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## CELL POLARITY REGULATES ORGAN GROWTH THROUGH THE HIPPO PATHWAY

Chiao-Lin Chen

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**CELL POLARITY REGULATES ORGAN GROWTH THROUGH  
THE HIPPO PATHWAY**

A

DISSERTATION

Presented to the Faculty of  
The University of Texas  
Health Science Center at Houston  
and  
The University of Texas  
M. D. Anderson Cancer Center  
Graduate School of Biomedical Sciences

in Partial Fulfillment

of the Requirements  
for the Degree of

DOCTOR OF PHILOSOPHY

by

**Chiao-Lin Chen, M.S.**

Houston, Texas

May 2011

# **CELL POLARITY REGULATES ORGAN GROWTH THROUGH THE HIPPO PATHWAY**

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# **CELL POLARITY REGULATES ORGAN GROWTH THROUGH THE HIPPO PATHWAY**



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# CELL POLARITY REGULATES ORGAN GROWTH THROUGH THE HIPPO PATHWAY

Publication No. \_\_\_\_\_

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Defects in apical-basal cell polarity and abnormal expression of cell polarity determinants are linked to human cancer. Loss of polarity is highly correlated with malignancy. In *Drosophila*, perturbation of apical-basal polarity, including overexpressing the apical determinant Crumbs, can lead to uncontrolled tissue growth. Cells mutant for the basolateral determinant *scribble* overproliferate and can form neoplastic tumors. Interestingly, *scribble* mutant clones that arise in wild-type tissues are eliminated and therefore do not manifest their tumorigenic potential. However, the mechanisms by which cell polarity coordinates with growth control pathways in developing organs to achieve appropriate organ size remain obscure.

To investigate the function of apical determinants in growth regulation, I investigated the mechanism by which the apical determinant Crumbs affects growth in *Drosophila* imaginal discs. I found that *crumbs* gain and loss of function cause overgrowth and induction of Hippo target genes. In addition,

Crumbs is required for the proper localization of Expanded, an upstream component of the Hippo pathway. Furthermore, we uncoupled the cell polarity and growth control function of Crb through structure-functional analysis. Taken together, our data identify a role of Crb in growth regulation specifically through modulation of the Hippo pathway.

To further explore the role of polarity in growth control, I investigated how cells mutant for basolateral determinants are eliminated by using patches of cells mutant for *scribble* (*scribble* mutant clones) as a model system. We found that competitive cell-cell interactions eliminate tumorigenic *scribble* cells by modulation of the Hippo pathway. The regulation of Hippo signaling is required and sufficient to restrain the tumorous growth of *scribble* mutant cells. Artificially increasing the relative fitness of *scribble* mutant cells unleashes their tumorigenic potential. Therefore, we have identified a novel tumor-suppression mechanism that depends on signaling between normal and tumorigenic cells. These data identify evasion of cell competition as a critical step toward malignancy and illustrate a role for wild-type tissue in eliminating abnormal cells and preventing the formation of tumors.

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# **Chapter 1:**

## **Introduction**

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## Overview

How is the size of an animal determined? How is homeostasis achieved? These are some of the most fundamental questions in developmental biology. Significant amounts of time and effort have been committed to understanding the mechanisms that determine the size of an animal and its organs (Conlon and Raff, 1999; Edgar, 2006; Halder and Johnson, 2011; Johnston and Gallant, 2002). Studies have shown that proper cell polarity is important to ensure correct body size and allow for normal developmental processes to occur (Assemat et al., 2008; Dow and Humbert, 2007; Humbert et al., 2008; Tepass et al., 2001). Perturbations of apical-basal polarity can lead to tumor formation and a variety of diseases (Dow and Humbert, 2007; Humbert et al., 2008; Vaccari and Bilder, 2009). Interestingly, epithelial cells that lose polarity, a defect that is an obligatory step toward malignancy, are often eliminated from normal tissues despite their ability to overproliferate in tissues comprised entirely of mutant cells (Bilder et al., 2000; Brumby and Richardson, 2003; Igaki et al., 2009). However, the mechanisms through which cell polarity regulates growth in developing organs and the mechanisms through which homeostasis is maintained remain obscure.

### 1.1. *Drosophila* as a model organism for growth control research

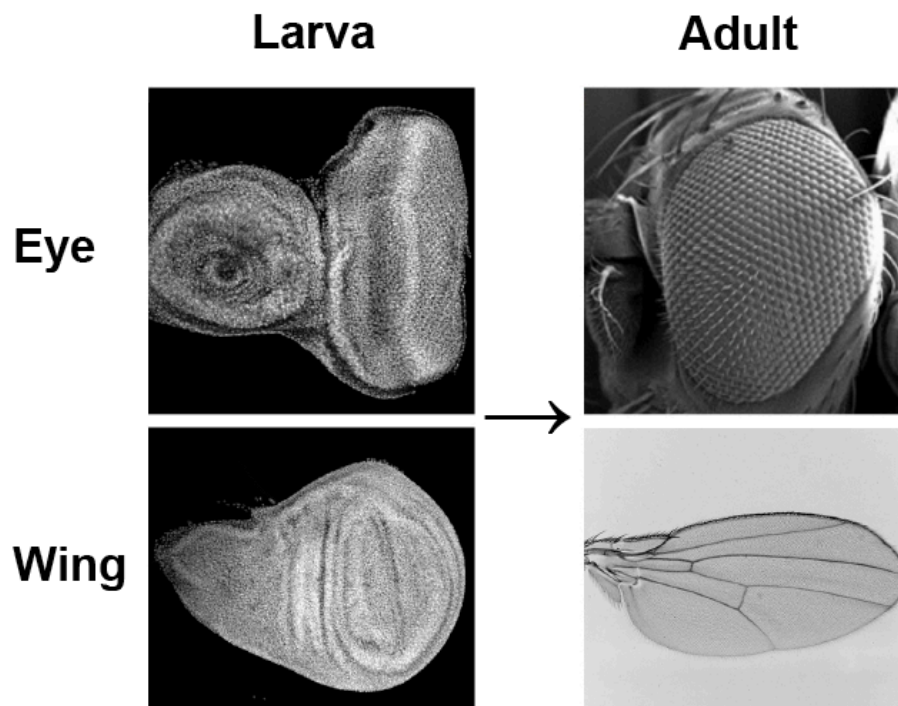
In our laboratory, we investigate the molecular mechanisms of organ size regulation via a genetic approach in the fruit fly *Drosophila melanogaster*.

Components of multiple signaling pathways that control basic developmental processes are functionally conserved between flies and humans (Brumby and Richardson, 2005). Although there is striking conservation in signaling pathways, in many cases there are multiple homologs or orthologs of a particular gene in humans where there is only one counterpart in flies. The complexity of vertebrate systems makes them difficult to be analyzed experimentally. In contrast, lack of redundancy in flies make them amenable as a model system. Moreover, over 80% of genes found to regulate organ size in *Drosophila* are conserved in vertebrates, and many have been implicated in human cancer and fly tumor formation (Brumby and Richardson, 2005; Oldham and Hafen, 2003; Pan et al., 2004; Prober and Edgar, 2001; Turenchalk et al., 1999). This demonstrates that not only normal processes but also abnormal processes appear to be conserved. The potential benefits of using *Drosophila* as a model organism to study human disease are many and the use of the fly system for this purpose has been reviewed extensively (Brumby and Richardson, 2005; Oldham and Hafen, 2003; Pan et al., 2004; Prober and Edgar, 2001; Turenchalk et al., 1999). Therefore, investigating growth regulation in *Drosophila* provides us with valuable information on organ size control and diseases related to uncontrolled growth, such as cancer.

