Epithelial polarity and growth signaling by *Drosophila* tumor suppressor genes

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Epithelial polarity and growth signaling by *Drosophila* tumor suppressor genes

Epitheliale polariteit en groei signalering door *Drosophila* tumor suppressor genen

(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht op gezag van de rector magnificus, prof.dr. H.R.B.M. Kummeling, ingevolge het besluit van het college voor promoties in het openbaar te verdedigen op woensdag 3 oktober 2018 des middags te 2.30 uur

door

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geboren op 27 december 1984
te Naaldwijk

Promotoren: Prof.dr. S.J.L. van den Heuvel

Prof.dr. D. Bilder

"Time flies like an arrow,
fruit flies like a banana"

-every Drosophilist ever

"Mmm... Hrmmmmm...
Whatever can be done?"

-Siegmeyer of Catarina

Table of contents

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CHAPTER 1:

General introduction

Geert de Vreede[1]

Epithelia

Epithelial tissues are one of the fundamental building blocks of multicellular animals, forming most of our major bodily organs. When the first primitive metazoan evolved from single-celled eukaryotes, its defining features were the formation of these epithelial cell layers and distinguishing the interior from the exterior of the animal. Although epithelia gained a multitude of specialized functions throughout the course of evolution, the core tissue architecture has been conserved accross animal species. Comparing for instance the basic epithelial cell layers of Cnidarians to the various types of epithelia in humans, many properties like the cell polarity, cellular junctions and basement membranes show high similarity. Understanding how epithelial cells are organized and what cellular signaling pathways control their shape, function and homeostasis is essential for understanding development and pathogenesis.

Form dictates function

The main role that epithelia play within an organism is forming the interface between different environments, such as the boundary to the outside world or the separation between distinct tissues. The specific interface that an epithelium lines determines its function, which means that a high degree of specialization is seen in these tissues (Alberts et al., 2014; Betts et al., 2016). Epithelial cells come in three particular shapes: squamous, cuboidal and columnar. Each of these shapes can organize in simple single cell monolayers or in stratified multilayered configurations (**Fig.1**). The different functions that epithelia perform are reflected in the forms and shapes of these configurations. For instance, the simple single

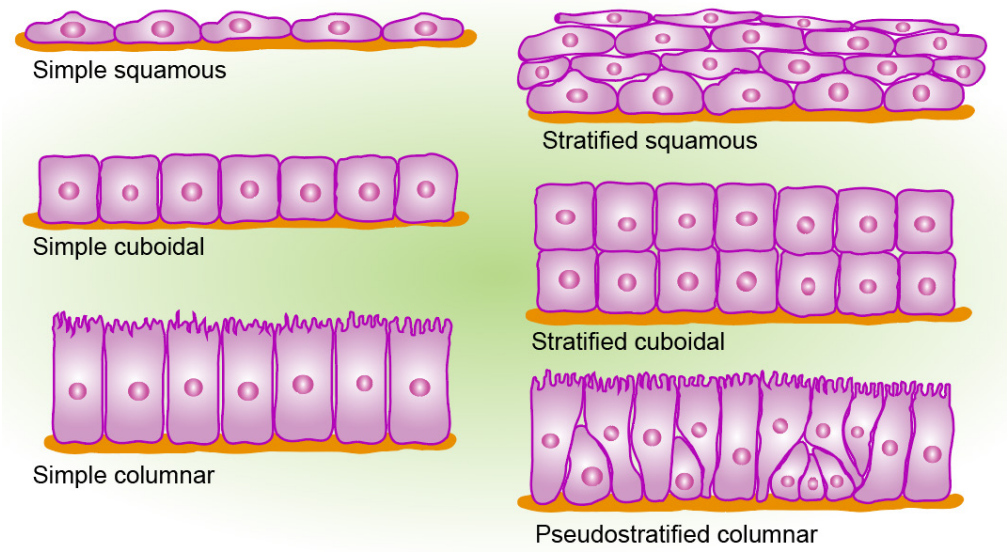

Figure 1: Types of epithelium. The different types of epithelial organization that can be found in organisms. The shape and layering of epithelia reflect the diverse functions that those tissues perform.

layered squamous epithelium is seen lining the alveoli in the lungs, where it facilitates efficient diffusion of gasses. In contrast, cuboidal cells are often seen in glands that are optimized for secretion. The columnar epithelial cells, which are ciliated in a number of cases, can absorb and secrete molecules, most obviously exemplified by the cells lining the digestive tract. Cilia protrude into the luminal space and can create a directional flow of the extracellular medium by pulsing movement. Multilayered, stratified epithelia are often observed where the interaction with the outer environment is especially abrasive. This generates protection from physical insults, but also dehydration, as is the case for the skin epithelium. When a higher degree of flexibility is required from the epithelium, cells modulate their shape depending on physical pressures that are exerted. This is a quality of transitional epithelia, which can be found in the bladder, ureter and urethra, where fluctuating fluid pressure dictates the cell shape. All these epithelial types indicate the diversification and specializations that this tissue has undergone, adapting to the conditions and demands of different organs. However, while there are many variations, epithelia share a common structure and organization, which is especially evident in their polarity.

Epithelial polarity

Epithelial cell membranes are divided in separate functional domains, which all have distinct properties (Macara et al., 2014; Wang et al., 2012). The cells have an apical, lateral and basal side. In general, the apical side is interacting with an outside environment, facing a lumen or the exterior of the animal. This side can be ciliated in certain epithelial cells, sensing and affecting the extracellular space, and absorb or secrete specific molecules, which requires very strict regulation of this domain. Cellular junctions on the lateral sides separate the apical and basal domains and tightly hold epithelial cells together. This forms a barrier to prevent harmful molecules and pathogens passing through the epithelium. The junctions determine adhesive characteristics of cells, and can relay information about physical forces acting on and within the tissue. The basal side of the epithelium faces the basement membrane, which forms a foothold for the epithelium. The basement membrane is formed by the secretion of extracellular matrix components, giving epithelial cells stability and signaling feedback from underlying connective tissue. Epithelial polarity at the cellular level implies tight regulation at the molecular level. Specific proteins and even plasma membrane lipids show asymmetry that needs to be established and maintained, through polarized trafficking, mutual exclusion and restrictive boundaries. The search for molecular master regulators of cell polarity led to the discovery of three major conserved protein modules controlling this process: the apical PAR and Crumbs complexes and the basolateral Scrib module (**Fig.2**). While each of these modules has its own domain and downstream signaling effectors within the cell, the crosstalk between the different units is equally essential for polarity regulation.

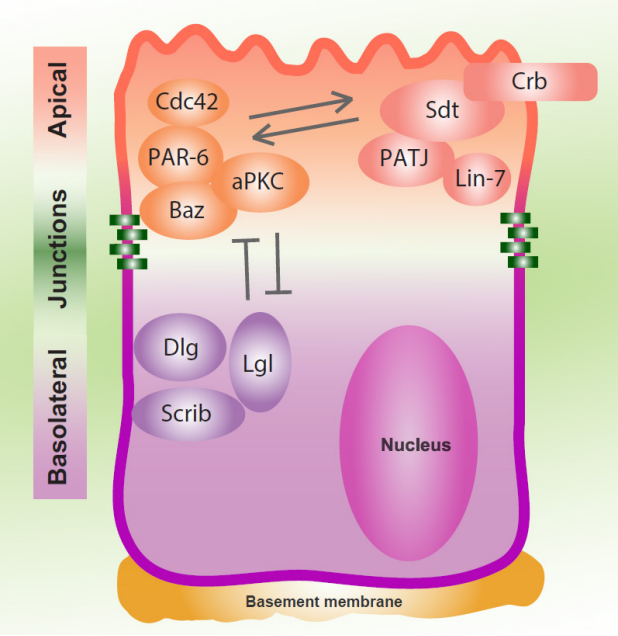

Figure 2: Polarity modules.
The three major polarity-regulating modules in epithelial cells: the apical PAR and Crumbs complexes and the basolateral Scrib module. The members of each module are depicted here, as well as the general interactions between the modules.

Regulation of epithelial polarity: the PAR complex

The major components comprising the PAR complex are PAR-3 (Bazooka in *Drosophila*), PAR-6 and atypical protein kinase C (aPKC). The original identification of the PAR genes happened in a *C. elegans* screen for early embryo mutants that fail to divide asymmetrically (Kemphues et al., 1988). These partitioning-defective mutants failed to distribute cellular determinants that are strongly polarized in wild-type zygotes, resulting in symmetric cell division. Both PAR-3 and PAR-6 proteins are localized to the anterior side of the embryo right before asymmetric division occurs (Etemad-Moghadam et al., 1995; Watts et al., 1996). aPKC (PKC-3 in worms) was discovered later to share this localization and mutant phenotype and subsequent physical interactions found between these three proteins tied them together as a complex (Lin et al., 2000; Tabuse et al., 1998). The importance of the PAR complex components is illustrated by the fact that their function is conserved across species, since polarity in multiple mammalian and *Drosophila* tissues has been shown to be dependent on them (Joberty et al., 2000; Kuchinke et al., 1998; Lin et al., 2000; Schober et al., 1999). This sparked the search for how the PAR complex regulates polarity on a molecular level. The key to this regulation is that the complex acts as a protein scaffold through its multiple interaction domains. Both PAR-3/Bazooka and PAR-6 contain PDZ (PSD-95, Discs large, ZO-1) domains, which facilitate protein-protein interactions (Etemad-Moghadam et al., 1995; Hung

and Kemphues, 1999). Not only do PDZ domains facilitate the interaction between these two PAR proteins, they also connect to other polarity regulators, like Crumbs complex proteins, and even to phosphatidylinositol lipid membranes to interact with polarized phosphoinositide signaling (Hurd et al., 2003; Wu et al., 2007). PAR-3/Bazooka also connects to junctional cell adhesion molecules like Nectin and JAM through its first PDZ domain, providing a mechanism for PAR-complex recruitments to the cell junctions (Ebnet et al., 2001; Itoh et al., 2001). PAR-6 and aPKC associate with their shared PB1 domain (Lin et al., 2000). In epithelial cells the phosphorylation of PAR-3/Bazooka is essential to define the apical/lateral border, for it prevents the recruitment of adherens junction components apically (Krahn et al., 2010a; Morais-de-Sá et al., 2010; Walther and Pichaud, 2010). This phosphorylation within the PAR-complex is not the only target of aPKC, for it has a multitude of polarity regulating substrates. This gives aPKC kinase activity a central role in controlling polarity, either stimulating the formation of apical protein complexes, or excluding basolateral components.

A key regulator of PAR complex activity is the Rho GTPase Cdc42, which is involved in cell division, migration, endocytosis as well as cell polarity. The activity of Cdc42 is seen as a crucial factor for polarity establishment in both *C. elegans* and *Drosophila* embryos. Both dominant-negative and dominant-active forms of Cdc42 disrupt epithelial polarity, and apical localization of PAR-6 is Cdc42-dependent (Hutterer et al., 2004). Cdc42-GTP association with PAR-6 activates aPKC which in turn phosphorylates PAR-3/Bazooka, promoting its apical exclusion (Gotta et al., 2001; Joberty et al., 2000; Nagai-Tamai et al., 2002; Qiu et al., 2000). This conservation of Cdc42 function throughout species highlights its importance for proper PAR complex function in polarity signaling.

The Crumbs complex

The members of the Crumbs polarity complex were all originally discovered in *Drosophila*. The eponymous transmembrane protein Crumbs (Crb) was the first to be discovered, showing severe epithelial defects when it is mutated in embryos (Jürgens et al., 1984; Tepass et al., 1990). The mutant embryonic epidermis secretes only crumbs of cuticle rather than a continuous sheet, explaining the name of the gene. Crb is an apically localized, type I transmembrane protein, containing a large extracellular domain (ECD) and a relatively small (37aa) cytoplasmic tail. The Crumbs ECD contains 29 epidermal growth factor (EGF)-like repeats, as well as four laminin-A globular domain-like repeats. The intracellular part consists of two highly conserved domains: a FERM-binding domain and a PDZ domain containing a C-terminal ERLI motif (Klebes and Knust, 2000). While this cytoplasmic tail is short, it is essential for polarity regulation by Crumbs. The tail has been shown to bind both PAR complex components aPKC and Par6 (Kempkens et al., 2006; Sotillos et al., 2004), as well as another core Crb complex component PALS1/Stardust (Bachmann et al., 2001). PALS1/Stardust acts as a central scaffold protein of the MAGUK family, containing L27, PDZ, SH3 and GUK domains. Loss of Sdt in *Drosophila* phenocopies loss of Crb (Bachmann et al., 2001). PALS1/Stardust is connected to the PAR complex through Baz (Krahn et al., 2010b), and directly binds to the remaining core components of the Crb complex: PATJ (previously described as Discs lost) and Lin-7 (Bachmann, 2004; Bhat et al., 1999; Roh et al., 2002). All four Crb complex members colocalize at the apical membrane

and co-immunoprecipitate, and show conservation throughout animal species. While its interaction with the PAR complex is a key part of its function, the Crb complex also ties to other cellular processes. One of those processes is actin cytoskeleton regulation, through FERM domain containing proteins Moesin and Yurt. Both of these proteins co-immunoprecipitate with Crb, and display Crb-dependent phenotypes (Laprise et al., 2006; Médina et al., 2002). Another interesting aspect connected to the Crb complex is growth regulation. Crb is implicated in affecting growth through the Hippo signaling pathway. This pathway has a multitude of inputs, but converges on the activity of Yorkie (YAP/TAZ in humans), which drives growth when active in the nucleus (Huang et al., 2005). Crb inhibits Yorkie growth signaling through its interaction with Expanded, another FERM domain protein (Ling et al., 2010; Robinson et al., 2010). This is seen as an important link between polarity regulation and tumor suppression.

The Scrib module

The basolateral Scribble module is made up of Discs Large (Dlg), Lethal Giant Larvae (Lgl) and Scribble (Scrib) (Bilder and Perrimon, 2000; Schneiderman and Gateff, 1967; Stewart et al., 1972). All of the Scrib components were originally characterized in *Drosophila*. Dlg and Lgl were found screening for giant larvae, a phenotype tied to epithelial disruption and tumorous overgrowth, while Scrib emerged out of a screen for disrupted embryonic epithelia. Each of the Scrib module members are thought to act as molecular scaffolds, as they all contain multiple protein-protein interaction domains. Dlg, like Sdt, is another member of the MAGUK family, containing PDZ, SH3, HOOK and GUK domains (Hough et al., 1997). Lgl has multiple WD repeats and phosphorylation sites that are necessary for proper function (Katoh and Katoh, 2004; Plant et al., 2003), and Scrib is a LAP family protein, which contain leucine rich repeats and PDZ domains (Bilder and Perrimon, 2000). All Scrib module members show strong conservation throughout metazoans and can be found in various tissues types. The particular protein domains are conserved, as well as localization of Scrib module components at the basolateral side of cells. Remarkably, direct physical interactions between these proteins have not been found, although they show colocalization, are dependent on each other to localize correctly, and share mutant phenotypes (Bilder et al., 2000). While some proteins have been found to directly associate with Scrib module members, the downstream effectors which mechanistically control polarity remain elusive. A possible connection lies in the control of cellular trafficking, since disrupted endocytosis in *Drosophila* show strong phenotypical resemblance to Scrib module mutants (Lu and Bilder, 2005; Vaccari and Bilder, 2005). Interestingly, the Scrib module also ties to Hippo signaling, since Scrib mutant tissue overgrowth is driven by Yki activity (Menendez et al., 2010; Sun and Irvine, 2011).

Interactions between complexes

The PAR, Crumbs and Scrib polarity regulators interact with each other as well as with their downstream effectors. The mutual antagonism seen between the apical and basal modules is the most straightforward example of these interactions. Mutual antagonism entails that the polarity complexes control and define their respective domains by excluding the components of the opposite side.

One direct antagonistic mechanism has been described in the molecular interactions between aPKC and Lgl. As mentioned before, the kinase activity of aPKC plays an important role in PAR polarity regulation, and Lgl is one of the target substrates of aPKC (Betschinger et al., 2003; Plant et al., 2003; Yamanaka et al., 2003). Lgl binding detracts aPKC from its apical substrates, while the phosphorylation of Lgl by aPKC triggers dissociation of Lgl from the membrane towards the cytoplasm, shutting down its inhibitory effect on the PAR complex components (Atwood and Prehoda, 2009; Bailey and Prehoda, 2015; Dong et al., 2015; Yamanaka, 2006). This direct kinase-substrate interaction drives the mutual inhibition between aPKC and Lgl. In addition, Lgl can bind to the PDZ domain of PAR-6, competing with PAR-3/ Bazooka thus inhibiting the formation of this apical complex (Wirtz-Peitz et al., 2008; Yamanaka et al., 2003). The presence of active Cdc42 at the apical membrane enhances aPKC phosphorylation activity towards Lgl, therefore strengthening the inhibition of this basolateral component on the apical side of the cell (Hutterer et al., 2004). This mutual exclusion by aPKC and Lgl is a strong driver of polarity maintenance. The PAR and Crumbs complex proteins also physically interact, albeit in a more cooperative fashion. aPKC has been reported to phosphorylate Crb directly, which is necessary for Crb function (Sotillos et al., 2004). In addition, the PDZ domain of PAR-6 binds to Crb as well as Stardust (Hurd et al., 2003; Kempkens et al., 2006; Lemmers et al., 2004), which is essential for apical exclusion of PAR-3/Bazooka to maintain a proper apical/lateral border (Harris and Peifer, 2005; Morais-de-Sá et al., 2010). These physical interactions between the apical polarity regulating modules are emphasized by their codependence, which means that the loss of function of one complex will cause mislocalization and functional disruption of the other (Harris and Peifer, 2005; Nam, 2003). No direct interactions between the Crb and Scrib regulators have been found, though a clear functional relation is evident from the fact that a loss of Crb complex can be mitigated by a loss of Scribble components (Bilder et al., 2002; Tanentzapf and Tepass, 2003).

Epithelial polarity and cancer

Pathologists have noted for centuries that there is a clear histological correlation between malignant tissue and epithelial disorganization, highlighting loss of polarity as one of the hallmarks of carcinoma. This is emphasized by the list of core PAR, Crumbs and Scrib module components being found aberrantly expressed or mislocalized in human tumor tissue (Halaoui and McCaffrey, 2015; Khursheed and Bashyam, 2014; Martin-Belmonte and Perez-Moreno, 2012; Saito et al., 2018). In line with findings designating them as tumor suppressors in *Drosophila*, the changes seen in the basolateral regulators are often a loss of expression or protein mislocalization (Gardiol et al., 2006; Nakagawa et al., 2004; Navarro et al., 2005; Schimanski et al., 2005). A similar conservation of function is seen regarding the PAR complex components where tumors in breast and liver cancer display overexpression of aPKC and PAR6 (Cunliffe et al., 2012; Kojima et al., 2008; Nolan et al., 2008; Tsai et al., 2000), and a loss of mammalian PAR3 in skin and esophageal cancer corresponds with its role in stabilizing junctions (Iden et al., 2012; Zen et al., 2009). For Crumbs however, overexpression of the membrane-tethered tail induces expansion of the apical domain and overproliferation in flies, (Lu and Bilder, 2005; Wodarz et al., 1995), while in vertebrates the loss of function has been associated with tumorigenesis (Karp et al., 2008; Storrs and Silverstein, 2007). This exemplifies that

findings in non-mammalian systems cannot always be directly extrapolated. The major polarity complexes are tied to numerous types of cancers, although the underlying molecular events that trigger the alteration of polarity regulation are poorly understood. Hints for this change could lie in the process of epithelial to mesenchymal transition (EMT). Cells becoming more malignant are undergoing EMT, and the dowregulation of polarity genes is a key step in the EMT process (Moreno-Bueno et al., 2008). While it is an attractive simplification, the straightforward attenuation of core polarity regulators does not account for all the intricacies of carcinogenesis. Primary tumors do display obvious mispolarization phenotypes, but also the later stages of cancerous progression like migration and invasion are tied to polarity regulation. For instance, migrating cells depend heavily on the actions of aPKC and Par6 at the leading edge, in concert with Cdc42 GTPase activity (Etienne-Manneville and Hall, 2001; Gérard et al., 2007). The Scrib complex component Dlg is also involved in controlling polarity of certain migrating cell types, and in this context aPKC and Par6 are needed to facilitate its specific localization (Etienne-Manneville et al., 2005). This implies that cancer cells have intricate modulation of polarity control depending on the state of malignancy and the type of cancer. It is therefore essential to understand more about the basic mechanisms of polarity signaling and how malignant cells hijack those mechanisms.

Fly tumor suppressors

The pioneering investigations into *Drosophila* tumor suppressor genes (TSGs) happened in the late 60s, early 70s, when several groups reported the identification of homozygous mutants that developed benign or malignant overgrowths in various larval tissues (Bryant and Schubiger, 1971; Gateff, 1978; Schneiderman and Gateff, 1967). These tissues included the central nervous system, blood cells and the imaginal epithelia.The mutants were divided in two distinct categories: neoplastic and hyperplastic (Bryant and Schmidt, 1990; Gateff, 1982). Neoplastic tumors have strongly altered tissue organization, fail to differentiate, display continuous proliferation and even invasiveness. Hyperplastic mutants, while still overgrown, show a more 'benign' character, retaining epithelial tissue polarity and the capacity to differentiate. Intriguingly, these different tumor characteristics reflect those seen in human tumors. It is important to note that fly tumors have a number of differences from true human tumors. The biggest difference compared to human cancer is that these *Drosophila* tumors can arise from mutations in single genes. Human malignancies arise mainly from cells carrying multiple genetic lesions, although there are a number of notable single-gene exceptions (Gutmann et al., 2017; Moline and Eng, 2011; Sparagana and Roach, 2000). This highlights an important distinction between human and fly tumors, though it also emphasizes opportunity, as *Drosophila* can help overcome the complexity involved with modeling the process of cancer. Below I will describe the known *Drosophila* TSGs that are relevant for this thesis, and the underlying signaling that links these genes and human tumors.

Scribble, Discs Large and Lethal Giant Larvae

As described before, the Scrib module mutants were discovered in screens for giant larvae and epithelial disruption. *Discs large* and *lethal giant larvae* were among the first TSGs to be characterized, (Schneiderman and Gateff, 1967; Stewart et al., 1972). In these initial studies, the mutants were easily recognized by their 'giant larvae' phenotype, where larva do not pupate after the L3 stage as in wild-type, but keep growing, bloat and eventually die. The imaginal discs and nervous tissue in these larvae show dramatic neoplastic overgrowth and loss of polarity. In addition, taking the tumors out of the animals and transplanting them into wild-type hosts showed that the mutant tissue keeps proliferating and can sometimes invade surrounding tissue (Woodhouse et al., 1994, 1998). Mutants for *scrib*, which was discovered later, share these phenotypes (Bilder and Perrimon, 2000; Bilder et al., 2000). The fact that these overlapping phenotypes could be observed in the larva is due to the strong maternal contributions allowing the mutants to develop into that stage. When removing this maternal product, Scrib module mutants die due to aberrant embryonic development. The embryonic epidermis of *scrib*, *dlg* and *lgl* embryos shows polarity defects like mislocalization of apical determinants in the basolateral domain and disruption of adherens junctions (Bilder et al., 2000). The epithelial overgrowth seen in Scrib module mutants highlights the link between the loss of polarity and over-proliferation. It is still an open question why cells aberrantly activate proliferation pathways upon losing polarity. An interesting explanation comes from the striking parallels between tumor growth and wound healing. The epithelial disruption caused by wounding requires immediate changes of cell polarity and growth. Wounds, as well as tumors, activate of stress signaling, immune cell recruitment, de-differentiation and the stimulation of proliferation by cytokines (Lee and Miura, 2014; Schäfer and Werner, 2008). In this sense tumors can be regarded as 'wounds that never heals'. Intriguingly, analysis of the transcriptional profile of *Drosophila* polarity mutant tumors also shows this overlap with wound healing processes (Bunker et al., 2015). Whether these wound healing factors have molecular ties to Scrib module components remains to be investigated.

Endocytic trafficking regulators

In *Drosophila*, endocytic trafficking pathway mutant tissues phenocopy Scrib module mutants in many ways, showing a loss of polarity and neoplastic overgrowth (Shivas et al., 2010). This similarity between mutant phenotypes suggests that endocytic trafficking and polarity regulation are linked processes, although the exact connection is unknown. Endocytic trafficking is part of the core cellular machinery and essential for viability, explaining the absence of endocytic mutants in the classical larval zygotic tumor suppressor screens. The advantages of the *Drosophila* model system were a key factor in the discovery of endocytic mutants involved in tumorigenesis. Using the mitotic recombination techniques described below allowed for generating mutant tissue in a heterozygous background, initially revealing *rab5*, *avalanche*, *vps25* and *tsg101* as tumor suppressors (Lu and Bilder, 2005; Moberg et al., 2005; Thompson et al., 2005; Vaccari and Bilder, 2005). In the following years this set of endocytic regulators was expanded with AP-2 subunits, Clathrin heavy chain, the Rab5 effector Rabenosyn, Sec1/Munc18-family protein Vps45, and a large set of components belonging to the

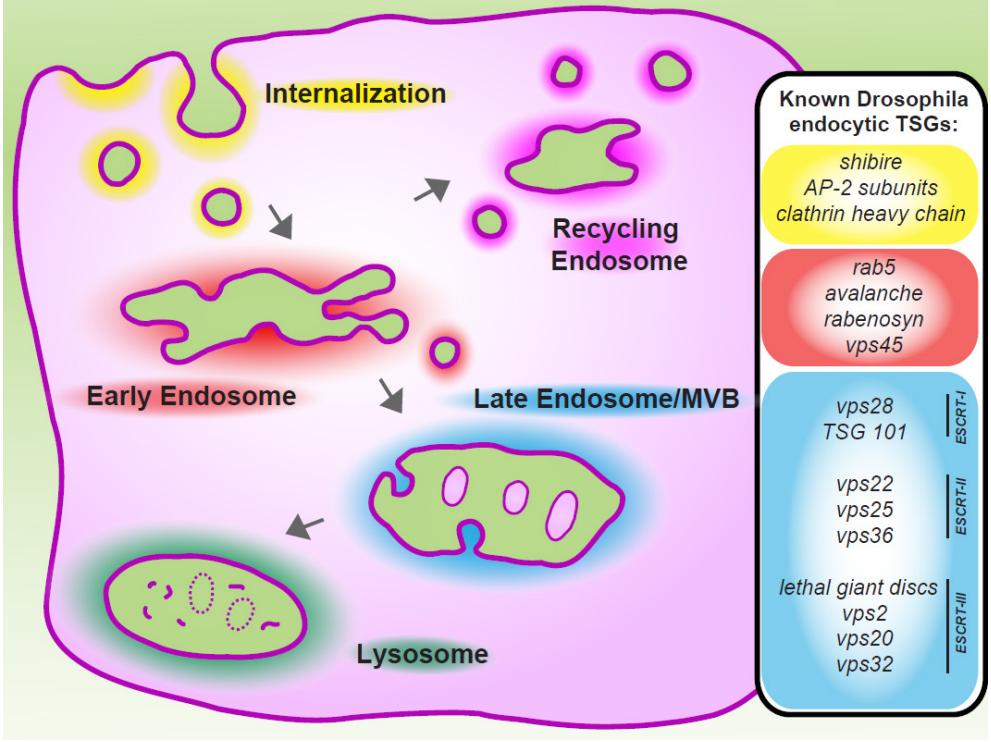

Figure 3: Endocytic sorting & fly TSGs. The canonical endocytic trafficking pathway, from initial internalization to lysosomal degradation. The known *Drosophila* endocytic TSGs are listed with their corresponding membrane trafficking process.

ESCRT (endosomal sorting complex required for transport) complexes (Herz et al., 2009; Morrison et al., 2008; Vaccari et al., 2009; Windler and Bilder, 2010). All these TSGs are involved in controlling a subset of compartments in the canonical endocytosis pathway, tying to initial internalization, early endosomal cargo allocation or late endosome/MVB sorting (**Fig.3**). Strikingly, no components regulating other endocytic itineraries like the recycling endosome or lysosomal transport were identified as TSGs. One attractive model as to how disrupted endocytosis leads to tumorigenesis is that these mutants accumulate numerous mitogenic signaling receptors because the cells are not able to facilitate proper turnover. This is supported by the observation that endocytic TSG mutant tissues accumulate receptors for the pro-growth EGF, Dpp and Notch signaling pathways (Childress et al., 2006; Lu and Bilder, 2005; Moberg et al., 2005; Morrison et al., 2008; Thompson et al., 2005; Vaccari et al., 2009; Windler and Bilder, 2010). Another factor that could contribute to the endocytic neoplastic phenotype is the link to polarity regulation. Mistrafficking of polarity proteins can disrupt proper polarity and therefore trigger neoplastic transformation. The transmembrane polarity regulator Crb is a candidate for being involved, since Crb levels and localization have shown to be altered in endocytic TSG mutants (Lu and Bilder, 2005).

Hippo pathway

The Hippo pathway is a major and conserved regulator of growth signaling that, surprisingly, was unknown until about twenty years ago, when it was discovered in *Drosophila*. Much like the endocytic pathway mutants, the generation of mosaic tissues fueled the discovery of components of the Hippo growth regulating kinase cascade. When generating these clones in a tissue that is not required for viability (e.g. the eye), it is easy to score for overgrowth phenotypes, even if these are subtle. Mutants for *hippo*, *warts*, *mats* and *salvador* all share hyper-proliferative phenotypes in a clonal context (Harvey et al., 2003; Jia et al., 2003; Justice et al., 1995; Kango-Singh, 2002; Pantalacci et al., 2003; Tapon et al., 2002; Udan et al., 2003; Wu et al., 2003; Xu et al., 1995). The overgrowths are hyperplastic in nature, as they retain polarity and still form an epithelial monolayer. The key downstream component that drives the proliferation in Hippo pathway mutants is the transcriptional co-activator Yorkie (Yki). Nuclear Yki triggers pro-growth transcription, while phosphorylation by the Warts kinase inhibits Yki activity through cytoplasmic retention that prevents it from entering the nucleus (Huang et al., 2005) (**Fig.4**). Expression of an activated (non-phosphorylatable) form of Yki displays hyperplastic overgrowth, therefore phenocopying the loss of Hippo components (Oh and Irvine, 2008; Ziosi et al., 2010). A number of components upstream

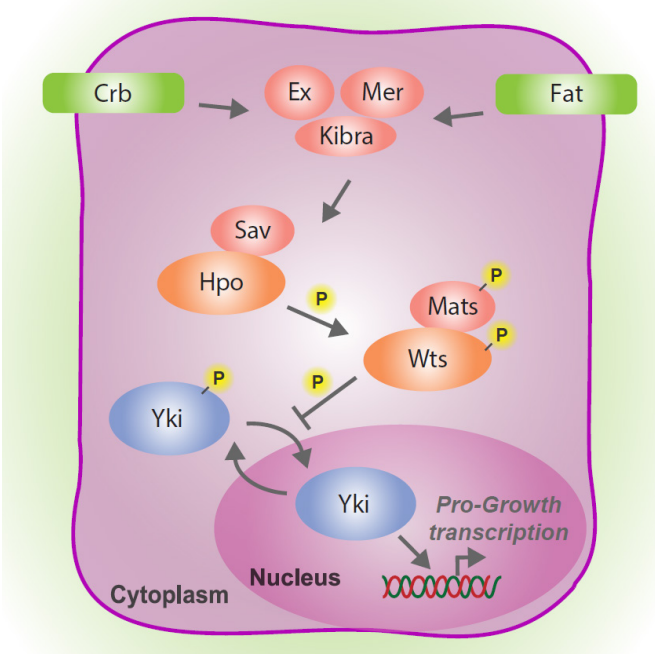

Figure 4: The Hippo pathway in ***Drosophila*****.** The major components of the Hippo growth signaling pathway in *Drosophila* are depicted here. This pathway revolves around the control of transcriptional co-activator Yki, which triggers growth-promoting target gene induction in the nucleus. Through multiple upstream inputs the Wts and Hpo kinases can be activated to inhibit nuclear localization of Yki, down-regulating Yki-dependent growth signaling.

of the core Hippo signaling module also act as TSGs. Mutant clones for the cadherin superfamily member *fat* overgrow and a number of alleles generate hyperplastic imaginal discs (Bryant et al., 1988; Willecke et al., 2006). It is believed that Fat acts through the FERM domain containing protein Expanded (Silva et al., 2006). Expanded and Merlin, another FERM protein, have been connected to upstream Hippo regulation, as double mutant clones highly resemble loss of Hippo function, and overexpression triggers Warts phosphorylation (Hamaratoglu et al., 2006). As previously mentioned, polarity protein Crumbs affects Hippo signaling by exerting its tumor suppressive function through Expanded (Ling et al., 2010; Robinson et al., 2010). In addition, the *Drosophila* TSG *kibra*, which encodes a WW-domain protein, has been associated with this regulation (Genevet et al., 2010; Yu et al., 2010). The plethora of upstream effectors feeding into Yki activity indicates strict control of the Hippo pathway, underlining its importance in normal growth signaling and tumorigenesis.

Signaling in *Drosophila* TSGs and human malignancies

A major factor in the interest in fly TSGs lies in the possibility that we can extrapolate the findings towards human tumors. While using flies for modeling cancer has obvious disadvantages like the lack of adaptive immunity and the open circulatory system, the ease of use, high percentage of gene homology and many available genetic techniques significantly outweigh these cons. A great example of this is the Hippo signaling pathway described above. While it was shown in mice that overexpression of YAP (the mammalian Yki homolog) induced liver overgrowth and hepatocellular carcinoma (Dong et al., 2007), initially the involvement of Hippo signaling in cancer was not believed to be very substantial, since mutations in Hippo pathway components are rare. However, several studies indicate that Hippo signaling is often altered in a multitude of malignancies (Pan, 2010). These alterations include the down-regulation of Hpo ortholog Mst1/2 and Wts ortholog Lats1/2, which leads to pro-growth signaling (Minoo et al., 2007; Seidel et al., 2007; Takahashi et al., 2005). This emphasizes the importance of discoveries in *Drosophila*, drawing attention to pathways that could be implicated in cancer. It is beyond the scope of this chapter to summarize all the contributions that *Drosophila* studies have made towards cancer research, and this has been reviewed in many other works (Christofi and Apidianakis, 2013; Gonzalez, 2013; Rudrapatna et al., 2012; Sonoshita and Cagan, 2017). Here the focus will be more on the signaling pathways in addition to Hippo that underlie the *Drosophila* tumor phenotypes and are also implicated in human malignancies, highlighting those that play a role in the following thesis chapters.

