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, JNK, and Notch signalling 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) signalling pathways. Additionally, we demonstrated that the ‘nutrient sensing’ kinases, Salt 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.
INTRODUCTION

Apical-basal polarization of the epithelium is essential to maintain tissue architecture and restrict organ growth (Elsum and Humbert, 2013). Epithelial cell polarity arises due to creation of distinct membrane domains (apical, basal and basolateral) via the coordinated activity of three major polarity complexes, conserved from flies to humans. Specifically, epithelial cell polarity is coordinated by: 1) the Crumbs (CRB) complex, comprised of the transmembrane protein Crb and associated proteins Stardust and Patj, localized at the subapical region; 2) the Scribble module (Scribble (Scrib), Disc-large (Dlg) and Lethal (2) giant larvae (Lgl)), localized to septate junctions in Drosophila (basolaterally in mammals) to promote basolateral membrane identity; and 3) the Partitioning defective (PAR) complex (atypical protein kinase C (aPKC), Bazooka (PAR3) and PAR6)), which promotes separation of basolateral and subapical membrane domains (Tepass, 2012). Dynamic and reciprocal interactions between these polarity complexes determine cellular membrane identity and epithelial organization (McCaffrey and Macara, 2011).

A critical determinant of cell polarity is the activity of the PAR complex and aPKC, which has dual roles in cell polarity. aPKC directly phosphorylates 1) Crb to allow binding of the Std/Patj complex (Sotillos et al., 2004), and 2) Lgl to result in exclusion from the apical membrane (Betschinger et al., 2005; Plant et al., 2003). Additionally, these apical-basal cell polarity regulators also control tissue growth. Drosophila lgl , scrib and dlg are termed ‘junctional scaffold neoplastic tumor suppressor genes, mutations of which are associated with loss of cell polarity and characterised by imaginal disc epithelial and neural tissue overgrowth, impaired differentiation and the formation of transplantable tumors (Hariharan and Bilder, 2006). Despite detailed knowledge of the molecular interactions between the Crb, PAR and Lgl complexes in the establishment and maintenance of cell polarity, how these mutually exclusive polarity modules interact to coordinate epithelial organization with tissue growth is less well understood.
Thus, we have been investigating how these polarity regulators control tissue growth, and have discovered that Lgl’s, but not Scrib or Dlg, role in tissue growth control occurs via regulation of signalling pathways and this function is independent of Lgl’s role in cell polarity (Doggett et al., 2011; Doggett et al., 2015; Grzeschik et al., 2007; Grzeschik et al., 2010a; Parsons et al., 2014a; Portela et al., 2015; Richardson and Portela, 2017). Of particular relevance here, our previous studies have shown that loss of lgl and the concomitant increase in aPKC activity, or increased levels of Crb, impairs the Hippo tissue growth control pathway and is associated with ectopic cell proliferation, decreased apoptosis and subsequent tissue overgrowth in the Drosophila eye (Grzeschik et al., 2010a; Grzeschik et al., 2010b; Parsons et al., 2014a; Parsons et al., 2010; Parsons et al., 2014b; Portela et al., 2015; Richardson and Portela, 2017).

To gain insights into the relationship between epithelial structure and organ growth we utilized cell polarity phenotypes in the adult Drosophila eye and undertook a boutique genetic screen using RNA interference (RNAi). Due to the critical role phosphorylation plays in regulating the activity of numerous cellular signalling processes and growth pathways, we screened a collection of kinase and phosphatase RNAi lines. By screening for modification of the adult eye phenotypes due to overexpression/activation of Crb or aPKC (using GMR > crbintra or GMR > aPKCC4) we identified 185/365 genes that were capable of modifying these phenotypes. To further explore the ability of these cell polarity modifier genes to regulate tissue growth, we extended our analysis to screen for modification of a tissue overgrowth phenotype associated with knockdown of lgl in the adult Drosophila wing (using en > lgl-RNAi (lgl))). From this secondary screen of the 185 genes from the primary screen, we identified 18 genes that also modified the en > lgl adult Drosophila wing size, compared with en > alone. Of the 18 genes that modified cell polarity phenotypes in the adult Drosophila eye and wing, several modulated signalling pathways involved in tissue growth control, such as the Hippo, Wingless/Wnt and
inositol phosphate signalling pathways. We also identified stress responsive genes, such as members of the ‘nutrient sensing’ or AMP activated protein kinases (AMPKs), dSik2 and dSik3 (Shackelford and Shaw, 2009). In summary, our genetic screens identified genes and biological processes that provide entry points to investigate the molecular mechanisms that coordinate epithelial structure and tissue growth control during development.
**MATERIALS AND METHODS**