Like mammals, *Drosophila* undergoes regulative development (Bryant and Simpson, 1984) and retains an innate cellular plasticity. Cells in developing

organs respond to extrinsic cues by adjusting their proliferation rate and inducing additional cell proliferation or cell death to produce organs with appropriate sizes (de la Cova et al., 2004; Johnston and Gallant, 2002; Neufeld et al., 1998). For example, when some cells are ablated or irradiated in precursor epithelial tissues of flies, called imaginal discs, the wounded tissues are able to regenerate and form organs of normal size (Bryant, 1975). Several lines of evidence indicate that wounded tissues can signal to neighboring cells to undergo additional cell divisions, referred to as compensatory proliferation, to replace the damaged cells (Bergmann and Steller, 2010; Day and Lawrence, 2000; Johnston and Gallant, 2002). Unlike the roundworm *C. elegans* in which the loss of the ablated cells is not compensated for by extra proliferation of remaining cells, development of fly organs is not restricted to a fixed cell lineage (Bryant and Simpson, 1984; Deppe et al., 1978). Therefore, *Drosophila* provides a suitable *in vivo* system to analyze cell-cell interactions and their effects on cell growth and tissue homeostasis.

I used the developing eye and wing of the fly to investigate how proper organ size is achieved (Figure 1.1). The *Drosophila* life cycle includes embryonic, larval, pupal, and adult stages. Most organs of an adult fly, including eyes and wings, are derived from primordial epithelial tissues called imaginal discs. Imaginal discs develop from clusters of about 20-50 cells during embryogenesis (Baker, 2001; Cohen, 1993). During larval development, the



**Figure 1.1. Development of *Drosophila melanogaster*.**

Like most other external structures in *Drosophila*, adult fly eyes and wings (**Right**), are derived from primordial tissues in the larva, called imaginal discs (**Left**). Imaginal discs from third instar larva are stained for DAPI to reveal nuclei.



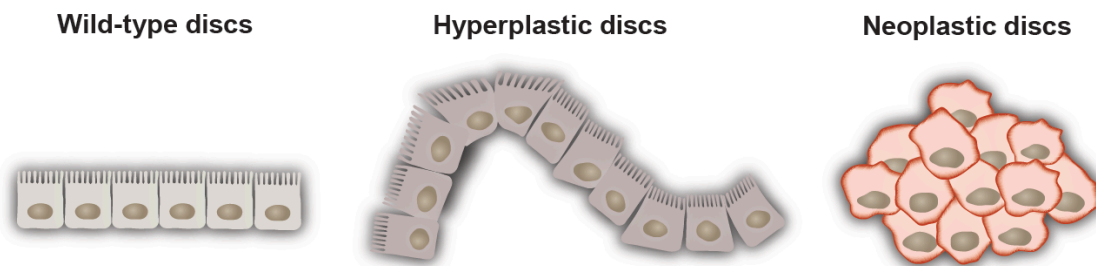
disc cells undergo a massive amount of cell proliferation such that cell number increases exponentially. Upon pupation, discs can reach about 30,000 to 100,000 cells. During the third instar and pupal stages, these discs begin to differentiate into their corresponding adult tissues. At metamorphosis in the pupal stage, the imaginal discs evert and fuse to form the adult structures. This relatively short life cycle, around 10 days at 25°C, is another attractive feature of *Drosophila* that makes it an excellent genetic model organism.

Additionally, many sophisticated tools and techniques for experimental analysis in *Drosophila* have been well established. These features make flies amenable for both reverse and forward genetic screens with intensive genetic manipulation. In the field of growth control, the search to identify tumor suppressor genes started from studies of loss of function mutations in genes that result in tumorous growth in homozygous animals (Gateff, 1982). Afterwards, multiple screens were performed to identify genes that are essential for growth regulation by screening for overgrown tissues. Mutations in genes that cause overgrowth phenotypes are more likely to be specific for growth regulation than mutations in genes leading to reduced tissue size, because cell lethality can be caused by mutations in housekeeping genes that are unrelated to growth control. With the improvement of genetic techniques, different methods of genetic screens were developed and performed, including dominant modifier screens and mosaic screens. Mosaic screens utilized the FRT/FLP

system, adopted from the yeast system, to generate tissue-specific homozygous mutants in otherwise heterozygous animals (Golic and Lindquist, 1989; Xu and Rubin, 1993). This system permitted identification of growth regulatory genes that are also essential during early development by allowing researchers to bypass requirements in viability. Recently, the method of using RNA interference has been developed and improved to knock down the expression of target genes in flies efficiently. In addition to classic EMS screens, several RNAi-based forward genetic screens have been used to identify genes that regulate growth and other functions (Cronin et al., 2009; Mummery-Widmer et al., 2009; Pospisilik et al., 2010). These advanced genetic techniques have contributed to the discovery and characterization of many growth-controlling pathways, including the Hippo pathway.

## **1.2. Organ Size Regulation in *Drosophila***

In early studies, mutations with overgrown discs were isolated from screens or arose spontaneously (Gateff, 1982). The overgrowth phenotypes are classified as hyperplastic overgrowth and neoplastic overgrowth based on their distinct characteristics (Figure 1.2). Cells in hyperplastic discs overproliferate, but retain apical-basal polarity, the ability to differentiate, and monolayered cellular organization, which often result in a folded structure. After extensive research, most mutations that are characterized as hyperplastic tumor suppressors have been grouped into different pathways, including the insulin



**Figure 1.2. Different types of overgrowth phenotypes**

Cartoon pictures depict cells in different discs. **(Left)** In wild-type discs, cells form a sheet of epithelial cells. **(Center)** Cells in hyperplastic discs overproliferate, but retain polarity and monolayered cellular organization, which often results in extra folding of the disc. **(Right)** In contrast, cells in neoplastic discs lose their morphology structure and pile on top of each other.

receptor pathway and the Hippo pathway (Edgar, 2006; Badouel et al., 2009; Halder and Johnson, 2011; Reddy and Irvine, 2008). The insulin receptor pathway responds to nutrient conditions and regulates cell size, while the Hippo pathway regulates cell number by inhibiting cell proliferation and promoting apoptosis. While both cell number and cell size are important aspects for organ size regulation, we are focusing on understanding mechanisms that regulate cell number. Phenotypes, target genes, cellular components, and the functional conservation of the Hippo pathway will be discussed more in detail in the next section.

Discs homozygous mutant for neoplastic tumor suppressor genes have defects in apical-basal polarity and thus exhibit disrupted epithelial architecture and multilayered epithelia. In addition, cells in neoplastic discs overproliferate, fail to differentiate, and have the ability to invade into other tissues. When a small piece of a neoplastic disc is transplanted into wild-type animals, mutant cells show metastatic behaviors, including forming secondary tumors (Woodhouse et al., 1998). Additionally, expressing an activated form of Ras (Ras<sup>V12</sup>) has been shown to synergistically interact with mutations in neoplastic tumor suppressor genes to promote tumor growth and invasion (Brumby and Richardson, 2003; Wu et al. 2010). Notably, these neoplastic phenotypes are characteristics of primary malignant tumor diagnosis (Hanahan and Weinberg, 2011).

Two broad classes of genes associated with neoplastic overgrowth phenotypes have been identified. The first group of genes encode components of basolateral cell polarity determinants, including *lethal giant larvae (lgl)*, *discs large (dlg)*, and *scribble (scrib)*. Mutation in these genes leads to loss of cell polarity accompanied by overproliferation in epithelial cells. The role of apical-basal cell polarity in growth regulation will be discussed in greater detail in the following section.

