-I). In D, the arrowhead indicates ectopic cross veins. Results represent individual wings ± SD. *** P < 0.0001. RNAi, RNA interference

Cell polarity complexes and Salt Inducible Kinase 3 interact to restrict tissue growth

Of particular interest, our cell polarity gene screens identified two members of the AMP-related kinase family, Salt Inducible Kinase 3 (SIK3) (Class 3.4) and SIK2 (Class 3.3), which have important roles in nutrient-dependent signaling (Choi et al. 2011, 2015; Wang et al. 2011), but had not previously been connected to cell polarity regulation. SIK3 was identified in our screen as a negative regulator of tissue growth affecting aPKC, Crb, and Lgl phenotypes (Tables S4, S6, and S7 in File S1). Although depletion of SIK3 alone did not noticeably affect adult eye morphology or size (Figure 6A), SIK3 knockdown caused glassiness and bulging of retinal tissue in adult eyes in the crbnTRA or aPKCC42 background (Figure 6, B and C respectively, compared with Figure 1, C and D). Wing size was unaltered following depletion of SIK3 alone in the posterior wing compartment via RNAi (en – GAL4 driven SIK3i), expression of a kinase dead transgene (SIK3K70M), or reduced SIK3 gene dosage using SIK3 heterozygotes (null allele SIK3722/+). However, reduced SIK3 activity, in combination with lgl depletion, resulted in a significant increase in wing size (compare Figure 6, D and E, Figure 6, F and G, and Figure 6, H and I; quantified in Figure J). Thus, SIK3 modulates the activity of the cell polarity complexes and growth pathways to restrict tissue growth.

Intriguingly, our data support a requirement for SIK2 activity in promoting normal and lgl-dependent wing growth (Tables S4, S6, and S7 in File S1), while reduced SIK3 activity increased wing growth in the Lgl loss-of-function background. In Drosophila, Salt Inducible kinases have been implicated in tissue growth via Hippo pathway signaling (Wehr et al. 2013). SIK2 and SIK3 phosphorylate and inactivate Salvador (SAV), a core component of the Hippo kinase complex, leading to activation of the Yki transcriptional program and increased
tissue growth (Wehr et al. 2013). Furthermore, codepletion of SIK2 and SIK3 reduced tumor growth in a Drosophila tumor model (activated Src + RasVT12) (Hirabayashi and Cagan 2015). Given the observation that SIK2 and SIK3 differentially modify Lgl-dependent tissue growth, but are both required for Hippo pathway inactivation and Src + RasVT12-driven tumor growth, future studies are required to determine how the SIKs interact with Lgl to control wing growth.

Conclusions

Genetic screens in Drosophila remain a powerful tool for identifying and unraveling gene function in specific signaling pathways and cellular processes. We undertook a boutique genetic screen, specifically using RNAi lines targeting kinases and phosphatases, to identify novel signaling pathways involved in the regulation of epithelial structure with tissue growth. Analysis of the hits that genetically interacted with lgl, aPKC, and/or crb cell polarity genes revealed that they were associated with Hippo, Notch, JNK, Dpp, Hh, Wg, Ras, lipid/PI3K, and unknown signaling pathways. Future studies determining the molecular relationships between cell polarity proteins and the modifiers identified will be required to determine whether these interactions are direct. Recent advances in proteomics through the generation of the Drosophila and human Protein Interaction Map, and studies coupling genetic manipulation to the analysis of kinase–phosphatase networks, will considerably advance our capacity to define the in vivo function of these genes (Guruhrasha et al. 2011; Huttlin et al. 2015; Sopko et al. 2014). Of relevance to the novel pathways revealed in this study, further exploration of the roles of SIK2 and SIK3 in Drosophila development, and mammalian cancer models, is required to unravel the intricacies between cell polarity protein complexes and nutrient sensing kinases in normal development and cancer.

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