**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 engrailed (en - GAL4 or en - GAL4, UAS - GFP (II)) express GAL4 predominantly in larval eye or wing discs respectively (obtained from Bloomington Stock Center). We constructed three ‘cell polarity’ stocks: GMR - GAL4 was recombined with UAS - aPKC^{CAAXWT} (Sotillos et al., 2004) or UAS - crb^{intra-38.1.2b} (Klebes and Knust, 2000) and the en > lgli stock was generated by standard genetic techniques. UAS - lgl - RNa{\textsc{i}}^{51249} 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.

\[ \text{GMR} > \text{aPKC}^{CA} : \text{GMR - GAL4, UAS - aPKC}^{CAAXWT}/\text{CyO}. \]

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

\[ \text{en} > \text{lgli} : \text{en - GAL4, UAS - GFP /CyO; UAS - lgl - RNa}{\textsc{i}}^{51249}/\text{TM6B}. \]

\[ \text{UAS - SIK3}^{K70M} \text{ and null allele SIK3}^{72} \text{ were obtained from M. Montminy, Salk Institute for Biological Studies, California, USA.} \]

\[ \text{UAS - 40D (UAS}^{90D}) \text{ was obtained from VDRC (60101).} \]

\[ \text{naked-lacZ (P(ry}^{[+7.2]=PZ)nkd[04869a]) \text{ and independent SIK3 RNAi stock TRiP.JF03002 were obtained from Bloomington Stock Centre (#25111 and #28366 respectively).} \]

**Genetic Screens**

Genetic screens are described in Figure 1 and Figure 3. Two virgin females from \text{GMR} > \text{aPKC}^{CA} \text{ or } \text{GMR} > \text{crb}^{intra} \text{ stocks were crossed to two males carrying UAS - RNa}{\textsc{i}} \text{ transgenes.}
Two virgin females from $en > lgli$ stocks were crossed to two males from $UAS - RNAi$ lines that showed a modification of $GMR > aPKC^{CA}$ and/or $GMR > crb^{intra}$ phenotype. RNAi fly stocks were obtained from National Institutes of Genetics (NIG-Fly, Japan) or VDRC (Supporting Information, Table 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 $GMR > aPKC^{CA}$ adult eyes or $en > lgli$ adult wings were rescreened 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 $GMR > aPKC^{CA}$ 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.

**Analysis of wing size**

F1 progeny from crosses of $en > lgli$ 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 in 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 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.

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 gene ontology (GO) term finder was used.

Signalling 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) www.pantherdb.org and KEGG (Kyoto Encyclopedia of Genes and Genomes) 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 (Rockland, 1:500) and rabbit anti-Lgl (Dennis Strand, Johannes Gutenberg University, Germany. 1:500), Alexa Fluor conjugated 561, (Abcam, 1:500). Confocal images were taken with Olympus FV 1000, processed through Fiji and Adobe Photoshop CS6, assembled in Adobe Illustrator CS6.

Drosophila stocks and antibodies are available upon request or from stock centers as listed in the Materials and Methods. File S1 contains all supplementary material, including four supplementary Figures and seven supplementary 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 groups of epithelial retinal cells (Figure 1A). The organized structure of the *Drosophila* retina, makes it sensitive to defects in epithelial structure and tissue growth, and therefore an ideal system for genetic modifier screens. When compared with control (*GMR* - *GAL4*/+, Figure 1A, A’), overexpression of the transmembrane-intracellular domain of *crb* (*UAS - crb*\textsuperscript{intra}) 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 1B, B’) (Grzeschik and Knust, 2005; Grzeschik et al., 2010a; Johnson et al., 2002; Robinson et al., 2010). Expression of membrane-tethered constitutively-active *aPKC* (*aPKCCAAXWT*, hereafter referred to as *aPKC*\textsuperscript{CA}) via *GMR* - *GAL4* resulted in a small and rough adult eye, with necrosis (arrowhead Figure 1C, C’). We and others have previously demonstrated the utility of *GMR* > *crb*\textsuperscript{intra} (crb\textsuperscript{intra}) and *GMR* > *aPKC*\textsuperscript{CA} (aPKC\textsuperscript{CA}) to detect genes capable of modifying cell polarity phenotypes (Grzeschik et al., 2010a; Ogawa et al., 2009; Parsons et al., 2014a; Parsons et al., 2010; Parsons et al., 2014b; 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{intra} and/or *aPKC*\textsuperscript{CA} adult eye phenotype 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 (Supporting Information, Table S1 and Table S2 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{intra} and/or *aPKC*\textsuperscript{CA} 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* \(^{\text{intra}}\) and/or *aPKC* \(^{\text{CA}}\) and/or *GMR – GAL4* alone. Class 1 genes only modified *aPKC* \(^{\text{CA}}\) (e.g. *Dp110* (*Pi3K92E*) Figure 2A, A’ and A’’). Class 2 genes only modified *crb* \(^{\text{intra}}\) (e.g. calcium calmodulin regulated kinase *PhK*\(^{\gamma}\); Figure 2B, B’ and B’’). Class 3 genes modified both *aPKC* \(^{\text{CA}}\) and *crb* \(^{\text{intra}}\) but did not generate a phenotype with *GMR - GAL4* alone (e.g. membrane-associated guanylate kinase *CASK*; Figure 2C, C’ and C’’). Class 4 hits not only interacted with *crb* \(^{\text{intra}}\), *aPKC* \(^{\text{CA}}\) but their knockdown alone with *GMR-GAL4* resulted in a visible phenotype (e.g. Jun kinase (JNK) pathway member *misshapen* (*msn*) Figure 2D, D’ and 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* \(^{\text{intra}}\) and/or *aPKC* \(^{\text{CA}}\) see Supporting Information, Table S3. 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, GS3K, CLK), comprising protein kinases involved in the MAPK cascade and mitotic cell cycle (Table 2), is most highly enriched, suggesting that these kinases might play important roles in aPKC and/or Crb function.