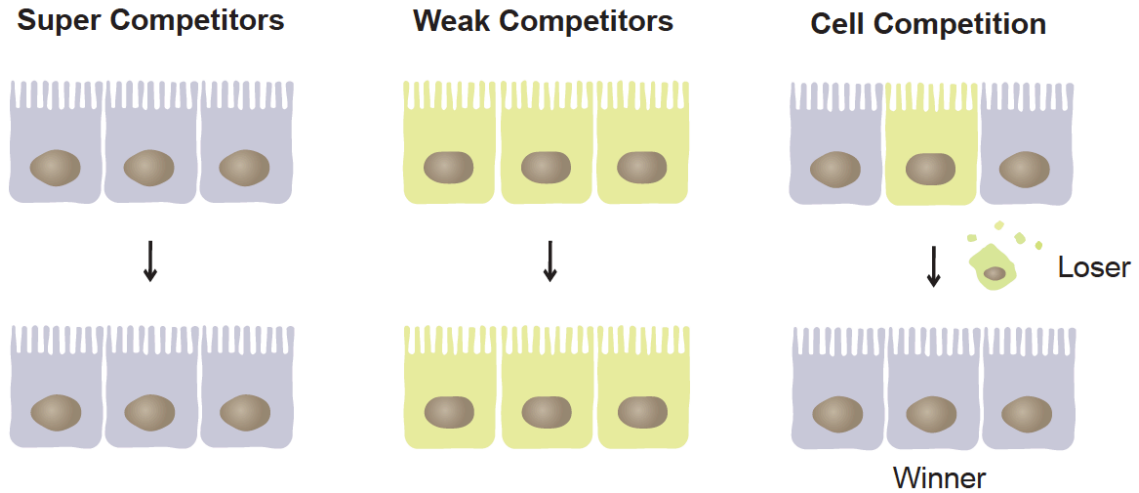
The second group of genes in the neoplastic class encode components with functions in the endocytic trafficking pathway, including *avalanche (avl)*, *Rab5*, *vps25*, and *vps23* which is also known as *erupted* or *Tsg101* (Herz et al., 2006; Lu and Bilder, 2005; Moberg et al., 2005; Thompson et al., 2005; Vaccari and Bilder, 2005; Wucherpfennig et al., 2003). Endocytosis is a multistep process that cells use to engulf extracellular substances, recycle transmembrane proteins and lipids, and regulate signaling pathways (Gagliardi et al., 2008; Giebel and Wodarz, 2006; Shivas et al., 2010; Vaccari et al., 2008). Part of the plasma membrane is internalized to form endosomes, and transmembrane proteins are transported from the cell surface into the cellular compartment by the internalization. Avl and Rab5 are localized in early endosomes and function at the sorting step of endocytic trafficking (Lu and Bilder, 2005; Wucherpfennig et al., 2003), while Vps25 and Vps23 participate at later stages of endocytosis (Herz et al., 2006; Moberg et al., 2005; Thompson et

al., 2005; Vaccari and Bilder, 2005). *vps25* and *vps23* encode components of the ESCRT (Endosomal Sorting Complex Required for Transport) complex that regulates the sorting of ubiquitinated proteins, which are directed to lysosomes for degradation rather than being recycled to the cell surface (Giebel and Wodarz, 2006; Herz et al., 2006; Moberg et al., 2005; Thompson et al., 2005; Vaccari and Bilder, 2005). Mutations in *avl* and *vps23* mutants lead to the accumulation of the apical polarity determinant Crumbs through out the cell (Lu and Bilder, 2005; Moberg et al., 2005). Therefore, the neoplastic transformation caused by endocytosis defects may be a secondary consequence of the upregulation of Crumbs and the disruption of cell polarity. However, further studies will be needed to investigate this hypothesis.

*vps25* and *vps23* mutant cells exhibit another interesting characteristic of the overgrowth phenotype, termed non-autonomous overgrowth (Hariharan and Bilder, 2006). This type of overgrowth has been discovered in a screen using the mosaic analysis strategy. Cells with mutations in *vps25* and *vps23* induce overproliferation in neighboring cells rather than cause their own proliferation, and thus non-cell-autonomously regulate cell proliferation (Herz et al., 2006; Moberg et al., 2005; Thompson et al., 2005; Vaccari and Bilder, 2005). In *vps25* mutant clones, apoptosis is increased cell-autonomously (Herz et al., 2006; Thompson et al., 2005; Vaccari and Bilder, 2005). A proposed model for uncontrolled proliferation of discs containing *vps25* is that the failure of recycling

the receptors of growth promoting pathways, such as Notch, leads to constitutively active signals to these pathways (Herz et al., 2006; Thompson et al., 2005; Vaccari and Bilder, 2005). This non-autonomous overgrowth phenotype implies that cell-cell interaction and extrinsic signals may play important roles in regulating growth and maintaining tissue homeostasis.

A homeostatic mechanism that has been implicated to ensure tissues achieve their proper sizes is called cell competition. Cell competition is a phenomenon that was originally observed and characterized in *Drosophila*. When a tissue is comprised of two populations of cells with different growth ability, the cells that grow more slowly are progressively eliminated and consequently the adult tissue is mainly composed of the faster growing cells (Morata and Ripoll, 1975), even though tissues comprised of only the slow growing cells are able to give rise to a normal adult organ (Figure 1.3). Studies have shown that the composition of adult tissue is not only determined by the growth rates of different cell populations but also through induced apoptosis and additional proliferation. Notably, the less competitive cells are eliminated by induced apoptosis and the more competitive cells are induced to undergo extra rounds of proliferation and engulf the dying cells. Blocking cell death is sufficient to prevent these weaker cells from dying and being outcompeted (de la Cova et al., 2004; Li et al., 2009; Moreno and Basler, 2004). Therefore, it appears that the growth properties of cells are changed in a context dependent manner. As a



**Figure 1.3. A schematic diagram of cell competition**

Cells with different growth ability can give rise to normal tissues in homotypic situations, but would otherwise be considered to be “super competitors” or “weak competitors” when compared to each other. When a tissue is comprised of two different populations of cells, the slower growing ones are eliminated and thus called “Loser” cells. The remaining cells are called “Winner” cells.



result of this competitive cell interaction, cells that compose the adult tissue are called “winner cells” or “super competitors” while cells that are eliminated from the tissue are defined as “loser cells” or “weak competitors”.

One well-known example of a losing genotype is *Minute*, a group of dominant mutations in genes that encode ribosomal proteins and result in reduced translational efficiency and a lower growth rate in heterozygous tissue. Even though *Minute* cells are viable in a homotypic situation, they are eliminated when wild-type cells are present. Notably, apoptosis is induced inside the patches of *Minute* cells, also known as *Minute* clones, around the clone boundary (Li et al., 2009; Martin et al., 2009). Blocking apoptosis by overexpressing p35, a cell death inhibitor, rescues these *Minute* cells from elimination (Li et al., 2009). On the other hand, additional proliferation is induced in wild-type cells (Simpson, 1979; Simpson and Morata, 1981). Consequently, adult structures are composed of mainly wild-type cells with few *Minute* cells occasionally present. Therefore, wild-type cell are winners when they are surrounded by cells with *Minute* mutations.