JNK signaling

The c-Jun NH2-terminal kinase (JNK) signaling pathway is one of the major players in *Drosophila* tumorigenesis that is also associated with human cancer. JNK was discovered in the early 90s as the kinase that phosphorylates c-Jun, a transcription factor and proto-oncogene that, along with c-Fos, makes up the Activator Protein (AP-1) family (Hibi et al., 1993). The pathway was delineated as a cascade of MAP kinases that can be triggered by multiple upstream inputs, mainly cell intrinsic stresses like UV radiation

and heat shock, but also extrinsic pro-inflammatory cytokines like Tumor Necrosis Factor α (TNFα) and interleukins (**Fig.5**)(Kyriakis et al., 1994; Rouse et al., 1994; Sluss et al., 1994; Yan et al., 1994). The wide variety of cellular responses that JNK controls includes proliferation, differentiation, migration and apoptosis. A role for JNK signaling in cancer has long been acknowledged, although the complexity of downstream effects have produced conflicting data, supporting both a pro-oncogenic and anti-tumorigenic function depending on context (Tournier, 2013; Wagner and Nebreda, 2009). In multiple knockout mice models, the loss of JNK made the animals more prone to develop several tumor types (Cellurale et al., 2012; Girnius et al., 2018; Hübner et al., 2012; Johnson et al., 1996; She et al., 2002), while others showed that JNK activity was needed to promote oncogenesis (Cellurale et al., 2011; Nielsen et al., 2007; Sakurai et al., 2006; Shibata et al., 2008). The outcome of JNK signaling in these cases seems to be dependent on tissue type and upstream input, though a role for JNK signal strength and duration has also been implied (Ventura et al., 2006). In *Drosophila*, the initial investigations into JNK signaling were primarily into its role in early morphogenesis, where its signaling is required in dorsal closure (Kockel et al., 1997; Riesgo-Escovar et al., 1996). Interestingly, the first connection to tumorigenesis in flies was shown in tumors induced by polarity loss (Brumby and Richardson, 2003; Igaki et al., 2006). In these models, neoplastic tumors are generated through the Ras cooperation strategy, which combines overexpression of an active form of Ras with polarity loss. The growth and invasiveness of these tumors is dependent on JNK signaling. Downstream of JNK activity the overgrowth has been shown to be at least partially driven by Hpo-dependent Yki activation (Menendez et al., 2010; Robinson and Moberg, 2011; Sun and Irvine, 2011), while invasiveness is tied to the activity of JNK target Matrix Metalloprotease 1 (MMP1) (Uhlirova and Bohmann, 2006). The duality of JNK activity also comes forth in this system, since generating polarity deficient clones without Ras expression leads to JNK-dependent elimination of these cells, indicating that JNK can function as an anti-tumor agent. The role that JNK adapts thus looks to be dependent on the social context of tumorigenic cells, where in completely mutant tissues JNK promotes growth, and in genetic mosaics JNK stimulates apoptotic elimination (Brumby and Richardson, 2003; Igaki et al., 2006; Leong et al., 2009; Ohsawa et al., 2011; Wu et al., 2010a).

JAK/STAT signaling

The Janus Kinase/Signal Transducers and Activators of Transcription (JAK/STAT) pathway is a conserved signaling cascade that is activated by cytokines and growth factors, generally promoting proliferation and cell survival. In humans, aberrant activation has been mostly associated with hematological malignancies (Chen et al., 2012; Vainchenker and Constantinescu, 2012), though roles in solid tumors have also emerged (Thomas et al., 2015). In contrast to the multiple paralogs in mammals, the *Drosophila* genome encodes a single JAK and a single STAT that are activated by a single cytokine receptor, simplifying the investigations into the pathway (**Fig.5**)(Luo et al., 1997; Yan et al., 1996). In flies, the previously mentioned endocytic regulators were one of the first TSG classes linked to JAK/STAT signaling (Moberg et al., 2005; Vaccari and Bilder, 2005). Mutant clones were shown to activate JAK/STAT in surrounding wild-type cells through upregulation of its secreted ligand Unpaired (Upd), promoting overgrowth. In these cases Notch signaling is the major driver of Upd gene activation, though it has also

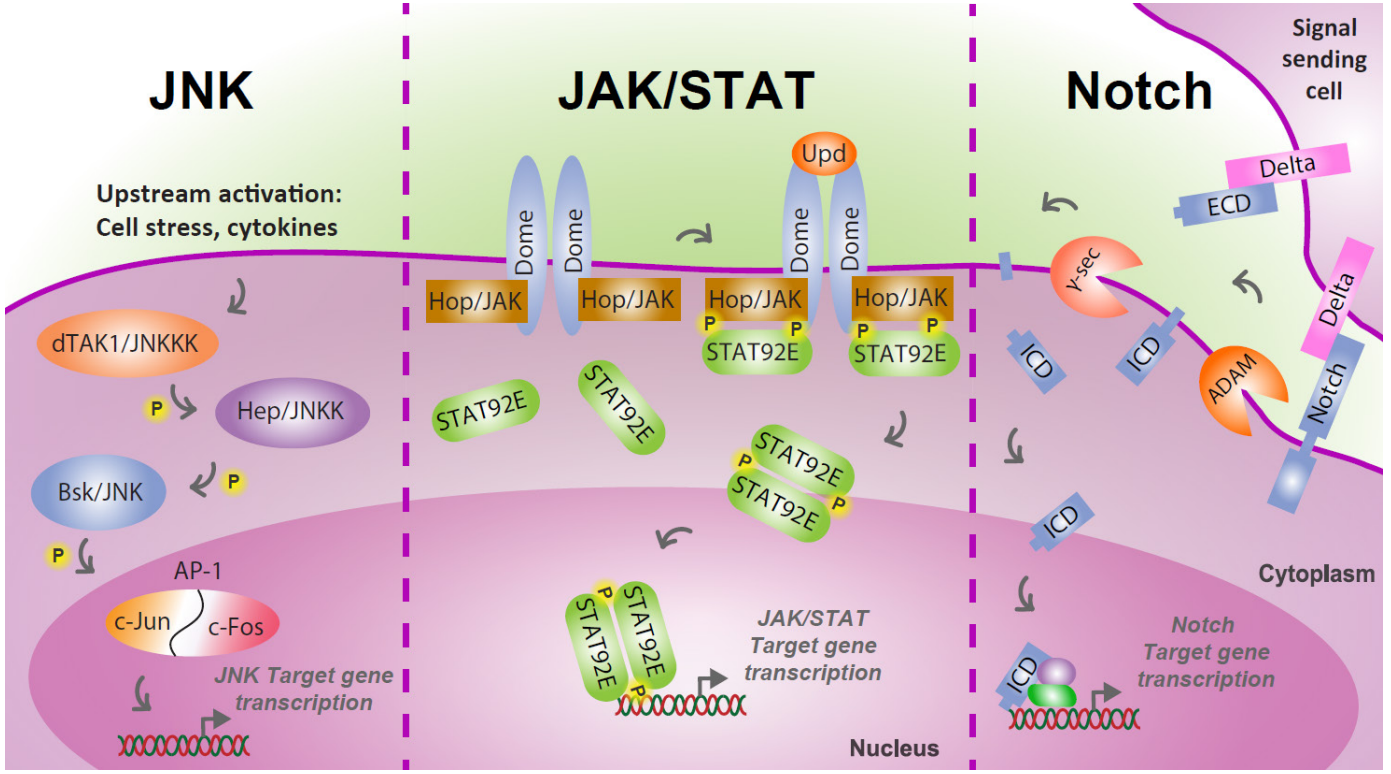

Figure 5: Signaling pathways in *Drosophila* tumors . The JNK, JAK/STAT and Notch signaling pathways in *Drosophila*. Cell stress or specific cytokines activate the JNK pathway. A kinase cascade leads to the downstream activation of the AP1 complex, which promotes transcription of target genes like MMP1 and puc. The JAK/STAT pathway is initiated by binding of Unpaired (Upd) ligand to receptor Domeless (Dome). Ligand-mediated receptor multimerization brings two JAKs (Hopscotch in *Drosophila*) in close proximity, which activates both JAKs through transphosphorylation. Subsequently each JAK phosphorylates its substrate transcription factor STAT (STAT92E in Drososphila). Phosphorylated STAT forms dimers that can enter the nucleus and induce transcription. The transmembrane protein Notch is activated by ligands like Delta binding to its extracellular domain (ECD). This triggers proteolytic cleavage of Notch ECD, followed by the release of its intracellular domain (ICD) into the cytoplasm. Cytoplasmic Notch ICD enters the nucleus and controls Notch target gene transcription.

been shown that polarity deficiency is sufficient to trigger JAK/STAT via JNK (Wu et al., 2010). It is suggested that this JAK/STAT activation normally promotes compensatory proliferation when suboptimal cells are being eliminated from the epithelium, which goes awry when these cells gain a proliferative advantage like activated Ras. In addition to this Notch- regulated non-autonomous overgrowth, neoplastic TSG mutants have been shown to activate autonomous tissue proliferation by Upd activation through JNK and Yki (Bunker et al., 2015). Together this indicates that endogenous JAK/STAT signaling harbors elevated tumorigenic potential, which has been show in the *Drosophila* imaginal wing disc, where the regions that overlap with the characteristic JAK/STAT expression pattern coincide with so-called "tumor hotspots"(Tamori et al., 2016).

Notch signaling

Notch signaling, which was originally discovered in *Drosophila* studies (Kidd et al., 1986; Wharton et al., 1985), centers around the activation and cleavage of Notch upon binding to a ligand from a signal-sending cell, followed by nuclear translocation of its intracellular-domain resulting in downstream transcriptional activation (Fig.5) (Andersson et al., 2011). Similar to JNK signaling, both tumor suppressive and tumor promoting functions have been ascribed to the pathway. The first evidence for Notch as an oncogene came from research in T cell acute lymphoblastic leukemia (T-ALL) (Ellisen et al., 1991), where NOTCH1 is the most frequently mutated gene (Weng et al., 2004). Subsequently Notch has been found to be overly active in solid tumor types, including breast, colorectal, pancreatic and lung cancer (Büchler et al., 2005; Chu et al., 2010; Pece et al., 2004; Reedijk et al., 2005). In many of these studies, the oncogenic form of Notch is an aberrantly truncated version missing all or part of the extracellular domain, leading to constitutive signaling activation. Conversely, a loss of Notch signaling has been tied to bladder, oesophageal, gliomal and other cancer types, suggesting a role as a tumor suppressor (Network et al., 2015; Nowell and Radtke, 2017; Rampias et al., 2014; Song et al., 2014). In flies, Notch is seen as one of the big contributors of non-autonomous overgrowth in endocytic mutant tumorigenesis. It shows either elevated levels at the plasma membrane or is trapped in intracellular compartments (Childress et al., 2006; Lu and Bilder, 2005; Moberg et al., 2005; Morrison et al., 2008; Thompson et al., 2005; Vaccari et al., 2009). One of the downstream targets of Notch signaling is the JAK/STAT ligand Upd, which explains the JAK/STAT driven overgrowth seen in many of these mutants. Intriguingly, Notch has also been tied to the polarity regulating neoplastic TSGs. Comparable to Ras cooperation, clones of activated Notch combined with polarity loss results in massive neoplastic overgrowth (Brumby and Richardson, 2003). In addition, *lgl* has been associated with regulating Notch signaling independent of its function in polarity, through altered trafficking and vesicle acidity (Parsons et al., 2014; Portela et al., 2015).

The *Drosophila* model system

The fruit fly *Drosophila melanogaster* is a powerful model organism, being used for laboratory experiments for over a century. The short life cycle, easy maintenance in a lab and genetic accessibility have made it a staple for many branches of research. Our understanding of a multitude of genes that play significant roles in human physiology and disease comes from their initial discovery in *Drosophila* (Bilder and Irvine, 2017). In my thesis work I have used fruit flies to study epithelial polarity and tumor suppressor genes. Flies have a number of epithelia that are useful for these studies, including the embryonic epidermis, the follicle cells, the gut and the imaginal discs. The focus below will be on the imaginal disc epithelium, since it is involved in most of the experiments described in this thesis.

The imaginal disc epithelium

As a holometabolous insect, *Drosophila* goes through a larval and pupal stage before reaching adulthood. During the larval phases the tissues that will give rise to adult structures are known as 'imaginal' tissues. Some of these tissues are small groups of cells, like histoblast nests or the salivary gland imaginal rings, though most form larger epithelial sac-like structures called imaginal discs. Within the larva, there are a total of 19 discs, of which nine bilateral pairs form the epidermal structures, and one medial disc that gives rise to the genitalia (Aldaz and Escudero, 2010; Beira and Paro, 2016). The pairs consist of the labial, clypeolabral and eye-antennal discs forming the head,

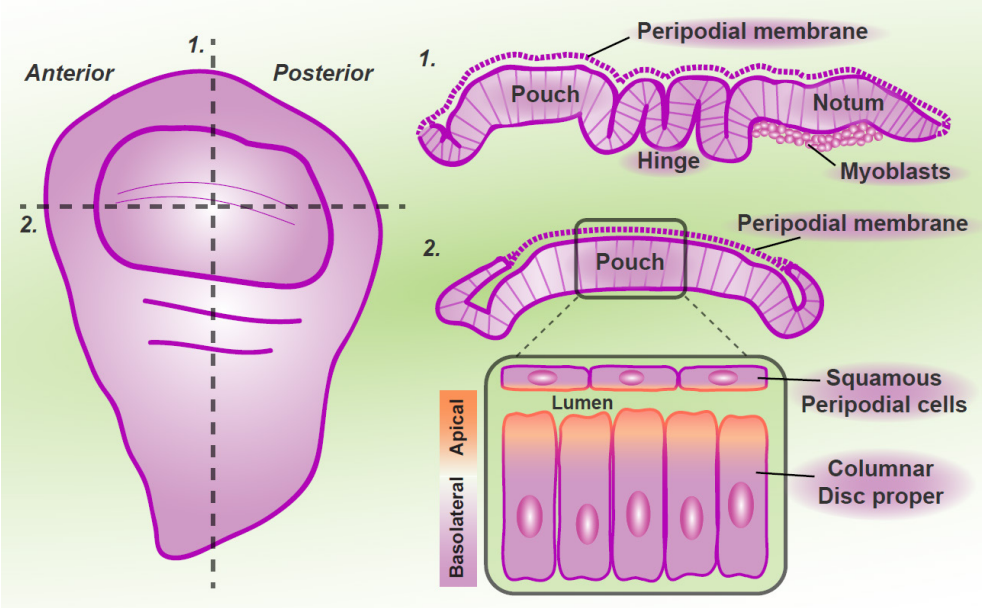

Figure 6: Imaginal wing disc epithelium. The *Drosophila* wing imaginal disc consists of a single layer of columnar epithelial cells connected to a sheet of squamous peripodial cells. Cross sections highlight different regions of the disc, as well as the polarity of the epithelium.

the humeral, wing and haltere discs forming the thorax, wings and halteres, and six leg discs. All the imaginal discs arise form a small number of embryonic cells, about 20-70 for the larger ones including the wing and eye-antennal discs (Mandaravally Madhavan and Schneiderman, 1977). During larval development these cells divide exponentially and late in the third instar, the final larval stage just before pupariation, disc cell numbers range from 10,000 to 50,000 cells (Morata and Ripoll, 1975). This makes imaginal discs ideal for studying tissue growth mechanisms. They are also very convenient for studying epithelia, since they are mostly made up of a single columnar cell layer which is easily accessible and manipulable (**Fig.6**). These epithelial cells form the disc proper, while an exterior layer of squamous cells form the peripodial membrane. The peripodial cells contribute relatively little to adult structures, but are involved in disc patterning and disc morphogenesis (Gibson and Schubiger, 2000; Haynie and Bryant, 1986; Pastor-Pareja et al., 2004). In addition, discs generally contain a population of mesodermal myoblasts, tracheal (respiratory) tissue and neurons.

Techniques: Gal4-UAS

A specific advantage of using *Drosophila* as a model organism are the genetic techniques available and the ease with which they are employed. A common but essential component in the broad toolkit of *Drosophila* genetics is the GAL4/UAS system. This technique employs the yeast protein GAL4, a transcriptional activator, and its specific binding target sequence UAS (Upstream Activation Sequence) to generate spatiotemporal expression of any genetic construct of interest (**Fig.7**)(Brand and Perrimon, 1993; Fischer et al., 1988; Webster et al., 1988). GAL4 transcription is coupled to regulatory elements of gene of interest (the driver) so that it is expressed only in the tissue where this gene is active. For example, this could be a wing disc-specific driver or even a gene just active in a subset of wing disc cells. Since GAL4 is non-native to fruit flies, it does not affect the cells expressing it on its own. However, when it is expressed in cells that also contain a UAS construct, GAL4 will bind this sequence and activate transcription of that construct. These UAS constructs can range from GFP reporters to double-stranded RNA for targeted knock-downs and even toxic enzymes to ablate specific cell populations. On its own GAL4/UAS is already a powerful technique, and it becomes even more potent in combination with other tools like clonal induction.

Clonal techniques

Many genes are important in early development. Zygotic mutations in these genes often result in embryonic lethality, therefore preventing the analysis later in development. To circumvent this problem, it is possible to generate patches of cells (clones) within tissues. These clones are homozygous mutant for genes of interest (**Fig.7**). The workhorse of this principle is the FLP-FRT system (Golic and Lindquist, 1989). The presence of Flp recombinase enables mitotic recombination between homologous chromosomes that contain site-specific Flippase Recognition Targets (FRTs) for this enzyme. By putting a mutation of interest on a chromosome with an FRT site, FLP-mediated recombination can generate clones of mutant cells in an otherwise heterozygous animal. Often the mutant clones and their wild-type 'twin spot' can be distinguished by having GFP or another marker on one of the chromosomes. Commonly Flp is under control of a heat

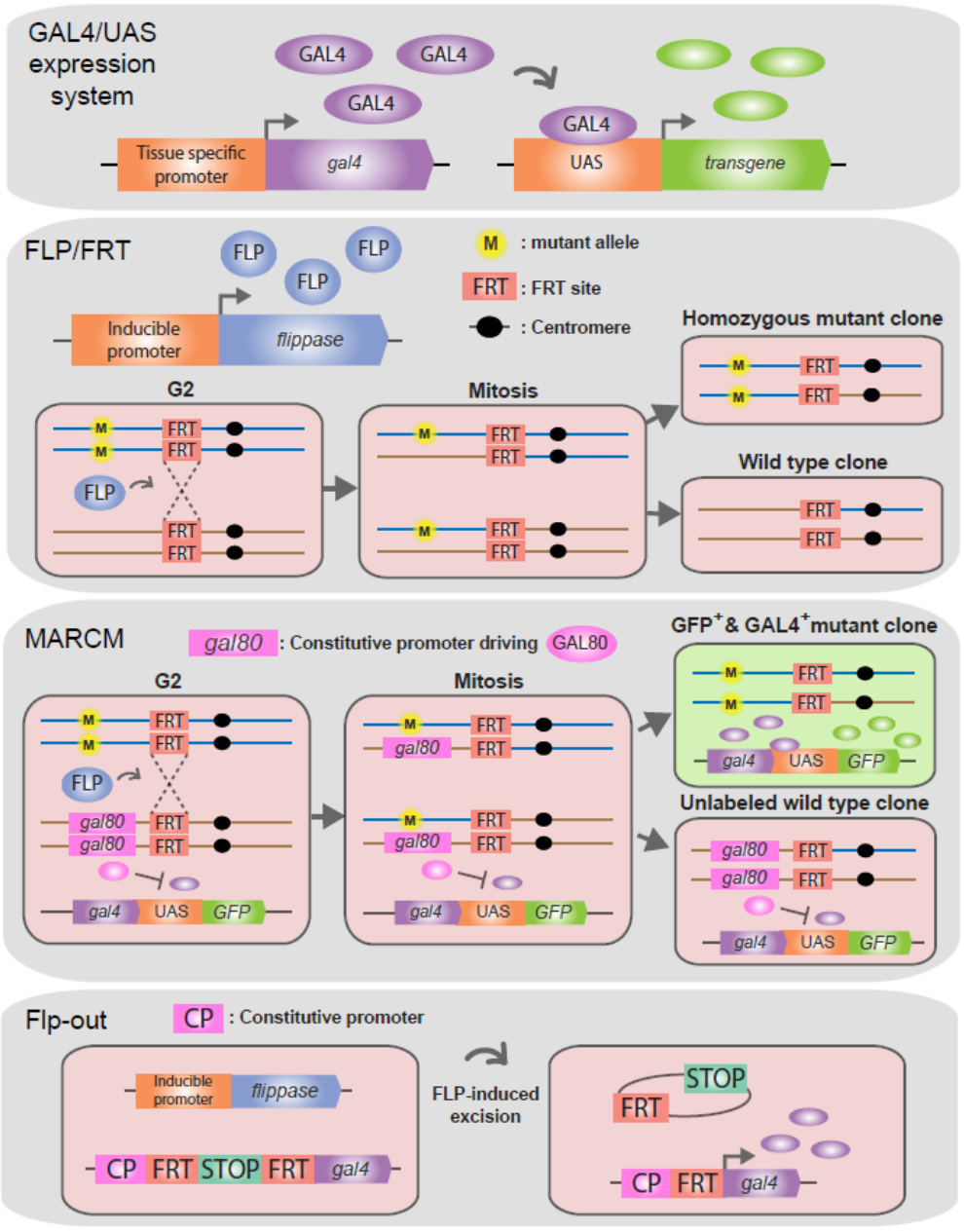

Figure 7: GAL4/UAS and clonal techniques. Different genetic techniques that are employed in *Drosophila*. The GAL4/UAS system facilitates spatiotemporal expression of any transgene of interest using the non-native GAL4 protein that binds UAS promotor sequences. FLP/FRT mediated recombination allows for the generation of homozygous mutant clones of cells in an otherwise heterozygous mutant animal. MARCM combines FLP/FRT with GAL4 and its suppressor GAL80 to enable transgene expression specifically within mutant clones. Clonal induction with Flp-out circumvents the need for mitotic recombination and generates clones expressing GAL4, through excision of an inhibitory element that blocks a constitutive promoter.

shock inducible promotor, though later versions of this system in flies also employ tissue specific drivers like *ey* for the eye-antennal disc and *Ubx* for the wing disc (St Johnston and Johnston, 2002). Building upon this technique of generating clones, MARCM (Mosaic Analysis with a Repressible Cell Marker) is a variation where the mutant clones are also expressing GAL4 (Lee and Luo, 2001). This is accomplished by having constitutive expression of GAL80, which suppresses GAL4, from the homologous chromosome. During mitotic recombination, the generated mutant cells will lose GAL80 expression, therefore activating GAL4. This is often coupled with UAS-GFP to positively mark the mutant cells, though any other UAS construct can be driven in the mutant population as well. All of these techniques depend on mitotic recombination. A way to avoid the dependence on cell division is using Flp-out clones (Bischof and Basler, 2008). This revolves around the insertion of a cassette containing a transcriptional stop signal flanked by two FRT sites that are in close proximity on the same chromosome. This cassette generally sits between a constitutively active promotor and its target, therefore blocking expression in the absence of Flp. The presence of Flp, in this case often controlled by heat shock, induces the excision of the FRT cassette, enabling transgene expression in a clonal context. These techniques have been applied to resolve the function of many genes essential in normal development, but also to study processes where developmental pathways are deregulated, like tumorigenesis.

Summary and Scope of this thesis

The *Drosophila* model system has been at the forefront of many scientific discoveries. Studies of tumor suppressor genes in fruit flies have provided major insights towards polarity and growth signaling, while also aiding the understanding of complex signaling in cancer and other human diseases. The research in this thesis describes and discusses findings regarding a multitude of *Drosophila* tumor suppressor genes, putting the results of these studies in a broader context of epithelial growth, polarity and cell signaling.

In **Chapter 1** I provide an overview of epithelial polarity and its regulators, including the *Drosophila* genes that are involved in controlling epithelial integrity and cause tumorous phenotypes when disrupted. The mutations that cause tumorigenesis in flies can provide clues to what cellular processes are connected to aberrant proliferation and allows us to interrogate signaling pathways that underlie malignant transformation. While many *Drosophila* mutants are known that generate epithelial overgrowth, the links between the different categories of genes are largely unknown. **Chapter 2** establishes a connection between two of these major gene groups: the Scrib polarity module and endocytic trafficking regulators. This work shows that components of the retromer trafficking pathway genetically interact with the Scrib module, and retromer pathway cargo traffic is defective in Scrib mutant tissue. The Scrib module is necessary for proper retromer function and we propose that one of the major ways in which Scrib regulates polarity is through controlling endocytic itineraries of retromer- dependent cargo. **Chapter 3** identifies a new player in *Drosophila* epithelial polarity regulation. Here we show that null mutants for a substrate adaptor of an SCF-class E3 ubiquitin ligase, called *supernumerary limbs* (*slmb*), display a loss of polarity and dramatic overproliferation in the imaginal discs of *Drosophila* larvae. Normally Slmb is required to antagonize the polarity regulator aPKC by restricting its activity to the apical membrane, independent of endocytic trafficking and parallel to the Scrib module. This implicates targeted protein degradation as a new mechanism involved in regulating *Drosophila* apicobasal polarity. In **Chapter 4** we investigate molecular signaling underlying a known *Drosophila* TSG that has been relatively understudied. While many fly TSGs provided insight into the regulation of epithelial growth, polarity and architecture, some have remained obscure. An example of this is *tumorous imaginal discs* (*tid*), which was discovered along with many other classical tumor suppressors, though the functional mechanisms behind *tid* have remained elusive. We report that tid was originally miscloned, and the phenotypes are driven by the loss of the N-linked glycosylation pathway component ALG3. Tumorous growth is driven by altered Hippo growth signaling, which is induced by excessive JNK pathway activity. Surprisingly this JNK activation is caused by the defective glycosylation of a single protein - the fly TNF receptor homolog- inducing increased affinity for TNF. Our findings indicate that N-linked glycosylation can modulate TNFR signaling by adjusting the dynamic interaction threshold with its ligand, providing tissues with a mechanism to respond to altered physiological inputs.

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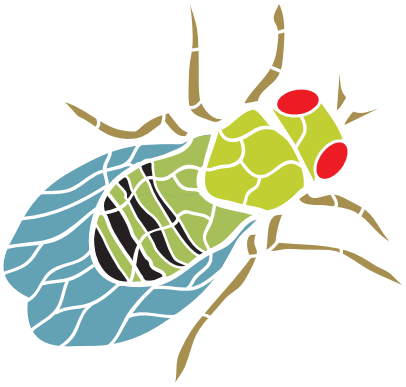

CHAPTER 2:

The Scribble module regulates retromer-dependent endocytic trafficking during epithelial polarization

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ABSTRACT

Scribble (Scrib) module proteins are major regulators of cell polarity, but how they influence membrane traffic is not known. Endocytosis is also a key regulator of polarity through roles that remain unclear. Here we link Scrib to a specific arm of the endocytic trafficking system. *Drosophila* mutants that block AP-2-dependent endocytosis share many phenotypes with Scrib module mutants, but Scrib module mutants show intact internalization and endolysosomal transport. However, defective traffic of retromer pathway cargo is seen, and retromer components show strong genetic interactions with the Scrib module. The Scrib module is required for proper retromer localization to endosomes and promotes appropriate cargo sorting into the retromer pathway via both aPKC-dependent and -independent mechanisms. We propose that the Scrib module regulates epithelial polarity by influencing endocytic itineraries of Crumbs and other retromer-dependent cargo.

INTRODUCTION

The polarized distribution of proteins is central to biological function. Foundational work has identified several multiprotein modules that act as key polarity regulators throughout vertebrates and invertebrates (St Johnston and Ahringer, 2010). Polarity control must ultimately impact vesicular trafficking to achieve a restricted protein distribution at the plasma membrane (PM), but how specific polarity-controlling modules influence the general process of membrane traffic is a long-standing mystery.

Two polarity modules, called the Par and Crumbs (Crb) modules, specify the apical membrane domain. In the *Drosophila* Par module, Bazooka (Baz; also known as Par-3) and Par-6 serve as scaffolding proteins that direct aPKC kinase activity to appropriate targets in response to a Cdc42-GTP-mediated cue (Goldstein and Macara, 2007). One potential target is the transmembrane protein Crb, which can specify apical identity via a poorly characterized aPKC-dependent pathway (Bulgakova and Knust, 2009).

A third module, called the Scribble (Scrib) module, is a major regulator of the basolateral domain, where it serves to exclude apical protein localization. In *Drosophila* this module consists of Scrib, Discs-large (Dlg) and Lethal giant larvae (Lgl) (Yamanaka and Ohno, 2008), which are 'junctional scaffolds' that contain multiple protein-protein interaction motifs. Lgl shows reciprocal negative regulation with aPKC, but how it and other Scrib module proteins interface with membrane trafficking machinery is not known. Polarity control by the Scrib module is also required to prevent malignant overgrowth in several fly tissues (Bilder, 2004), leading Scrib module genes to be described as 'neoplastic' tumor suppressor genes (TSGs). Evidence suggests the conservation of a tumor suppressive role in mammals (Martin-Belmonte and Perez-Moreno, 2011; Pearson et al., 2011) as well as an important role in influencing the Hippo pathway (Cordenonsi et al., 2011). Currently there is thus much interest in understanding the fundamental activity of the Scrib module.

An intriguing hint comes from recent work revealing that certain canonical regulators of endocytic trafficking also act as fly neoplastic TSGs (reviewed by Shivas et al., 2010). For instance, loss of Rab5 or endosomal sorting complex required for transport (ESCRT) components results in disorganized overgrowth of imaginal epithelia, whereas mutations that disrupt subsequent stages of endocytic trafficking do not. Rab5 and ESCRT also regulate apical polarity in mammalian epithelia (Dukes et al., 2011; Zeigerer et al., 2012), while Par mutations in several systems can cause defects in cargo internalization and endolysosomal traffic (reviewed by Shivas et al., 2010). Here we investigate the hypothesis that Scrib mediates polarity through influencing endocytic itineraries. We show that the Scrib module regulates retromer-dependent sorting events that can return internalized cargo to the cell surface, thereby linking this conserved polarity-regulating module to a specific, bona fide vesicle trafficking pathway.

RESULTS

AP-2-dependent endocytosis is required for epithelial organization and proliferation control

We recently reported the isolation of null mutations in *Drosophila* genes encoding regulators of endocytosis from the cell surface. These include subunits of the AP-2 adaptor complex, the Dynamin ortholog Shibire (Shi) and the Clathrin heavy chain (Chc). When imaginal discs consist predominantly of cells mutant for these genes, the tissues are severely disorganized and show up regulation of Matrix metalloprotease 1 (Mmp1) (Windler and Bilder, 2010). Mutant eye discs are also larger than their wild-type (WT) counterparts, lose neuronal differentiation and epithelial monolayering, and display disrupted cell shapes (Fig.1A-D; supplementary material Fig. S1A-D). Mutant clones in the adult follicle epithelium also lose epithelial organization (supplementary material Fig. S1I-L). These phenotypes confirm that *AP-2* subunits, *shi* and *Chc* act as neoplastic TSGs (Windler and Bilder, 2010).

Similar cortical polarity defects in endocytic and Scrib module mutant cells

We analyzed PM polarity in these endocytic mutants, first assessing proteins that are peripherally associated with the cell cortex. The apical markers aPKC and Par-6 and the basolateral marker Dlg are found in separate but contiguous domains in WT epithelial cells (Fig. 1E,I). In *AP-2* follicle cells, aPKC is mislocalized around the cortex (Fig. 1J), both interspersed and overlapping with Dlg. This phenotype reflects a severe perturbation of apicobasal polarity. A similar distribution is seen in *lgl* follicle cells (Fig. 1K) and with Par-6 in *dlg* and *scrib* imaginal discs (Fig. 1F). Follicle cells and discs behave similarly, and *dlg*, *lgl* and *scrib* genotypes were indistinguishable (data not shown). When we compared Scrib module and *AP-2* mutant discs with cortical markers, no consistent differences were detected. These results demonstrate that proper restriction of the apical membrane domain requires AP-2-dependent endocytosis, and further suggest that polarization of the cell cortex might be controlled similarly by both the endocytic regulators and the Scrib module.

The Scrib module regulates Crb trafficking after endocytic internalization

Because endocytosis acts primarily on integral membrane proteins, we then analyzed the transmembrane proteins Crb and Neuroglian (Nrg), which are restricted to apical and basolateral domains, respectively, in WT cells (Fig. 1G,L). In *AP-2* cells, Crb shows a fragmented distribution around the PM (Fig. 1M), with regions of overlap as well as complementary distribution with Nrg. Mislocalization, exclusion and overlap between Crb and Nrg are also seen in *lgl* cells. Strikingly, whereas Nrg was exclusively PM localized and indistinguishable between the two genotypes, Crb showed significantly reduced PM localization in *lgl* as compared with AP-2 tissue, accompanied by a hazy, subcortical distribution (Fig. 1N,P,R). Subcortical Crb was also seen in *dlg* discs (Fig. 1H and Fig. 2D). Therefore, while Scrib module and *AP-2* mutants phenocopy each other in most respects, they show a specific difference in Crb subcellular localization.

Subcortical Crb could result from defects in exocytic delivery to the PM, or from defects in endocytic traffic. To distinguish between these possibilities, we examined cells depleted simultaneously of *AP-2* and *lgl*. In contrast to *lgl*-depleted cells, these dual depleted cells show levels of Crb cortical association comparable to cells depleted of *AP-2* alone (Fig. 1O-R). The epistasis suggests that, whereas AP-2 is required for Crb internalization, Scrib module mutants are defective in post-internalization trafficking of Crb.

Scrib module mutations do not alter AP-2-dependent internalization or lysosomal trafficking

The evidence for endocytic trafficking defects in Scrib modulemutant cells, as well as the polarity phenotypes shared with endocytic mutant cells, raised the possibility that the Scrib module controls epithelial polarity by regulating general endocytic traffic.We directly analyzed endocytosis using the cargo Notch. InWTdiscs, Notch is internalized by AP-2 and degraded after 5 h (supplementary material Fig. S2A) (Lu and Bilder, 2005). This

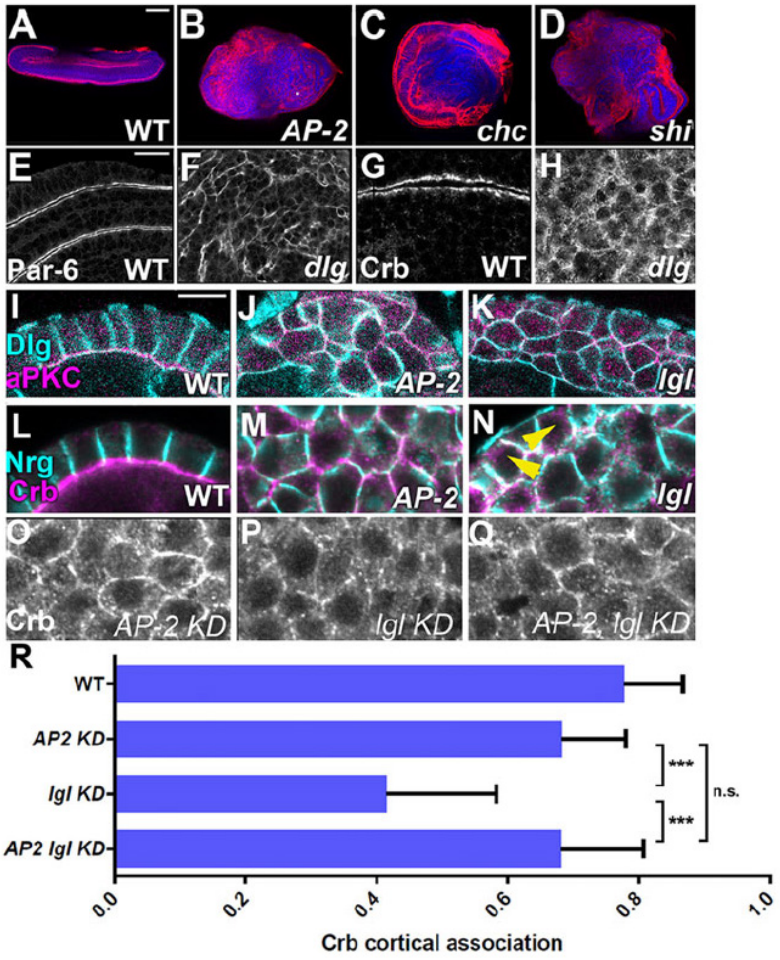

Figure 1: Comparison of Scrib module and endocytic polarity phenotypes.
Compared with WT (A), eye discs mutant for *AP-2α* (B), *Chc* (C) or *shi* (D) are overgrown, disorganized and multilayered [x-z sections; F-actin, red; nuclei, blue (DAPI)]. Par-6 (E) and Crb (G) are apical in WT. In *dlg*, both are mislocalized, but Par-6 remains at the plasma membrane (PM) (F) whereas Crb also shows a hazy subcortical distribution (H). Separate domains of apical aPKC (magenta) and basolateral Dlg (cyan) in WT follicle cells (I) are lost in *AP-2* (J) and *lgl* (K). Separate domains of apical Crb (magenta) and basolateral Nrg (cyan) in WT (L) are lost in *AP-2* (M) and *lgl* (N), but *lgl* displays a subcortical haze of Crb (arrowheads). Crb localization is shown in follicle cells knocked down for *AP-2* (O), *lgl* (P) and for *AP-2* and *lgl* (Q). (R) Quantitation of Crb cortical association; 1.0 reflects full association. $n \geq 200$ cells from at least five samples for each. ***$P<0.001$; n.s., not significant; individual *P*-values are given in supplementary material Table S1. Error bars indicate s.d. Scale bars: 100 µm in A; 25 µm in E; 10 µm in I.

process is intact in discs mutant for *dlg, scrib* or *lgl* (supplementary material Fig. S2B-D), in contrast to discs mutant for *AP-2* (supplementary material Fig. S2F) or *Rab5* (Lu and Bilder, 2005). We found no evidence of a decreased rate of endocytosis (supplementary material Fig. S2G-I), and the endocytic tracer Dextran was also internalized and degraded (supplementary material Fig. S2K). Because Notch internalization and degradation, like epithelial polarity and proliferation control, require AP-2 (Windler and Bilder, 2010), we conclude that the Scrib module does not regulate polarity via general control of AP-2-dependent internalization or endolysosomal traffic.