**Gene Ontology Analysis of adult *Drosophila* eye cell polarity modifier genes**

As the starting population of genes for this boutique screen was highly enriched for gene
ontology (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 analysed (Table 1). Genes associated with the GO term ‘establishment or maintenance of cell polarity’ were only found in Class 3, highlighting the sensitivity of both the \textit{aPKC}^{Ca} and \textit{crb}^{intra} adult eye phenotypes to changes in cell polarity network activity. Although all classes showed association with the GO term signal transduction, only classes with \textit{aPKC}^{Ca} modifiers showed enrichment for MAPK signalling pathway genes. Furthermore, depletion of genes associated with ‘regulation of cellular response to stress’ was only observed in Class 1 (\textit{aPKC}^{Ca}). Taken together these genetic screens demonstrated that cell polarity network activity was sensitive to several cellular processes, including proliferation, stress and signalling 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 \textit{aPKC}^{Ca} and/or \textit{crb}^{intra} (Classes 1 - 4) revealed numerous genetic interactors that might be new genes involved in cell polarity regulation of tissue growth (Supporting Information, Table S3). 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*)) resulted in a 10% increase in total adult wing area (Figure 3B and C, overlayed in D; quantified in E). 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 (Supporting Information, Table S4). 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 analysed. Class 2 included 10 genes that produced adults following co-knockdown of *lgl*, but the crumpled wing phenotype precluded measurement. The most informative class, Class 3, comprised 42 genes, where either co-depletion with *lgl* and/or depletion alone altered adult wing size. Essentially all Class 3 genes, except *Tao-1* and *dSIK3*, suppressed adult wing growth associated with *lgl* depletion.