Another model used extensively in cell competition studies is Myc, a homolog of the proto-oncogene c-Myc (de la Cova et al., 2004; Moreno and Basler, 2004; Portela et al. 2010; Rhiner et al. 2010). Myc promotes ribosomal biogenesis and growth by inducing the expression of ribosomal genes (Grewal

et al., 2005). Ectopically expressing Myc can turn cells into super competitors (de la Cova et al., 2004; Moreno and Basler, 2004). Wild-type cells that are juxtaposed to cells with high levels of Myc are outcompeted, becoming loser cells in this scenario.

Several models have been proposed to explain this interesting competitive cell-cell interaction phenomenon (Johnston, 2009). The activation of Jun kinase (JNK) signaling is often associated with the out-competed cells (Moreno and Basler, 2004; Moreno et al., 2002). Blocking JNK signaling inhibits the cell death that occurs in loser cells and prevents their elimination (Moreno and Basler, 2004; Moreno et al., 2002). Therefore, JNK activation has been implicated in the elimination of loser cells by inducing apoptosis. Even though it is an appealing explanation for cell competition, several lines of evidence argue against this model. First, blocking JNK activity is not enough to inhibit the cell competition induced by Myc overexpression (de la Cova et al., 2004). Second, upon the overexpression of Myc, JNK is induced in the winner cells rather than wild-type loser cells (de la Cova et al., 2004). Another alternative model is that cells are competing for Decapendaplegic (Dpp), which promotes cell survival and tissue growth, to gain a growth advantage and thus become winner cells (Moreno and Basler, 2004; Moreno et al., 2002). The Dpp competing model is based on the finding that *Minute* cells have lower Dpp signaling activity, which in turn activates the JNK pathway to trigger apoptosis (Moreno et al., 2002).

However, overexpressing Myc in a wild-type background does not affect Dpp activity and Dpp activity is not required to prevent the elimination of *Minute* cells surrounded by normal cells (de la Cova et al., 2004; Tyler et al., 2007). While the molecular mechanisms of cell competition remain controversial, the relative fitness of different cell populations is thought to be important for cell competition. Currently, a role for cell competition in cancer biology has been proposed (Baker and Li, 2008; Moreno, 2008; Rhiner and Moreno, 2009; Vidal and Cagan, 2006), and the cell competition phenomenon has begun to be characterized in the mammalian system (Bondar and Medzhitov, 2010; Oertel et al., 2006; Oliver et al., 2004). Given the oncogenic role of Myc in mammals, cell competition may function as a critical homeostatic mechanism in growth control and cancer formation.

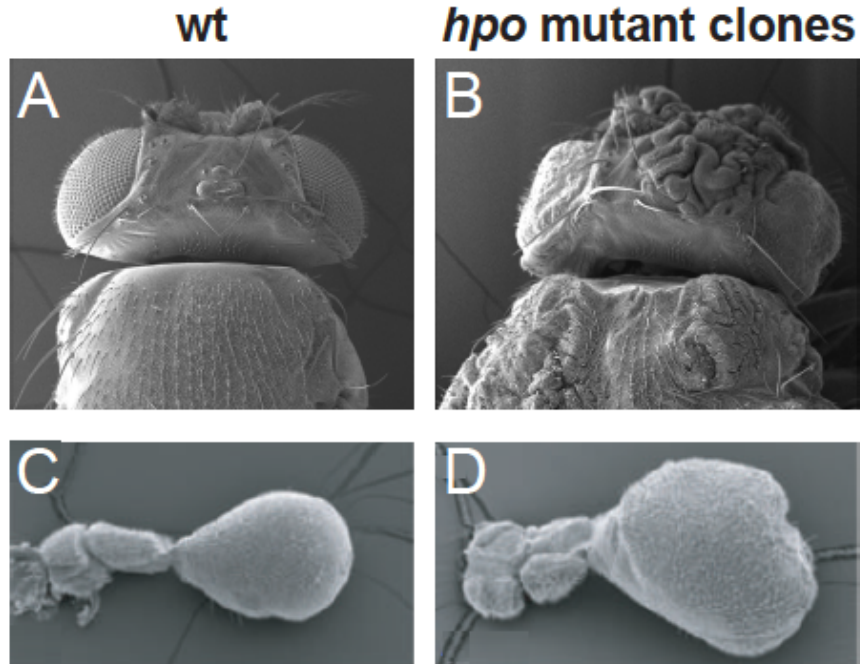
### **1. 3. The Hippo signaling pathway**

Genetic screens in *Drosophila* have identified Hippo signaling as a tumor suppressor pathway (Badouel et al., 2009; Halder and Johnson 2010; Reddy and Irvine, 2008; Zhao et al., 2010). Hippo signaling coordinately regulates cell proliferation and apoptosis, processes that are critical for the proper determination of organ size. Cells lacking Hippo pathway activity evade cell death, grow faster, undergo excess proliferation, and are thought to be super-competitors and able to rescue *Minute* mutants from being outcompeted (Neto-

Silva et al. 2010; Tyler et al., 2007; Ziosi et al. 2010). Therefore, flies with inactive Hippo signaling exhibit dramatic overgrowth phenotypes in imaginal discs and corresponding adult structures (Figure 1.4). Similarly, mouse livers that are mutant for *hpo* homologs, *Mst1* and *Mst2*, overgrow, showing that this function is conserved between phyla. Notably, depleting Hippo pathway activity in fly tissues leads to massive overgrowth, but with little patterning defects or cell size changes.