Altered trafficking of retromer cargo in Scrib module mutants

To reconcile the altered endocytic traffic of Crb (Fig. 1) with the normal degradative traffic of Notch (supplementary material Fig. S2) in Scribmodulemutant cells,we considered whether these cells might be defective in a distinct post-internalization route. An alternative to endolysosomal transport is traffic through the retromer pathway from endosomes to Golgi. Crb transits this pathway, which promotes its recycling to the PM (Pocha et al., 2011; Zhou et al., 2011), as the retromer does with other cargo (Grant andDonaldson, 2009; Johannes and Popoff, 2008). Strikingly, whereas the canonical retromer cargo Wntless (Wls) (Eaton, 2008) is found at steady state at the PM of WT discs, in *dlg* discs it shows an additional, substantial subcortical distribution (Fig. 2A,B,K). By contrast, E-cadherin (Ecad; Shotgun – FlyBase) and transgenic CD8, as well as other transmembrane proteins, remain PM associated in *dlg* discs as in WT (Fig. 2E-H,K; supplementary material Fig. S3), demonstrating that subcortical trapping is seen only with specific cargo, is not due to general exocytic defects, and is not an artifact of overexpression. Altered localization of Wls resembled that of Crb (Fig. 2C,D,K), which colocalized poorly with the vesicular markers examined (supplementary material Fig. S4). Moreover, treatment with lysosomal inhibitors revealed increased lysosomal accumulation of both Wls and Crb, but not Ecad, specifically in *dlg* tissue (Fig. 2L-Q; supplementary material Fig. S5). The demonstration that Wls, like Crb, is defectively trafficked in *dlg* discs suggests that the Scrib module is required for proper sorting into and/or transit of endocytic cargo through the retromer pathway.

Disrupting retromer trafficking enhances Scrib module phenotypes

If endocytic sorting into the retromer pathway is functionally involved in polarization by the Scrib module, then genes regulating the two processes should genetically interact. Mild knockdown of *lgl* in the dorsal wing disc leads to ruffling of the adult wing (Fig. 3A,B). This phenotype is enhanced when flies are heterozygous for *scrib* (Fig. 3C), but not *shi* or *AP-2* subunits (Fig. 3D), demonstrating that it represents a specifically sensitized background. Strikingly, mild knockdown of the retromer subunits *Vps35* and *Vps26*, which have little effect on WT wings (Fig. 3E,F), dramatically enhanced the effect of mild *lgl* knockdown, resulting in a lethal 'giant larvae' phenotype with mispolarized and tumorous discs when *Vps26* and mild *lgl* knockdown are combined (100%, n=43; Fig. 3G,H). These genetic interactions are consistent with a model in which Scrib module proteins regulate polarity by influencing endocytic sorting into retromer pathways.

The Scrib module influences retromer-dependent sorting

We sought further evidence for Scrib module involvement by examining vesicular trafficking compartments. Antibodies and tagged transgenes showed that, although polarized distribution is lost, the overall morphology of exocytic and endolysosomal

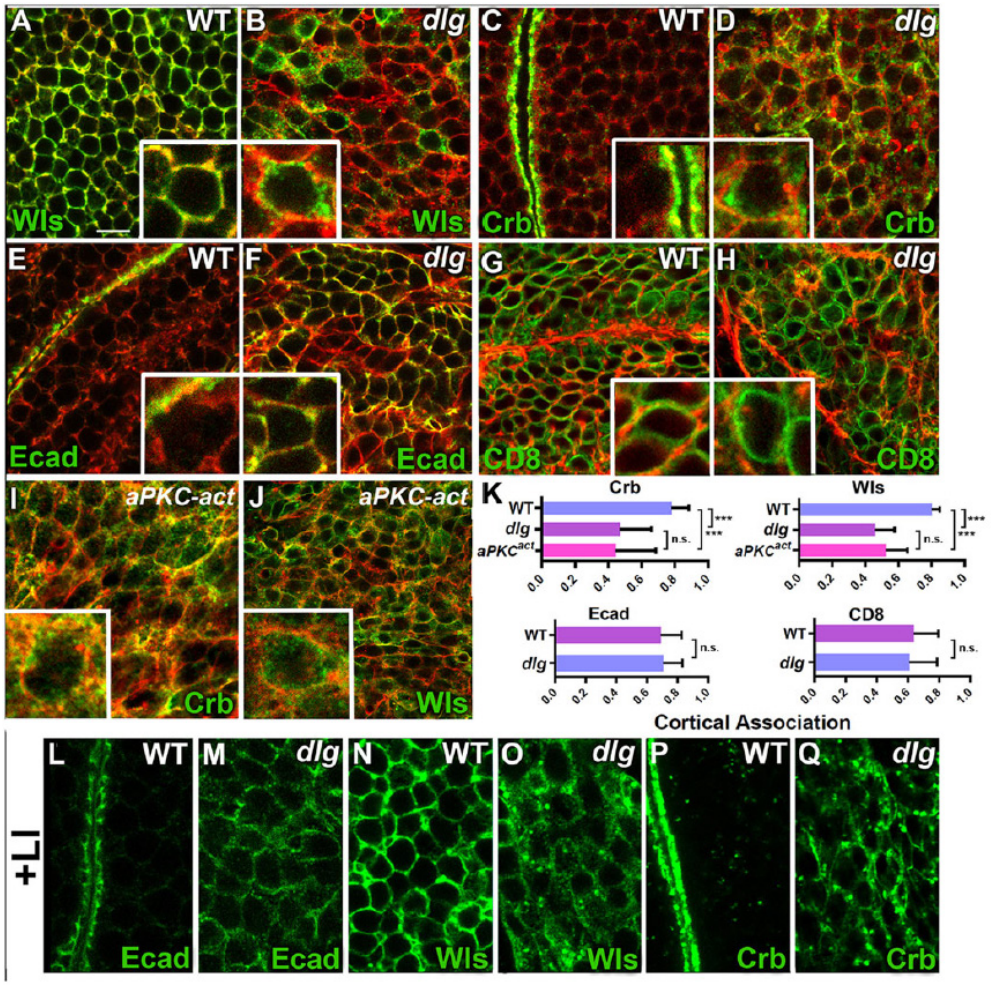

Figure 2: Defective traffic of retromer cargo in Scrib module mutant discs.
(A-J) Wing imaginal discs were stained with phalloidin (red). (A-D) Transgenic Wls and endogenous Crb are enriched subcortically in *dlg* but not WT. (E-H) Endogenous Ecad and transgenic CD8 localize to both WT and *dlg* PMs. (I,J) Activated aPKC is sufficient to induce Crb and Wls subcortical localization. (K) Quantitation of cortical association; 1.0 reflects full association. ***$P<0.001$; individual P-values are given in supplementary material Table S1. Error bars indicate s.d., $n \geq 200$ cells from at least five samples for each. (L-Q) In discs cultured with lysosomal inhibitors (LI), Ecad accumulation in *dlg* does not differ from WT, whereas the accumulation of Crb and Wls is increased. Scale bar: 10 µm.

compartments in *dlg* discs is similar to WT (supplementary material Fig. S6). A marker for the recycling endosome, Rab11, is also not obviously changed. By contrast, *dlg* tissue shows clear alterations of two markers associated with retromer sorting compartments: Rab9 and Vps29 (Burgess et al., 2012; Dong et al., 2013). The restricted and punctate localization of these markers seen in WT is replaced by widespread and diffuse staining in *dlg* mutant cells (Fig. 3I-N). Vps29 and Rab9 colocalize with endosomal and Golgi markers in WT cells (Burgess et al., 2012; Dong et al., 2013), but as these compartments are unaltered in *dlg*

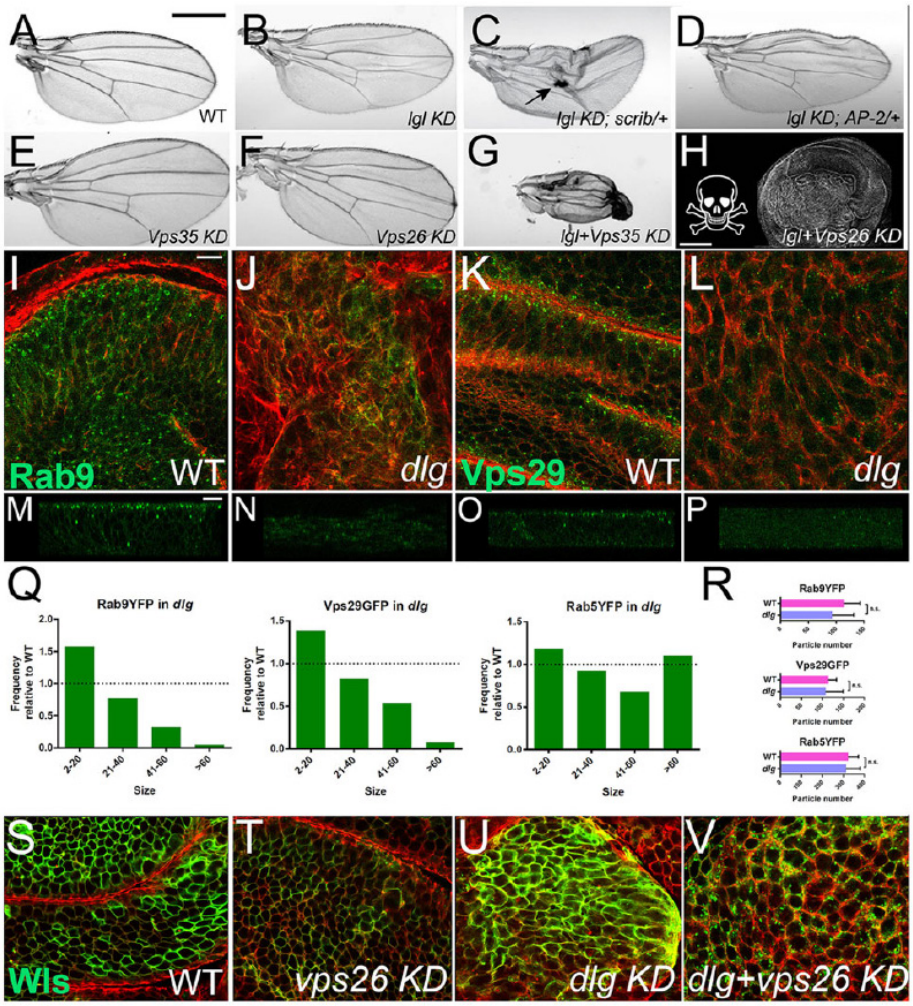

Figure 3: Scrib module proteins control polarity via retromer.
The WT wing (A) wrinkles upon mild *lgl* knockdown in the dorsal compartment (B). Heterozygosity for *scrib* (C) but not *AP-2* (D) enhances this phenotype. Mild *Vps35* (E) or *Vps26* (F) knockdown has little effect alone, but strongly enhances mild *lgl* knockdown (G), including neoplastic transformation and pupal death (H). WT punctate localization of Rab9-YFP and Vps29-GFP (I,K) is dispersed in *dlg* discs (J,L). Quantitation (M) reveals that large Rab9-YFP and Vps29-GFP puncta are strongly depleted, whereas Rab5-YFP puncta are much less affected. Puncta numbers (N) do not change significantly. *P*-values are given in supplementary material Table S1. Error bars indicate s.d. $n \geq 200$ cells from at least five samples for each. PM levels of Wls-V5 in WT (O) are reduced upon *Vps26* knockdown (P). Compared with *dlg* knockdown alone (Q), *Vps26 dlg* double knockdown shows reduced cortical and subcortical localization and enhanced punctate trapping (R) of Wls-V5. (I-L,O-R) Phalloidin staining is in red. Scale bars: 400 µm in A; 100 µm in H; 10 µm in I.

tissue (supplementary material Fig. S6) the data suggest that the
Scrib module specifically controls the enrichment of retromer at sites of endocytic sorting. We further investigated the relationship between the regulation of retromer sorting and the Scrib module by carrying out doubledepletion experiments. Wls traffics via retromer (Eaton, 2008), and strong RNAi-mediated knockdown of *Vps26* in

otherwise WT cells reduces steady-state PM levels (Fig. 3O,P). When compared with *dlg* knockdown alone (Fig. 3Q), simultaneous depletion of *Vps26* with *dlg* prevents Wls from reaching the PM and achieving a subcortical distribution, and Wls is found instead in endosomal puncta (Fig. 3R). These data, showing that the *dlg* trafficking phenotype requires retromer activity, are consistent with the genetic interactions uncovered above and suggest that the Scrib module normally regulates trafficking via retromer.

aPKC-dependent and -independent trafficking regulation by the Scrib module

The above data indicating specific and functionally relevant retromer defects raise the question of exactly which cargo is mistrafficked to alter apicobasal polarity. Crb is mistrafficked in Scrib module mutant cells (Fig. 1H,N and Fig. 2D) and is basolaterally mislocalized when endosomal entry is blocked (Lu and Bilder, 2005). Mislocalization of Crb is also sufficient to specify apical character on PMs (Wodarz et al., 1995) and to induce neoplastic growth (Lu and Bilder, 2005).We tested whether Crb was the single relevant cargo by completely removing it from Scrib module mutant cells using a null allele. However, discs and follicle cells completely lacking Crb and the Scrib module, or Crb and an endocytic regulator, remained mispolarized and neoplastic (supplementary material Fig. S1Q-X) (Leong et al., 2009). These data rule out Crb as the sole polarity-regulating cargo that requires Scrib module-dependent trafficking. We considered whether other apical regulators might be controlled by Scrib-influenced trafficking. Baz, Par-6 and aPKC

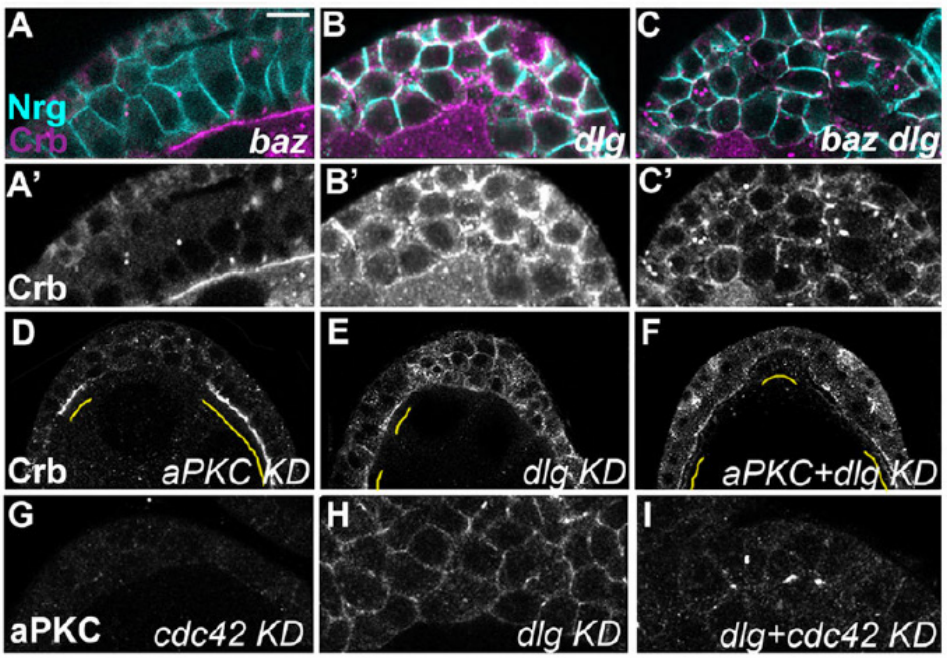

Figure 4: Scrib module trafficking regulation requires Par activity.
(A-C') Mutating *baz* in *dlg* mutant follicle cells largely prevents the inappropriate localization of Crb (magenta, with Nrg in cyan; A'-C' show the Crb channel alone) and causes enhanced endosomal trapping. (D-F) Knocking down *aPKC* in *dlg* knockdown cells has a similar effect. Yellow lines mark WT cells. (G-I) Knocking down *Cdc42* in *dlg* cells causes loss of most apical PM identity (as revealed by aPKC in white). Scale bar: 10 μm.

remained at the PM in Scrib module mutant cells, and, unlike Crb (Moberg et al., 2005), they were not trapped in endocytic compartments in ESCRT mutant cells (Fig. 1F; data not shown). Antibodies and a tagged transgene (Fletcher et al., 2012; Harris and Tepass, 2008) revealed a significant cytosolic population of Cdc42 in WT cells, preventing an assessment of altered distribution in mutants. We then asked whether Par module activity was involved in Scrib-mediated trafficking. To test sufficiency, we expressed an activated form of aPKC and found that it induces subcortical trapping of Crb and Wls in imaginal discs (Fig. 2I,J). To test necessity, we analyzed mutant follicle cells in which both the Par and Scrib modules are inactivated. In these cells, the subcortical haze of Crb is eliminated (Fig. 4A-F) and cells almost completely lack an apical domain (Fig. 4G-I). However, double-mutant and depleted cells do not show the extensive degradation of Crb seen when the Par module alone is inactivated (Fig. 4A,D). Instead, Crb accumulates in internal puncta, and some residual PM localization is evident (Fig. 4C,F). This incomplete epistasis with respect to cargo localization contrasts with the strong epistasis with respect to polarity (Bilder et al., 2003; Tanentzapf and Tepass, 2003), revealing that the Scrib module regulates endosomal trafficking in part through an aPKC-independent mechanism. However, the necessity and sufficiency experiments together indicate that excess Par module activity in Scrib module mutant cells is a major contributor to defective trafficking, suggesting that the Scrib module influences trafficking of aPKC-regulating cargo in addition to Crb.

DISCUSSION

Our data identify a specific trafficking role for the Scribmodule, a core player in the conserved polarization machinery. The evidence that the Scrib module has an endocytic mechanism integrates two major pathways that control cell polarity. Our results rule out AP-2- dependent endolysosomal transport and instead identify a role for the Scrib module in sorting cargo that passes through the retromer. The data further indicate that this relationship is direct and specific, given the requirement of the Scrib module for retromer organization on endosomes and the strong genetic interaction seen with retromer subunits.

Our data point to complexity in the action of the Scrib module. It is clearly not a positive regulator of retromer activity, as the depletion of PM Crb and Wls, their shunting to the lysosome and the loss of apical polarity seen in retromer mutants (Pocha et al., 2011; Zhou et al., 2011) are largely opposite to the Crb misdistribution seen in Scrib module mutants. However, the Scrib module does not simply negatively regulate retromer sorting, since reducing retromer function potently enhances Scrib module hypomorphic phenotypes, and the Scrib module null phenotype induces defects in retromer component localization and retromer-dependent trafficking. A recent paper describes a role for mammalian Scrib in stabilizing the Ecad-p120 interaction and in preventing retromer sorting of lysosomally destined Ecad (Lohia et al., 2012); however, our data, which show that Scrib module mutant cells display PM-localized Ecad, lysosomal Wls and Crb, and no evident Golgi trapping, demonstrate that a different mechanism is at work in the fly.

One possibility is that Scrib module mutants cause neither a wholesale gain nor loss of retromer activity, but rather inappropriate sorting that results in cargo ectopically returning to an incorrect PM domain. In addition to retromer-dependent retrograde transport and ESCRT-dependent lysosomal targeting, cargo can also exit the sorting endosome via Rab11 recycling, and Crb is known to pass through Rab11 compartments

(Blankenship et al., 2007; Fletcher et al., 2012; Roeth et al., 2009); cargo could be aberrantly shunted into this route when Scrib module loss alters retromer activity. Because Rab11 is also involved in biosynthetic transport (Ang and Fölsch, 2012), rendering its inhibition toxic, and Rab11-dependent recycling cargoes are not well-validated in fly epithelia, we are currently unable to test this model. An activity of Scrib module proteins in influencing the sorting and subsequent destination of transcytotic cargo, which can involve retromer activity (Su et al., 2010; Vergés et al., 2004), would be consistent with many of the results reported here. The Scrib module could influence transcytotic sorting by regulating cargo modifications at the basolateral surface in a manner distinct from the apical surface [for instance, via Lgl-mediated inhibition of aPKC (Yamanaka and Ohno, 2008)]. Alternatively, the requirement for proper Rab9 and Vps29 localization on endosomes points to Scrib affecting more general aspects of retromer function. Overall, a model consistent with our data is that Scrib regulates polarity by influencing sensitive sorting steps within endosomes, specifically the itinerary of apically destined proteins that can transit the retromer pathways.

As data demonstrate that polarity regulators can influence endocytic trafficking of distinct cargo in different ways (Shivas et al., 2010), strict tests of these hypotheses must await identification of the specific polarity-regulating cargoes involved. Crb is one of these, and our data build on recent advances in understanding Crb trafficking (Fletcher et al., 2012; Pocha et al., 2011; Roeth et al., 2009; Zhou et al., 2011). However, studies of double mutants show that Crb is not the sole cargo responsible for polarity control. The Scrib module phenotypes show a strong requirement for the Par module, consistent with previous data pointing to Cdc42/Par endosomal sorting activity (reviewed by Harris and Tepass, 2010), although the data also reveal a Par-independent role. Overall, our findings point to an additional Par-regulating cargo that undergoes AP-2-dependent, retromer-mediated recycling to specify the apical surface; the identification of this cargo will open the door to defining the precise molecular mechanisms by which Scrib controls its trafficking.

MATERIALS AND METHODS

Fly stocks and genetics

Mutant eye discs and follicle cell clones were generated as described (Lu and Bilder, 2005). Follicle cell knockdown employed *traffic jam-Gal4* (Tanentzapf et al., 2007) to drive expression of RNAi stocks, except for Fig. 4D-G, which used *hsFLP; act>STOP>GAL4 UASGFP* with 5′ induction. WT control flies were *w* or isogenized *FRT* stocks. Owing to the similar mutant phenotypes of AP-2 complex subunits, representative experiments carried out with the $AP\text{-}2\alpha^{40\text{-}31}$ allele (Windler and Bilder, 2010) are labeled *AP-2*. Additional alleles used include shi^{FL54}, Chc^{3}, $lgl^{4,}$ baz^{Xl106}, fab^{121}, $Vps25^{A}$, $scrib^{1}$, $dlg^{40.2}$, $Vps45^{GG11}$, crb^{11A22}. Other transgenes included *tub-Rab5-YFP, tub-Rab7-YFP, tub-Vps29-GFP, en-Gal4, Ms1096-Gal4, UAS-CD8-GFP,* $UAS\text{-}aPKC^{WTCAAX}$, *UAS-wls-V5, UAS-Rab-YFP, UAS-Vps26-myc, UAS-cdc42-V5*. RNAi constructs were created by the Transgenic RNAi Resource Project (TRiP) (*Cdc42-IR, Vps26-IR*), Vienna *Drosophila* Resource Center (VDRC) (*AP-2a-IR 15566, dlg-IR 41134*), the D.B. lab (*lgl-IR 'weak'*) or provided by X. Lin (Cincinnati Children's Hospital, OH, USA) [*Vps35-IR (III vp2), Vps26-IR 'mild'*]. Mutant eye discs were generated as described (Menut et al., 2007). Descriptions of *Drosophila* stocks can be found on FlyBase. The crb coding region was PCR-amplified and sequenced from heterozygous crb^{11A22} adults; on the mutant chromosome, the nucleotide change C902T replaces amino acid Q950 within the EGF repeats to create a stop codon.

Immunohistochemistry and microscopy

Ovaries and L3 larvae were dissected in PBS, fixed in 4% formaldehyde in PBS for 20 min at roomtemperature, and stained using standard procedures (Bilder and Perrimon, 2000). The following primary antibodies were used: rat anti-Elav (7E8A10, 1:50), mouse anti-Notch^{ECD} (C458.2H, 1:25), mouse anti-Dlg (4F3, 1:100), rat anti-Ecad (DECAD2, 1:25), mouse anti- MMP1 (5H7B11, 3B8D12 and 3A6B4, 1:100) (all obtained from Developmental Studies Hybridoma Bank), rabbit anti-PKCζ (1:200; sc216, Santa Cruz Biotechnology), rat anti-Crb (1:1000; U. Tepass, University of Toronto, Canada and E. Knust, MPI-CBG, Dresden, Germany), rabbit anti-Cdc42 (1:1000; U. Tepass), mouse anti-Nrg (1:200; 1B7, M. Hortsch, University of Michigan, Ann Arbor, USA), rabbit anti-Lva (J. Sisson, UT Austin, TX, USA), guinea pig anti-Scrib (1:500). TRITC-phalloidin (1:500, Sigma) was used to visualize the cell cortex. Secondary antibodies (1:250) were from Molecular Probes. Fluorescent images are confocal sections acquired on a Leica TCS or Zeiss 700 confocal microscope. Follicle sections at stages 6-7 are taken at the equator; eye and wing disc sections are taken below the peripodium. Note that mispolarized disc cells do not show a clear apical domain; images presented are representative. Eye discs imaged in transverse section were mounted in 2-hydroxyethylagarose (Sigma); other tissues were mounted in SlowFade (Molecular Probes). Adult female wings were mounted dorsal side up in 3:1 Canada balsam:methyl salicylate and imaged using a Z16 APO microscope (Leica), with a Planapo 2.0× lens, fitted with a DFC300 FX camera. Images were assembled with Adobe Photoshop CS5.

Quantitation and disc culture

For cortical association analysis, pixel intensity profileswere generated in Fiji (Schindelin et al., 2012). Correlation coefficients between transmembrane protein immunoreactivity and F-actin were calculated to determine the degree of cortical association. For particle analysis of endocytic markers, Ilastik software (Sommer et al., 2011) was used for thresholding, segmentation and generating binary images. Particle size and number were quantified using Analyze Particles in Fiji. Data were analyzed using a twosample *t*-test assuming unequal variances. For lysosomal inhibition, discs were cultured for 5 h in *Drosophila* cell medium (M3, Sigma) containing 200 µm Leupeptin and 50 mM NH4Cl. Notch and Dextran trafficking experiments were carried out as described previously (Lu and Bilder, 2005), and endocytic puncta per cell volume were counted in confocal stacks analyzed in ImageJ (Schindelin et al., 2012) using the LOCI Bio-Formats Importer and Image 5D plugins.

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Author contributions

G.d.V., J.D.S. and D.B. designed the research, analyzed the data and wrote the manuscript; G.d.V., J.D.S., S.L.W., H.M. and H.L. performed experiments.

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SUPPLEMENTARY FIGURES

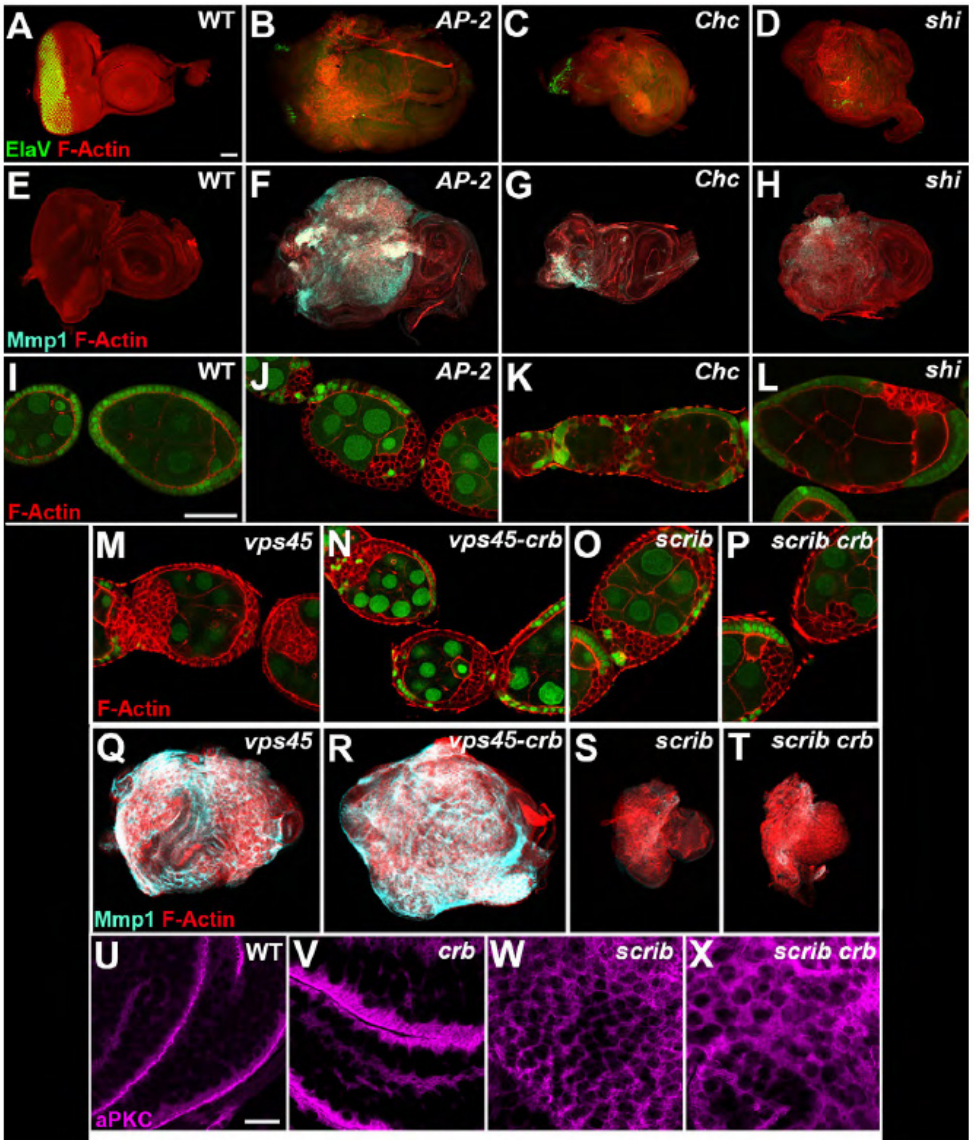

Figure S1.
WT (A, E, I), *AP-2* (B, F, J), *Chc* (C, G, K) and *shi* (D, H, L) mutant tissue stained to assess hallmarks of neoplastic transformation; F-actin staining is red in all images. A-D: Neuronal differentiation (anti-Elav, green) is lost in mutant eye discs. E-H: Mmp1 (cyan) is elevated in mutant eye discs. I-L: mutant follicle cell clones (lacking GFP) are round and form multilayers. Crb activity is not required for neoplastic transformation of Scrib module or endocytic mutants in follicle cells (MP) or imaginal discs (Q-T). Removing Crb does not suppress *Vps45* or *scrib* phenotypes. F-actin (red); Mmp1 (cyan), GFP represents WT cells. Double mutant discs still show ectopic apical polarity, demonstrated by aPKC staining (violet) (U-X). Scale bars: A, I 50 µm, U 10 µm.

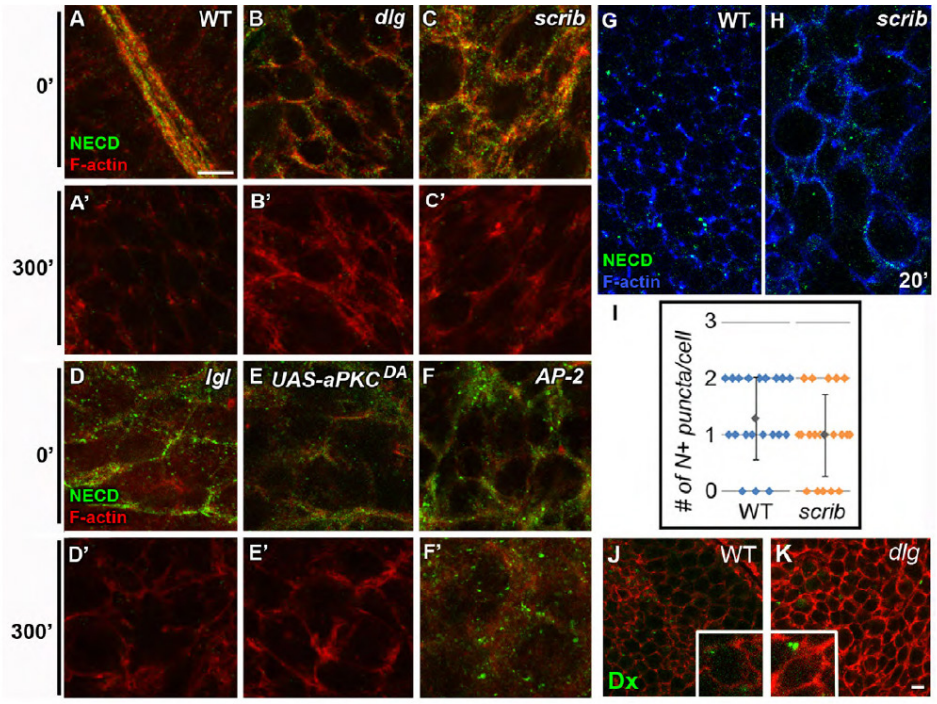

Figure S2.
Scrib module mutant cells exhibit AP-2-dependent endocytosis. Notch localization (green) from live trafficking experiments in mutant eye discs (A-D, F) and wing discs expressing *aPKC*^{CA} (E). Notch at the cell surface labeled at time point zero (A-F) is no longer seen after 300 minutes (A-F') except in *AP-2*. 20' time point shows comparable numbers of Notch-containing endosomes in WT (G) and *scrib* (H) cells. (I) Quantitation of endocytosed Notch at 20' time point in WT (blue points) and *scrib* (red points). (J, K) Dextran is endocytosed by both WT and *dlg* cells. Scale bar: 5 µm.