After we had completed our screens, we were alerted to a recent report suggesting that approximately 25% of the VDRC KK RNAi collection can generate false positive enhancement of impaired Hippo pathway signalling, due to ectopic expression of the *tiptop (tio)* transcription factor 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\text{UAS}). We observed modification of the GMR > crb\text{intra}, en - GAL4 and en > lgli, but not GMR - GAL4 or GMR > aPKC\text{CA} with the tio tester stock (40D\text{UAS}) (Supporting Information Figure S2). We note that in some instances where two or more RNAi lines for a given gene were tested for modification of crb\text{intra} and aPKC\text{CA} the genetic interaction produced opposite results (e.g. wunen (wun), Supporting Information Table S3) this may be due to a false positive interaction of the KK line with crb\text{intra}, off target effects or the ability of different RNAi’s to efficiently suppress target genes. As KK lines interacting with both crb\text{intra} and lgli may represent false positives, we intersected crb\text{intra} and lgli modifiers, which revealed four interactors CG1830 (PhKγ), CG10417, CG8866, CG32484 (Sk2) (Figure 4A, Supporting Information Table S5). Three of these interactors CG10417, CG8866, 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 > lgli compared with en – GAL4 interactors in Class 3, revealed four subclasses: Subclass 3.1 interactors only modified en > lgli; Subclass 3.2 modifiers affected en – GAL4 and en > lgli wing size equivalently; Subclass 3.3 en > lgli wings were smaller than en – GAL4 modified wings and Subclass 3.4 en > lgli wing size larger than en – GAL4 wings. Since genes in Subclass 3.2 modified en – GAL4 and en > lgli wing size equivalently, these genes were ruled out as being specific en > lgli interactors, leaving 18 genes in the remaining classes as Lgl modifiers. Additionally, as both en - GAL4 and en > lgli crossed to 40D\text{UAS} wings displayed an approximate 10% decrease in wing size (Supporting Information Figure S2) 15 Subclass 3.2 wing modifiers (where equivalent reduction in en > lgli and en wing growth was observed) may also be false positives (indicated with *, Supporting Information Table S6). In summary, the en > lgli screen identified 18 kinases and phosphatases where
knockdown of the modifier gene only showed modification of wing growth with \textit{lgli} but not with \textit{en}, or modified \textit{en > lgli} wing size more than \textit{en} alone (Supporting Information Table S6). These genes include \textit{CG9784} (\textit{IPP}, lipid phosphatase) and \textit{PpV} (predicted Wnt pathway regulator (Swarup \textit{et al.} 2015)), which dramatically modified Lgl-depleted wings, but had little effect or approximately 10\% reduced growth following knockdown alone (Figure 4B & C, D & E respectively, quantified in F). Thus, although screening the VDRC KK RNAi collection can generate false positive genetic interactions and some modifier genes require further validation (see Supporting Information Table S6) 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 \textit{en > lgli} wing size Classes 3.1, 3.3 and 3.4 (Supporting Information Figure S3 and Table S6, S7) revealed that 15 of the Lgl wing size modifier genes interacted with both aPKC and Crb, suggesting that these genes were general cell polarity tissue growth regulators. Three genes, \textit{Btk29A}, \textit{Ror} and \textit{PpY-55A}, did not interact with \textit{Crb}, suggesting that these genes might be specific for the \textit{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 signalling pathways**

Analysis of the 18 cell polarity-tissue growth interactors (Classes 3.1, 3.3 and 3.4) for their links to signalling pathways (Supporting Information Table S7 and Figure 5), revealed that many were associated with signalling pathways that Lgl, aPKC or Crb have previously been shown to regulate in tissue growth control; the Hippo, Notch and Jun Kinase (JNK) pathways (Grzeschik \textit{et al.}, 2010a; Parsons \textit{et al.}, 2014a; Parsons \textit{et al.}, 2014b; Portela \textit{et al.}, 2015; Sun and Irvine,
2011; Zhu et al., 2010). Notably, other Lgl interacting genes were associated with signalling pathways not previously linked to the negative regulation of tissue growth by Lgl; Wingless (Wg), Decapentaplegic (Dpp), Hedgehog (Hh), Src, Ras and Lipid signalling/PI-3-Kinase (PI3K), (Supporting Information Table S7 and Figure 5). However, three Lgl interacting genes, the protein kinase genes, \textit{Ror}, \textit{CAMKIIB} and the phosphatase gene, \textit{PpY-55A}, have been poorly studied and not yet been associated with known signalling pathways.

Several of the cell polarity-tissue growth interactors affected the Hippo signalling pathway; depletion of \textit{Tao-I} (Class 3.4) (Boggiano et al., 2011; Poon et al., 2011), \textit{HIPK} (Class 3.3) (Chen and Verheyen, 2012), \textit{dco} (Class 3.1) (Milton et al., 2010; Sopko et al., 2009), \textit{SIK2} (Class 3.3) and \textit{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, \textit{hpo} and \textit{wts} (Class 3.1) (Harvey and Tapon, 2007), also interacted with \textit{en>lgli}, resulting in pupal lethality, although at lower temperatures \textit{hpo-RNAi} produced adults with wing size defects (Supporting Information Table S4). 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 \textit{en > lgli} screen demonstrates that this phenotype is sensitive to modifier genes that regulate tissue growth.

We have previously demonstrated that Notch signalling is impaired in \textit{lgli} 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 signalling in the eye (Richardson and Pichaud, 2010). \textit{HIPK}, identified as an \textit{lgli}, \textit{aPKC} and \textit{crb} interactor, in addition to its regulation of Hippo pathway signalling also acts a positive regulator of Notch signalling in eye development (Lee et al., 2009). HIPK also regulates JNK signalling in the \textit{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 signalling in the wing tissue and in Notch signalling 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 (Supporting Information Table S3), knockdown of Tak1 or Msn were lethal with en>lgli and en>, precluding analysis of their specific interaction with lgl, whilst Bsk or Takl2 knockdown did not noticeably modify the en>lgli phenotype (Supporting Information Table S4).