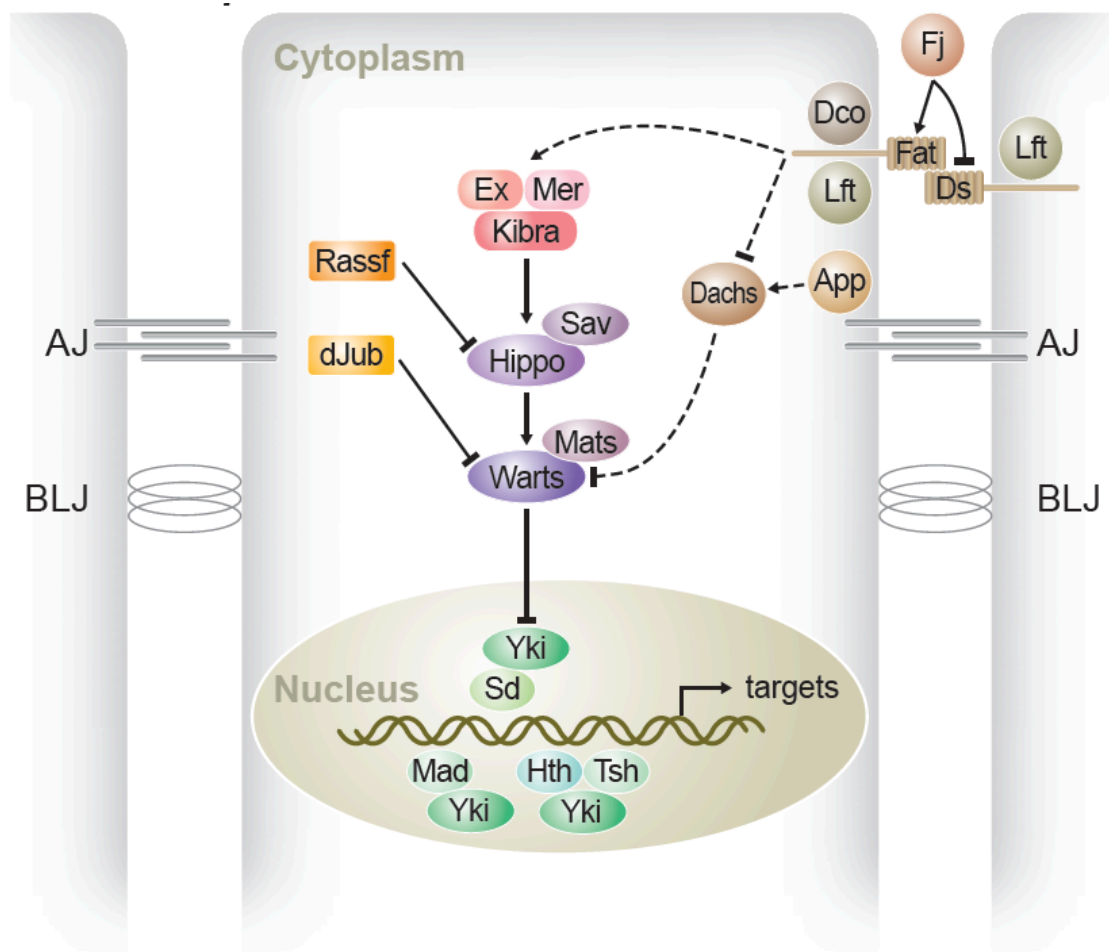
### **Hippo Pathway Components**

Many components of the Hippo pathway have been identified, and define a signal transduction cascade from the plasma membrane to the nucleus (Figure 1.5.) (Badouel et al., 2009; Halder and Johnson 2010; Reddy and Irvine, 2008; Zhao et al., 2010). Fat is an atypical cadherin and a potential receptor for a growth-regulating signal (Bennett and Harvey, 2006; Silva et al., 2006; Willecke et al., 2006). Fat has been shown to bind a protocadherin cadherin, Dachsous (Ds), and the heterophilic interaction between Fat and Ds has been proposed to function as an extrinsic cue to regulate Hippo signaling and promote phosphorylation of the Fat intracellular domain by Disc overgrown (Dco), a homolog of Casein Kinase 1 $\epsilon$  (Feng and Irvine, 2009; Matakatsu and Blair, 2004; Matakatsu and Blair, 2006; Willecke et al., 2008; Sopko et al., 2009). The intracellular domain of Fat has been reported to bind to Lowfat (lft), a



**Figure 1.4. The Hippo pathway regulates tissue size.**

Scanning electron micrographs of **(A)** a wild-type head and **(B)** a head with *hpo* mutant clones resulting in massive tissue overgrowth. **(C,D)** A haltere with *hpo* mutant clones is larger than a wild-type haltere. (Modified from Udan, et al. 2003).



**Figure 1.5.**

**Figure 1.5. A schematic diagram of the current Hippo pathway.**

Solid lines represent interactions confirmed by either multiple groups or in multiple systems. Dashed lines represent single reports or contradictory results. The atypical cadherin Fat transduces a signal to activate Hippo signaling. Two FERM-domain-containing proteins, Mer and Ex, can form a complex with the WW containing protein Kibra to activate Hpo kinase. Activated Hpo, together with Sav and Rassf, phosphorylates and activates Wts kinase. Wts, together with Mats, phosphorylates and inhibits the transcriptional coactivator, Yki. Unphosphorylated Yki is localized in the nucleus and able to form complexes with transcription factor Sd, Hth, Tsh, or Mad to drive the expression of target genes. Other molecules have been reported to regulate the Hippo pathway, such as dJub, Fj, Ds, Lft, Dco App, and Dachs. Modified from Halder and Johnson, 2011.

cytoplasmic protein that is required for proper localization of Fat at the sub-apical region (Mao et al., 2009). In contrast to Dco, Four-jointed (Fj), an extracellular kinase, phosphorylates the extracellular domains of Fat and Ds and thus regulates the binding affinity between Fat and Ds (Ishikawa et al., 2008; Simon et al., 2010).

Fat transduces a growth regulatory signal to an unconventional myosin, Dachs (D) and a FERM domain containing adaptor protein Expanded (Ex) via unknown mechanisms (Feng and Irvine, 2009; Hamaratoglu et al., 2006; Reddy and Irvine, 2008; Sopko et al., 2009). Ex is a FERM domain protein that localizes at the subapical region of the plasma membrane where it forms a complex with another FERM domain containing protein, Merlin (Mer), and a WW domain protein, Kibra, to regulate Hippo pathway activity (Baumgartner et al., 2010; Genevet et al., 2010; Hariharan and Bilder, 2006; Yu et al., 2010; Hamaratoglu et al., 2006). While it is unclear how Fat activates Hippo signaling, D is shown to mediate the activity of Fat (Cho et al., 2006; Mao et al., 2006). Removing D in *fat* mutant tissue suppresses *fat* mutant phenotypes, including overgrowth and the deregulation of Ex (Cho et al., 2006; Mao et al., 2006). In addition, D can physically interact with Warts (Wts), a downstream kinase in the Hippo pathway (Cho et al., 2006). The localization and activity of D is regulated by a palmitoyltransferase, Approximated (App), that has been shown to play a role in planar cell polarity and has been implicated in Fat signaling (Matakatsu



and Blair, 2008). Similarly, removing *App* can restore *fat* mutant overgrown discs to wild-type size (Matakatsu and Blair, 2008). Further studies of *dachs*, and *App* will be required to define the mechanisms by which they influence Hippo signaling and whether or not they are pathway members.

The core components of Hippo signaling form a kinase cascade. Hippo (Hpo) is a serine/threonine kinase that associates with Salvador (Sav) to phosphorylate and activate another serine/threonine kinase, Warts (Wts) (Harvey et al., 2003; Jia et al., 2003; Justice et al., 1995; Kango-Singh et al., 2002; Pantalacci et al., 2003; Polesello et al., 2006; Udan et al., 2003; Wu et al., 2003; Xu et al., 1995). *Rassf* has been shown to compete with Sav for binding to Hippo and may thus function as a negative regulator of the Hippo pathway (Polesello et al., 2006). The other reported negative regulator of Hippo signaling is *djub*, an adaptor protein, that can bind to Sav and Wts. Wts kinase together with its cofactor Mats (Mob as a tumor suppressor) phosphorylates and inhibits the activity of a transcriptional co-activator Yorkie (Yki) by regulating its localization (Huang et al., 2005; Wei et al., 2007). Phosphorylated Yki can bind to 14-3-3, a phosphopeptide binding protein, and remains in the cytoplasm whereas unphosphorylated Yki is thought to translocate into the nucleus and can form complexes with different transcription factors, including Scalloped (Sd), Homothorax (Hth), Teashirt (Tsh), and Mad (Mothers against Dpp) (Oh and

Irvine, 2011; Peng et al., 2009; Wu et al., 2008; Zhang et al., 2008b) to induce the expression of target genes.