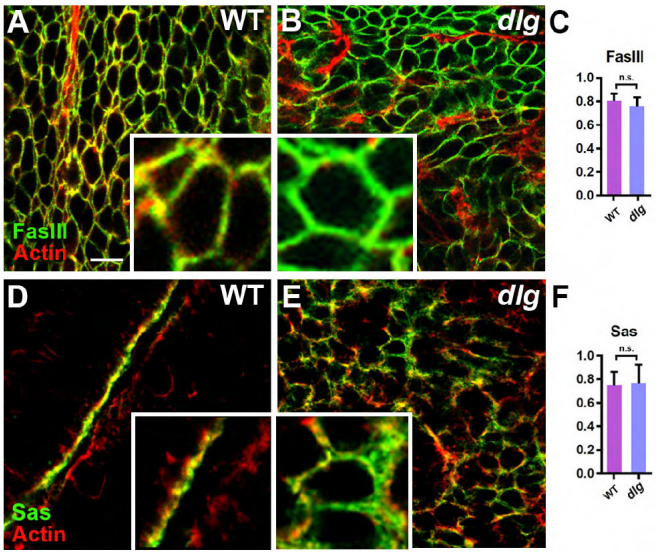

Figure S3.
Sas (A, B) and FasIII (D, E) remain cortical in *dlg* imaginal discs. Quantitation (C, F) reveals comparable cortical localization.Scale bar: 10 µm.

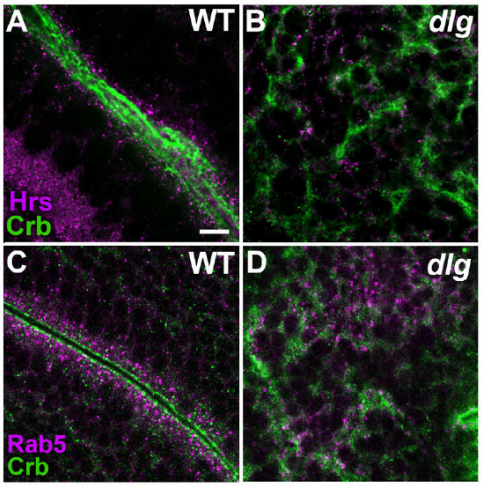

Figure S4.
Crb does not colocalize well with Hrs (A, B) and Rab5 (C, D) in *dig* discs. Scale bar: 10 µm.

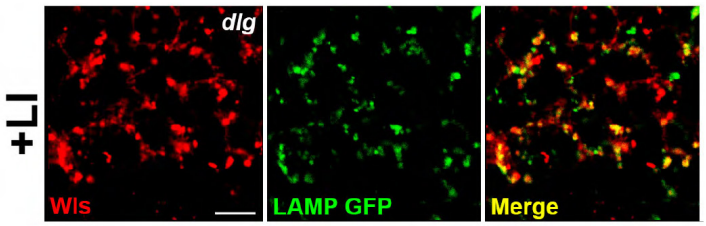

Figure S5.
Wls is found in lysosomal puncta in *dlg* discs treated with a lysosomal inhibitor. Scale bar: 5 µm.

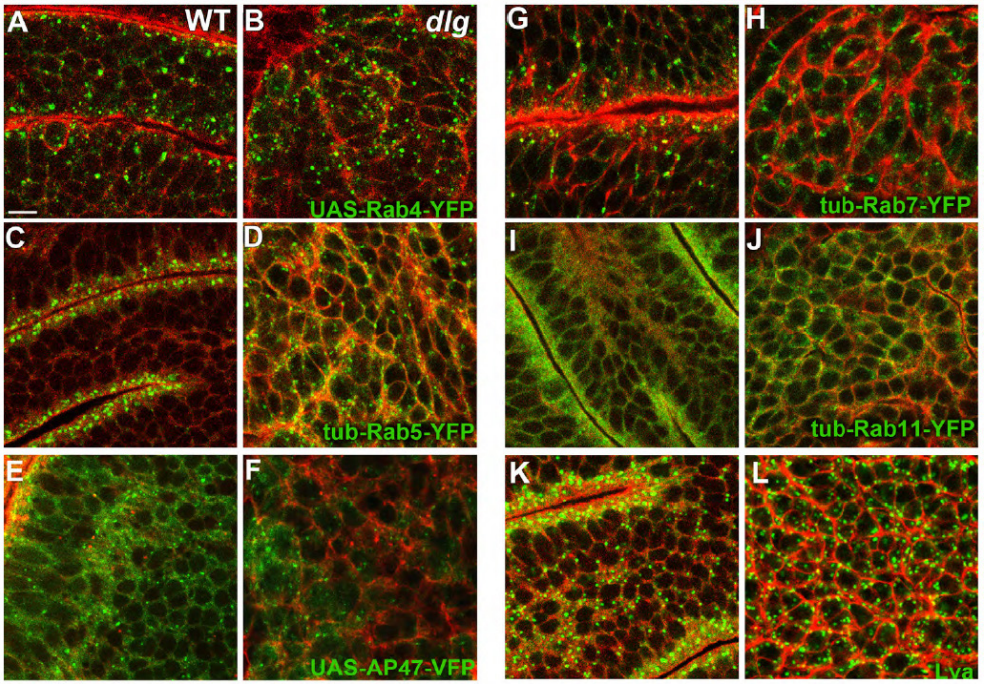

Figure S6.
Though polarization is lost, the organization of many membrane-bound subcellular compartments are not grossly altered in *dlg* mutant wing discs (phalloidin in red marks cortical actin). Scale bar: 10 µm.

SUPPLEMENTARY TABLE S1: *P*-values

Fig. 1 R	P-value
WT vs *AP2 KD*	2.16E-05
WT vs *lgl KD*	3.84E-13
WT vs *AP2 lgl KD*	1.13E-04
AP2 KD vs *lgl KD*	1.60E-09
AP2 KD vs *AP2 lgl KD*	9.81E-01
lgl KD vs *AP2 lgl KD*	2.35E-09

Fig. 2 K	P-value
Crb: WT vs *dlg*	3.19E-08
Crb: WT vs *aPKC act*	1.04E-07
Crb: dlg vs *aPKC act*	7.40E-01
Wls: WT vs *dlg*	3.21E-14
Wls: WT vs *aPKC act*	1.45E-09
Wls: *dlg* vs *aPKC act*	7.77E-02
Ecad: WT vs *dlg*	7.12E-01
CD8: WT vs *dlg*	6.21E-01

Fig. 3 N	P-value
Rab9YFP WT vs *dlg*	4.00E-01
Vps29GFP WT vs *dlg*	7.66E-01
Rab5YFP WT vs *dlg*	4.43E-01

p<0.05
p>0.05

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CHAPTER 3:

The F-box protein Slmb restricts the activity of aPKC to polarize epithelial cells

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ABSTRACT

The Par-3/Par-6/aPKC complex is the primary determinant of apical polarity in epithelia across animal species, but how the activity of this complex is restricted to allow polarization of the basolateral domain is less well understood. In *Drosophila*, several multiprotein modules antagonize the Par complex through a variety of means. Here we identify a new mechanism involving regulated protein degradation. Strong mutations in *supernumerary limbs* (*slmb*), which encodes the substrate adaptor of an SCF-class E3 ubiquitin ligase, cause dramatic loss of polarity in imaginal discs accompanied by tumorous proliferation defects. Slmb function is required to restrain apical aPKC activity in a manner that is independent of endolysosomal trafficking and parallel to the Scribble module of junctional scaffolding proteins. The involvement of the Slmb E3 ligase in epithelial polarity, specifically limiting Par complex activity to distinguish the basolateral domain, points to parallels with polarization of the *C. elegans* zygote.

INTRODUCTION

Polarization is a fundamental feature of animal cells, from newly fertilized zygotes to dividing stem cells to homeostatic epithelia. This common feature is controlled by a conserved set of regulators, which segregate the single plasma membrane into several discrete domains. The most broadly used polarity regulators comprise the Par complex, consisting of the PDZ-containing scaffolds Par-3 and Par-6, which associate with Cdc42-GTP and the atypical protein kinase (aPKC) (Goldstein and Macara, 2007; St Johnston and Ahringer, 2010). In the *C. elegans* zygote and the *Drosophila* oocyte, these proteins localize to and specify the anterior cortex. In most epithelial cells and neural stem cells, they localize to and specify the apical plasma membrane, and in migrating cells they define and act at the leading edge. The Par complex thus serves as a 'master regulator' for many types of cell polarity.

To achieve and maintain polarity, the Par complex must be restrained to distinguish a complementary membrane domain. In contrast to the pre-eminent role of the Par complex, multiple protein modules that limit Par activity have been identified in different contexts (Tepass, 2012). In the *C. elegans* zygote, the protein kinases PAR-1 and PAR-4 act downstream of the RING finger protein PAR-2 to antagonize Par localization and define the posterior cortex (St Johnston and Ahringer, 2010; Zonies et al., 2010). Par-1 and Par-4 (Lkb1 – FlyBase) are also key regulators of fly oocyte polarization, but often have less central roles in other polarized cell types (Haack et al., 2013; Partanen et al., 2013). Instead, in many of these tissues a second group of proteins, the Scribble (Scrib) module, acts to restrict the Par complex. In the Scrib module, Scrib and Dlg are basolaterally localized PDZ-containing scaffolds that regulate Lgl, a syntaxin- and myosin-binding protein that can directly antagonize aPKC (Bilder, 2004; Elsum et al., 2012). Yet another module, the Yurt/Coracle (Cor) complex, specifies the basolateral domain in mid-stage *Drosophila* embryos and zebrafish photoreceptors (Laprise and Tepass, 2011). Rac and PI3 kinase also play a role at this stage (Chartier et al., 2011). Further, in fly epithelia but not neuroblasts, AP-2-mediated endocytosis restricts apical polarity regulators to their appropriate surface; endocytosis also plays a crucial role in polarization of the worm zygote (Halbsgut et al., 2011; Shivas et al., 2010). The mechanisms by which this diverse set of proteins – which we will call Par or apical antagonists – negatively regulate the Par complex is an active field of investigation. In none of these cases is the mechanism well understood, nor how they coordinate with each other.

Our incomplete knowledge of the mechanisms of Par antagonists raises the possibility that additional regulators of basolateral polarity remain to be identified. Here we report that strong mutations in the F-box protein Slmb, a substrate adaptor for SCF E3 ubiquitin ligases, result in excess Par complex activity in *Drosophila* imaginal discs, thereby expanding the apical membrane domain. Our results indicate that Slmb-mediated protein degradation acts in parallel to the Scrib module to oppose aPKC activity and thus specify the epithelial basolateral membrane.

RESULTS AND DISCUSSION

Slmb is a novel *Drosophila* neoplastic tumor suppressor gene

To identify new regulators of basolateral polarity, we analyzed mutants isolated in a genetic screen for *Drosophila* tumor suppressor genes (TSGs). The screen utilized mitotic recombination to generate imaginal discs predominantly populated by homozygous mutant cells growing in an otherwise heterozygous larva. Mutations in a small set of genes cause larval or pupal lethality in this context; many of these show a set of tumor-like phenotypes collectively called 'neoplastic' (Menut et al., 2007). Discs mutant for one uncharacterized complementation group, *MENE(3R)-B*, show multiple hallmarks of neoplastic transformation. Monolayered organization is lost, disc size is dysregulated, F-actin levels are elevated and differentiation is prevented (Fig. 1A-F). In addition, Matrix metalloproteinase 1 (Mmp1), a mediator of tissue invasion, is upregulated (Fig. 1A,B). These phenotypes closely resemble those of the Scrib module, suggesting that *MENE(3R)-B* identifies a gene with similar function.

MENE(3R)-B alleles fail to complement mutants in *slmb*, which encodes an F-box and WD40 repeat protein homologous to vertebrate β-TrCP (Btrc) that functions as a specificity factor in a Skp–Cullin– F-box (SCF) E3 ubiquitin ligase complex (Frescas and Pagano, 2008; Jiang and Struhl, 1998; Theodosiou et al., 1998). Sequencing identified coding region lesions, including early and late truncations as well as missense mutations, in all six alleles (Fig. 1G), and a *slmb* transgene rescues the neoplastic phenotype (supplementary material Fig. S1). The existing alleles *slmb*^{1} and *slmb*^{2} have been widely used, and epithelial organization phenotypes have not been reported. Sequencing revealed that these contain missense mutations in the fifth and seventh WD40 domains

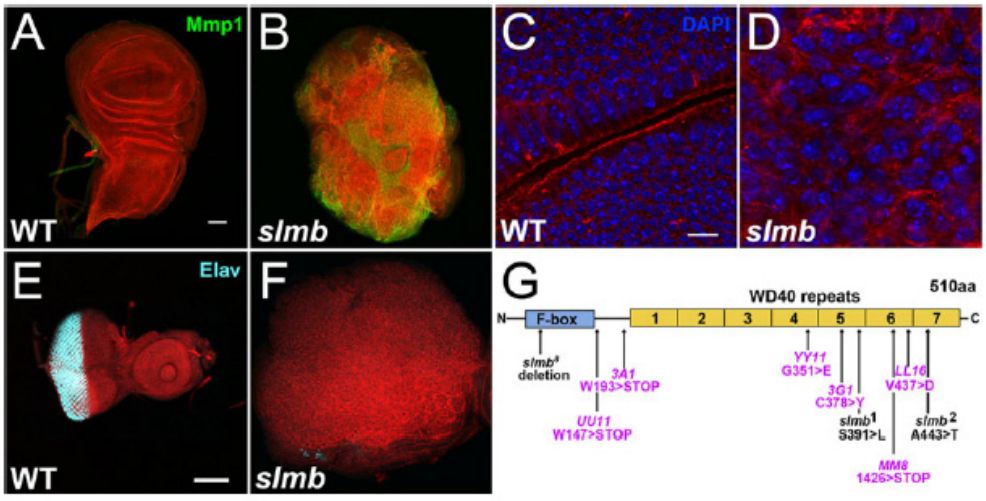

Figure 1: *slmb* is a novel neoplastic tumor suppressor gene.
(A-D) *slmb* wing discs show upregulation of Mmp1 (green) and loss of epithelial organization. DNA (DAPI), blue; F-actin (Phalloidin stain) is red in all figures. (E,F) *slmb* eye discs fail to differentiate. Elav, cyan. (G) Domain structure of Slmb and identified lesions. *MENE(3R)-B* alleles in violet text. Scale bars: 50 μm in A,E; 10 μm in C.

(Fig. 1G), indicating that both may be hypomorphic. We confirmed that *slmb*^{1} and *slmb*^{2} mutant discs show no and only a limited degree of neoplastic transformation, respectively (supplementary material Fig. S1). However, discs predominantly mutant for the deletion allele *slmb*^{8} (Milétich and Limbourg- Bouchon, 2000) show neoplasia, confirming that this phenotype is induced only by strong alleles. Null mutations in *Roc1a*, which encodes a frequent component of the SCF^{Slmb} complex (Noureddine et al., 2002), also show neoplasia (supplementary material Fig. S1). These data demonstrate that *slmb* functions as a new neoplastic TSG, and suggest that it does so via its role in the SCF^{Slmb} E3 ligase.

Slmb restricts apical polarity

Known neoplastic TSGs regulate epithelial polarity. We therefore analyzed *slmb* tissue with markers for polarized membrane domains. In wild-type (WT) imaginal epithelia, the transmembrane proteins Cadherin 87 (Cad87) and Fasciclin III (FasIII) occupy complementary apical and basolateral membrane domains. In *slmb* cells, Cad87 is distributed ectopically around the cell circumference in discontinuous domains that sometimes overlap with FasIII (Fig. 2A,B). Similar effects are seen with the polarized

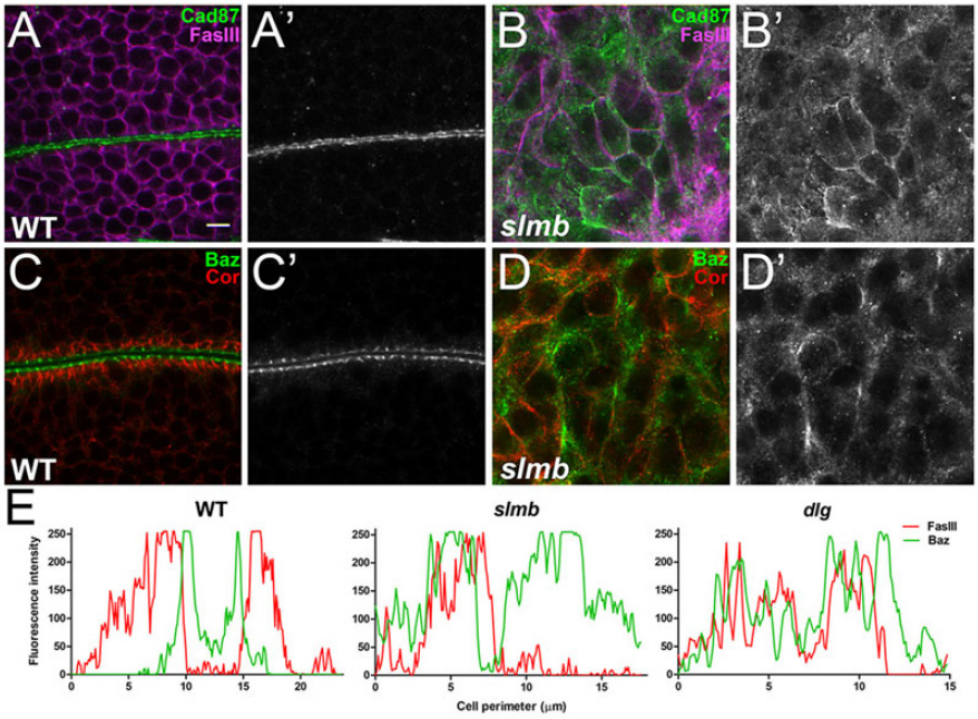

Figure 2: Slmb represses apical polarity.
(A-D') WT and *slmb* wing discs stained for the transmembrane proteins Cad87 (apical, green) and FasIII (basolateral, magenta) (A,B) or peripheral proteins Baz (apical, green) and Cor (basolateral, red) (C,D). In the absence of Slmb, distinct domains are lost and apical proteins expand around the cell cortex. A'-D' show single-channel images of apical markers. (E) Quantitation of Baz and FasIII staining along plasma membrane profiles of representative WT, *slmb* and *dlg* cells documents apical expansion. The *x*-axis is the length along the cell perimeter; 0 is the arbitrary spot where the measurement began. Scale bar: 5 μm.

peripheral membrane proteins Bazooka (Baz; *Drosophila* Par-3) and Cor (Fig. 2C,D). Expanded apical domains of *slmb* tissue resemble those of Scrib module mutants (Fig. 2E), and indicate that Slmb also acts as an apical antagonist.

To explore the breadth of Slmb function in cell polarity, we attempted to generate clones of strong alleles in other tissues. We were generally unable to recover clones in the follicle cell epithelium, and females carrying germline clones failed to produce eggs, preventing analysis of embryonic epithelia (data not shown). Clones in the larval central nervous system showed defective optic lobe organization. Intriguingly, clones derived from type I neuroblasts frequently contained multiple neuroblast-like cells, suggesting that Slmb might also be required for asymmetric cell division (supplementary material Fig. S2) (Li et al., 2014).

Slmb does not regulate endolysosomal trafficking

Of the known Par antagonists, only the Scrib module and endocytic components have been shown to strongly regulate imaginal basolateral polarity, raising the possibility that *slmb* might act primarily via one of these pathways. We first asked whether either Scrib or endocytic components promote Slmb-mediated protein degradation. However, Armadillo (Arm) levels (Jiang and Struhl, 1998) are not increased in *scrib* or *AP-2σ* depleted tissue (Fig. 3A-C), nor was there evidence for misregulation of other Slmb targets (supplementary material Fig. S3). We next asked whether Slmb might act through either the Scrib module or endocytic regulators to restrain apical polarity. To test whether Slmb interferes with AP-2- mediated endocytosis, we analyzed Notch trafficking and used a lysosomal inhibitor to monitor accumulation in the endolysosomal pathway (Windler and Bilder, 2010). Endocytic mutants prevent this accumulation (Fig. 3E), but in *slmb*, as in WT and Scrib module mutant discs, Notch is internalized and trafficked appropriately to endolysosomal compartments (Fig. 3D,F,G). Consistent with a lack of involvement in endocytic regulation, heterozygosity for *slmb* does not enhance a weak *avalanche* (*Syntaxin 7*) RNAi phenotype, which is sensitive to the dosage of endocytic regulators of polarity (Morrison et al., 2008). Taken together, these data fail to support a general endocytic role for Slmb.

Slmb and Scrib regulate polarity via distinct but parallel pathways

To test whether Slmb might directly influence Scrib module activity, we examined protein localization. A distinctive feature of *scrib* and *dlg* mutants is that, although many proteins are mispolarized, Dlg is specifically lost from the plasma membrane of *scrib* mutants, as is Scrib in *dlg* mutants (Bilder et al., 2000). By contrast, examination of *slmb* mutant cells reveals that both Scrib and Dlg retain tight, albeit dysregulated, cortical localization (Fig. 3H-M). Additionally, heterozygosity for *slmb* does not enhance weak *lgl-RNAi* nor *lgl* or *dlg* hypomorphic phenotypes, all of which are sensitive to the dosage of Scrib module components (H. A. Morrison and B. D. Bunker, PhD theses, University of California Berkeley, 2010). Thus, despite the many phenotypic similarities, the failure to control Scrib and Dlg membrane recruitment and the lack of genetic interaction suggest that Slmb regulates polarity in parallel to the Scrib module.

Misregulation of known substrates cannot account for the *slmb* null phenotype

The strong neoplastic phenotype seen in *slmb* tissue points to the existence of a polarity-regulating substrate, the levels of which must be controlled. We therefore examined known Slmb substrates to see whether any could account for this phenotype (supplementary

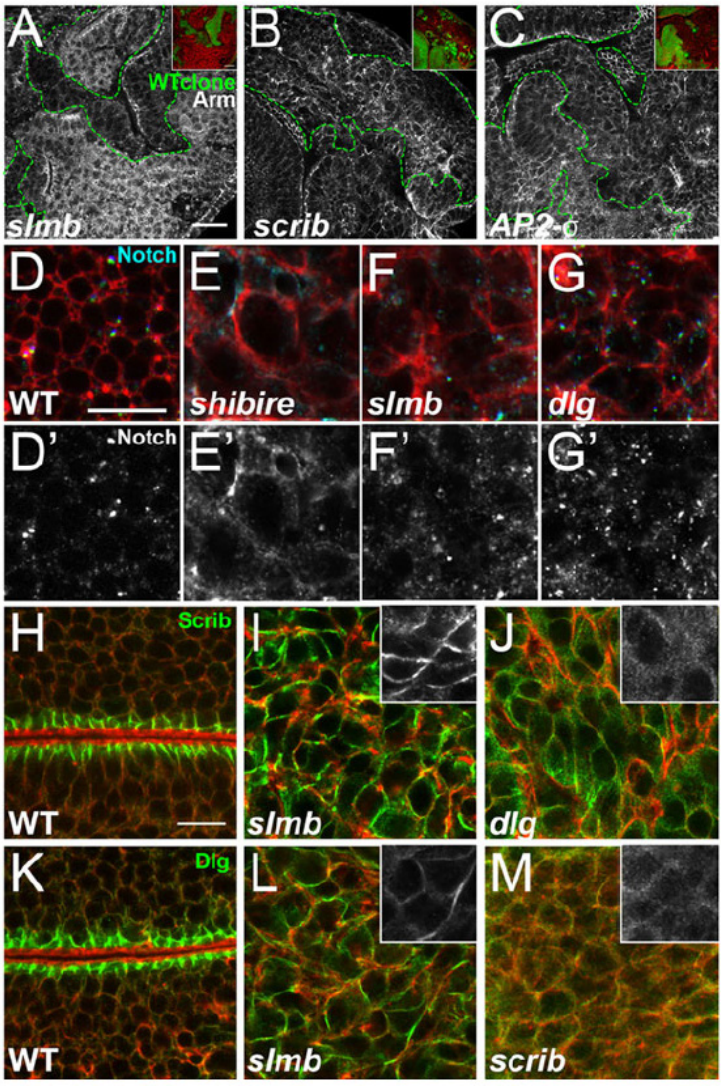

Figure 3: Slmb is not a regulator of endolysosomal traffic and acts in parallel to the Scrib module. (A-C) *slmb* cells exhibit increased Arm, whereas *dlg* and *AP-2σ* cells do not. Inset shows clonal boundaries. (D-G') Wing discs cultured with lysosomal inhibitor and stained for Notch. Notch is trapped prior to the lysosomal accumulation in *shibire* tissue, but in *slmb* or *dlg* tissue is trafficked similar to in WT. D'-G' show Notch (cyan) and F-actin (red) staining. (H-M) Scrib and Dlg remain cortical in *slmb* mutants, but are lost from the plasma membrane in *dlg* or *scrib* mutants. Insets show higher magnification of Scrib (I) or Dlg (L) localization. Scale bars: 25 µm in A; 10 µm in D,H.

material Fig. S4). Both Arm and Cubitus interruptus (Ci) are subject to proteolytic regulation by Slmb. Cells individually expressing or co-expressing active, non-degradable forms of Arm and Ci display a degree of hyperplastic overgrowth, consistent with known roles in the imaginal disc, but retain normal polarity and tissue architecture and do not upregulate Mmp1 (supplementary material Fig. S4A). Overexpression of stabilized Plk4 (Sak kinase –

FlyBase) and Cap-H2 (Buster et al., 2013; Rogers et al., 2009) caused no growth or polarity phenotypes (supplementary material Fig. S4B,C). Overexpression of a stabilized form of the polarity kinase Par-1, recently shown to be an Slmb substrate at *Drosophila* synapses (Lee et al., 2012), also failed to phenocopy *slmb* loss. This suggests that an unidentified Slmb substrate normally regulates epithelial organization in imaginal discs.

aPKC is required for slmb-mediated neoplastic transformation

An attractive candidate target of Slmb-mediated polarity regulation is the Par complex component aPKC. Overexpression of activated aPKC is sufficient to expand the apical domain and confer neoplastic phenotypes similar to those of *slmb* tissue (Fig. 4O) (Eder et al., 2005). Intriguingly, one predicted isoform of aPKC (aPKC-G) contains two Slmb-binding degrons and, when expressed in S2 cells, is degraded in a *slmb*-dependent manner (supplementary material Fig. S4). However, *aPKC-G* transcripts are present at very low levels in L3 discs, and expression of a degron-lacking aPKC-G had no effect on disc polarity or growth (supplementary material Fig. S4). We then used an antibody directed against a shared protein region to analyze total aPKC and found that it is mispolarized in *slmb* tissue and more widely distributed around the plasma membrane (Fig. 4A,B). However, aPKC levels are not obviously elevated, as assessed by immunohistochemistry, when compared with neighboring WT cells, in contrast to the evident elevation seen with Arm (Fig. 3A). We quantitated western blots of disc lysates which indicated a modest but not significant elevation of aPKC in *slmb* versus WT(Fig. 4C). These data suggest that, although aPKC is mislocalized in *slmb* cells, it is unlikely to be a target of *slmb*-mediated degradation.

Despite the absence of elevated levels of aPKC, we found multiple signs of increased aPKC activity in *slmb* discs. Excessive aPKC activity drives neoplastic overgrowth in part through upregulation of JAK-STAT pathway ligands, mediated by the Yorkie (Yki) transcription factor (Doggett et al., 2011;Robinson and Moberg, 2011; Sun and Irvine, 2011). *slmb* discs show robust activation of a STAT signaling reporter, and their neoplastic phenotype is sensitive to the dosage of *yki* (Fig. 4D-G). A second aPKC-regulated process is seen upon overexpression of the Crb intracellular domain in photoreceptors (Fig. 4H,I) (Tanentzapf and Tepass, 2003); the resulting morphogenetic defects are dependent on aPKC (Fig. 4J). Although heterozygosity for *slmb* does not enhance endocytic or Scrib module phenotypes, it does robustly enhance Crb overexpression (Fig. 4K). Finally, elevated aPKC activity is sufficient to induce trafficking defects of the retromer-dependent transmembrane cargoWntless (Eaton, 2008), leading to a distinctive subcortical trapping phenotype (Fig. 4L,N; plasma membrane localization, 73±12% for WT versus 52±13% for $aPKC^{Act}$) (de Vreede et al., 2014); *slmb* mutant tissue phenocopies this trapping (Fig. 4M; plasma membrane localization, 44±25%; $P<2\times10-5$ versus WT, $P=0.14$ versus $aPKC^{Act}$).

To directly test the functional involvement of hyperactive aPKC, we reduced aPKC activity in *slmb* cells using a weak dominant-negative construct ($aPKC^{DN}$) that has no effect on WT cells but can suppress phenotypes driven by elevated aPKC activity (Sotillos et al., 2004). Strikingly, expression of $aPKC^{DN}$ in *slmb* clones strongly suppressed tumorous growth (Fig. 4P-S). The resultant clones were smaller than WT clones, suggesting that aPKC activity also promotes *slmb* survival in this context, perhaps because tumorous *slmb* cells are 'addicted' to oncogenic aPKC, the excess activity of which allows them to survive in the presence of other misregulated *slmb* substrates. This reliance demonstrates a specific requirement for aPKC in *slmb* tissue and, along with the above results, reveal that Slmb acts as a negative regulator of aPKC activity.

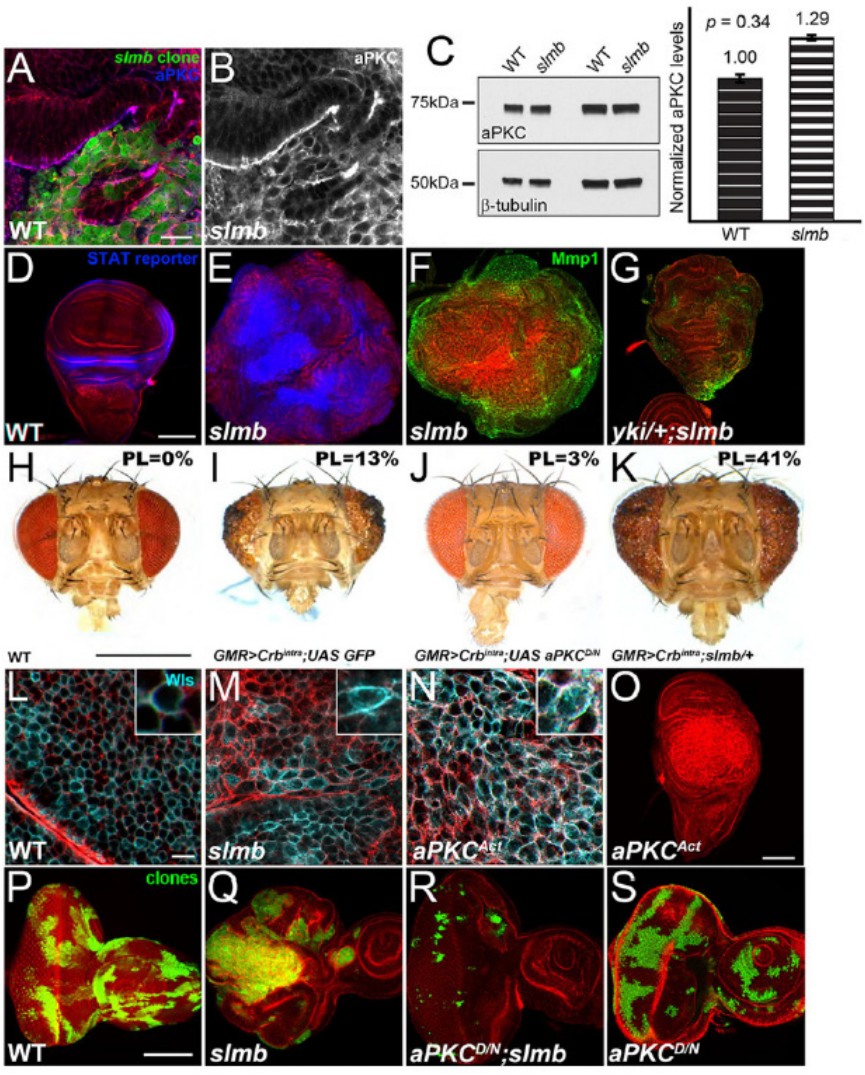

Figure 4: Slmb limits aPKC activity to prevent neoplasia.
(A,B) aPKC localization is expanded in *slmb* cells. (C) Western blots of WT and *slmb* wing discs. aPKC levels are normalized to β-tubulin and quantitated. Error bars indicate s.e. (D,E) A JAK/STAT pathway reporter (blue) is elevated in *slmb*. (F,G) The *slmb* neoplastic phenotype is sensitive to levels of *yki*. (H-K) Crb^{intra} expression in developing photoreceptors causes eye defects. This phenotype reflects excess aPKC activity and is enhanced by loss of one copy of *slmb*. PL, degree of pupal lethality. (L-N) Wntless (Wls, cyan) is cortical in WT cells but accumulates subcortically in *slmb* mutant and activated aPKC-expressing cells. (O) Neoplastic transformation driven by excess aPKC activity in the wing pouch. (P-S) *slmb* MARCM clones (GFP positive) grow larger than WT clones but are strongly reduced when $aPKC^{DN}$ is expressed. $aPKC^{DN}$ alone does not affect growth or survival. Scale bars: 10 µm in A,L; 100 µm in D,O,P; 400 µm in H.

Conclusions

Here, we extend the mechanisms involved in epithelial polarity to include a new function: targeted protein degradation. Targeted degradation can create spatial asymmetries in protein distributions, and there is precedent for roles of E3 ubiquitin ligases, including SCF^{Slmb} (Li et al., 2014; Morais-de-Sá et al., 2013), in polarizing different aspects of cells. The involvement of Slmb in *Drosophila* apicobasal polarity has gone unnoticed due to the previous use of hypomorphic alleles. The strong alleles described here display potent expansion of the apical pole of imaginal epithelia, demonstrating that Slmb is a new polarity regulator that functions to restrict the apical domain.

Loss of Slmb phenocopies the polarity defects associated with mutations in two classes of 'apical antagonists': the Scrib module of core polarity regulators, and endocytic regulators that control trafficking through the early endosome. Despite the similar polarity defects, *slmb* mutations do not alter endolysosomal cargo traffic, nor do they display protein recruitment defects characteristic of Scrib module mutants; furthermore, no genetic interactions are seen with either pathway. Nevertheless, the downstream consequences of polarity misregulation – including tumor-like transformation and the upregulation of specific target genes – are again shared between *slmb* and the other apical antagonists, and, moreover, *slmb* and Scrib module mutant cells share a distinctive trafficking defect associated with elevated aPKC activity. We therefore suggest that Slmb acts in parallel to the Scrib module to antagonize the Par complex and other apical regulators.