Our analysis of the Lgl interacting genes, revealed several novel signalling pathways (Dpp, Wg, Hh, Src, Ras, Lipid-PI3K) 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)) signalling 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 signalling (Li et al., 1995), so may genetically interact with Lgl, by affecting Dpp signalling. However, since Dpp positively regulates wing tissue growth (Brumby and Richardson, 2005), and Lgl depletion would be expected to decrease Dpp signalling, this is unlikely to directly account for the wing size increase. Furthermore, knockdown of Pka-C1 should lead to increased Dpp signalling, as well as Hedgehog and Wingless signalling in the wing epithelium (Li et al., 1995) and therefore it is difficult to understand how Pka-C1 leads to a reduction in en>lgli 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 signalling in the wing leading to the
reduced tissue growth of en>lgli 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) signalling 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 signalling reporter (Tyler and Baker, 2007; Zeng et al., 2000), we found that Wg signalling was upregulated in lgl27S3 mutant clones relative to the surrounding wildtype cells in larval eye discs (Supporting Information Figure S4). Consistent with Lgl modulating Wg signalling, two cell polarity interactor genes are implicated in Wg signalling (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 signalling in Drosophila (Swarup et al., 2015), revealed Dco and three other kinases (VRK (Class 3.3), HIPK (Class 3.3) and Eip63E (Class 3.1)), as well as five phosphatases (Wun2, PP2c1, PpV, Wdb (all Class 3.3), PP2C (Class 3.1)) (Supporting Information Table S6, S7; Figure 5). These genes therefore might regulate Wg signalling 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 signalling pathway and Lgl/aPKC in Drosophila embryo epithelial morphogenesis (Dollar et al., 2005; Kaplan et al., 2011; Kaplan et al., 2009; Kaplan and Tolwinski, 2010) and in Xenopus (Choi and Sokol, 2009). Thus, cell polarity regulation of the Wg signalling might be important in coordinating epithelial structure and organ growth. However, it should also be noted that the Wingless signalling 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 signalling pathways associated with the cell polarity-tissue growth interactor genes, were Hh (dco, HIPK, Pka-C1), Src (Btk29A), Ras (dco, PpV, Eip63E, VRK, Pka-C1), and Lipid-PI3K (wdh, CG9784 (IPP), wun2 (Supporting Information Table S6, S7; Figure 5). With the cell polarity tissue growth interactors that are associated with Hh signalling, dco (Class 3.1) (Shi et al., 2014)), HIPK (Class 3.3) (Swarup and Verheyen, 2011) and Pka-C1 (Class 3.3) (Kiger and O'Shea, 2001), two of these genes are also regulators of the Hippo pathway, and indeed Hh signalling has been linked to Hippo pathway regulation (Kagey et al., 2012). Thus, the link between these cell polarity tissue growth interactors and the Hh pathway may ultimately affect the Hippo pathway in the modulation of Lgl, aPKC or Crb phenotypes. Likewise, the link between the cell polarity tissue growth interactors and the Src and Ras pathways, might be also related to Hippo signalling (Enomoto and Igaki, 2013; Reddy and Irvine, 2013), as detailed below.

Brk29A (Class 3.1) is regulated by Src signalling in tissue growth (Read et al., 2004), and interestingly Src signalling has previously been shown to interact with Lgl mutant cell extrusion and invasion phenotypes in the wing epithelium (Ma et al., 2013). Recent studies have revealed that overexpression of Src regulates tissue growth via JNK-dependent repression of the Hippo pathway (Enomoto and Igaki, 2013), and thus the suppression of the en>lgli wing overgrowth by Brk29A might be due to restored Hippo pathway signalling.

Cell polarity tissue growth interactors were also associated with the Ras pathway; dco (Class 3.1),
PpV (Class 3.3), Eip63E (Class 3.1 (Friedman et al., 2011), VRK (Class 3.3) (Ashton-Beaucage et al., 2014), Pka-C1 (Class 3.3) (Zeng et al., 2010). Since elevated Ras signalling is driver of tissue growth through promoting cell proliferation and inhibiting apoptosis (Brumby and Richardson, 2005), but has been also linked to Hippo pathway impairment (Reddy and Irvine, 2013) these interacting genes by regulating Ras signalling might indirectly affect Hippo signalling to modulate the Lgl, aPKC and Crb phenotypes.