### **Target genes of the Hippo pathway**

Several downstream target genes that drive cell proliferation and cell survival are transcriptionally regulated by Hippo signaling. These target genes include *CyclinE* (*CycE*), and *diap1* (*Drosophila* Inhibitor of Apoptosis Protein-1), *bantam* microRNA, and Myc (Hamaratoglu et al., 2006; Neto-Silva et al., 2010; Nolo et al., 2006; Thompson and Cohen, 2006; Ziosi et al., 2010). *CycE* is a limiting factor for S phase entry and overexpression of *CycE* is sufficient to drive cell division (Neufeld et al., 1998). *DIAP1* is an antiapoptotic protein, and extra *DIAP1* can protect cells from apoptosis induced during development (Hay et al., 1995) by inhibiting the activity of downstream caspases. *bantam* microRNA is another critical target of the Hippo signaling pathway (Nolo et al., 2006; Thompson and Cohen, 2006). Overexpressing *bantam* is sufficient to rescue the cell lethal phenotype of *yki* mutant cells. Moreover, tissues that overexpress *bantam* are drastically overgrown compared to wild-type tissue. Therefore, Hippo signaling acts as a tumor suppressor by negatively regulating Yki driven expression of growth promoting target genes. Another downstream target of Yki is Myc, a growth promoting transcription factor Myc (Neto-Silva et al. 2010; Ziosi et al. 2010). It has been shown that the expression of Myc can be elevated by Yki overexpression and potential binding sites of Yki-Sd complex are found

in the regulatory region of Myc (Neto-Silva et al. 2010; Ziosi et al. 2010). Notably, the expression of some Hippo pathway components, such as *fj*, *ex*, and *kibra*, are transcriptionally upregulated in cells lacking Hippo pathway activity, potentially providing negative feedback on Hippo signaling and thus maintain homeostasis of Hippo pathway activity (Genevet et al., 2010; Hamaratoglu et al., 2006; Willecke et al., 2006; Yu et al. 2010). During the course of study on Hippo signaling, multiple useful transgenic reporter genes have been identified or generated, including *ex-lacZ* and *diap1-GFP*. These reporter genes can be used as readouts for the activity of Hippo signaling in imaginal discs.

### **The Hippo pathway is conserved in mammals and involved in tumorigenesis**

In mammals, counterparts of most Hippo pathway components can be found (Table 1.1), often with growth-related functions (Reddy and Irvine, 2008) Halder and Johnson 2011; Vidal and Cagan, 2006). Similar to the kinase cascade module in *Drosophila*, Hippo homologs, MST1 and 2 (Mammalian Ste20 like kinase) phosphorylate Wts homologs LATS1 and 2 (Large tumor suppressor 1,2) to inhibit the Yki homologs, Yap and Taz (Chan et al., 2005; Hao et al., 2008; Lei et al., 2008; Oka et al., 2008; Zhang et al., 2008a; Zhao et al., 2007). Moreover, Yap overexpression in the adult mouse liver mimics Hippo

**Table 1.1. – Components of the Hippo pathway and their homologs**

Most Hippo pathway members are conserved in vertebrates, except that a direct homolog of Dachs is not known (Halder and Johnson).

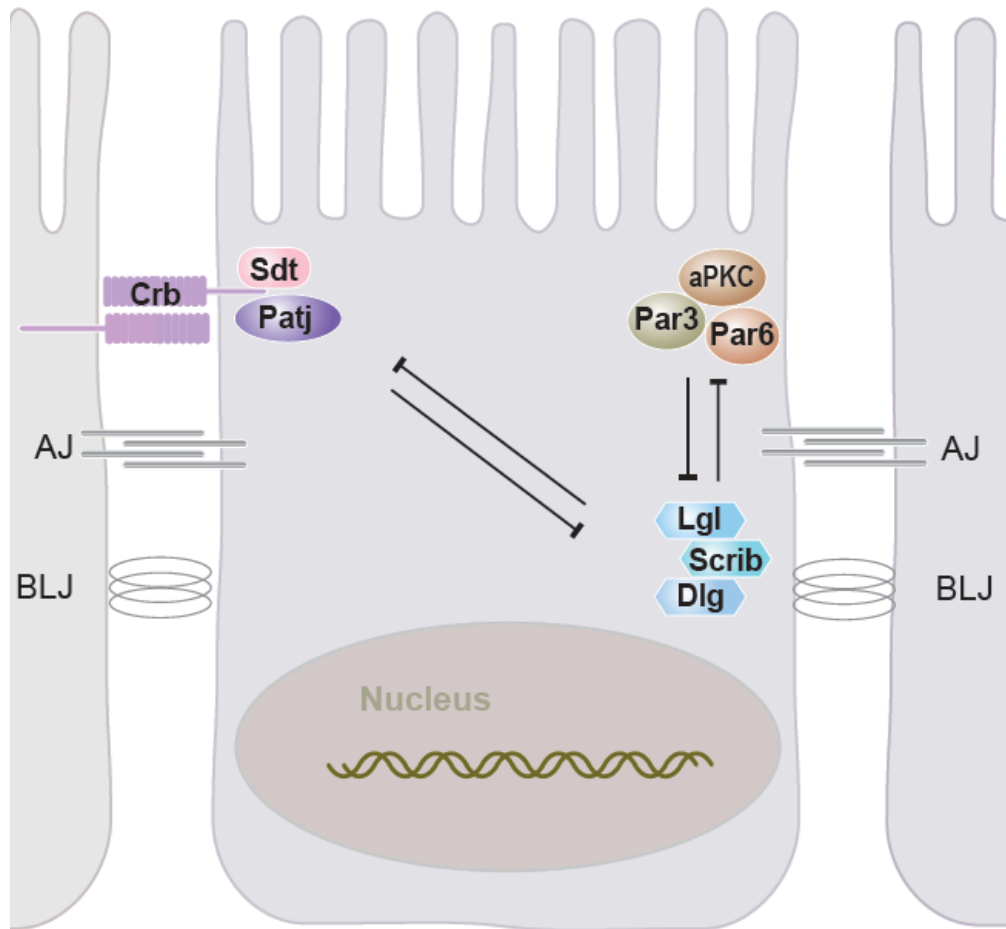
<b>Fly component</b>	<b>Mouse homolog(s)</b>	<b>Protein type</b>
Fat (Ft)	Fat4	Atypical cadherin
Dachsous (Ds)	Dchs1-2	Atypical cadherin
Four-jointed (Fj)	Fjx1	Ser/Thr kinase
Discs overgrown (Dco)	CKI $\delta$ , CKI $\epsilon$	Kinase
Lowfat (Lft)	Lix1, Lix1-L	Adaptor protein
Dachs (D)	N/A	Unconventional myosin
Approximated (App)	ZDHC9, -14, -18	DHHC palmitoyltransferase
Expanded (Ex)	Ex1/FRMD6, Ex2	FERM-domain protein
Merlin (Mer)	Neurofibromatosis 2 (NF2)	FERM-domain protein
Kibra	Kibra	WW-domain protein
dRassf	Rassf1-6	Adaptor protein
dJub	Ajuba, LIMD1, WTIP	Adaptor protein
Hippo (Hpo)	Mammalian sterile-20 like 1-2 (Mst1-2)	Ser/Thr kinase
Salvador (Sav)	Sav1/WW45	WW-domain protein
Warts (Wts)	Large tumor suppressor 1-2 (Lats1-2)	Ser/Thr kinase
Mob as tumor suppressor (Mats)	Mob1A, Mob1B	Adaptor protein
Yorkie (Yki)	Yes-associated protein (Yap), Taz	Transcriptional co-activator
Scalloped (Sd)	TEAD1-4	Transcription factor
Teashirt (Tsh)	Tshz1-3	Transcription factor
Homeothorax (Hth)	Meis1-3, Prep1-2	Transcription factor
Mothers against Dpp (Mad)	Smad	Transcription factor

pathway inactivation and leads to a dramatic increase in liver mass (Camargo et al., 2007; Dong et al., 2007). Later studies demonstrated that Mst1/2 and Sav1 restrict liver growth postnatally through Yap phosphorylation (Lee et al., 2008; Lu et al., 2010; Song et al., 2010; Zhou et al., 2009). It appears that Hippo signaling is crucial for regulating organ size in mammals as well, and the core components of the mammalian Hippo pathway act together as their counterparts do in *Drosophila* (Halder and Johnson, 2011; Reddy and Irvine, 2008). Notably, many vertebrate homologs of Hippo pathway components are involved in cancer formation (Chan et al., 2010; Fernandez and Kenney, 2010; Zeng and Hong, 2008; Zhao et al., 2010). For example, LATS1 and 2 are human tumor suppressor genes (Li et al., 2003; Xia et al., 2002), and loss of the human Mer homolog, NF2 causes Neurofibromatosis (Lallemand et al., 2003; McClatchey et al., 1998). Furthermore, YAP, the Yki homolog, acts as an oncogene in humans (Dong et al., 2007). Evidence indicating involvement of the Hippo pathway in cancer is rapidly accumulating (Chan et al., 2010; Fernandez and Kenney, 2010; Zeng and Hong, 2008; Zhao et al., 2010). These studies indicate that this pathway may function as a critical regulator of tissue size in humans as well. Thus, understanding how Hippo signaling is regulated in *Drosophila* will have direct implications for understanding normal tissue development and the molecular causes underlying cancer in humans.