The role for Slmb defined here points to the existence of an apical polarity-regulating protein substrate, the levels of which must be controlled. We have ruled out a number of validated Slmb substrates as the relevant target. Bioinformatic scans of *Drosophila* proteins for Slmb degron sequences suggest other candidates, including Expanded (Ex), but overexpression of Ex is not sufficient to induce polarity defects resembling those of *slmb* (Blaumueller and Mlodzik, 2000; Fernández et al., 2011). Although we cannot rule out a contribution from the elevation of multiple substrates, *slmb*-like polarity phenotypes can be induced by the elevated activity of individual proteins, including Crb or aPKC. Despite evidence that aPKC undergoes ubiquitin-mediated degradation in embryos (Colosimo et al., 2009), neither aPKC nor Crb levels appear to be controlled by Slmb-mediated degradation in imaginal discs. Nevertheless, our data together suggest that the substrate of Slmb in polarity regulation will function as a positive regulator of aPKC-driven outcomes.

Our demonstration that Slmb limits aPKC activity to distinguish the epithelial basolateral domain reveals intriguing parallels to polarization of the worm zygote. In this context, Par-2 is the primary antagonist that restricts aPKC/Par activity, while Lgl homologs function in a parallel, redundant role. Par-2 contains a RING finger domain that is characteristic of single-subunit E3 ligases, but Par-2 homologs have not been identified outside of nematodes, Par-2 does not affect aPKC/ Par levels, and a degraded substrate in polarity regulation has yet to be identified (St Johnston and Ahringer, 2010; Zonies et al., 2010). The discovery of a *Drosophila* E3 ligase with a similar function to Par-2 raises the possibility of a conserved molecular logic to polarity in these two paradigmatic systems; determination of the relevant substrate will shed further light on this question.

MATERIALS AND METHODS

Fly stocks and genetics

Predominantly mutant imaginal discs were generated as described previously (Menut et al., 2007). *slmb* mutant images represent *slmb*^{UU11} unless otherwise specified. For further details of fly stocks and genetics and the generation of mutants and clones, see supplementary Materials and Methods.

Immunohistochemistry and microscopy

Western blots loaded with equivalent protein concentrations were probed with anti-β-tubulin (E7, Developmental Studies Hybridoma Bank) and anti-aPKC (sc-216, Santa Cruz Biotechnology). Two biological replicates for each of six WT and four mutant technical replicates were quantitated.

Wandering L3 imaginal discs, larval CNS and ovaries were dissected in PBS on ice, fixed in 4% formaldehyde in PBS for 20 min at room temperature and stained using standard procedures (Bilder and Perrimon, 2000). Primary and secondary antibodies and stains are described in the supplementary Materials and Methods. Fluorescent tissues were mounted in SlowFade (Molecular Probes). Images are single sections taken with Leica TCS or Zeiss 700 confocal microscopes except for adult heads, which were frozen, mounted in agar and photographed using a Leica Z16 APO microscope and DFC300 FX camera. Images were assembled with Adobe Photoshop or Illustrator. Image quantitation used Fiji (Schindelin et al., 2012): cortical fluorescence intensity plots measured gray values along single-cell tracings; Wls cortical localization measured correlation coefficients with phalloidin staining.

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Author contributions

L.C.S., S.L.W. and D.B. designed the research; L.C.S., G.d.V., G.C.R. and S.L.W. performed the experiments; L.C.S., G.d.V., S.L.W., G.C.R. and D.B. analyzed the data; L.C.S., G.d.V., S.L.W. and D.B. wrote the manuscript.

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SUPPLEMENTARY MATERIALS AND METHODS

Fly stocks and genetics

Mutants used included *slmb*^{1}, *slmb*^{2}, *slmb*^{8}, *dlg*$^{40\text{-}2}$, *Roc1a*^{G1}, *AP-2sigma* and *yki*^{B5} (described in FlyBase). Transgenes included *tub>slmb-myc* (Ko et al., 2002), *UAS-Ci*$^{M1\text{-}4}$ (Chen et al., 1999), *UAS-Arm*^{S10} (Pai et al., 1997), *UAS-Plk4*^{SBM} (Rogers et al., 2009), *UAS-CapH2*^{SBM} (Buster et al., 2013), *UAS-Par1*^{T408A} (Lee et al., 2012), *UAS-aPKC*$^{CAAX\text{-}DN}$ (Sotillos et al., 2004) and UASaPKC$^{\Delta N}$ (Betschinger et al., 2003) driven by *MS1096-GAL4*, as well as *hs-Wls-V5* and *UAS-Wls-V5* (Belenkaya et al., 2008). Entirely mutant wing discs were generated using *UbxFLP/FM7; cl FRT82B/TM6B* and entirely mutant eye discs were generated using e*yFLP cl GMRhid FRT82B/TM6B*. MARCM clones in the eye and neuroblast were generated with *eyFLP* and *hsFLP* stocks, respectively. Follicle cell clones were generated as described (Lu and Bilder, 2005).

Immunohistochemistry

The following primary antibodies were used: mouse anti-Mmp1 (1/100), mouse anti-Arm (N27A1, 1/100), mouse anti-Dlg (4F3, 1/100), mouse anti-Coracle (1/100), mouse anti-FasIII (7G10, 1/20), mouse anti-Notch^{ECD} (C458.2H, 1/50), mouse anti-Lamin (1/100), rat anti-Elav (9F8A9, 1/50) (all from Developmental Studies Hybridoma Bank, see references therein), rat anti-Crb (1/750; U. Tepass, E. Knust), guinea pig anti-Cad87E (1/1000; U. Tepass), guinea pig anti-Scrib (1/200), rabbit anti-PKCζ (sc-216, Santa Cruz Biotechnology, 1/200), rabbit anti- Miranda (1/500), mouse anti-Prospero (1/100). TRITC-phalloidin was used to visualize F-actin (1/400, Sigma) and either TO-PRO-3 (1/400) or DAPI (1/3000) was used to visualize DNA. Secondary antibodies were from Molecular Probes.

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SUPPLEMENTARY FIGURES

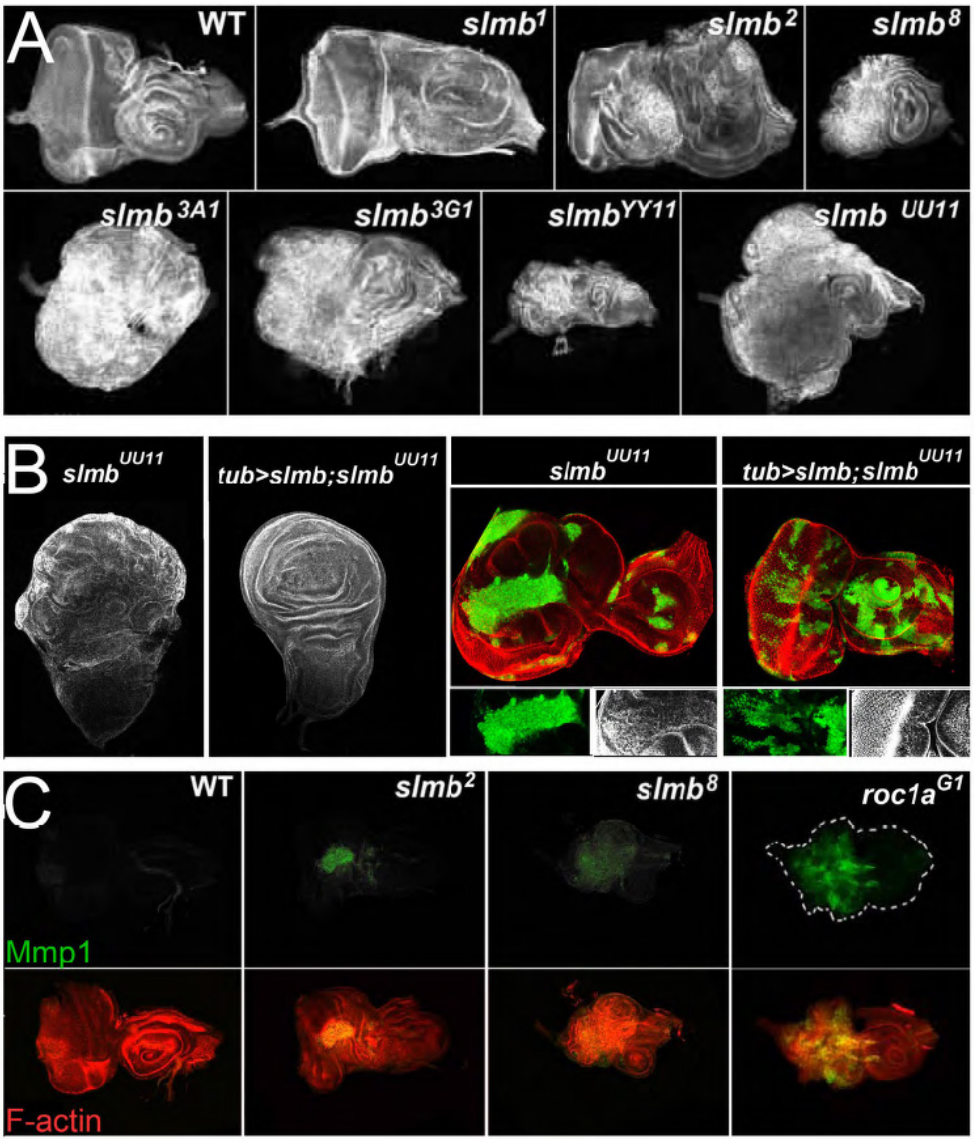

Figure S1: Analysis of *slmb* allelic series.
(A) Phalloidin staining of *slmb* mutant eye discs demonstrates that strong alleles show the most severe neoplastic transformation. (B) A *slmb* transgene rescues the neoplastic phenotypes of *UU11* in predominantly mutant wing discs and GFP-marked eye disc mosaics. (C) Discs derived from the deletion allele *slmb*^{8} and null mutation in the SCF core component *roc1a* also display hallmarks of neoplasia, including disrupted F-Actin and upregulation of Mmp1.

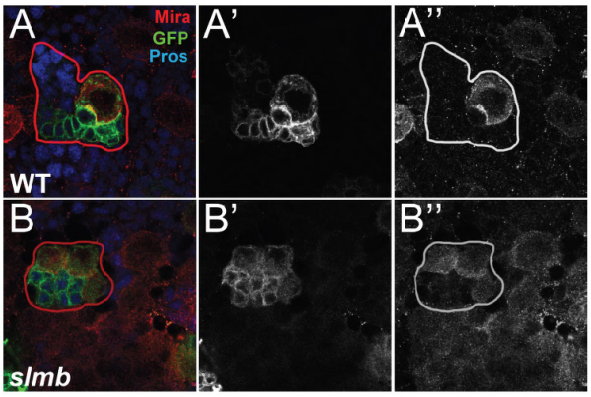

Figure S2: Effect of loss of slmb in neuroblasts.
(A, B) GFP marks clones generated using the MARCM system. Larval type I neuroblasts divide asymmetrically to produce a new Miranda-positive neuroblast (red) and a smaller ganglion mother cell that will differentiate into a neuron or glia (Prospero positive, blue). *slmb* mutant neuroblasts display defects in asymmetric cell division, with a fraction of clones containing multiple Miranda- positive neuroblast-like cells.

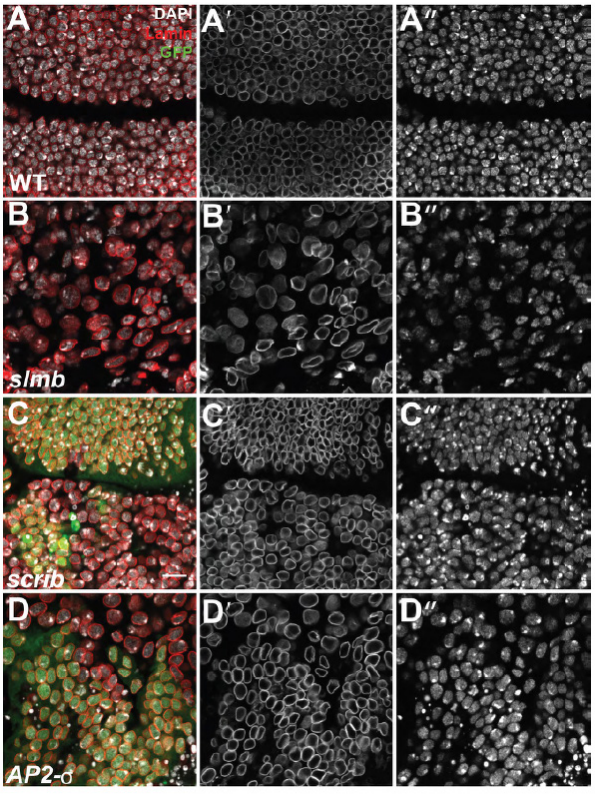

Figure S3: Junctional scaffold and endocytic class TSGs do not regulate Slmb activity.
(A, B) Cells mutant for strong *slmb* alleles show chromosome condensation defects leading to a swollen nuclear lamina, reflecting misregulation of Condensin components. (C,D) In contrast, *scrib* and *AP2-sigma* mutant cells have WT nuclei and lamina size. Presence of GFP marks mutant cells. Scale, 10 µm.

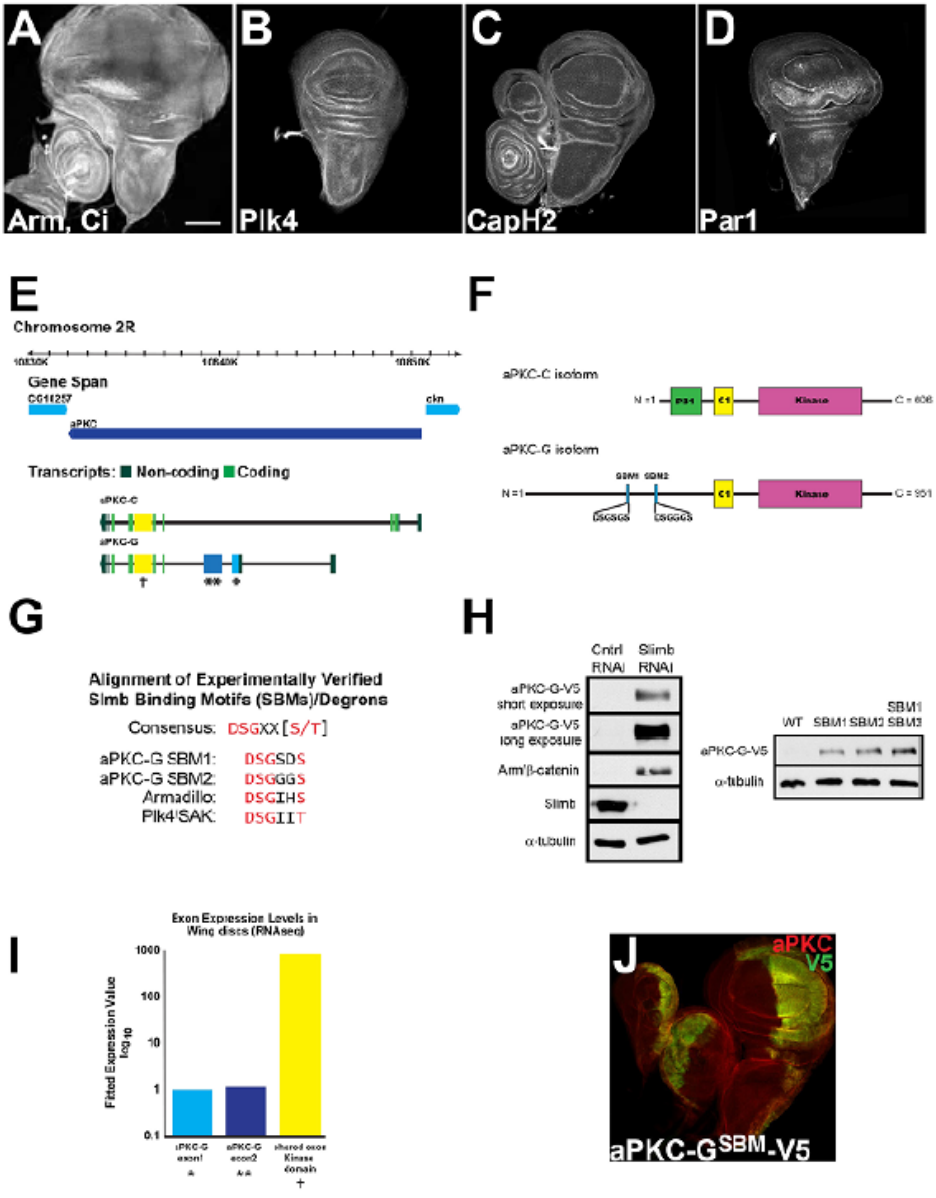

Figure S4: Misregulation of known substrates cannot account for the slmb phenotype.
(A-D) Overexpression of stabilized versions of known Slmb substrates throughout the presumptive wing pouch and notum using *MS-1096GAL4* does not phenocopy loss of *slmb*. (E, F) Gene and protein models comparing a common aPKC isoform C with the G isoform containing two Slmb binding motifs (SBM). (G) Alignment of aPKC-G SBMs with experimentally validated SBM degrons from other Slmb targets. (H) Western blots demonstrating that RNAi mediated knockdown of *slmb* in S2 cells results in stabilization of the aPKC-G isoform, as does mutation of the SBMs. (I) RNA-seq data from third instar wing discs comparing levels of the unique aPKC-G exons with an exon encoding the shared Kinase domain; values shown are derived from RPKM. (J) Overexpression of a stabilized version of aPKC-G in the posterior domain of the wing disc (*en>GFP*, green) does not affect polarity or growth. Scale bar: 100 µm in A

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CHAPTER 4:

A *Drosophila* Tumor Suppressor Gene Prevents Tonic TNF Signaling through Receptor N-Glycosylation

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ABSTRACT

Drosophila tumor suppressor genes have revealed molecular pathways that control tissue growth, but mechanisms that regulate mitogenic signaling are far from understood. Here we report that the *Drosophila* TSG *tumorous imaginal discs (tid)*, whose phenotypes were previously attributed to mutations in a DnaJ-like chaperone, are in fact driven by the loss of the N-linked glycosylation pathway component ALG3. *tid/alg3* imaginal discs display tissue growth and architecture defects that share characteristics of both neoplastic and hyperplastic mutants. Tumorous growth is driven by inhibited Hippo signaling, induced by excess Jun N-terminal kinase (JNK) activity. We show that ectopic JNK activation is caused by aberrant glycosylation of a single protein, the fly tumor necrosis factor (TNF) receptor homolog, which results in increased binding to the continually circulating TNF. Our results suggest that N-linked glycosylation sets the threshold of TNF receptor signaling by modifying ligand-receptor interactions and that cells may alter this modification to respond appropriately to physiological cues.

INTRODUCTION

Tumorigenesis is ultimately driven by dysregulated cellular signaling that promotes unchecked proliferation (Hanahan and Weinberg, 2011). Proliferation-regulating signaling pathways in animals are therefore normally under tight control, to prevent aberrant growth. The primary mechanism of signaling regulation is limited availability of ligand, although levels of receptor can also be regulated, as can receptor availability on the plasma membrane or even its polarized localization. A full understanding of the mechanisms that limit mitogenic signaling is an important goal of both basic biology and cancer research.

Major insight into growth regulation has arisen from research in model organisms such as *Drosophila melanogaster*. For instance, *Drosophila* studies revealed key steps of receptor tyrosine kinase signaling and uncovered the phenomenon of cell competition (Amoyel and Bach, 2014; Duffy and Perrimon, 1994; Shilo, 1992; Simpson, 1979; Simpson and Morata, 1981). Additional insight into growth regulatory mechanisms has come from the analysis of fly tumor suppressor genes (TSGs) (Hariharan and Bilder, 2006; Richardson and Portela, 2017). Disruption of a single fly TSG is sufficient to cause overproliferation in epithelial organs of the larva called imaginal discs. Initial genetic screens identified several classes of fly TSGs. The neoplastic TSGs (*discs large, lethal giant larvae*, and *scribble*) (Bilder and Perrimon, 2000; Schneiderman and Gateff, 1967; Stewart et al., 1972) revealed an intimate link between cell polarity and cell proliferation control, a principle also relevant to human cancers. The hyperplastic TSGs, including *hippo, warts,* and *salvador*, uncovered the novel Hippo (Hpo) signal transduction pathway, which is now recognized as a conserved growth control mechanism (Harvey et al., 2003; Jia et al., 2003; Justice et al., 1995; Kango-Singh et al., 2002; Pantalacci et al., 2003; Tapon et al., 2002; Udan et al., 2003; Wu et al., 2003; Xu et al., 1995). Even less prominent *Drosophila* TSGs such as *lethal giant discs* have demonstrated important biological concepts (Buratovich and Bryant, 1995; Klein, 2003).

One classic *Drosophila* TSG that remains understudied is *tumorous imaginal discs* (*tid*) (Gateff, 1978; Loffler et al., 1990). Imaginal discs of *tid* homozygous larvae develop into overgrown masses (Kurzik-Dumke et al., 1995). Genetic mapping and cytogenetic analyses attributed this phenotype to loss of a conserved molecular chaperone of the DnaJ family (Kurzik-Dumke et al., 1995). Evidence for a tumor-suppressive role for a mammalian homolog, hTid-1, has been presented (Chen et al., 2009; Copeland et al., 2011; Kurzik-Dumke et al., 2008). However, the exact molecular mechanism through which *tid* could regulate cell and tissue proliferation remains mysterious.

We report here that the *tid* gene was cloned incorrectly. Aberrant cell proliferation in the *Drosophila* mutant arises not from disruptions to the DnaJ homolog but rather to an adjacent gene that encodes the mannosyltransferase ALG3, involved in N-linked glycosylation. We show that overgrowth in *tid/ALG3* mutants is caused by mis-glycosylation of a single transmembrane protein, the *Drosophila* tumor necrosis factor (TNF) receptor homolog Grindelwald, which results in downstream activation of Jun N-terminal kinase (JNK) and inactivation of the growth-suppressing Hpo pathway. Our results suggest that this post-translational modification modulates ligand-receptor affinity in the TNF receptor (TNFR) pathway and thus provides a regulatory mechanism setting a dynamic threshold for JNK-mediated stress signaling and growth control.

RESULTS

Tumorous Phenotypes of tid Mutants

The mutant phenotype of the classic *Drosophila* TSG *tumorous imaginal discs* (*tid*) was described in 1992 (Kurzik-Dumke et al., 1992). *tid* was reported to encode a DnaJ-like molecular chaperone (Kurzik-Dumke et al., 1995) but has received little attention since, prompting a reinvestigation. To characterize the loss of function phenotype, we generated tid^{1}/tid^{2} transheterozygous animals. As previously described, these mutants develop into "giant" L3 larvae bearing imaginal disc tumors. The disc proliferation rate is slow, such that *tid* discs are initially smaller than discs from comparably aged wild-type (WT) larvae. However, mutant larvae delay puparium formation up to 11 days, during which growth continues. *tid* discs show clear organizational defects, displaying abnormal thickness and tissue folding; cells have altered shape and F-actin levels are elevated (Figures 1A, 1B, S1A, and S1B). Similar phenotypes are observed in *tid* hemizygous animals. We directly counted dissociated wing disc cells and found that, prior to pupation, *tid* mutants contain almost double the amount of cells as WT (Figure 1C). Although more limited than the ~5-fold increase of a neoplastic mutant (*dlg*) or the ~3-fold increase of a hyperplastic mutant (*wts*) (Figures S1C– S1E), the increase demonstrates that loss of *tid* indeed causes tumorigenic overgrowth. To assess proliferative capacity beyond the larval stage, we transplanted *tid* discs into the abdomens of WT adult hosts. As previously described (Kurzik-Dumke et al., 1992), tumors recovered from these hosts after 17 days are extremely overgrown and display a strong increase in architectural disorganization (Figures 1D and 1E). The unchecked imaginal disc proliferation caused by *tid* mutations confirm that *tid* is a *bona fide* TSG.

tid Displays Characteristics of Neoplastic and Hyperplastic TSGs

To further investigate the mutant phenotype, we assessed mitotic clones. When homozygous *tid* eye discs in an otherwise heterozygous larva were generated, animals displayed the pupal lethal phenotype also seen when animals carry neoplastic TSG mutant eye discs (Menut et al., 2007). The eye discs display mild phenotypes compared with fully mutant animals (Figures S1F–S1I), suggesting that perdurance of the *tid* gene product or its substrates is too strong to effectively deplete using clonal strategies.

Unlike neoplastic mutants, apicobasal polarity markers showed no obvious disruption, although *tid* cells are more cuboidal than the columnar epithelium of WT or hyperplastic TSG mutant discs (Figures S1J–S1L). Other distinct properties of various TSG mutants include susceptibility to cell competition and cooperativity with oncogenes (Brumby and Richardson, 2003; Pagliarini, 2003). Clonal loss of neoplastic but not hyperplastic TSGs induces cell competition and elimination of the mutant cells; however, when combined with expression of oncogenic RasV12, neoplastic clones show dramatically synergistic overgrowth as well as tissue invasion. Using the MARCM (mosaic analysis with a repressible cell marker) system (Lee and Luo, 2001), we induced *tid* clones with or without RasV12 expression (Figures 1F–1I). *tid* clones were recovered but at an abundance lower than WT, indicating that they are partially outcompeted (Figure S1M). When *tid* clones also expressed RasV12, cooperative overgrowth was clearly observed, albeit less extreme than that between *scrib* and RasV12 and lacking invasion (Figure 1I). Thus, *tid* shares characteristics of both neoplastic and hyperplastic TSGs.

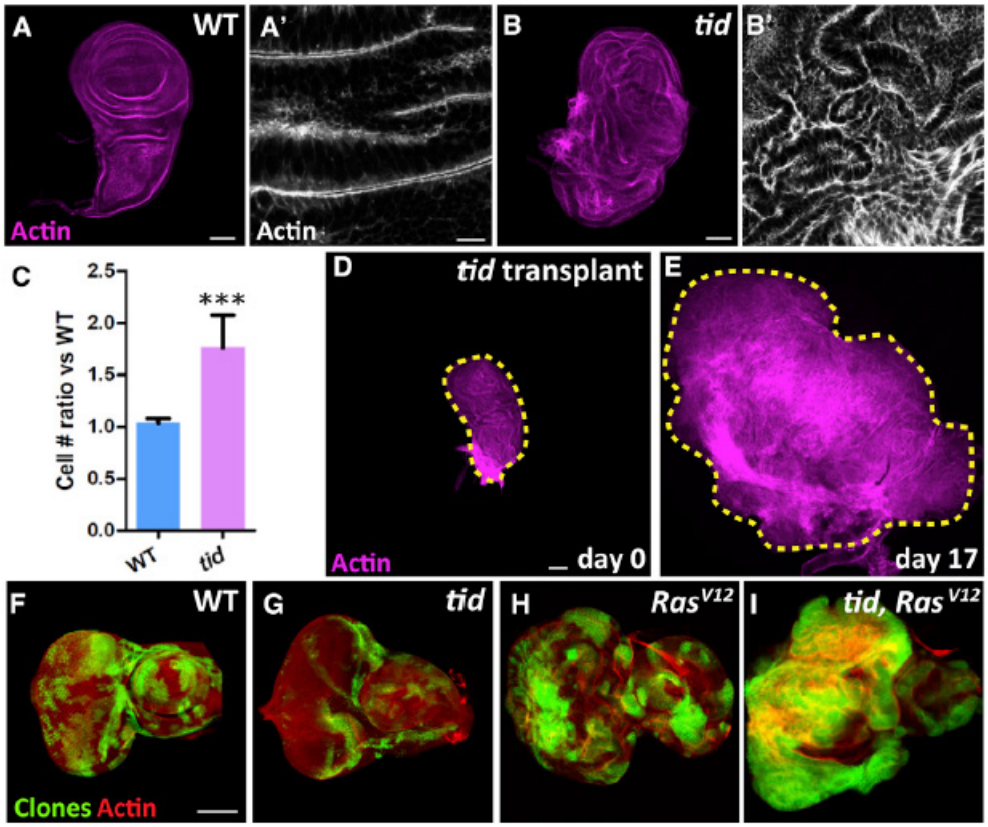

Figure 1: *tid* Is a Tumor Suppressor.
(A–E) Compared with WT (A), *tid* mutant wing imaginal discs (B) show disorganized tissue architecture and moderate overgrowth, quantified in (C). When *tid* tumors from a 10-day-old larva are transplanted into the abdomen of an adult host (D) they continue proliferating and overgrow dramatically, with more severe architectural defects (E). (F and G) *tid* clones (F) in the eye disc are partially outcompeted compared with WT (G). (H and I) *tid* mutations (H) cooperate with RasV12 (I) to strongly enhance overgrowth. ***p<0.001; error bars indicate SD. Images are representative of n≥10 animals per genotype. Scale bars: 100 μm in (A), (B), (D), and (F); 10 mm in (A'). See also Figure S1.

tid Mutants Disrupt *CG4804*

The original sequencing analysis of the tid^1 and tid^2 alleles failed to identify molecular lesions within any protein coding sequence. These experiments did identify a lesion in the 5′ UTR of the DnaJ-encoding *CG5504* in tid^2, leading to the suggestion that this lesion disrupted *CG5504* transcription (Kurzik- Dumke et al., 1995). However, the *CG5504* open reading frame (ORF) is found in the intron of a second ORF, *CG4084* (also called *neighbor of tid* [*l(2)not*]) (Figure 2A). Our sequencing of tid^1 and tid^2 identified lesions in the *CG4084* ORF in both alleles. tid^1 induces a nonsense mutation at glutamine 375, resulting in a truncation of the remaining 135 amino acids of the predicted CG4084 protein. In tid^2, we confirmed the previously reported molecular lesions: a 24 bp deletion and a single base pair insertion. Within the *CG4084* ORF, these lesions induce an in-frame deletion that removes amino acids 36–43, and a frameshift leading to premature

termination of the protein after 192 amino acids (Figure 2A). These data raise the possibility that loss of *CG4084* function, rather than *CG5504*, may in fact be responsible for the *tid* phenotype. To rigorously test this, we performed a rescue assay using constructs driving the *CG4084* ORF alone under either direct (*tubulin*) or upstream activating

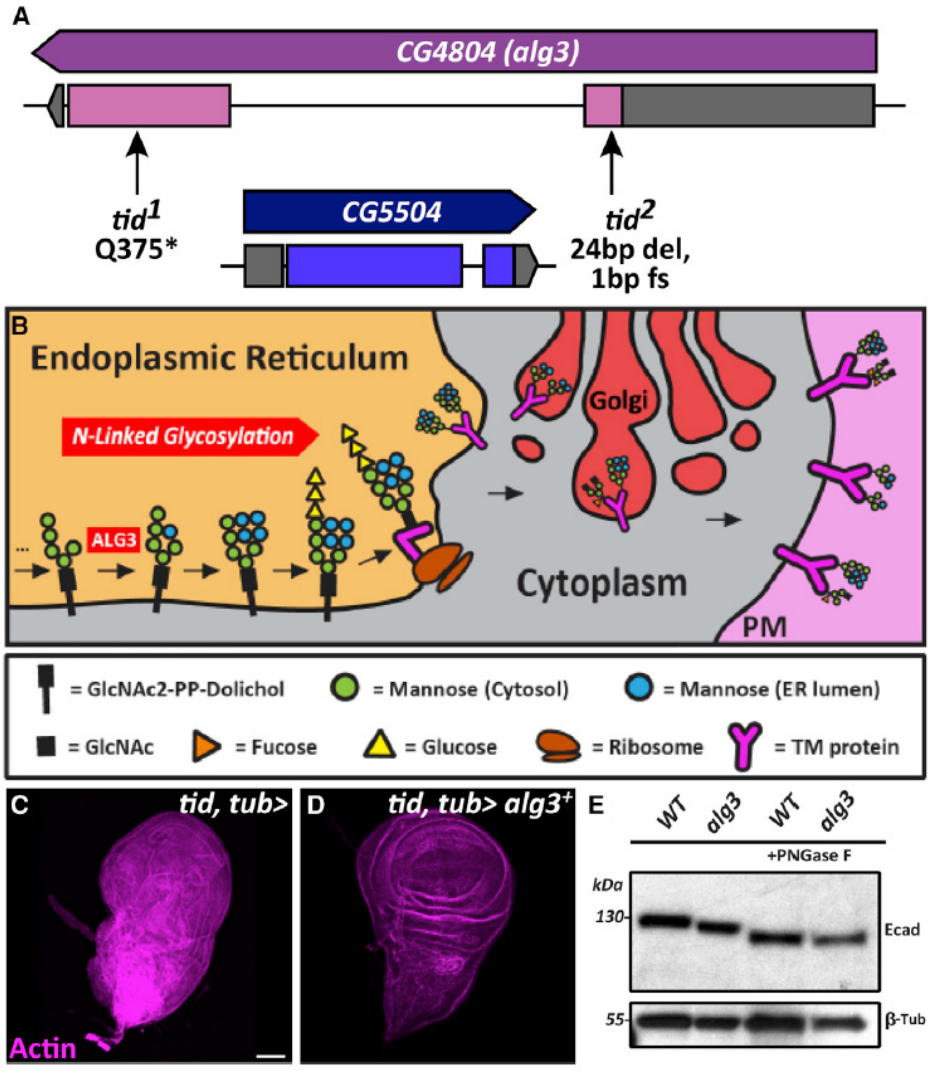

Figure 2: *tid* Mutants Disrupt *CG4804*, Encoding *Drosophila* ALG3.
(A) Schematic displaying the location of *tid*[1] and *tid*[2] lesions, both lying within the *CG4804* ORF. (B) Steps of lipid-linked oligosaccharide synthesis in the N-linked glycosylation pathway. ALG3 is the first mannosyltransferase that acts within the ER lumen. After sequential addition of mannose and glucose monomers, the core glycan is transferred to the target protein, which is transported to the plasma membrane after further glycan trimming and modification within the Golgi. PM, plasma membrane. (C and D) Ubiquitous expression of *CG4804* rescues the *tid* tumorous phenotype. (E) E-cadherin western blots reveal a mobility shift in *alg3* mutants relative to WT. PNGase treatment of E-cadherin generates equivalent shifts in both genotypes, indicating a partial glycosylation defect in *alg3*. Images are representative of n≥10 animals per genotype. Scale bar: 100 μm in (C). See also Figure S2.

sequence (UAS) control. When these constructs were expressed in either *tid*^{1} or *tid*^{2} flies, they rescued lethality, as well as both the imaginal disc (Figures 2C and 2D) and the pupal lethal phenotypes. We conclude that the *tid* gene was originally miscloned, and that the observed tumorous phenotype is actually due to loss of *CG4804* function.

CG4804 Encodes *Drosophila* ALG3, and Mutants Show Defective N-Glycosylation

We next sought to identify the molecular function of *CG4084*. BLAST searches revealed that *CG4084* is the single fly homolog of the yeast and human *Asparagine-Linked Glycosylation-3* (*ALG3*) genes. *ALG3* encodes a mannosyltransferase required for the biosynthesis of lipid-linked oligosaccharides in the ER N-glycosylation pathway, which is conserved from yeast to humans (Figure 2B). To avoid confusion with the previous annotation, we will refer hereafter to the *CG4084* gene as *alg3*, and to the *tid*^{1} and *tid*^{2} alleles as mutations in *alg3*. To confirm that *alg3* mutants are defective in N-glycosylation, we turned to biochemical analysis of E-cadherin (Ecad), which has four N-glycosylation sites (Figure 2E). In western blots, Ecad from *alg3* discs runs at a lower molecular weight than in WT discs. Following treatment with peptide-*N*-glycosidase (PNGase), an enzyme that completely removes N-linked glycans, the band shift in *alg3* discs is increased and is equivalent to that in PNGase-treated WT discs (Figure 2E). This demonstrates that *alg3* mutants have aberrant but not a complete lack of N-glycosylation, consistent with the role of Alg3 within the N-glycosylation pathway (Figure 2B). Related phenotypes were observed with mutants in *alg9*, which acts immediately after *alg3* in the N-glycosylation pathway (Figures S2A–S2E). These data indicate that proper N-glycosylation is required for normal epithelial growth and architecture.