Cell polarity tissue growth interactors were also associated with lipid/PI3K signalling (Supporting Information Table S6, S7): CG9784 (IPP, encoding a Inositol phosphate phosphatase, which are involved in membrane trafficking and lipid signalling, Class 3.1) (De Craene et al., 2017; Erneux et al., 2016; Liu and Bankaitis, 2010; Morrison et al., 2000), wdb (encoding PP2A-B’ subunit, which modulates PI3K-Akt signalling, Class 3.3) (Vereshchagina et al., 2008) and wun2 (encoding a lipid phosphatase involved in glycerolipid metabolism, which is required for septate junction formation in the larval tracheal system, Class 3.3) (Ile et al., 2012).

Of relevance, deregulation of phospholipid metabolism is linked to cell polarity in both mammalian cells and Drosophila (Claret et al., 2014; Shewan et al., 2011). Furthermore, Lgl has been recently shown to bind to PIP2 and PI4P phospholipids, which targets Lgl in Drosophila and mammalian cells to the plasma membrane (Dong et al., 2015). Thus, our data supports the growing body of research that links plasma membrane lipid domains and cell polarity. However, PI3K phosphorylates the phospho-inositol, PIP2, to generate PIP3, which signals via Akt to regulate mTor (mechanistic target of rapomycin) activity in tissue growth control (Yu and Cui, 2016), and mTor signalling has been recently shown to modulate target gene accessibility of the Hippo pathway effector, Yki (Parker and Struhl, 2015). Thus, these lipid/PI3K signalling regulators might also indirectly affect the Hippo pathway to mediate their interactions with the
Lgl, aPKC or Crb phenotypes.

Altogether, our analyses reveal several known and novel signalling pathways linking cell polarity modules to multiple regulatory networks controlling tissue growth. Many of these signalling pathways are also linked to the regulation of Hippo pathway, therefore their interaction with Lgl, aPKC and/or Crb may reflect their modulation of Hippo signalling. However, the precise mode by which these cell polarity tissue growth interacting genes modulate these signalling pathways and in turn how they might be regulated by the polarity regulators requires further investigation.

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

Of particular interest, our cell polarity screens identified two members of the AMP-related kinase family, *Salt Inducible Kinase 3* (*dSIK3*) (Class 3.4), and *dSIK2* (Class 3.3) that have important roles in nutrient-dependent signalling (Choi et al., 2011; Choi et al., 2015; Wang et al., 2011), but had not previously been connected to cell polarity regulation. *dSIK3* was identified in our screen as a negative regulator of tissue growth affecting aPKC, Crb and Lgl phenotypes (Supporting Information Tables S4, S6, S7). Although depletion of *dSIK3* alone did not noticeably affect adult eye morphology or size (Figure 6A), *dSIK3* knockdown caused glassiness and bulging of retinal tissue in adult eyes in the *crb* intra or *aPKCC* A background (Figure 6B & C respectively, compared with Figure 1C & D). Wing size was unaltered following depletion of *dSIK3* alone in the posterior wing compartment via RNAi (*en–GAL4* driven *dSIK3i*), expression of a kinase dead transgene (*dSIK3*K70M) or reduced SIK3 gene dosage using *dSIK3* heterozygotes (null allele *SIK3* 72/+). However, reduced SIK3 activity, in combination with *lgl* depletion, resulted in a significant increase in wing size (compare Figure 6D &E; 6F & G; 6H & I, quantified in J). Thus, *dSIK3* modulates activity of the cell polarity complexes and growth
pathways to restrict tissue growth.

Intriguingly, our data support a requirement for \textit{dSIK2} activity in promoting normal and \textit{lgl}-dependent wing growth (Supporting Information, Tables S4, S6, S7), while reduced \textit{dSIK3} activity increased wing growth in the Lgl loss-of-function background. In \textit{Drosophila}, Salt Inducible kinases have been implicated in tissue growth via Hippo pathway signalling (Wehr \textit{et al.} 2012). 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 \textit{et al.}, 2013). Furthermore, co-depletion of \textit{dSIK2} and \textit{dSIK3} reduced tumor growth in a \textit{Drosophila} tumor model (activated \textit{Src + Ras}^{V12}) (Hirabayashi and Cagan, 2015). Given the observation that \textit{dSIK2} and \textit{dSIK3} differentially modify Lgl-dependent tissue growth, but are both required for Hippo pathway inactivation and \textit{Src + Ras}^{V12}-driven tumor growth, future studies are required to determine the how SIKs interact with Lgl to control wing growth.