#### **1. 4. Apical-basal polarity and growth control**

Apical-basal cell polarity is characterized by asymmetrical localization of cellular components within epithelial cells. Proper establishment and maintenance of apical-basal cell polarity in epithelial tissues is essential for developmental processes, including morphogenesis, proliferation, differentiation, and exchanging molecules between cells (Assemat et al., 2008; Dow and Humbert, 2007; Humbert et al., 2008; Tepass et al., 2001). Defects in apical-basal polarity are often associated with human cancer (Dow and Humbert, 2007; Humbert et al., 2008; Vaccari and Bilder, 2009).

The formation and maintenance of proper epithelial cell polarity relies on the concerted action of three conserved complexes: the Crumbs (Crb), atypical Protein Kinase C (aPKC), and Scribble (Scrib) polarity modules (Figure 1.6) (Assemat et al., 2008; Dow and Humbert, 2007; Humbert et al., 2008; Tepass et al., 2001). The Crumbs complex, composed of Crb, Patj, and Stardust (Sdt), and the aPKC complex, composed of aPKC, Par6, and Bazooka (Baz), localize to the subapical region of the plasma membrane and are important for the establishment and maintenance of the apical domain (Assemat et al., 2008; Dow and Humbert, 2007; Humbert et al., 2008; Tepass et al., 2001). The Scrib module contains Scrib, Disc large (Dlg), and Lethal giant larvae (Lgl), and is localized in the basolateral region.



**Figure 1.6. A schematic diagram of apical-basal polarity complexes.**

Apical-basal polarity is regulated by the concerted action of three conserved complexes. The Crumbs and aPKC complexes localize apically and direct the formation of the apical domain. The Discs large complex localizes basolaterally and inhibits the formation of the apical domain.

The asymmetrical distribution of these three complexes is mutually dependent upon each other in various tissues, such as in follicular cells and embryonic cells (Assemat et al., 2008; Dow and Humbert, 2007; Humbert et al., 2008; Tepass et al., 2001). aPKC has been reported to phosphorylate Crb and Lgl and thus functionally link the three polarity complexes (Sotillos et al., 2004; Tian and Deng, 2008). The physical interaction and phosphorylation of Crb by aPKC is required and sufficient for Crb localization at the apical domain (Sotillos et al., 2004). The phosphorylation of Lgl by aPKC is important to prevent Lgl from associating with the plasma membrane and thus inhibit its apical localization (Tian and Deng, 2008). Moreover, expressing an unphosphorylated form of Lgl is not able to rescue the polarity defects in *lgl* mutants suggesting that the phosphorylation of Lgl is required for its role in polarity (Tian and Deng, 2008). However, the mechanism by which the basolateral complex inhibits the apical identity remains unclear.

While the hierarchy of genes that control polarity remains an open question, disruption of either apical complex is known to cause the loss of apical markers and the expansion of the basolateral domain (Assemat et al., 2008; Dow and Humbert, 2007; Humbert et al., 2008; Tepass et al., 2001). In contrast, disruption of the basolateral Scrib complex or ectopic expression of apical determinants results in the loss of basolateral markers and the expansion of the apical domain (Assemat et al., 2008; Dow and Humbert, 2007; Humbert



et al., 2008; Tepass et al., 2001). Notably, perturbation of apical-basal polarity is often associated with cancer progression in vertebrates and can lead to the development of neoplastic tumors in *Drosophila* imaginal discs (Dow and Humbert, 2007; Humbert et al., 2008; Vaccari and Bilder, 2009). Imaginal discs that are homozygous mutant for *scrib*, *dlg*, or *lgl* lose their apical-basal polarity and severely overgrow (Assemat et al., 2008; Dow and Humbert, 2007; Humbert et al., 2008; Tepass et al., 2001). Similarly, overexpression of the apical determinant Crb causes overgrowth of *Drosophila* imaginal discs in addition to causing defects in cell polarity and expansion of apical domain markers to the basolateral domain (Lu and Bilder, 2005). Thus, both promotion of the apical domain and loss of basolateral determinants generate similar overgrowth phenotypes in imaginal discs. This implies that the overabundance of the apical domain or mislocalization of polarity complexes cause the overgrowth phenotypes that are associated with polarity defects. Interestingly, *scrib* mutant cells surrounded by wild-type cells are eliminated while homozygous *scrib* mutant discs display neoplastic overgrowth phenotypes (Figure 1.7). Nevertheless, depleting aPKC activity, but not removing Crb, in *scrib* mutant clones can partially rescue the *scrib* mutant phenotypes (Leong et al., 2009). Therefore, different polarity components may have specific inputs into growth regulation.

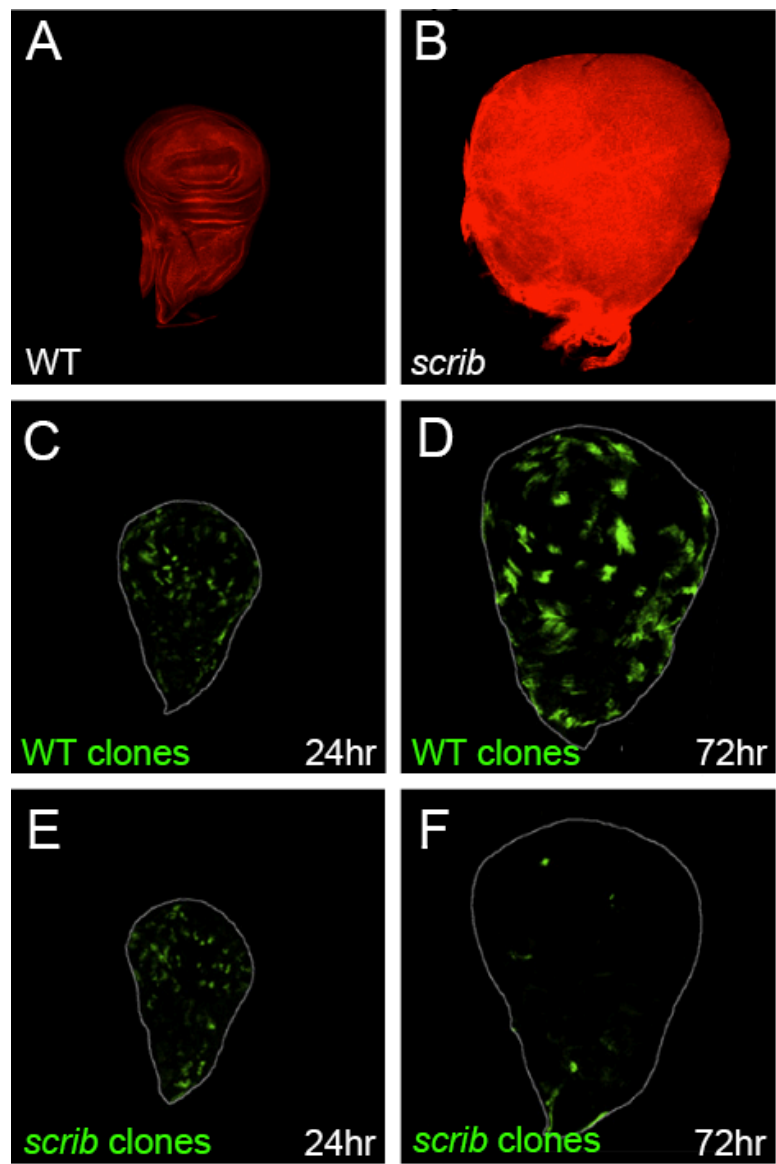


Figure 1.7.