Hpo-Dependent Yki Activation in *alg3* Mutants Drives Tumorous Overgrowth

In order to determine how N-glycosylation ensures normal disc growth, we investigated mitogenic pathways that might be activated in *alg3* tumors. Reporters for STAT, Notch, and Wg signaling, pathways that drive disc overproliferation in other mutants (Bach et al., 2003; Classen et al., 2009; Moberg et al., 2005; Pellock et al., 2007; Thompson et al., 2005; Vaccari and Bilder, 2005), were largely normal in *alg3* discs (Figures S3A–S3F). Mispolarization of aPKC, a hallmark of neoplastic TSG mutants, was also not seen (Figure S1K), and reducing aPKC levels did not affect the *alg3* phenotype (Figures S3G and S3H). N-glycosylation aids protein maturation in the ER, and perturbation of this process can trigger the unfolded protein response (UPR) (Davenport et al., 2008). A transgenic UPR reporter was strongly induced in *alg3* tissue, but inhibiting the UPR did not notably change its size or morphology (Figures S3K–S3M). Because both neoplastic and hyperplastic TSG mutants inhibit Hpo pathway kinases and induce pro-growth Yki activity (Menendez et al., 2010; Robinson and Moberg, 2011; Sun and Irvine, 2011), we investigated whether this pathway plays a role in *alg3* tumors. Interestingly, reporters for Yki activity were clearly upregulated in *alg3* as well as *alg9* mutant tissue (Figures 3A, 3B, and S2F–S2I). We then reduced Yki activity, using overexpression of the inhibitory kinases Hpo and Wts under conditions that do not affect WT disc growth. These manipulations significantly reduced *alg3* tumor size, albeit without restoring defective disc morphology (Figures 3C–3E), suggesting that *alg3* mutant overgrowth is driven by altered Hpo pathway activity.

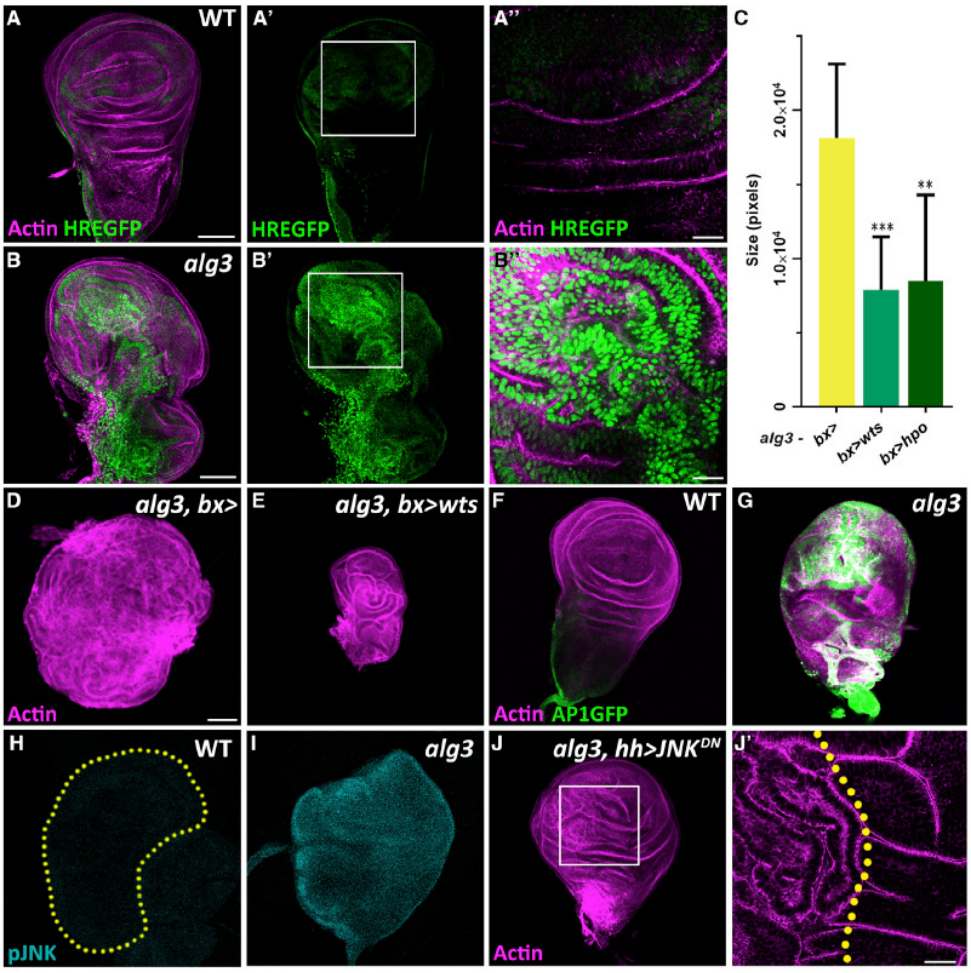

Figure 3: Hippo and JNK Drive the *alg3* Phenotype.
(A and B) The Hippo pathway reporter HREGFP is elevated in *alg3* discs (B) compared with WT (A). (C) Reducing Hpo growth signaling through Wts or Hpo overexpression shows a robust tumor size reduction. *alg3-bx>* n=28 discs, *alg3-bx>wts* n=31 discs, *alg3-bx>hpo* n=24 discs. (D and E) Expressing Wts in *alg3* discs rescues tumorous overgrowth but not architecture defects. (F–I) The JNK pathway reporters AP1GFP (F) and pJNK (H) are elevated in *alg3* discs (G and I). The WT wing disc is outlined in yellow. (J) Blocking JNK signaling in the posterior half of *alg3* discs reduces overgrowth and restores tissue architecture. Yellow line indicates antero-posterior compartment border. ***p<0.001; **p<0.01; error bars indicate SD. Images are representative of n≥10 animals per genotype. Scale bars: 100 μm in (A), (B), and (D); 10 μm in (A''), (B''), and (J'). See also Figures S2 and S3.

alg3 Mutants Inhibit Hpo Pathway via Excessive JNK Signaling

To assess how defective N-glycosylation could reduce Hpo pathway kinase activity, we considered candidate Hpo regulators, focusing on those altered in both neoplastic and hyperplastic tumors. The transmembrane apical polarity regulator Crumbs (Crb) has been implicated in Hpo signaling in multiple tumor types (Hamaratoglu et al., 2009; Robinson et al., 2010). However, depletion of Crb failed to influence the *alg3* phenotype (Figures

S3I and S3J). An alternative route by which Yki is activated is through the induction of the JNK pathway (Fernandez et al., 2011; Ohsawa et al., 2012; Robinson and Moberg, 2011; Sansores-Garcia et al., 2011; Sun and Irvine, 2011). Strikingly, a transgenic reporter for JNK signaling was strongly upregulated in *alg3* discs (Figures 3F and 3G). Elevated JNK activation was confirmed by immunostaining for phosphorylated JNK, which was clearly increased compared with WT (Figures 3H and 3I). To test the functional relevance of excessive JNK activity, we blocked signaling in one-half of *alg3* discs with a dominant-negative form of JNK. While this does not affect WT discs (Figure S3N), in *alg3* it resulted in a potent rescue of the phenotype (Figure 3J). Blocking JNK signaling not only inhibited tumorous overgrowth, it also restored local tissue architecture, reduced F-actin levels, and negated the extended larval stages of *alg3* larvae. The data suggest that Hpo-regulated Yki activation in *alg3* mutant discs is caused by excessive JNK signaling.

Glycosylated TNFR Drives JNK-Dependent Overgrowth

We next examined how the JNK signaling cascade could be triggered by aberrant N-glycosylation. Since N-glycosylation acts mainly on transmembrane and secreted proteins, one candidate route is through the *Drosophila* TNFR. Remarkably, RNAi-mediated depletion of the major fly TNFR (Flybase: *grindelwald*, *grnd*; Andersen et al., 2015) in one half of *alg3* discs induced a strong reversion of the tumorous phenotype, resembling the reversion induced by dominant-negative JNK (Figures 4A and 4B). Rescue was also induced by overexpression of the Grnd extracellular domain, which acts in a dominant-negative fashion in other contexts (Figures S4B and S4C) (Andersen et al., 2015). In accordance with a general role during defective N-glycosylation, RNAi depletion of *grnd* also rescued *alg9* mutant defects (Figure S2C). By contrast, depletion of a second *Drosophila* TNFR homolog (Flybase: *wengen*; Kanda et al., 2002) did not rescue *alg3* tumors (Figure 4C).

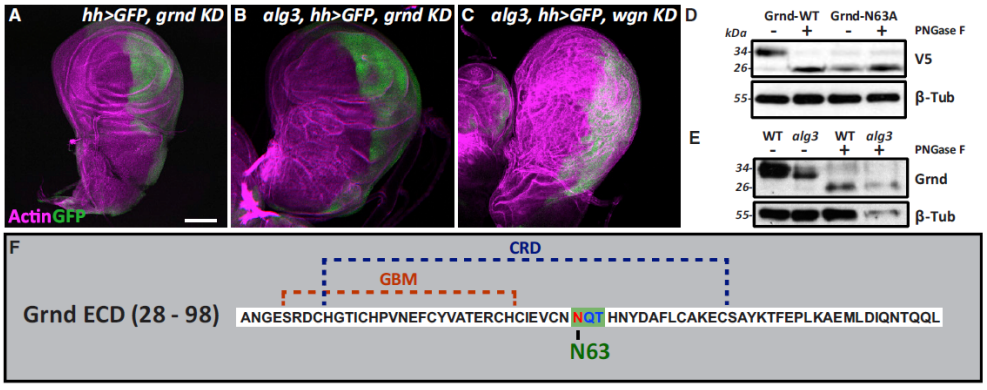

Figure 4: Glycoprotein Grnd/TNFR Activates JNK Signaling in *alg3*.
(A and B) Knockdown of Grnd in the posterior half of *alg3* discs rescues overgrowth and tissue architecture. (C) Knockdown of Wgn does not rescue *alg3*. (D) Western blotting of V5-tagged transgenic Grnd. Grnd N63A shows altered mobility on western blot corresponding to PNGase-treated WT Grnd. (E) Western blotting with an antibody for endogenous Grnd in *alg3* shows a mobility shift compared with WT, and PNGase treatment causes equivalent shifts of both genotypes. Note the loading disparity in the fourth lane, and see Figure S4A for quantitation. (F) The Grnd-ECD contains a single high-confidence predicted glycosylation site at N63 (NetNGlyc). CRD, cysteine-rich domain; GBM, glycosphingolipid-binding motif. Images are representative of n≥15 animals per genotype. Scale bar: 100 µm in (A). See also Figures S2 and S4.

To determine whether Grnd could be a direct target of Alg3 function, we first assessed whether Grnd was N-glycosylated *in vivo*. Western blotting of Grnd in imaginal tissue showed a significant molecular weight reduction when extracts were treated with PNGase to remove N-linked glycans (Figures 4D, 4E, and S4A). Grnd from *alg3* discs showed a higher molecular weight than those from PNGase-treated WT discs, but this difference was eliminated when *alg3* discs were also treated with PNGase (Figures 4E and S4A). Analysis of Grnd protein sequence suggested a single high-confidence N-glycosylation site, at asparagine 63 (Figure 4F). This site lies within the cysteine-rich domain (CRD) of Grnd and close to the glycosphingolipid-binding motif (GBM), which are characteristic for TNFR family members. Consistent with this analysis, replacement of asparagine 63 with alanine in transgenic Grnd (GrndN63A) produced a protein running at a molecular weight identical to that of PNGase-treated WT Grnd (Figure 4D), and this was not further altered by PNGase treatment. These results demonstrate that Grnd, which shows excessive signaling in *alg3* mutants, is normally glycosylated at N63.

Circulating TNF Produced in the Fat Body Activates TNFR

TNFR signaling is triggered by binding to the secreted ligand TNF (Brenner et al., 2015). Because the *Drosophila* genome encodes a single TNF homolog (Flybase: Eiger, Egr) (Igaki et al., 2002), we investigated the role of Egr in *alg3* mutants. Interestingly, depleting Egr in *alg3* discs did not result in any amelioration of the phenotype (Figures 5A and 5B). This suggested that either TNFR hyperactivation in this context was ligand independent or that the TNF involved is produced in a separate, non-imaginal tissue. Consistent with the latter, ubiquitous depletion of Egr reverts the *alg3* tumor phenotype (Figure 5C). One candidate source for Egr production is hemocytes, which are attracted to neoplastic tumors and locally secrete Egr (Bidla et al., 2007; Parisi et al., 2014; Perez et al., 2017). However, hemocyte-specific depletion of Egr in *alg3* larvae also did not affect the tumor phenotype (Figures S4D and S4E), nor could we detect hemocyte attachment to *alg3* discs. An alternate source for Egr is the fat body (Agrawal et al., 2016; Mabery and Schneider, 2010). Strikingly, fat body-specific depletion of Egr in *alg3* larvae led to a strong rescue of the tumor phenotype (Figure 5D), establishing that circulating Egr produced in a remote tissue activates Grnd in imaginal discs to trigger excessive JNK signaling.

Like Grnd, Egr is a glycoprotein (Kauppila et al., 2003) and thus could also be regulated by N-glycosylation. We used local expression of a *UAS-alg3* rescue construct to determine the tissue requirement for N-glycosylation in suppression of these JNK-driven tumors. Restoring Alg3 function to the fat body did not alter the *alg3* mutant phenotype, while restoring Alg3 function to imaginal discs clearly rescued the tissue (Figures 5E and 5F). This strongly suggests that, while Egr is necessary for aberrant JNK activation in *alg3* mutants, its N-glycosylation does not play a role in the phenotype.

N-Glycosylation Reduces TNFR-TNF Binding to Regulate Signaling

Given the evidence for a direct regulatory role of Grnd N-glycosylation, we examined whether N-glycosylation-deficient Grnd exhibits excess JNK signaling activity. Overexpression of GrndN63A in imaginal discs alone did not produce an evident phenotype, suggesting that any increase in JNK activity was low (Figure S4F). Similarly, overexpression of Egr in the fat body to elevate circulating levels of the ligand had no effect on WT discs (Figure S4G). However, elevation of circulating Egr induced a strong enhancement of the *alg3* phenotype, accompanied by increased JNK signaling and cell

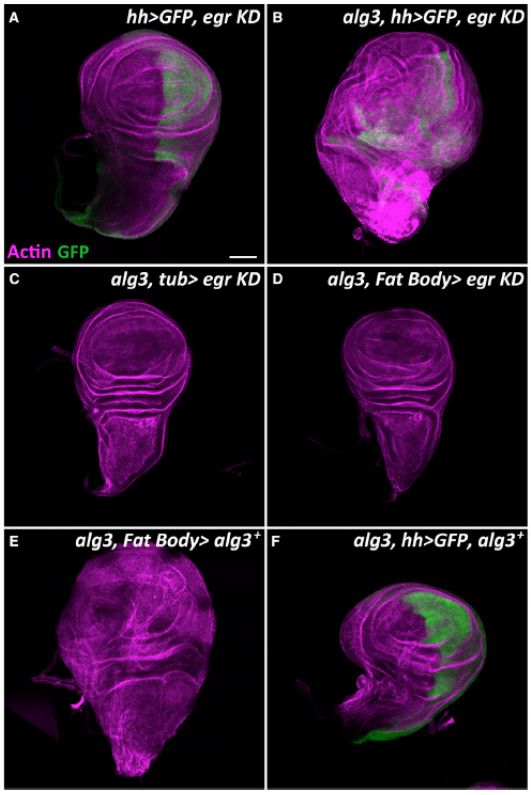

Figure 5: Systemic Egr/TNF-α Produced by theFat Body Activates Grnd/TNFR in the Wing Disc.
(A and B) Local RNAi depletion of Egr in the wing imaginal disc does not rescue *alg3* discs. (C and D) Ubiquitous knockdown of Egr rescues *alg3* discs (C), and depletion in the fat body (D) recapitulates that phenotype. (E and F) Driving *alg3* expression in the fat body does not rescue *alg3* discs (E), while local expression within discs rescues overgrowth and architecture defects (F). Images are representative of n≥10 animals per genotype. Scale bar: 100 μm in (A). See also Figure S4**.**

death (Figures S4J, S4K, 6B′, and 6C′). We also assayed an independent tissue in which Grnd is active. Egr- and Grnd-dependent JNK signaling is activated in the insulin-producing cells (IPCs) of the larval brain to limit overall growth of the animal in conditions in which nutrient consumption is low (Agrawal et al., 2016). In line with these findings, overexpression of the Grnd intracellular domain in the IPCs, which induces ectopic JNK activation, reduces animal size, assayed by pupal volume (Figures 6K and 6L). Interestingly, overexpression of GrndN63A in IPCs also resulted in a clear reduction of pupal volume, whereas overexpression of WT Grnd had no effect (Figure 6L). Together these data indicate that preventing N-glycosylation of Grnd induces increased ligand sensitivity to activate JNK.

How could N-glycosylation of Grnd enhance its signaling? Glycosylation can influence protein trafficking, but the WT apical localization of Grnd remains intact in *alg3* tumors (Figures S4H and S4I). In addition, GrndN63A remains apically localized and does not perturb disc architecture, arguing against changes in receptor or ligand accessibility (Figures S4F, S4L, and S4M). Because the N-glycosylation site in Grnd is located in the predicted TNF-binding domain, an attractive alternative possibility is

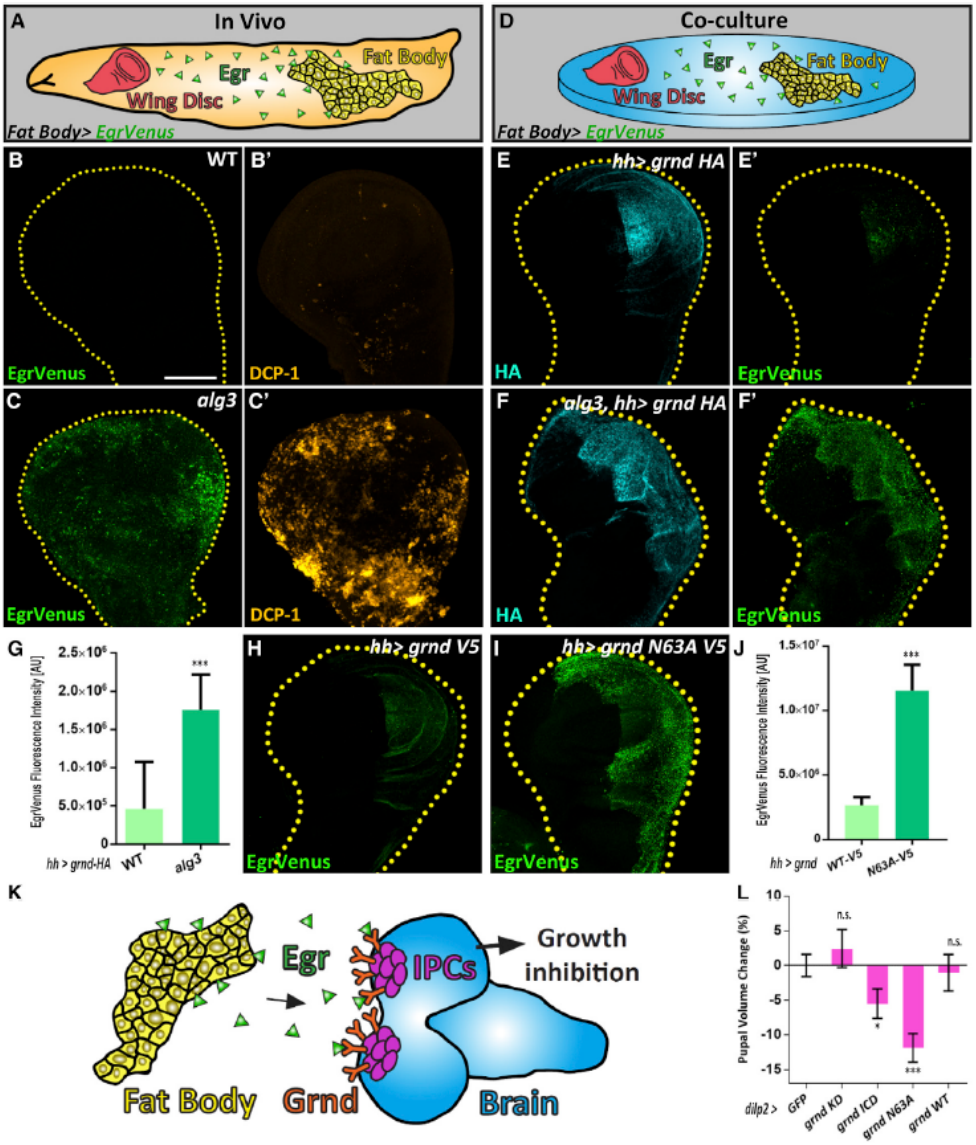

Figure 6: Grnd N-glycosylation Reduces Ligand Binding.
(A–C) (A) Schematic representation of the experiments in (B) and (C): Venus-tagged Egr is expressed in the fat body of WT or *alg3* larvae. Egr-Venus binding and cell death (DCP-1) are elevated in *alg3* compared with WT. (D–J) (D) Schematic representation of *ex vivo* co-culture using Egr-Venus expressing fat body tissue. When WT (E) and *alg3* (F) discs expressing Grnd in the posterior compartment are co-cultured, Egr-Venus binds more strongly to *alg3*: quantified in (G), *hh>grnd-HA WT* n=26 discs, *hh>grnd-HA alg3* n=30 discs. When discs expressing WT Grnd (H) and Grnd-N63A (I) are co-cultured, Egr-Venus binds more strongly to Grnd-N63A: quantified in (J), *hh>grnd-WT-V5* n=33 discs, *hh>grnd-N63A-V5* n=46 discs. (K) Circulating Egr binds Grnd on IPCs in the larval brain, activating JNK to inhibit pupal growth. (L) Changes in pupal volume caused by expression of Grnd constructs in IPCs. Grnd-N63A inhibits growth analogous to the dominant-active Grnd ICD. *dilp2>GFP* n=97, *dilp2>grnd KD* n=61, *dilp2>grnd ICD* n=64, *dilp2>grnd N63A* n=75, *dilp2>grnd WT* n=68. ***p < 0.001; *p < 0.05; n.s., not significant; error bars indicate SEM. Images are representative of n≥15 animals per genotype. Scale bar: 100 µm in (B). See also Figure S4.

that N-glycosylation alters receptor-ligand affinity. To explore Egr-Grnd interactions, we expressed Venus-tagged Egr in the fat body and monitored its accumulation in the imaginal disc. While WT discs show little Egr association in this assay, *alg3* discs extensively accumulated Egr, despite the fact that Grnd levels are not increased (Figures 6A–6C and S4N–S4Q). This result suggests that impaired glycosylation could increase the affinity of Grnd for Egr. In an *ex vivo* system where Egr-Venus-expressing fat body is cultured alongside imaginal discs (Figure 6D), cells that express transgenic Grnd bound significantly more Egr when they were mutant for *alg3* than when they were WT (Figures 6E– 6G). An even stronger increase of bound Egr was seen in WT cells expressing GrndN63A, which is predicted to lack all N-glycans (Figures 6H–6J). Importantly, Grnd protein levels at the membrane remain comparable in both co-culture experiments, indicating that increased Egr binding is not simply caused by elevated receptor abundance (Figures S4R and S4S). These data suggest that physiological N-glycosylation regulates TNFR ligand binding, specifically by reducing its affinity for TNF.

DISCUSSION

We have shown that mutations in the classic *Drosophila* TSG *tumorous imaginal discs* (*tid*) disrupt the *ALG3* homolog *CG4084*, altering the lipid-linked biosynthetic pathway that generates oligosaccharides for protein N-linked glycosylation. Although altered glycosylation affects many proteins and can induce a UPR, we find that the growth control phenotype of *alg3* can be ascribed to a single target and a single mechanism. This target is the *Drosophila* TNFR homolog, whose proper modification at a single extracellular site is required to prevent inappropriate TNF binding, subsequent JNK activation, and downstream Yki-driven overproliferation. We postulate that N-glycosylation can act as a mechanism to modulate JNK signaling in response to cellular stresses.

The *alg3* mutations studied here were originally identified for their overgrowth phenotype in imaginal discs (Kurzik-Dumke et al., 1992). Like most other *Drosophila* TSGs, this phenotype is caused by changes in Hpo-regulated Yki activation, but *alg3* mutants differ in both upstream regulation and downstream targets. Mutations in core Hpo signaling components result in rapid proliferation of disc cells, while the slow growth of *alg3* mutant tissue resembles that of the neoplastic TSGs. Nonetheless, the STAT pathway, which is a major mitogenic effector in neoplastic mutants (Bunker et al., 2015; Gilbert et al., 2009; Wu et al., 2010), is not elevated in *alg3* tissue. Upstream, JNK-dependent Yki activity is seen in both *alg3* and neoplastic mutants (Menendez et al., 2010; Sun and Irvine, 2011). However, JNK activation in neoplastic mutants has been suggested to occur either through ligand-independent Grnd activation caused by alteration to apicobasal polarity (Andersen et al., 2015) or through Grnd-independent mechanisms (Muzzopappa et al., 2017). In *alg3* mutants, polarity is intact and overgrowth entirely relies on a Grnd-Egr axis, specifically the increased sensitivity of misglycosylated Grnd for endocrine Egr. Thus, TNFR signaling induced by altered N-glycosylation seems to define distinct consequences for downstream Hpo-mediated growth control.

While we have not tested biochemical affinities directly, our data are consistent with a model where TNF-binding properties are directly regulated by glycosylation of TNFR. Partial or complete removal of the glycan at N63, within the ligand-binding domain of Grnd, leads to an increase of bound Egr, indicating that N-glycosylation normally limits Grnd engagement and downstream signaling. In *Drosophila* larvae, Egr

is continuously transcribed in the fat body for secretion into the hemolymph, bathing Grnd-expressing tissues, including imaginal discs and IPCs in ligand (Agrawal et al., 2016). Our results suggest that proper N-glycosylation of Grnd sets a threshold that prevents tonic signaling in these and other tissues under normal circumstances. This raises the intriguing possibility that cell-autonomous changes in N-glycosylation, perhaps induced by stress inputs, could modulate ligand affinity, allowing a rapid and local response to this endocrine signal under different physiological conditions.

The modulation of Grnd ligand binding suggested here echoes the regulation of Notch by the glycosyltransferase Fringe (Stanley and Okajima, 2010; Takeuchi and Haltiwanger, 2014). However, the obligate role of Alg3 in all N-glycan synthesis is fundamentally distinct from Fringe's substrate-specific elaboration of a particular O-glycan. In the case of Notch, the specific sugar residues added by Fringe alter receptor selectivity for one ligand over another (Brückner et al., 2000; Moloney et al., 2000). Since either aberrant or absent Grnd N-glycosylation results in increased ligand binding and ectopic signaling, evidence for specific glycan structures in modulating the ligand-receptor interface does not currently exist. Whether the glycan could provide a simple steric obstacle to ligand binding or may regulate it through more complex interactions will await structural studies.

Grnd shows strong homology to vertebrate TNFR family members in its extracellular TNF-binding domain, although downstream signaling in the fly acts mainly through JNK (Andersen et al., 2015; Igaki et al., 2002), in contrast to mammalian homologs that also signal through nuclear factor κB (NF-κB), p38, and caspases (Dempsey et al., 2003). Among the 29 mammalian TNFR superfamily members, at least seven have predicted N-glycosylation sites in their extracellular domains. Several of these sites have been studied, and their proposed roles vary from promoting signaling to inhibiting it or being functionally neutral (Charlier et al., 2010; Han et al., 2015; Klıma et al., 2009; Shatnyeva et al., 2011; Vaitaitis and Wagner, 2010). Our results motivate analyses of the receptors BCMA and DR4, which are closely related to Grnd and whose predicted N-glycosylation sites each lie in an analogous location within the ligand-binding domain (Andersen et al., 2015).

The data presented above, which highlight a new mechanism for restraining TNF signaling, hint at pathogenic mechanisms for several human diseases. Altered glycosylation is emerging as a frequent hallmark of cancer, in which JNK signaling is increasingly implicated (Bubici and Papa, 2014; Pinho and Reis, 2015; Vajaria and Patel, 2017). Moreover, mutations in the extracellular domain of human TNFR1, including predicted N-glycosylation sites, can cause the autoinflammatory disease TRAPS (TNFRassociated periodic syndrome) (Cantarini et al., 2012). Because the erroneous activation of Grnd in *alg3* mutants is akin to an autoinflammatory response, defective N-glycosylation could be an additional mechanism for hyperactive TNFR1 signaling. Finally, mutations in N-glycosylation pathway enzymes including Alg3 result in recessive genetic diseases called type I congenital disorders of glycosylation (CDG-I) (Jaeken, 2012; Scott et al., 2014). CDG patients exhibit a variety of poorly characterized symptoms associated with multiple organs, and the etiology of CDG is largely unknown. Our finding of altered inflammatory TNFR/JNK signaling in analogous fly mutants provides a new avenue to investigate.

MATERIALS AND METHODS

Drosophila Stocks and Genetics

Drosophila melanogaster stocks were kept on cornmeal molasses food at room temperature and experimental crosses were raised at 25°C. *OreR* flies were used for WT controls. *alg3*^{tid1} *FRT42*, *alg3*^{tid2} *Kr FRT42* and *alg3*^{tid2} *FRT42* were used to generate transheterozygous animals, mitotic clones and transgenic recombinants. Other alleles and their sources are listed in the **Key Resources Table**. For imaginal disc samples third instar stage larvae were used. Since these larval imaginal discs are not sexually dimorphic, male and female samples were grouped together.

Sequencing tid Alleles

Genomic DNA was isolated from 50 hemizygous larvae using the 30 Fly Prep protocol (BDGP). The *tid/not* genomic region was amplified with Phusion high fidelity DNA polymerase (NEB) using the following primers: 5'-TTAATTTTCGCCGGTTATCA-3' (*l(2) not*-F) and 5'-ACTCAGACCATTTTACTGCA-3' (*l(2)not*-R). Amplicons were gel-purified and sequenced. Sequence data were aligned and analyzed using ContigExpress (Vector NTI); sequences from mutant larvae were compared to the FlyBase sequences for *l(2)not* and *tid*.

Grnd Antibodies

For generating monoclonal antibodies against the extracellular domain of Grnd a peptide with the sequence ANGESRDCHGTICHPVNEFCYVATERCHPCIEVCNNQTHNY DAFLCAKECSAYKTFEPLKAEMLDIQNTQQ, corresponding to amino acids 28-98 of Grnd was fused to the N-terminus of His-MBP and used to inoculate mice. The resulting hybridoma candidates were screened for IHC and Western Blot reactivity. Grnd antibodies 7D9 and 6F10 were selected for IHC and Western Blot detection respectively.

Immunofluorescence and Microscopy

Larval imaginal discs were dissected in PBS, and fixed for 20 minutes in 4% PFA. After rinsing in PBS, samples were blocked for one hour in PBST containing 5%NGS (Gibco) and 1%BSA (Gibco). Primary and secondary staining was done overnight in block at 4°C. The primary antibodies and dilutions are listed in the **Key Resources Table**. Secondary fluorophore-conjugated antibodies (Molecular Probes) were used at 1:250. Confocal images were obtained on either a Leica TCS SP2 Scanning confocal microscope or a Zeiss LSM 700 confocal microscope. For every experiment at least ten discs were imaged, and representative images for each experiment were chosen. Images were processed in either Adobe Photoshop CC or Fiji (Schindelin et al., 2012).

Cell Counts and Pupal Volume Measurements

WT, *alg3, dlg* and *wts* imaginal discs containing either ubiquitously expressed GFP or RFP were collected and transferred to a polystyrene tube containing 500ml of 9x Trypsin-EDTA (Sigma), 1x PBS and 0.5µg/ml Hoechst 3334. At least 20 discs per genotype were collected, and counting experiments were repeated at least five times. After ~4hrs of nutation at room temperature, proper cell dissociation was confirmed using light microscopy. Cells were counted on a hemocytometer, and by pairing off GFP and RFP positive genotypes, cell number ratios compared to WT were calculated. For the assessment of pupal sizes, larvae were staged and numbers were controlled to prevent

crowding. At least 60 pupae per genotype were collected and imaged using a Leica Z16 APO microscope. Pupal length (L) and width (W) were measured using Fiji (Schindelin et al., 2012), and used to calculate pupal volume with the formula $4/3\pi(L/2)(W/2)^2$

Western Blotting and PNGase Assay

For Western blots, larvae were dissected in PBS, using carcasses (15 larva per sample) without guts and fat body, or just the imaginal discs (60 discs per sample). Cell lysates were homogenized in 1X RIPA with protease inhibitors (Roche). 40 µg of lysate was loaded into wells. Proteins were electrophoresed at 200V for 45 minutes and blotted at 100V for 20-60 minutes depending on protein size. Membranes were blocked for an hour in 5% NFDM. For staining, the primary antibodies and dilutions are listed in the **Key Resources Table**. HRP-conjugated secondary antibodies were used at 1:10,000. Blot was developed with standard ECL reagents (Advansta). The protein de-glycosylation assay was done using PNGaseF (NEB) according to the user suggested guidelines. Each Western Blot experiment has been repeated at least three times.

Co-Culture Experiments

Dissected larval fat bodies expressing Venus-tagged Egr (*R4>EgrVenus*) were cultured in a small volume of Schneider's medium (Gibco) containing 10% FBS (Gibco) and 1% Pen/Strep (Caisson Labs) for 3 hours at 29°C. At least 12 larval imaginal discs of the genotype of interest were added to this mixture and co-cultured for 5 hours at 29°C. After culturing the discs are rinsed in PBS, and fixed for 20 minutes in 4% PFA. Each Co-Culture experiment has been repeated at least three times.

Quantification and Statistical Analysis

Fiji (Schindelin et al., 2012) was used to collect pupal dimensions, fluorescence intensity, Western Blot quantification and disc/clone size measurements. Graphpad Prism 5.03 and Excel (Microsoft Office) were used for statistical analysis and graphical representations. Column graphs show the mean with error bars indicating standard deviation, unless indicated otherwise. All experiments were repeated at least three times. Specific n value per experiment indicated in the Method Details section. The Student's t-test was used to determine statistical significance, as well as the F-test for determining the equality of variances in two-sample comparisons.

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Author contributions

Conceptualization and Methodology, G.d.V., H.A.M., and D.B.; Investigation, G.d.V., H.A.M., A.M.H., and R.M.B.; Resources, J.C., D.A., and R.M.B.; Writing – Original Draft, G.d.V. and D.B.