\textbf{Concluding remarks}

Genetic screens in \textit{Drosophila} remain a powerful tool for identifying and unravelling gene function in specific signalling pathways and cellular processes. We undertook a boutique genetic screen, specifically using RNAi lines targeting kinases and phosphatases, to identify novel signalling pathways involved in the regulation of epithelial structure with tissue growth. Analysis of the hits that genetically interacted with \textit{lgl}, \textit{aPKC} and/or \textit{crb} cell polarity genes, revealed they were associated with Hippo, Notch, JNK, Dpp, Hh, Wg, Ras, lipid/PI3K and unknown signalling 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 \textit{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 in vivo function of these genes (Guruharsha 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 role of dSIK2 and dSIK3 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.

ACKNOWLEDGEMENTS:
We thank all members of our laboratories for helpful discussions during the course of this study. We thank Flybase for their wealth of information. This work was funded by CASS Foundation Science and Medicine Grant (SM-14-5398) awarded to L.M.P. and National Health and Medical Research Council (NHMRC) Project Grant 628401 awarded to H.E.R and N.G. H.E.R. was supported by a NHMRC fellowship (1020056) and funds from La Trobe University and the La Trobe Institute of Molecular Science.
LITERATURE CITED


TABLES

Table 1. Frequency analysis of GO terms Class 1 - 4 modifiers of \( GMR > crb^{intra} \) and \( GMR > aPKC^{CA} \)

<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>
<th>Fold Enrichment</th>
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<tr>
<td>phosphorous metabolic process</td>
<td>GO:0006793</td>
<td>86.7</td>
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<td>21.1</td>
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<td>1.3-1.6</td>
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<td>11.6</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1.8</td>
</tr>
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</table>

Parsons et al., Table 1
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 analysed). 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 signalling networks.
FIGURES

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Figure 1

A
Control

A'

B
GMR > crb$^{\text{intra}}$

B'

C
GMR > aPKC$^{\text{CA}}$

C'

D

GMR-GAL4, UAS-crb$^{\text{intra}}$ and GMR-GAL4, UAS-aPKC$^{\text{CA}\times\text{WT}}$

UAS-RNAi
(370 genes known or predicted to have a role in protein phosphorylation)

F1 progeny screened for modification of parental adult eye phenotype
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, B and C. White scale bar represents 250µM.

(A, A’) Control (*GMR-GAL4*) adult eyes displaying highly organized, regular, lattice like organization of ommatidia to form the adult retina.

(B, B’) *GMR > crb\textit{intra}* adult eyes are slightly larger and rougher than control eyes.

(C, C’) *GMR > aPKC\textit{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 of cell polarity complexes in the developing eye (*GMR > crb\textit{intra} or GMR > aPKC\textit{CA}*) were crossed to males carrying *UAS-RNAi* transgenes to deplete proteins with roles in protein phosphorylation. F1 progeny were scored for modification of parental adult eye phenotypes.
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 > crb intra and/or GMR > aPKC CA and if RNAi expression alone via GMR-GAL4 also resulted in an aberrant eye phenotype. Classes of interactors are indicated.

(A, A’ and A’’) Class 1: Modifier genes only interacted with GMR > aPKC CA. (A) Adult eyes expressing UAS-PI3Ki (VDRC 38985) showed no interaction with GMR-GAL4 or (A’) GMR > crb intra but modified (A’’) GMR > aPKC CA to generate a small, glassy eye with decreased necrotic areas.

(B, B’ and B’’) Class 2: Modifier genes only interacted with GMR > crb intra. (B) Adult eyes expressing UAS-PhK γi (VDRC 33054) showed no interaction with GMR-GAL4 or (B’’) GMR > aPKC CA but modified (B’) GMR > crb intra to generate a larger eye with slight ruffling at the posterior edge (arrowhead).

(C, C’ and C’’) Class 3: Modifier genes interacted with both GMR > crb intra and GMR > aPKC CA. (C) Adult eyes expressing UAS-CASKi (VDRC 104793, 34184) showed a normal phenotype with GMR – GAL4 alone but modified (C’) GMR > crb intra to generate slightly larger eyes and (C’’) GMR > aPKC CA to reduce necrosis.

(D, D’ and 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 > crb intra to produce elongated rough adult eyes and (D’’) modified GMR > aPKC CA to generate small, glassy eyes with reduced areas of necrosis.