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KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Mouse anti-Gmd 1:500 (IHC)	Bilder lab	7D9
Mouse anti-Gmd 1:4 (Western)	Bilder lab	6F10
GP anti-Grnd 1:1000 (Western)	kindly provided by P. Leopold	Andersen et al., 2015
GP anti-Grnd 1:500 (IHC)	kindly provided by P. Leopold	Andersen et al., 2015
Mouse anti-Dlg 1:100 (IHC)	DSHB	4F3 RRID:AB_528203
Mouse anti-Wg 1:100 (IHC)	DSHB	4D4; RRID:AB_528512
Rabbit anti-HA 1:500 (IHC)	Thermo	71-5500; RRID:AB_2533988
Mouse anti-V5 1:500 (IHC)	Thermo	R960-25; RRID:AB_2556564
Mouse anti-V5 1:1000 (Western)	Thermo	R960-25; RRID:AB_2556564
Rat anti-DCAD1 1:100 (Western)	kindly provided by T. Uemura	Uemura et al., 1996
Mouse anti-Tubulin 1:1000	DSHB	E7; RRID:AB_528499
Rabbit anti-DCP-1 1:100 (IHC)	Cell signaling	9578; RRID:AB_2721060
Mouse anti-pJNK 1:100 (IHC)	Cell Signaling	9255S; RRID:AB_2307321
Rabbit anti-aPKC 1:200 (IHC)	Santa Cruz Biotech	SC-937; RRID:AB_632229
Rb anti-GFP 1:500 (IHC)	Torrey Pines	TP401; RRID:AB_10013661
Rb anti-β-gal 1:1000 (IHC)	Abcam	AB616; RRID:AB_305327
Chemicals, Peptides, and Recombinant Proteins		
Phalloidin-TRITC 1:500	Sigma-Aldrich	P1951; RRID:AB_2315148
DAPI 1:1000	Thermo	D1306, RRID:AB_2629482
Experimental Models: Organisms/Strains		
tid^{1}	Kurzik-Dumke et al., 1995	Flybase ID: FBal0009359
tid^{2} *kr*	Kurzik-Dumke et al., 1995	Flybase ID: FBal0009360
dlg$^{40-2}$	Mendoza-Topaz et al., 2008	Flybase ID: FBal0240608
scrib^{1}	Bilder and Perrimon, 2000	Flybase ID: FBal0103577
wts^{e2e-1}	Tao et al., 1999	Flybase ID: FBal0044522
UAS-Egr-venus	Keller et al., 2011	Flybase ID: FBal0325733
UAS-Wgn-RNAi	Keller et al., 2011	Flybase ID: FBal0138107
Egr Gal4	Keller et al., 2011	Flybase ID: FBti0147782
10XStat92E-GFP	Bach et al., 2007	Flybase ID: FBtp0036675

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
UAS-Egr	Igaki et al., 2002	Flybase ID: FBtp0017228
HREx8-GFP	Wu et al., 2008	Flybase ID: FBtp0072376
SU(H)-GFP	Liu and Posakony, 2014	Flybase ID: FBtp0083637
Xbp1-dsRed	Ryoo et al., 2013	Flybase ID: FBal0290717
ex-LacZ	Boedigheimer and Laughon, 1993	Flybase ID: FBti0003139
UAS-Wts	Jia et al., 2003	Flybase ID: FBal0151322
UAS-Hpo	Pantalacci et al., 2003	Flybase ID: FBtp0019872
AP1-GFP	Chatterjee and Bohmann, 2012	Flybase ID: FBtp0072196
UAS-grnd-FL-HA	Andersen et al., 2015	Flybase ID: N/A
UAS-grnd-ECD	Andersen et al., 2015	Flybase ID: FBal0316441
UAS-grnd-ICD	Andersen et al., 2015	Flybase ID: FBal0316440
Diap1-GFP	Zhang et al., 2008	Flybase ID: FBtp0051047
Act>y+>Gal4 UASGFP; Tub-Gal4 FRT82 Tub-Gal80 (MARCM82)	Lee and Luo, 2001	Flybase ID:N/A
Act>y+>Gal4 FRT42D; Tub-Gal80 Tub-Gal4 Uas-CD8GFP (MARCM42)	Lee and Luo, 2001	Flybase ID:N/A
UAS-Egr RNAi	Vienna Drosophila RNAi Center	Flybase ID: FBst0466011
UAS-Grnd RNAi	Vienna Drosophila RNAi Center	Flybase ID: FBst0476396
UAS-Xbp1-RNAi(A)	Vienna Drosophila RNAi Center	Flybase ID: FBst0451809
UbiGFP	Bloomington Drosophila Stock Center	Flybase ID: FBst0001691
CG11851^{c02021} *(alg9)*	Bloomington Drosophila Stock Center	Flybase ID: FBti0040996
w; FRT82	Bloomington Drosophila Stock Center	Flybase ID: FBti0002074
w; FRT42	Bloomington Drosophila Stock Center	Flybase ID: FBti0141188
UAS-RasV12	Bloomington Drosophila Stock Center	Flybase ID: FBst0064196
UbiRFP	Bloomington Drosophila Stock Center	Flybase ID: FBst0030852
Tubulin-Gal4	Bloomington Drosophila Stock Center	Flybase ID: FBst0005138
Actin-Gal4	Bloomington Drosophila Stock Center	Flybase ID: FBtp0019245
yw eyFLP; FRT42 cl	Bloomington Drosophila Stock Center	Flybase ID: FBst0005617
Bx-Gal4 (MS1096)	Bloomington Drosophila Stock Center	Flybase ID: FBst0008860
UAS-ANFEMD	Bloomington Drosophila Stock Center	Flybase ID: FBst0007001
En-Gal4	Bloomington Drosophila Stock Center	Flybase ID: FBst0030564
Hh-Gal4	Bloomington Drosophila Stock Center	Flybase ID: FBal0121962
R4-Gal4	Bloomington Drosophila Stock Center	Flybase ID: FBst0033832
CG-Gal4	Bloomington Drosophila Stock Center	Flybase ID: FBst0007011
He-Gal4	Bloomington Drosophila Stock Center	Flybase ID: FBst0008699
HmlΔ-Gal4	Bloomington Drosophila Stock Center	Flybase ID: FBst0030141
Dilp2-Gal4	Bloomington Drosophila Stock Center	Flybase ID: FBst0037516
UAS-sip3	Bloomington Drosophila Stock Center	Flybase ID: FBst0020333
UAS-JNK^{DN}	Bloomington Drosophila Stock Center	Flybase ID: FBst0009311
UAS-aPKC RNAi	Bloomington Drosophila Stock Center	Flybase ID: FBst0035140
UAS-Xbp1 RNAi(B)	Bloomington Drosophila Stock Center	Flybase ID: FBst0036755
UAS-Crb RNAi	Bloomington Drosophila Stock Center	Flybase ID: FBst0040869
Software and Algorithms		
Fiji	ImageJ	http://fiji.sc/
Illustrator	Adobe	www.adobe.com/uk/products/illustrator.html
Photoshop	Adobe	www.adobe.com/uk/products/photoshop.html
Excel	Microsoft	https://products.office.com/en-us/excel
Prism	Graphpad	www.graphpad.com/scientificsoftware/prism/
Leica Confocal Software	Leica	www.leica-microsystems.com/products/microscope-software/

SUPPLEMENTARY FIGURES

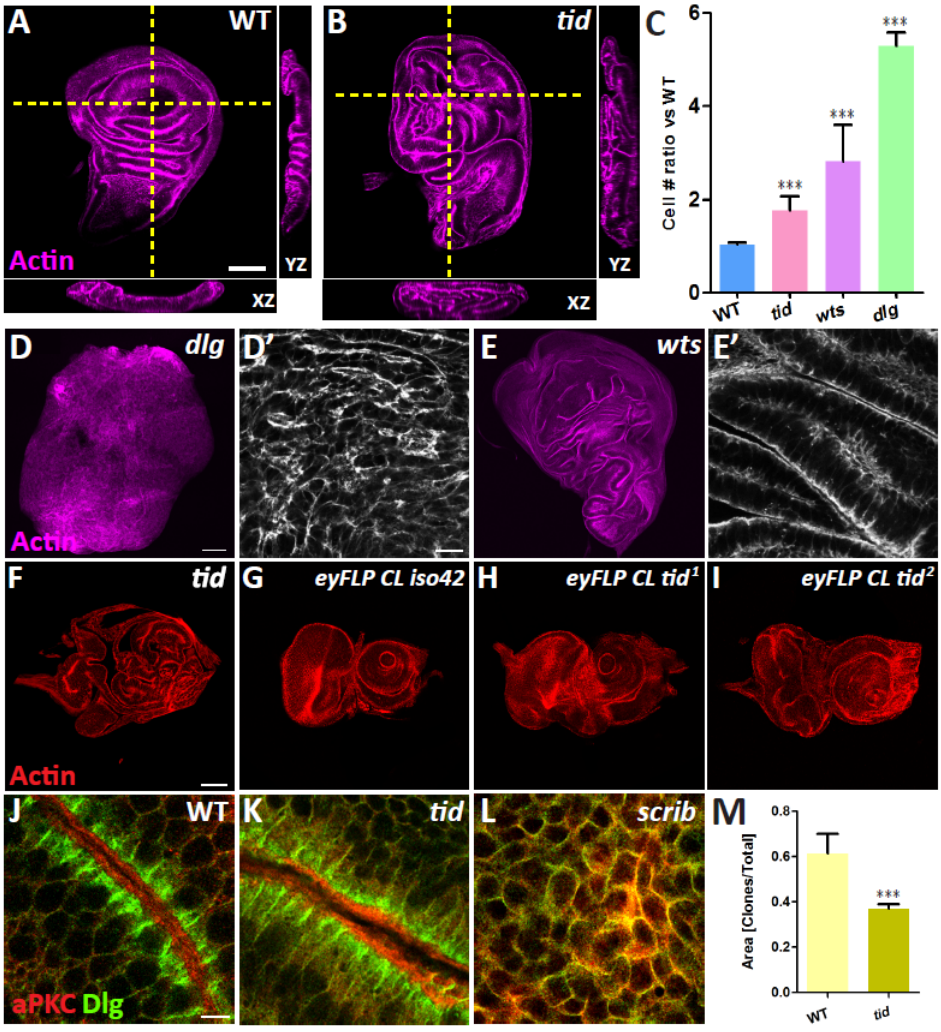


Figure S1: *tid* mutants show neoplastic and hyperplastic phenotypes.
(A,B) *tid* discs show altered tissue architecture that results in a thicker cross-section than WT. (C) Cell counts of neoplastic *dlg* and hyperplastic *wts* tumors, compared to WT and *tid* discs. (D,E) Comparison of disc size and architecture defects in representative neoplastic (*dlg*) and hyperplastic (*wts*) mutants. (F-I) Mutant eye discs generated in heterozygous animals show mild phenotypes compared to completely mutant animals. (J-L) Apicobasal polarity is intact in WT and *tid* discs, compared to the neoplastic mutant *scrib*. (M) Quantification of WT and *tid* clonal area in the eye discs. WT n=17 discs, *tid* n=16 discs. ***P <0.001; Error bars indicate s.d. Images are representative of n≥10 animals per genotype. Scale bars: 100 μm in A, D and F; 10 μm in D'; 5 μm in J.

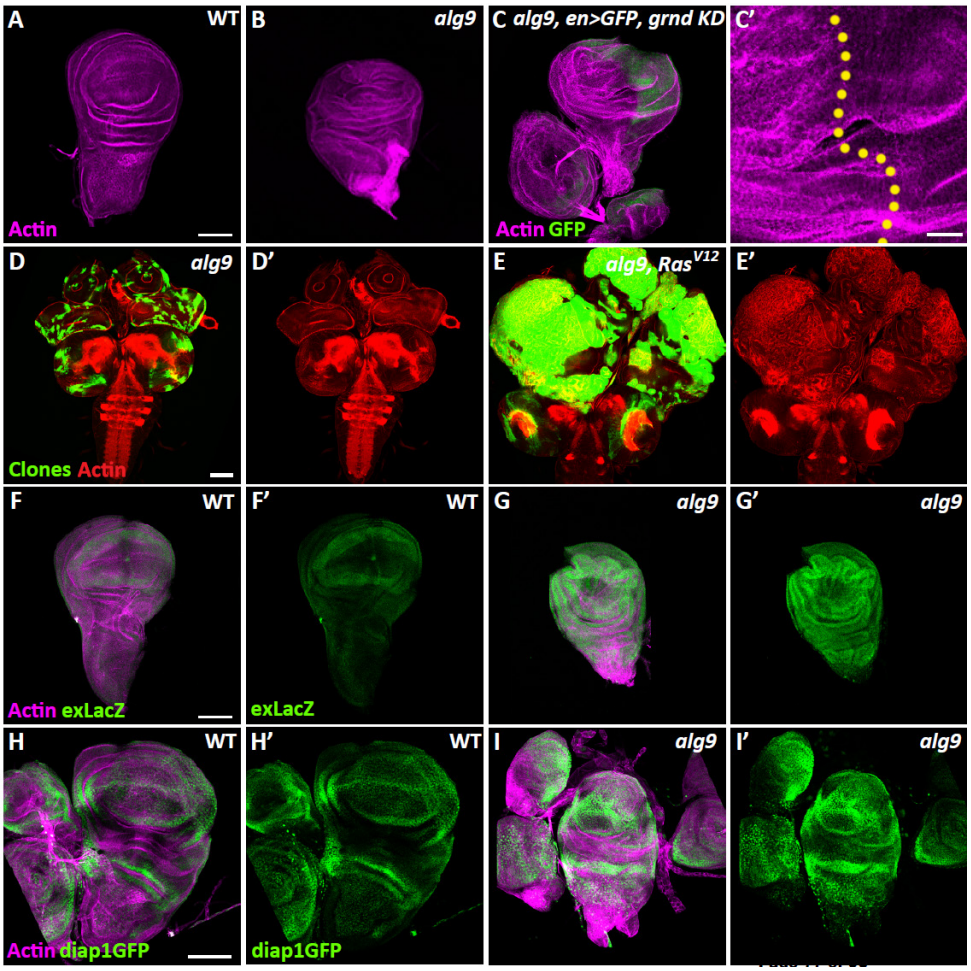


Figure S2: Mutants for the mannosyltransferase *alg9* partially phenocopy *alg3*.
(A,B) Discs mutant for *alg9* show architecture defects similar to *alg3*, but do not overgrow compared to WT. (C) Knock-down of Grnd rescues the *alg9* architectural defects. (D,E) Eye disc clones of *alg9* cooperate with RasV12 to generate overgrowth. The Hippo pathway reporters *exLacZ* (F,G) and *diap1-GFP* (H,I) are activated in *alg9* discs. Images are representative of n≥10 animals per genotype. Scale bars: 100 µm in A, D, F and H; 10 µm in C'.

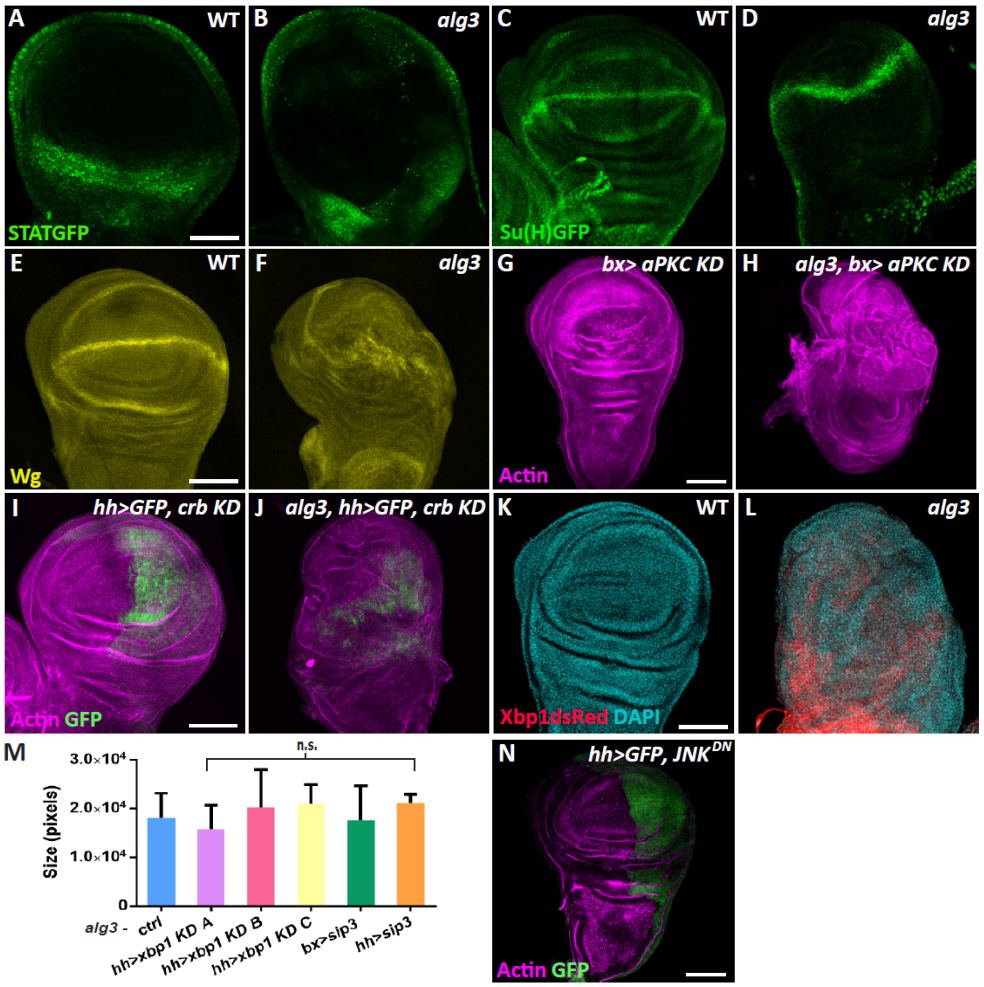
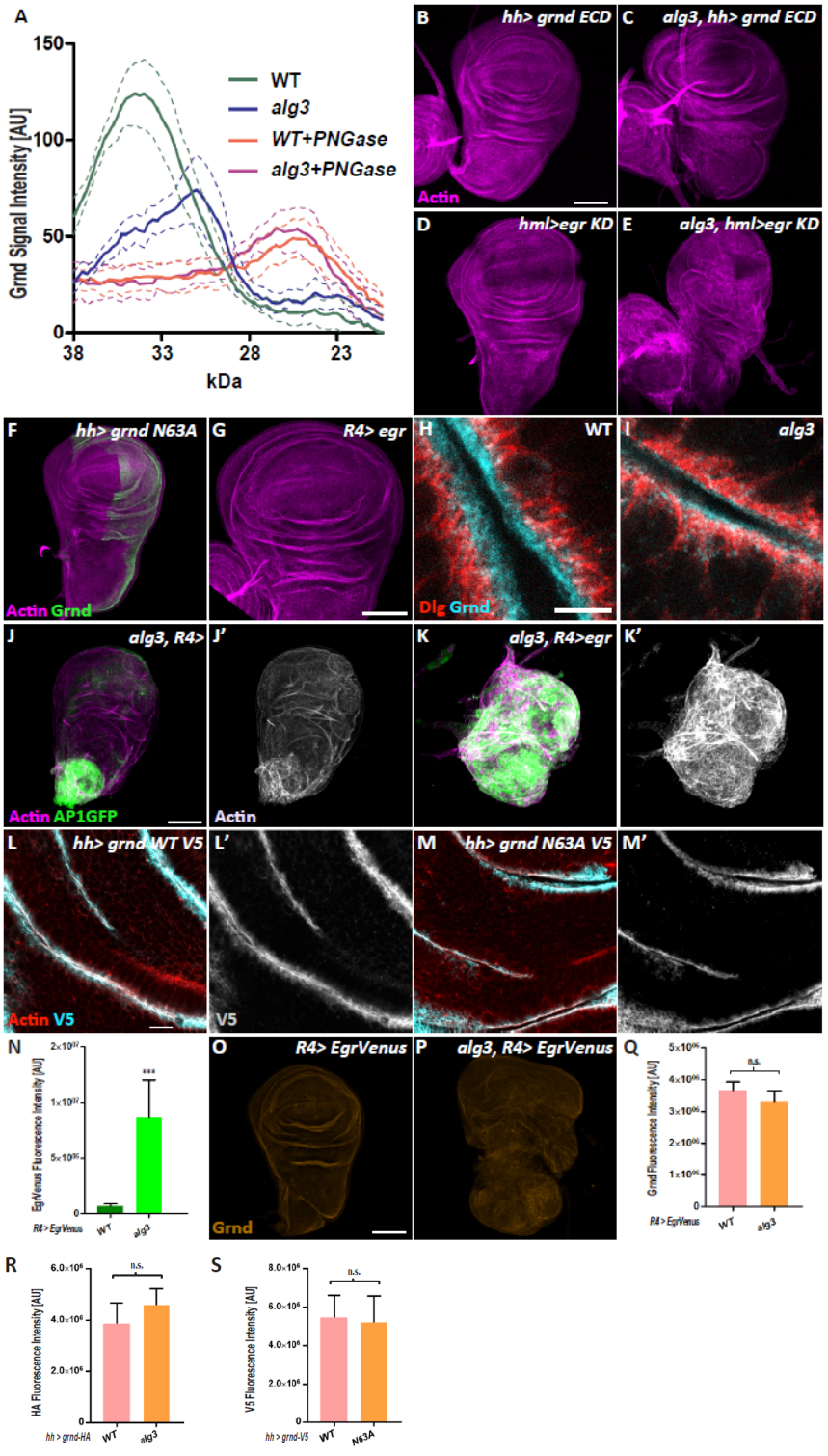


Figure S3: Signaling pathways in *alg3* mutants.
STATGFP (A,B), Su(H)GFP (C,D) and Wingless (E,F) patterning and intensity are not dramatically altered in *alg3* tumors compared to WT. Reducing levels of apical polarity regulators aPKC (G,H) and Crb (I,J) does not rescue the *alg3* phenotype. (K,L) A transgenic reporter for UPR is activated in *alg3* discs, though reduction of UPR does not rescue *alg3* overgrowth (M), *alg3*-ctrl n=21 discs, *alg3-hh>xbp1 KD A* n=18 discs, *B* n=21 discs, *C* n=16 discs, *alg3-bx>sip3* n=17 discs, *alg3-hh>sip3* n=16 discs. Blocking JNK signaling in the posterior half of a WT disc (N) does not cause any phenotype. ***P <0.001; n.s., not significant; Error bars indicate s.d.; Images are representative of n≥10 animals per genotype. Scale bars: 100 µm in A, E, G, I, K and N.


A
Grnd Signal Intensity [AU]
150
100
50
0
38
33
28
23
kDa
WT
alg3
WT+PNGase
alg3+PNGase
B hh> grnd ECD
Actin
C alg3, hh> grnd ECD
D hml>egr KD
E alg3, hml>egr KD
F hh> grnd N63A
Actin Grnd
G R4> egr
H WT
Dlg Grnd
I alg3
J alg3, R4>
Actin AP1GFP
J'
Actin
K alg3, R4>egr
K'
L hh> grnd WT V5
Actin V5
L'
V5
M hh> grnd N63A V5
M'
N
EgrVenus Fluorescence Intensity [AU]
***
R4> EgrVenus
WT
alg3
O R4> EgrVenus
Grnd
P alg3, R4> EgrVenus
Q
Grnd Fluorescence Intensity [AU]
n.s.
R4> EgrVenus
WT
alg3
R
HA Fluorescence Intensity [AU]
n.s.
hh > grnd-HA
WT
alg3
S
V5 Fluorescence Intensity [AU]
n.s.
hh > grnd-V5
WT
N63A

< Figure S4: Grnd localization, levels, and activity in *alg3* mutants.
(A) Western Blot quantification of endogenous Grnd signal intensity relative to molecular weight. Note that the peak density in WT has a higher molecular weight than in *alg3*, while PNGase treatment of both samples produces equivalent peak densities. Data shown represent an average of four separate Western Blot experiments like that shown in Figure 4E. Dotted lines indicate s.e.m. (B,C) Expression of the extracellular domain (ECD) of Grnd rescues *alg3* mutant defects in the imaginal discs. (D,E) Depletion of Egr in the larval hemocytes does not show any rescue. (F) Expression of Grnd-N63A in the imaginal disc does not induce a phenotype. (G) Fat body expression of Egr does not affect WT imaginal discs. (H,I) Apical localization of Grnd remains intact in *alg3* mutant tissue. (J,K) Excess Egr driven from the fat body increases actin levels and JNK reporter AP1GFP in *alg3* discs. (L,M) Localization of Grnd N63A does not change compared to wild-type Grnd. (N) Quantification of fat body driven Venus-tagged Egr in wing imaginal discs of WT and *alg3* larva, *R4>EgrVenus WT* n=13 discs, *R4>EgrVenus alg3* n=17 discs. Grnd levels in these animals are not altered (O,P), quantified in (Q), *R4>EgrVenus WT* n=13 discs, *R4>EgrVenus alg3* n=17 discs. (R) Transgenic Grnd expressed in WT versus *alg3* discs does not show any significant difference in levels, *hh>grnd-HA WT* n=26 discs, *hh>grnd-HA alg3* n=30 discs. The levels of WT Grnd versus Grnd-N63A (S) also do not show any significant difference, *hh>grnd-WT-V5* n=16 discs, *hh>grnd-N63A-V5* n=19 discs. ***P <0.001; n.s., not significant; Error bars indicate s.e.m. Images are representative of n≥10 animals per genotype. Scale bars: 100 µm in B, G, J and O; 5 µm in H; 10 µm in L.

SUPPLEMENTARY TABLE S1: Detailed genotypes

Figure	Panel	Genotype
1	A	*OreR*
	B	*alg3^{tid1}/ alg3^{tid2}*
	D	*alg3^{tid1}/ alg3^{tid2}*
	E	*alg3^{tid1}/ alg3^{tid2}*
	F	*eyFLP; Act>y+>Gal4 FRT42D/FRT42D; Tub-Gal80 Tub-Gal4 Uas-CD8GFP*
	G	*eyFLP; Act>y+>Gal4 FRT42D/ tid^{2} FRT42D; Tub-Gal80 Tub-Gal4 Uas-CD8GFP*
	H	*eyFLP; Act>y+>Gal4 FRT42D/FRT42D; Tub-Gal80 Tub-Gal4 Uas-CD8GFP/UAS-RasV12*
	I	*eyFLP; Act>y+>Gal4 FRT42D/ tid^{2} FRT42D; Tub-Gal80 Tub-Gal4 Uas-CD8GFP/UAS-RasV12*
2	C	*alg3^{tid1}/ alg3^{tid2};Tubulin-Gal4*
	D	*alg3^{tid1}/ alg3^{tid2};Tubulin-Gal4 UAS-alg3*
3	A	*HREx8-GFP/+*
	B	*alg3^{tid1}/ alg3^{tid2}; HREx8-GFP/+*
	D	*Bx Gal4; alg3^{tid1}/ alg3^{tid2}*
	E	*Bx Gal4; alg3^{tid1}/ alg3^{tid2}; UAS-wts/+*
	F	*AP1-GFP*
	G	*alg3^{tid1}/ alg3^{tid2} AP1-GFP*
	H	*OreR*
	I	*alg3^{tid1}/ alg3^{tid2}*
	J	*alg3^{tid1}/ alg3^{tid2}; Hh-Gal4 /UAS-JNK^{DN}*
4	A	*UAS Grnd RNAi; Hh-Gal4 UAS-GFP/+*
	B	*alg3^{tid1}/ alg3^{tid2} UAS-Grnd RNAi; Hh-Gal4 UAS-GFP/+*
	C	*alg3^{tid1}/ alg3^{tid2}; Hh-Gal4 UAS-GFP/ UAS-Wgn RNAi*
5	A	*Hh-Gal4 UAS-GFP/ UAS-Egr RNAi*
	B	*alg3^{tid1}/ alg3^{tid2}; Hh-Gal4 UAS-GFP/ UAS-Egr RNAi*
	C	*alg3^{tid1}/ alg3^{tid2}; Tubulin-Gal4/ UAS-Egr RNAi*
	D	*alg3^{tid1}/ alg3^{tid2}; R4-Gal4/UAS-Egr RNAi*
	E	*alg3^{tid1}/ alg3^{tid2}; R4-Gal4/UAS-alg3*
	F	*alg3^{tid1}/ alg3^{tid2}; Hh-Gal4 UAS-GFP/ UAS-alg3*
6	B	*UAS-Egr-venus/+; R4-Gal4/+*
	C	*alg3^{tid1}/ alg3^{tid2} UAS-Egr-venus; R4-Gal4/+*
	E	*Hh-Gal4/ UAS-grnd-FL-HA*
	F	*alg3^{tid1}/ alg3^{tid2}; Hh-Gal4/ UAS-grnd-FL-HA*
	H	*Hh-Gal4/ UAS-grnd-V5/+*
	I	*Hh-Gal4/ UAS-grnd-N63A-V5/+*
S1	A	*OreR*
	B	*alg3^{tid1}/ alg3^{tid2}*
	D	*dlg$^{40-2}$/Y*
	E	*wts^{e26-1}/ wts^{e26-1}*
	F	*alg3^{tid1}/ alg3^{tid2}*
	G	*eyFLP; FRT42D Cell lethal/ FRT42D*
	H	*eyFLP; FRT42D Cell lethal / alg3^{tid1} FRT42D*
	I	*eyFLP; FRT42D Cell lethal / alg3^{tid2}FRT42D*

S1	**J**	*OreR*
	K	*alg3^{tid1}/ alg3^{tid2}*
	L	*scrib^{1}/ scrib^{1}*
S2	**A**	*OreR*
	B	*alg9 ^{c02021}/ alg9 ^{c02021}*
	C	*En Gal4/UAS-Grnd RNAi; alg9 ^{c02021}/ alg9 ^{c02021}*
	D	*eyFLP; Act>y+>Gal4 UASGFP; Tub-Gal4 FRT82 Tub-Gal80/ alg9 ^{c02021} FRT82*
	E	*eyFLP; Act>y+>Gal4 UASGFP/UAS-RasV12; Tub-Gal4 FRT82 Tub-Gal80/ alg9 ^{c02021}FRT82*
	F	*ex-LacZ/+*
	G	*ex-LacZ/+ ; alg9 ^{c02021}/ alg9 ^{c02021}*
	H	*Diap1-GFP/+*
	I	*Diap1-GFP/+; alg9 ^{c02021}/ alg9 ^{c02021}*
S3	**A**	*10XStat92E-GFP/+*
	B	*alg3^{tid1}/ alg3^{tid2}; 10XStat92E-GFP/+*
	C	*SU(H)-GFP/+*
	D	*alg3^{tid1}/ alg3^{tid2}; SU(H)-GFP/+*
	E	*OreR*
	F	*alg3^{tid1}/ alg3^{tid2}*
	G	*Bx Gal4/+;;UAS-aPKC RNAi*
	H	*Bx Gal4; alg3^{tid1}/ alg3^{tid2}; UAS-aPKC RNAi*
	I	*Hh-Gal4 UAS-GFP/ UAS-Crb RNAi*
	J	*alg3^{tid1}/ alg3^{tid2}; Hh-Gal4 UAS-GFP/ UAS-Crb RNAi*
	K	*Xbp1-dsRed/+*
	L	*alg3^{tid1}/ alg3^{tid2}; Xbp1-dsRed/+*
	N	*Hh-Gal4 UAS-GFP/UAS-JNK^{DN}*
S4	**B**	*Hh-Gal4 / UAS-Grnd ECD*
	C	*alg3^{tid1}/ alg3^{tid2}; Hh-Gal4 / UAS-Grnd ECD*
	D	*HmlΔ-Gal4/ UAS-Egr RNAi*
	E	*alg3^{tid1}/ alg3^{tid2}; HmlΔ-Gal4/ UAS-Egr RNAi*
	F	*Hh-Gal4/ UAS-grnd-N63A-V5/+*
	G	*R4-Gal4/UAS-Egr*
	H	*OreR*
	I	*alg3^{tid1}/ alg3^{tid2};*
	J	*alg3^{tid1}/ alg3^{tid2} AP1-GFP; R4-Gal4/+*
	K	*alg3^{tid1}/ alg3^{tid2}; R4-Gal4/UAS-Egr*
	L	*Hh-Gal4/ UAS-grnd-V5/+*
	M	*Hh-Gal4/ UAS-grnd-N63A-V5/+*
	O	*UAS-Egr-venus/+; R4-Gal4/+*
	P	*alg3^{tid1}/ alg3^{tid2} UAS-Egr-venus; R4-Gal4/+*

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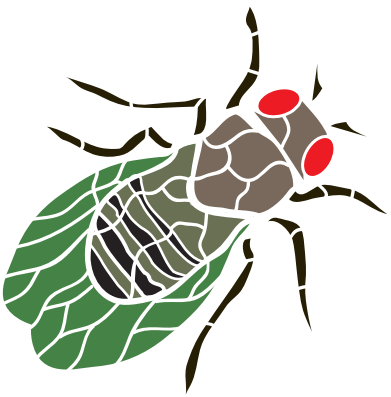

CHAPTER 5:

General discussion

Geert de Vreede[1]

Epithelia display a high degree of diversity and complexity throughout different organs and organisms. Despite these differences there is a strong conservation of not just the overall architecture, but also the signaling pathways underlying cell polarity, homeostasis and growth, showing the importance of this tissue type. Understanding epithelial biology is a key to understanding diseases that arise from epithelial signaling malfunction, especially in cancer. The fruit fly *Drosophila melanogaster* has an impressive track record as a genetic model system for many conserved developmental processes and human diseases. In this thesis, the fly has been used to study genes that trigger epithelial tumorigenesis upon disruption, focusing on the underlying signaling mechanisms that govern polarity and proliferation. Here I will discuss the results described in previous chapters in a broader context, with an emphasis on how the findings here fit with and expand our current understanding of epithelial polarity and growth control, in *Drosophila* as well as other models systems.

Apico-Basal Polarity regulation

As described in **Chapter 1**, a defining characteristic of epithelial cells is the distinct separation of domains along their apico-basal axis. Setting up and maintaining this polarity is pivotal to creating a functional cellular interface. The molecular machinery that controls polarity have been studied a multitude of model organisms. The proteins of the PAR, Crumbs and Scrib modules are the major players in this process. The components of these modules are strongly conserved, not only in controlling epithelial polarity, but also in cell migration, asymmetric cell division, axon guidance and antero-posterior polarity in the oocyte (Assémat et al., 2008; Goldstein and Macara, 2007; Lang and Munro, 2017; St Johnston and Ahringer, 2010; Suzuki, 2006; Tepass, 2012). While these three protein modules are well known and characterized, the downstream effectors and molecular interactions through which they control polarity remain poorly understood. We set out to uncover more about the mechanisms behind epithelial polarity by using the power of *Drosophila* genetics.

Previous work from this lab has described and built upon the roles of the basolateral polarity regulators of the Scrib module (Bunker et al., 2015; Menut et al., 2007; Zeitler et al., 2004). Genetic screening for phenotypes similar to Scrib mutants implicated the endocytic pathway in polarity control. This led to many contributions to uncovering the mechanisms of endocytic pathway components (Lu and Bilder, 2005; Morrison et al., 2008; Vaccari and Bilder, 2005; Vaccari et al., 2008, 2009, 2010; Windler and Bilder, 2010). The results described in **Chapter 2** connect endocytic trafficking and Scrib polarity regulation in *Drosophila*. The requirement of the Scrib module for proper retromer sorting indicates that Scrib can control polarity through manipulating endocytic itineraries of retromer cargo like Crumbs (Crb). With regard to epithelial polarity, the connection between retromer and apical polarity protein Crb is particularly interesting. Retromer function is required to localize and maintain Crb levels at the apical membrane in *Drosophila* epithelia (Pocha et al., 2011; Zhou et al., 2011). Loss of retromer leads to mislocalization of PAR complex proteins as well, and causes multilayering in the follicle epithelium (Pocha et al., 2011). Our analysis of retromer in *scrib* module mutants also displays these Crb defects, although we find the *scrib* mutant phenotype is not fully dependent on Crb function, indicating that other cargo are implicated in polarity control. The involvement of PAR

complex activity in retromer phenotypes of both Scrib and Crb modules hints towards a direct connection between PAR and retromer. Our observation in **Chapter 3** that aPKC activation is sufficient to induce subcortical trapping of retromer cargo Wls strengthens this case. How would the apical PAR complex control retromer trafficking? An attractive possibility is that retromer components can be directly phosphorylated by aPKC. Vps26 has been suggested as a prospective target for aPKC activity (Bailey and Prehoda, 2015). Future studies focusing on a relationship between PAR and retromer would be of particular interest to shed more light on retromer in epithelial polarity regulation.

A functional connection between Scrib and retromer has been shown in a mammalian system as well (Lohia et al.). In MDCK cells, Scrib is required to prevent retromer sorting of E-cadherin (Ecad) that is being trafficked to the lysosome. This results in aberrant Golgi accumulation of Ecad once Scrib is depleted. Since our *scrib* mutants do not show obvious changes in Ecad dynamics, the mechanism through which Scrib acts on retromer depends on the type of organism and epithelium. In line with this, a recent study found that in *C. elegans* intestinal cells the Scrib homologue LET-413 affects endocytic recycling through Rab10 activation, while not affecting retromer cargo (Liu et al., 2018). One of the potential explanations for these tissue-specific differences in retromer mechanism could come from the sorting nexins (SNX) protein family members (Gallon and Cullen, 2015). These sorting proteins interact with retromer and the specific SNX that associates with the retromer complex greatly affects the endocytic itinerary of the cargo in question. Determining which SNX family members and other associated proteins are involved in polarity-related retromer sorting is key to dissecting the various impacts on membrane trafficking in different tissues and species.

While our focus on endocytic trafficking regulation by the Scrib module was based on existing data and literature, a more unbiased approach to uncover novel regulators of epithelial polarity is through genetic screening. Forward genetic screens in *Drosophila* were one of the major drivers behind the discovery of polarity regulators. Especially the discovery of Crumbs and Scrib components was powered by these screens (Bilder et al., 2000; Jürgens et al., 1984; Schneiderman and Gateff, 1967; Stewart et al., 1972). The tumorous phenotypes that are a result of the polarity loss can also be used as a screening tool to uncover more polarity-regulating genes (Menut et al., 2007). This method of screening led to the discovery of Supernumerary limbs (Slmb) as a polarity regulator in **Chapter 3**. The F-box protein Slmb is a substrate adaptor for an E3 ubiquitin ligase complex and its function is required to restrict apical aPKC activity. At first glance the similarities between the tumorous phenotypes in **Chapter 2** and **Chapter 3** suggest that the mechanisms underlying their overgrowth and disrupted architecture are analogous. However, while mutants phenocopy especially the apical expansion, and both require aPKC activity, no genetic interaction between *slmb* and *scrib* module mutants was observed, and both Dlg and Scrib retain cortical localization in *slmb* tissue. This classifies targeted protein degradation as a distinct and separate pathway regulating epithelial polarity.

Our findings in *slmb* mutant eye and wing imaginal discs are not restricted to those specific epithelia, as a separate study has found very similar results in the *Drosophila* follicle epithelium (Morais-de-Sa et al., 2014). The main difference between the follicle and imaginal tissues is that aPKC levels in *slmb* mutant follicles are elevated, while in imaginal

discs aPKC is mislocalized rather than upregulated. Since most isoforms of aPKC lack a classic Slmb-dependent degron, our data suggest that aPKC is not the main polarity-regulating Slmb substrate. This mechanism echoes polarity regulation in the *C. elegans* zygote, where PAR-2 acts as a functional counterpart of Slmb, restricting aPKC activity (Zonies et al., 2010). While PAR-2 contains a RING finger domain characteristic of E3 ligases, it does not affect aPKC levels directly. For both Slmb and PAR-2 the possibility exists that an unknown substrate affecting aPKC activity is subject to degradative regulation. Identification of such a substrate would elucidate the roles of Slmb and PAR-2 in polarity signaling. In mammals, ubiquitin ligases have not been tied directly to aPKC, although protein degradation has been implicated in regulating other PAR complex members. In mammalian cells undergoing EMT as well as in neurons Par6 is a substrate for the E3 ubiquitin ligase Smurf1 (Cheng et al., 2011; Ozdamar et al., 2005). In addition, degradation of mammalian Par3 in cerebellar granule neurons has been linked to the activity of E3 ligase Siah1B (Famulski et al., 2010). Overall this points to targeted protein degradation a conserved mechanism for post-translational polarity control.

Our early investigations into *tid/alg3* in **Chapter 4** also involved assaying polarity defects. The overall morphology of *alg3* tumors resembled those of hypomorphic *scrib* alleles, previously described by our lab (Zeitler et al., 2004). While *alg3* mutants do not show clear polarity defects, the TNFR receptor Grnd that drives the *alg3* tumorous phenotype has been associated with coupling polarity loss to neoplastic overgrowth (Andersen et al., 2015). In this work Grnd knock-down rescues the JNK-dependent neoplastic tumor phenotype, analogous to what we observe when we reduce Grnd levels in *alg3* mutants. In this work by the Leopold lab neoplastic overgrowth is generated through depletion of the endocytic regulator *avalanche* as well as ectopic expression of the intracellular domain of Crb. This suggests Grnd could act downstream of polarity loss to trigger JNK signaling. However, a recent study has shown that polarity deficient neoplasms can develop in the absence of Grnd activity, arguing that Grnd activity is not sufficient to generate JNK-dependent tumors (Muzzopappa et al., 2017). The exact role that Grnd plays in polarity-impaired epithelial tumors remains an open question.

Clonal contexts

Many of our results regarding membrane trafficking phenotypes are either in completely mutant tissues or tissue-wide genetic depletions. Interestingly, results in clonal context show more variation. Mutant clones for *lgl* in the eye imaginal disc display Notch accumulation as well as an increase In early endosomal and MVB markers (Parsons et al., 2014; Portela et al., 2015). The *lgl* mutant clone phenotype can be rescued by reducing vesicle acidification which attenuates ectopic Notch signaling. This effect of Lgl on Notch signaling was independent of PAR complex activity (Parsons et al., 2014), in contrast with our data in both **Chapter 2** and **Chapter 3** showing that retromer cargo defects are tied to active aPKC. This suggests that Notch endocytic trafficking regulation by Lgl in clones acts through a mechanism independent of its role in polarity.

Endocytosis in a clonal context has also been addressed for *scrib* and *dlg* tissue. Mutant clones for both of these TSGs are eliminated from *Drosophila* epithelial tissues by the process of cell competition (Amoyel and Bach, 2014; Vivarelli et al., 2012). In *scrib*

clones an increase in endocytic markers has been reported, and it is suggested that elevated endocytosis is necessary to facilitate the JNK signaling activity required for the elimination of these clones (Igaki et al., 2009). Conversely, a recent study shows that *dlg* clones display normal bulk endocytosis, and although an aberrant accumulation of endocytic markers is seen, rescuing the polarity and trafficking compartment defects of *dlg* clones does not affect JNK activation and cells are still eliminated (Donohoe et al., 2018). In line with our results, these *dlg* clones displayed defective Crumbs trafficking. This study also separates aPKC activity upon polarity loss from JNK activation, similar to what is observed in whole mutant discs (Bunker et al., 2015). The fact that *slmb* clones do not undergo competitive elimination supports this model, as they are primarily driven by oncogenic aPKC signaling.

JNK-dependent elimination of polarity deficient clones by means of cell competition shows analogies to the ectopic JNK activation in *alg3* mutants in **Chapter 4**. As with the *alg3* tumorigenic phenotypes, the *Drosophila* TNF Egr and TNFR Grindelwald are required for JNK signaling in *dlg* and *scrib* clone elimination (Andersen et al., 2015; Igaki et al., 2009; Ohsawa et al., 2011). In addition, the source of Egr that triggers JNK activity in both situations is cell non-autonomous, although the exact tissue producing Egr differs depending on the context. While the circulating Egr that triggers ectopic JNK in *alg3* mutants is produced mainly in the larval fat body, in competitive elimination the ligand comes from either the surrounding wild-type cells or circulating hemocytes (Fogarty et al., 2016; Ohsawa et al., 2011; Pérez et al., 2017). The production of Egr coming from larval hemocytes that are attracted to tumorous tissue has been shown to reduce tumor size by triggering JNK-dependent cell death (Parisi et al., 2014), although we have not observed this mechanism in *alg3* mutants. Clonal phenotypes for *alg3* are mild compared to fully mutant animals, and *alg3* clones are not fully outcompeted like *scrib* or *dlg*. This could be due to a strong perdurance of the Alg3 protein, or because the phenotype of completely mutant animals is partially dependent on non-autonomous signaling, either from Egr or other pathways. The fact that TNF receptor Grnd is required cell-autonomously in both tumorigenesis and clonal competition raises the possibility that Grnd acts as a monitor for epithelial integrity. An intriguing avenue for future research would be to assess if Grnd is activated to cause JNK signaling upon any mechanism of epithelial disruption, for instance physical wounding.

Tumor growth signaling

The different tumor types described in this thesis all exhibit aberrant proliferation phenotypes that are driven by deregulated signaling pathways. The *scrib* module tumors in **Chapter 2** and slmb tumors in **Chapter 3** both arise from polarity disruption. In *Drosophila* polarity-deficient tumors the excessive proliferation is generally tied to the activation of two major pathways: the JNK pathway and aPKC dependent Yki activation (Brumby and Richardson, 2003; Bunker et al., 2015; Igaki et al., 2006; Menendez et al., 2010; Robinson and Moberg, 2011; Sun and Irvine, 2011). JNK dependent growth signaling is also essential for driving the *alg3* tumorous phenotype in **Chapter 4**.

As *slmb* discs show clear dependence on the dosage of *yki*, the Hippo (Hpo) pathway plays a central role in driving *slmb* tumorous overgrowth. However, *slmb* tumorous overgrowth is not just hyperplastic like *hpo* pathway mutants, and the activation of MMP1 indicates the the JNK pathway is at least partially involved in the *slmb* phenotype. The emphasis of **Chapter 2** is primarily on apicobasal polarity control. However, the retromer defects seen in *scrib* module mutants coincide with aberrant tumorous proliferation as well. As retromer cargo defects in both *slmb* and *scrib* module mutants are tied to aPKC activity, it would be interesting to see if mutants for *hpo* pathway components also display retromer defects. For example, the retromer cargo Crumbs feeds into the Hpo pathway through Expanded (Ling et al., 2010; Robinson et al., 2010).

Our results in **Chapter 4** indicate that Hpo mediated growth signaling contributes to the growth of *alg3* tumors. JNK-dependent Hpo pathway activation has been shown to drive regenerative growth as well as aberrant neoplastic growth in *Drosophila* (Fernandez et al., 2011; Ohsawa et al., 2012; Robinson and Moberg, 2011; Sansores-Garcia et al., 2011; Sun and Irvine, 2011). The exact link between JNK and Hpo signaling is not fully understood, but the Ajuba family proteins have been implicated in this activation of Yorkie through JNK (Sun and Irvine, 2013). The *alg3* tumors are similar to *hpo* pathway mutants in that they retain their polarity and activate Yki. However, we found this tumorous overgrowth to be independent of dJub. Therefore, the mechanism through which Hpo signaling gets activated downstream of JNK in *alg3* mutants remains elusive.

As described in **Chapter 1**, JNK signaling is a well-known player in tumorous overgrowth, in *Drosophila* as well as mammals. While the data in **Chapter 4** showing JNK activity driving a *Drosophila* tumor phenotype is not a novel mechanism, the means by which JNK gets activated in the *alg3* mutants is unlike any classic TSG. In addition, the change in glycosylation of the TNF receptor that modulates JNK activation has wider signaling implications than epithelial overgrowth in fruit flies. Glycosylation provides a post-translational mechanism through which any TNFR-dependent process can be modified. This points to an exciting direction for follow up studies addressing TNF/TNFR signaling events.

Concluding remarks

The data shown in this thesis provide examples of how studies of *Drosophila* tumor suppressors can expand our knowledge on apico-basal polarity control, epithelial growth regulation and signaling interactions. Our findings have linked established polarizing pathways together, described new regulators of cell polarity and proliferation, and even uncovered novel cell signaling mechanisms. Future *Drosophila* tumor suppressor studies will elaborate our understanding of tumorigenesis as well as the many signaling pathways and events that are tied to this process.

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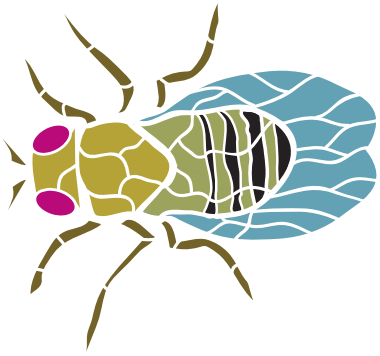

ADDENDUM:

Samenvatting voor niet-ingewijden
Curriculum Vitae
List of Publications
Dankwoord/Acknowledgements

Samenvatting voor niet-ingewijden

Het menselijk lichaam bestaat uit meerdere orgaansystemen. Dit zijn groepen van organen die samen een specifieke functie uitvoeren, zoals het zenuwstelsel of het verteringstelsel. De organen zelf bestaan weer uit verschillende weefsels, bijvoorbeeld zenuwweefsel, spierweefsel of bindweefsel. Weefsels zijn weer opgebouwd uit meerdere cellen. In mijn proefschrift ligt de focus op een speciaal soort weefsel: epitheelweefsel, ook wel dekweefsel genoemd. Epitheelweefsel bedekt alle vrije oppervlakken en holtes in het lichaam. De primaire functie van epitheelweefsels is het vormen van een beschermende cellaag tussen verschillende omgevingen. Vaak is dit een barrière tussen de buitenwereld en andere weefsels, zoals het geval is voor het grootste uitwendige menselijk orgaan, de huid, maar ook de epitheelcellen die het darmstelsel of de luchtwegen bekleden. Deze barrière ontstaat doordat epitheelcellen sterk aan elkaar gehecht zijn, waardoor zelfs moleculen ter grootte van een eiwit niet zomaar deze barrière kunnen passeren. Omdat epitheelcellen veelal twee compleet verschillende omgevingen begrenzen, zijn ze gepolariseerd. Deze polariteit houdt in dat de kant van de cellen die naar de buitenwereld is gericht (de apicale kant van de cel) sterk verschilt van de kant die zich aan de binnenkant van het lichaam bevindt (de basolaterale kant). Een speciale sub-groep van epitheelweefsels zijn de klierweefsels. Deze bestaan uit gespecialiseerde epitheelcellen die stoffen produceren en uitscheiden. Bekende voorbeelden hiervan zijn speekselklieren en melkklieren, maar ook grotere organen zoals de lever en de alvleesklier bestaan voornamelijk uit dit type epitheel. Epitheelcellen zijn dus overal te vinden, en niet alleen in de mens. Vrijwel ieder meercellig dier bevat epitheelweefsel. Dit komt ons goed van pas, omdat het de mogelijkheid geeft om de ontwikkeling, groei en de polariteit van epitheelcellen te bestuderen in ons favoriete modelorganisme, de fruitvlieg *Drosophila melanogaster*.

Waarom zijn we zo geïnteresseerd in epitheelcellen? Eén van de belangrijkste redenen is dat we een ziekte als kanker beter willen begrijpen en veel types kanker ontstaan in epitheelcellen. Elke klasse kwaadaardige tumor die wordt aangeduid met de term 'carcinoom' is ontstaan in epitheelweefsel. Voorbeelden hiervan zijn mammacarcinoom (de meest voorkomende vorm van borstkanker), adenocarcinoom (klierkanker) of basaalcelcarcinoom (huidkanker). Als we de epitheelcellen in deze carcinomen bekijken, zien we dat hun polariteit ernstig verstoord is en dat ze door ongecontroleerde celdelingen blijven groeien. We weten dat kanker ontstaat door een opeenstapeling van veranderingen (mutaties) in het DNA van de cel. Het DNA kan worden gezien als het instructieboek dat bepaalt hoe een cel wordt opgebouwd en welke functie deze cel verricht. In deze vergelijking zijn eiwitten de specifieke bouwstenen van de cel en de instructies voor ieder eiwit zijn gecodeerd in het DNA in de vorm van genen. Elke mutatie in een gen kan leiden tot verandering in het bijbehorende eiwit, waardoor een eiwit zijn functie vaak verliest, of soms juist veel actiever wordt dan normaal. Meerdere mutaties kunnen de eiwitten in een cel zodanig veranderen, dat het gedrag van de complete cel wordt aangepast. Cellen kunnen zich ongeremd gaan vermenigvuldigen, of zich verplaatsen naar andere weefsels in het lichaam, eigenschappen die beide kenmerken zijn van kanker. Dit gedrag is in gezond epitheelweefsel strak gereguleerd om te voorkomen dat cellen op hol slaan. Ons doel is om dit soort regulerende mechanismen te bestuderen en te begrijpen zodat we meer inzicht krijgen in wat er precies misgaat zodra mutaties een goedaardige cel in een kwaadaardige (maligne) veranderen.

Terug naar de fruitvlieg. Wat maakt *Drosophila* zo geschikt als modelorganisme voor ons onderzoek? Op het eerste gezicht lijken fruitvliegen ver verwijderd van de mens, maar als we kijken naar het DNA blijkt dat ongeveer 60% van de genen overeenkomen. Dit betekent dat van een meerderheid van de menselijke genen een soortgelijke versie in de fruitvlieg bestaat. Zo kunnen we mutaties in een fruitvlieg-gen maken, kijken wat voor effect de mutatie op de fruitvlieg heeft (het phenotype) en zo leren welke functie dit gen en het bijbehorende eiwit in de mens vervullen. *Drosophila* heeft als bijkomstig voordeel dat het al een lange tijd als proefdier in laboratoria gebruikt wordt. Zo zijn er veel technieken ontwikkeld om de genen aan te passen, door te knippen en plakken in het fruitvlieg DNA. We kunnen genen en eiwitten aan- en uitschakelen, of markeren om het gedrag binnen een cel te volgen. Daarbij is het ook mogelijk deze manipulaties exclusief in een specifiek orgaan of zelfs alleen in een klein groepje cellen uit te voeren, waarbij het tijdsframe van deze manipulaties gestuurd kan worden. Fruitvliegen hebben bovendien een korte levenscyclus (van bevrucht eitje naar volwassen vlieg duurt ongeveer 10 dagen), waardoor experimenten snel kunnen worden uitgevoerd. Dit alles maakt het voor ons makkelijk om experimenten uit te voeren in de epitheelweefsels van *Drosophila*. Daar komt bij dat bepaalde genetische mutaties in de fruitvlieg tumoren ontwikkelen in deze epitheelweefsels! Genen die door een mutatie hun werking verliezen wat leidt tot het ontstaan van tumoren noemen we tumor suppressor genen.

Het overkoepelende doel van mijn proefschrift is om tumor suppressor genen in *Drosophila* te bestuderen en zo meer te leren over hoe de groei en polariteit van epitheelcellen wordt gereguleerd. In **Hoofdstuk 1** beschrijf ik de tumor suppressor genen die bekend zijn in *Drosophila* en welke functie deze genen vervullen in epitheelcellen. Een belangrijke rol is hier weggelegd voor de genen en eiwitten die de polariteit van het epitheel beheren. Zoals hierboven beschreven is, hebben epitheelcellen een apicale en basolaterale kant. Om goed te kunnen functioneren moeten deze kanten van de cel strak begrensd en gescheiden blijven. Deze scheiding wordt binnen de cel gereguleerd door speciale polariteits-genen en bijbehorende eiwitten. Zodra deze polariteits-regelaars verstoord worden door bijvoorbeeld een mutatie, gaat de polariteit in de epitheelcel verloren. Wat fruitvliegen buitengewoon interessant maakt, is dat een enkele mutatie in de polariteits-genen die de basolaterale kant beheren ervoor kan zorgen dat er tumoren ontstaan in de epitheelweefsels! Deze basolaterale polariteits-genen zijn dus tumor suppressor genen. De apicale polariteits-genen blijken het omgekeerde effect te sorteren: zodra deze genen actiever dan normaal zijn, veroorzaken ze tumorgroei. Verstoorde polariteit blijkt dus zeer belangrijk voor het ontstaan van tumoren.

Een andere categorie tumor suppressor genen die ontdekt werden in *Drosophila* zijn de genen die endocytose reguleren. Endocytose is een belangrijk cellulair transportsysteem waarmee eiwitten van het celmembraan naar verschillende compartimenten binnen de cel worden vervoerd. Mutaties in endocytose-genen veroorzaken tumoren die erg veel lijken op de tumoren die we zien bij verstoorde polariteit. Het is dus waarschijnlijk dat deze twee types tumor suppressor genen met elkaar verbonden zijn, al was een direct verband nog niet gevonden. Daar kwam verandering in door ons werk in **Hoofdstuk 2**. Hier laten we zien dat hoewel polariteits-deficiënte tumoren een grotendeels functionerend endocytose transportsysteem bevatten, er defecten zijn in een specifiek onderdeel van dit transportsysteem. Eiwitten die dit specifieke

transportmechanisme nodig hebben komen op de verkeerde plek terecht zodra polariteit verloren gaat, wat betekent dat het mechanisme afhankelijk is van intacte polariteit. De manier waarop verschillende polariteits-regelaars hun functie in de epitheelcel uitoefenen is veelal onbekend, maar dit werk wijst erop dat de koppeling met het endocytose transportsysteem een belangrijke rol speelt.

Eén van de grote voordelen van de fruitvlieg is dat het makkelijk is om nieuwe, interessante genen te ontdekken door middel van een genetische screen. Zo een screen houdt in ons geval in dat we zelf mutanten genereren die dezelfde eigenschappen hebben als de tumor suppressor mutanten die we al kennen. De mutaties die we veroorzaken in onze vliegen zijn willekeurig, vandaar dat we onze mutanten moeten 'screenen', om te zien of ze interessante tumoren ontwikkelen en welk gen er precies gemuteerd is. Op deze manier kunnen we nieuwe tumor suppressor genen ontdekken en vervolgens bekijken hoe die genen en bijbehorende eiwitten passen in onze bestaande kennis. In **Hoofdstuk 3** beschrijven we een tumor suppressor gen dat we ontdekt hebben in een van onze genetische screens. Dit gen, genaamd *slmb*, speelt een rol in het beperken van het apicale domein van de epitheelcel. Zodra *slmb* gemuteerd is, zijn de eiwitten die dit apicale domein reguleren overmatig actief. Deze activiteit leidt, zoals ik hierboven ook beschreven heb, tot tumorgroei. De normale functie van *slmb* is dat het specifieke eiwitten aanwijst die door de cel moeten worden afgebroken. Dit is een zeer belangrijke functie, omdat het zorgt voor de balans van eiwitten in een cel. De *slmb* mutanten hebben dus een verstoorde balans van polariteits-eiwitten, waardoor epitheelcellen hun polariteit verliezen en tumoren kunnen ontwikkelen.

Niet iedere tumor suppressor is gelinkt aan polariteits-regulatie. In **Hoofdstuk 4** beschrijven we het gen *alg3*, waarvan de mutanten ook tumoren in de epitheelweefsels van *Drosophila* ontwikkelen, maar de polariteit van de cellen intact blijft. Dit gen is een vrij ongebruikelijke tumor suppressor, aangezien *alg3* een belangrijke stap vervult in een proces dat weinig aan tumoren en kanker gekoppeld wordt. Dit proces heet 'glycosylering'. Dit klinkt als een duur woord, maar glycosylering is een vrij algemeen proces wat in veel celtypes voorkomt en omvat alle stappen die nodig zijn om suikers aan eiwitten vast te plakken. Deze suikergroepen kun je zien als hulpstukken die ervoor zorgen de eiwitten beter functioneren. Eiwitten kunnen dus één of meerdere suikergroepen bevatten die aan het eiwit gekoppeld worden door glycosylering. De glycosylering in *alg3* mutanten is defect, waardoor suikers niet of beperkt aan eiwitten worden vastgezet. Maar waarom veroorzaakt dit defect in glycosylering tumorgroei? Wij hebben ontdekt dat de afwezigheid van suikers op bepaalde eiwitten ervoor zorgt dat de verbinding tussen deze eiwitten verandert. Hierdoor schakelen de epitheelcellen in *alg3* mutanten een groeisignaal in wat normaal gesproken niet actief is. Dit zorgt ervoor dat de epitheelcellen ongeremd blijven delen en zo tumoren ontwikkelen. Dit onderzoek is extra interessant omdat het proces van glycosylering niet eerder aan deze specifieke eiwitbinding en groeisignalering gelinkt is, waardoor het niet alleen van belang is voor kankeronderzoek, maar ook andere ziektes die te maken hebben met defecte glycosylering en eiwit-interacties.

In **Hoofdstuk 5** bespreek ik de resultaten van de voorgaande hoofstukken, plaats het in de context van wat bekend is en ook vooral van wat we nog niet weten en dus in de toekomst uit willen zoeken. Hoewel we veel weten over de regulatie van polariteit en groei in epitheelcellen is er nog veel meer dat we gewoon nog niet begrijpen. Het belangrijkste dat ik met proefschrift wil aantonen is dat we met het bestuderen van tumor suppressor genen in de fruitvlieg nieuwe dingen kunnen leren over hoe tumoren zich ontwikkelen en wat de onderliggende cellulaire mechanismen zijn. Zo begrijpen we beter hoe een gezond epitheelweefsel in elkaar steekt en functioneert, maar ook wat er misgaat als deze cellen een kwaadaardige transformatie ondergaan.

Curriculum vitae

Gerardus Adrianus Maria "Geert" de Vreede was born on December 27th 1984 in Honselersdijk, The Netherlands. He attended the "Westland College" in Naaldwijk in 1997, passing his VWO exam in 2003 for the profiles "Natuur & Gezondheid" and "Natuur & Techniek". In the same year he started his Bachelor studies in Electrical Engineering at Delft University of Technology. After finishing his "Propedeuse", his love for biology took over his fascination for engineering and he made a switch to studying Biology at Utrecht University in 2006. He completed the program in 2009, finishing with writing a Bachelor's thesis on "The role of the Scribble complex in setting up polarity and carcinogenesis" with dr. Mike Boxem. That year he started the Biomedical Sciences Master's program "Cancer, Genomics and Developmental Biology" at Utrecht University. For his major research project, Geert combined RNA interference techniques with live imaging to study the role of conserved polarity and cell division proteins in C. elegans, in the lab of prof. dr. Sander van den Heuvel under the supervision of dr Mike Boxem and dr. Marjolein Wildwater. For his minor research project, he worked in the lab of prof. dr. David Bilder at the University of California Berkeley on follicle cell rotation and glycosylation in *Drosophila*. Geert finished the Master's program with writing his Master's Thesis with prof. dr. Catherine Rabouille on "Endocytic control of tumor suppression in *Drosophila melanogaster*" in 2011. In February 2012 he started his PhD in the lab of prof. dr. David Bilder at the University of California Berkeley, under the supervision of prof. dr. Sander van den Heuvel, which resulted in the work described in this thesis.

List of Publications

de Vreede G, Morrison HA, Houser AM, Boileau RM, Andersen D, Colombani J, Bilder D (2018) A *Drosophila* tumor suppressor gene prevents tonic TNF signaling through receptor N-glycosylation. *Developmental Cell*, 45, 595–605, June 4

Skwarek LC, Windler SL, **de Vreede G**, Rogers GC, Bilder D. (2014) The F-box protein Slmb restricts the activity of aPKC to polarize epithelial cells. *Development*, 141:2978-83.

de Vreede G, Schoenfeld JD, Windler SL, Morrison H, Lu H, Bilder D. (2014) The Scribble module regulates retromer-dependent endocytic trafficking during epithelial polarization. *Development*, 141:2796-802.

Wildwater M, Sander N, **de Vreede G**, van den Heuvel S. (2011), Cell shape and Wnt signaling redundantly control the division axis of *C. elegans* epithelial stem cells. *Development*, 138: 4375-85.

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For the English-speaking readers, some will say this is the part of the thesis that most people will actually read. In that sense it is one of the most important parts. Here I'd like to thank everyone that helped me along the road to getting my PhD, not only scientifically, but also at a personal level.

First and foremost I'd like to thank my promotor and daily supervisor **David Bilder**. When I first came to your lab for my Masters' rotation in 2011, I immediately felt at home in your lab. It was an easy decision for me to come back a year later to start my PhD. Learning 'how to fly' under your mentorship was a wonderful experience and made me fall in love with fly genetics. The way you are engaged with everybody's project in the lab is exemplary and I'm still impressed by how easy you find the right words when writing just about anything. I'm glad our time together in the lab was productive and resulted in several beautiful publications. I'm sure the lab will continue doing great science and look forward to seeing what comes out of it in the future! Thanks for everything!

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Dear **Ale**, I'm so grateful for having you as a friend and a former labmate. Spending so many years together in the lab you go through both the good and the bad days. You were always there for me, whether it was through the wise words of your mom, starting a Cake cover band, providing Spanish lessons or just talking about "crazy Americans". Our trips to fly meetings in the US and Barcelona were some of the highlights of my time here. Watching you present at the meeting in Orlando made me see how much of a role model you are, not just as a scientist but also as a human being, maybe even more than you realize. I'm a little sad that our old Albany clan has scattered out over the Bay Area, but I'm positive we'll hang out often enough. I'm glad you're enjoying your work at Genentech, they are lucky to have you. Finalmente, yo quiero decir algunas palabras de cierre en tu lengua materna, pero mi español es insuficiente así que vamos a la playa con mi tortuga de la noche!

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we were trying to figure out. Keep kicking ass in the Perrimon lab! **Linda**, getting two labs to go to the gym is no small feat, I'd be in way worse physical shape if it weren't for you. I'm sure you are still putting many men in their place in Seattle. **Sa Kan**, crazy Japanese guy, I still get many updates on your life through Dong. Looks like your lab in Kobe is doing well, I hope to come visit sometime! **Robin**, my favorite fly meeting roomy! Your Brexit from the lab made me very sad, I really miss our complaining sessions and the snarky commentary on stupid Americans. Hopefully your interactions with the DMV and human resources are better in Arizona!

The people you know outside of the lab are just as important as the ones inside, to keep you grounded in reality and remind you that there's also a world outside of the big bubble that is science. This part is dedicated to them. My old roomy, **Carolyn**, thanks for taking me in I when I came back to the States. I always looked forward to your Murder Mystery parties, the Best Dressed Award is one of my proudest achievements in life. Have the dogs adjusted to Boston temperatures yet? **Patrik**, Mr P, P-spot, P-dog, the most Swedish of friends. I'm so glad I found someone here to talk about fotboll and play long romantic sessions of FIFA with. The Americans just don't understand. I'm a little sad you don't live down the street anymore, but every time I pet Bamse it makes the trip to Daly City worth it. Oh hi **Tim**, where Patrik fulfills my soccer needs, you are my go to for video games and bad movies. The late night Killing Floor sessions were really good for my scientific productivity, I'm sure. Now that I'm finished writing my thesis, I can finally catch up on playing Fallout 4!

Thijs, eigenlijk zou jij een eigen hoofdstuk in dit proefschrift krijgen, maar dat mocht niet van Sander, vandaar maar dit stukje hier. Ik ben zo blij dat jij ook het Amerikaanse avontuur bent aangegaan, al was het alleen al omdat je zo in een betere tijdzone woont om online te komen voor Dark Souls. Wij zijn zo vaak samen doodgegaan, dat schept echt een band. Onze belevenissen in Boston, LA en de Bay Area waren legendarisch, met als hoogtepunt wel die alligator die we in Berkeley hebben gegeten! Superlekker! Aangezien je binnenkort met je kersverse familie weer in Nederland te vinden bent zal ik ook bij het Academiegebouw een zeepje laten vallen. **Linda**, ook jij kwam hier terug voor je PhD. Stanford is dan ook geen verkeerde keuze (al zal de gemiddelde Berkeley undergrad het daar niet mee eens zijn). Onze (road)trips in Californië en daarbuiten waren super en het is altijd fijn om met iemand te praten die in hetzelfde promotieschuitje als jou zit. Veel succes met de laatste loodjes! **Rolo**, elke keer als ik je spreek vraag ik me weer af in welke uithoek van de wereld je je bevind. San Francisco is een stuk saaier zonder jou. Ik ben al veel te lang niet wezen filmhoppen en zonder jou had ik de echte Kerstman nooit ontmoet. Stockholm was supergezellig! Ik ben benieuwd naar je volgende bestemming! **Sam**, het spijt mij en Thijs nog steeds dat we je in een auto omsingeld met zombies hebben achtergelaten. We moeten weer eens gaan wijnproeven, zolang het naast een lange rechte weg is komt dat wel goed. **Tim**, ben je nog bomen aan het zagen? Les aan het geven in de bus? Ik hoop dat je ondertussen wat meer hebt uitgevogeld wat je met je leven wilt doen. **Suzanne** en **Vincent**, bedankt voor de hulp op afstand voor mijn promoveer-rompslomp. Ik ben benieuwd naar jullie toekomstige boekjes! **Tessa** en **Gwen**, de dagen van BancTec en de Das liggen al erg ver achter ons. Het is altijd mooi om herinneringen met jullie op te halen als ik in de buurt ben. Misschien een keertje in Californië doen? **Lisanne**, jij bent hier binnenkort, maar dan ben ik weer

weg! Veel plezier met je grote Amerika-trip, laat me weten wat je beleefd hebt. **Rob**, we moeten weer eens een wedstrijdje in de Kuip meepakken als ik in het land ben! Al is een poppen-musical een goed alternatief. **Paul**, Eto'Onings, misschien dat we met Rob erbij voor eens en altijd het eeuwige vraag kunnen beantwoorden: Burger King of McDonald's? Natuurlijk kan ik ook alle **Kantelaars** niet vergeten te bedanken; ook al zijn jullie allemaal ver weg, ik kan nog steeds slechte woordgrappen kwijt in de WhatsApp groep. Daarbij is het elke keer als ik weer in Nederland ben net alsof ik nooit weggeweest ben (al zijn er wel een hoop babies bij gekomen). Ook vond ik het supergaaf dat een aantal van jullie me hier bezocht hebben!

Uiteraard wil ik ook al mijn familie bedanken voor hun steun door de jaren heen. Alle ooms en tantes, neefjes en nichtjes, bedankt voor de interesse en gezelligheid als ik me weer eens in het Westland bevond. **Papa** en **mama**, **Anita**, **Corine** en **Marlène**, de afstand maakt het niet altijd even makkelijk. Bedankt voor alle lange skypesessies, voor het luisteren naar mijn geklaag en het uitleggen van de moeilijkheden van fruitvlieg-experimenten. Jullie hebben me al die jaren geholpen en bijgestaan, en ik ben blij en trots dat ik het eindresultaat met jullie kan vieren!

Jessica, I'm so happy to have had you by my side during the last couple of years of my PhD. Thanks for making sure I spend some time out of the lab (and out of the 'writing room'). You know I'm not a fan of clichés, but for you I will line a bunch of them up right here: You bring out the best in me and I could not have done this without you, your support really got me through all of it. I hope you will enjoy this Dutch adventure, and I'm looking forward to all the other adventures we'll have in the future!

"It always seems impossible
untill it is done "

-Nelson Mandela