(E) Venn diagram depicting the number of genes in each Class. Green shading denotes Class 1, GMR > aPKC CA. Pink shading represents Class 2, GMR > crb intra. Orange central region (arrowhead) overlapping Class 1 and 2 represents Class 3 genes that modified both GMR > crb intra and GMR > aPKC CA. Grey shading denotes Class 4, genes that interacted with GMR >
crb\textsuperscript{intra} and GMR > aPKC\textsuperscript{CA} and also produced an aberrant eye phenotype when expressed alone via GMR-GAL4.
A

en-GAL4, UAS-GFP;
UAS-Igign{51249}

×

UAS-RNai
(GMR > aPKC{CA} and/or
GMR > crb{intra} modifiers)

F1 progeny screened for modification of parental
wing phenotype

B

Control

C

overlay B/C

D

E

Wing area (Pixels)

1800000

1600000

1400000

1200000

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Figure 3

41
Figure 3. Depletion of lgl in the developing *Drosophila* wing increases adult wing size

(A) Genetic scheme of F1 cell polarity modifier screen. Virgin females expressing RNAi depletion of Lgl (*en > lgli*) were crossed to males carrying *UAS-RNAi* transgenes corresponding to the 185 genes identified in the primary screen. F1 progeny were scored for modification of parental adult wing size. Black scale bar represents 500µM.

(B, C, D) Adult female wings, anterior up, proximal to the left.

(B) Control adult wing (C) adult wing *en > UAS-GFP; UAS-lgl-RNAi* (VDRC 51249) displayed increased growth (D) overlay of (B) and (C) highlighting *en > lgli* overgrowth (grey).

(E) Quantification of total wing area in control (*en > GFP, RFP*) animals compared to *en > lgli*.

Results represent individual wings ± standard deviation (SD). ****p < 0.0001.
Figure 4. Modifier genes of en > lgli adult wing size

(A) Venn diagram depicting the number of modifier genes in Classes 1 - 4 from the adult eye screen (Total 185) and overlap between modifier genes identified in en > lgli wing size Class 3 (Total 42). Central yellow shading denotes en > lgli wing size Class 3 modifiers. Green shading denotes eye Class 1, GMR > aPKCCA. Pink shading denotes eye Class 2, GMR > crb intra. Purple denotes eye Class 3 genes that modified GMR > crb intra and GMR > aPKCCA. Grey shading denotes eye Class 4, genes that interacted with GMR > crb intra and GMR > aPKCCA but also show a phenotype when expressed alone via GMR-GAL4. The overlap with en > lgli (yellow) indicates the number of genes from each eye Class that also modified en > lgli wing size Class 3.

(B - E) Adult female wings, anterior up, proximal to the left.

(B) en > lgli specific modifier gene: en > GFP; CG9784i (VDRC 30098) wings show no change in wing size (C) en > lgli/CG9784i have decreased wing growth and holes.

(D) en > lgli modifier gene: en > GFP; PpVi (VDRC 101997) wings are slightly smaller than the control (less than 5%) yet significantly impacted (E) en > lgli adult wing growth.

(F) Quantification of total wing area (A-D) in control animals compared to en > GFP and en > lgli. Results represent individual wings ± standard deviation (SD). ****p < 0.0001.
Figure 5. Summary of cell polarity tissue growth interactors and the signalling pathways they are associated with.

Genes from the wing size Class 3 that modified \( en > lgli \), greater than \( en-GAL4 \) alone (Classes 3.1, 3.3, 3.4) are listed under the signalling pathways that they are associated with. Genes highlighted in blue or yellow were identified as kinases or phosphatase regulators of Wnt signalling respectively (Swarup et al., 2015).
Figure 6

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Figure 6. SIK3 genetically interacts with cell polarity complexes to negatively regulate organ size

(A - C) Adult female eyes, posterior is to the left. (D - I) Adult female wings, anterior up, proximal to the left.

(A) Expression of *UAS-SIK3-RNAi* (independent RNAi TRiP.JF03002) with *GMR-GAL4* in the developing eye has no effect on adult eye morphology. (B) Expression of *UAS-SIK3 RNAi* in conjunction with *GMR > crb^intra* or (C) *GMR > aPKC^Ca* @18°C increases adult eye size.

(D) Reduction of SIK3 activity by RNAi depletion *en > GFP; UAS-SIK3-RNAi* (F) overexpression of kinase dead transgene *en > GFP; UAS-SIK3^K70M* or (H) null allele *en > GFP/SIK3^72* has no effect on adult wing size (< 1%). (E) In conjunction with reduced lgl activity (*en > lgl*) decreased SIK3 activity by RNAi depletion *UAS-SIK3-RNAi* (G) overexpression of kinase dead transgene (*UAS-SIK3^K70M*) or (I) null allele SIK3^72* significantly increased wing size.

(J) Quantification of total wing area (D - I). Results represent individual wings ± SD. ****p < 0.0001.