**Figure 1.7. *scrib* mutant phenotypes in different backgrounds.**

**(A,B)** Wing imaginal discs are stained for phalloidin. **(A)** A wild-type disc **(B)** An overgrown disc that is comprised of homozygous *scrib* mutant cells. **(C,D)** Patches of wild-type cells are positively marked by GFP expression and induced after 24 and 72 hours respectively. **(E,F)** Patches of *scrib* mutant cells (*scrib* mutant clones) are positively marked by GFP expression and surrounded by normal cells. *scrib* mutant clones are generated after 24 hours **(E)**, but eliminated after 72 hours **(F)**. (A,B) are modified from Zeitler et al., 2004 and (C-F) are modified from Igaki et al., 2009.

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## **Chapter 2:**

### **Research Significance**

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## **2. 1. Rationale for studying *crumbs* and *scribble***

Millions of people die from cancer each year (Jemal et al., 2011). Most cancers arise from epithelial cells, a specialized cell type that exhibits apical-basal polarity (Humbert et al., 2008). Correct polarity is important for key physiological processes like proliferation. Apical-basal polarity is established and maintained by three conserved modules, including Crb, aPKC, and Scrib complexes. Loss of polarity is a hallmark of cancer cells and highly correlated with the invasive ability of cancer cells (Hanahan and Weinberg, 2011). In addition, polarity components are targeted by human papillomaviruses (HPV) in cervical cancer to initiate malignancy (Takizawa et al., 2006; Thomas et al., 2005). However, the mechanisms by which polarity defects contribute to tumor formation and metastasis remain unclear. In *Drosophila*, the proper regulation of cell polarity has been shown to be important for growth control (Hariharan and Bilder, 2006; Lu and Bilder, 2005; Rolls et al., 2003). Disruption of apical-basal polarity can lead to neoplastic transformation, which is often associated with the expansion of the apical domain and defects in endocytic trafficking (Shivas et al., 2010; Vaccari and Bilder, 2009). Several models have been proposed to explain how neoplastic transformation is caused by polarity defects. The accumulation of multiple signaling receptors has been suggested to cause overgrowth in mutant tissues as a general consequence of disrupted compartmentalization and/or endocytosis. Alternatively, the overgrowth phenotypes of polarity mutants may be due to misregulation of a component of a specific growth-regulating

pathway. To distinguish between these possibilities, I analyzed the effects of altering apical determinant Crb on growth by genetically manipulation.

In addition, mutations that disrupt the basolateral polarity complex components lead to different phenotypes in a context dependent manner. For example, *Drosophila* larvae that are homozygous mutant for *scribble* (*scrib*), a conserved basolateral polarity determinant, produce imaginal discs that grow into large and amorphous tumors capable of metastasis (Figure 1.7A,B) (Bilder et al., 2000). However, *scrib* mutant cells that arise in wild-type discs and are therefore surrounded by normal cells, are eliminated (Brumby and Richardson, 2003; Igaki et al., 2009). When neighboring cells are removed by induced apoptosis, *scrib* mutant cells are not eliminated and grow massively. These data suggest that the presence of normal cells is critical to determine the viability of *scrib* mutant cells. However, the role of neighboring wild-type cells in the elimination of cells mutant for neoplastic tumor suppressor genes is not well characterized. To understand the contribution of the cellular microenvironment in the elimination of pre-cancerous polarity mutant cells, we studied the *Drosophila* neoplastic tumor suppressor gene *scrib*.

## **2. 2. Dissertation research aims**

To address the connection between growth and polarity, I analyzed the apical determinant Crb and the basolateral determinant Scrib. Crb

overexpression phenotypes share some growth phenotypes with known Hippo pathway components, such as enlarged tissue size but without significant patterning defects. Therefore we sought to determine if the massive overproliferation phenotype seen by increasing apical determinants is due to misregulation of the Hippo pathway. To test this hypothesis, we assayed the effects of increasing Crb on Hippo pathway activity and whether Yki is required for the effects. Moreover, to investigate if the expansion of the apical domain is required for causing overgrowth, *crb* mutant phenotypes were characterized in detail. Since Crb and Hippo components each localize to the sub-apical region, we tested whether Crb specifically interacts with any Hippo pathway components by expression of those Hippo pathway components in *crb* mutant tissue and vice versa. Furthermore, to clarify the relationship between cell polarity and growth regulation, we performed functional analysis by using Crb deletion constructs containing different binding motifs. These data will be presented in Chapter 4.

In addition to determining the role of apical-basal polarity in growth regulation, I wanted to investigate how tumorigenic cells are eliminated by neighboring cells and the effects that the local environment has upon cell growth. Since the viability of cells mutant for the neoplastic tumor suppressor gene *scrib* has been shown to be dependent on its local environment, we use it as model system to address this question. Generation of genetic mosaics in

*Drosophila* permits the investigation of mechanisms by which cell-cell interactions suppress tumor formation.

Removal of *scrib*<sup>-</sup> mutant clones from tissues has been proposed to occur through cell competition, a process where different cell populations compare their fitness and determines the proportion of their contribution to the organ (Brumby and Richardson, 2003). Adult organs will consist mainly of cell with greater fitness because weaker cells are eliminated during development. To confirm this idea, we decreased the fitness of normal cells adjoining *scrib* mutant cells and examined the growth ability of *scrib* mutant cells by comparing the growth activity of *scrib* mutant cells in a competitive environment. Reciprocally, we tested whether increasing the fitness of *scrib* mutant cells can prevent them from being outcompeted. To further test the hypothesis that the survivability of *scrib* mutant cells determined by the relative cell fitness, we increased the fitness of both *scrib* mutant cells and juxtaposed normal cells and examined the growth ability of *scrib* mutant cells. Effects on the local environment mediated by neighboring cell population may also affect signaling pathways. To investigate which growth control pathway is required for *scrib* mutant cells to grow massively, we examined the activities of multiple growth control pathways, including the Hippo pathway. Furthermore, we tested whether oncogenes that cooperate with *scrib* mutant cells, such as Ras<sup>V12</sup>, prevents the



elimination of *scrib* mutant cells by modulating cell competition. These data will be presented in Chapter 5.

The completion of these studies will result in a thorough analysis of how cell polarity coordinates with tissue growth and illustrate a role for wild-type tissue in preventing the formation of cancers. Therefore, our work would broaden our understanding of an early step in oncogenesis.

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# **Chapter 3:**

## **Materials and Methods**

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### **3. 1. Immunostaining**

Antibody stainings of imaginal discs and BrdU incorporations were carried out as described (Hamaratoglu et al., 2006) with the exception of Crb stainings. For Crb staining, third instar larvae were incubated in acetone on ice for 10 minutes after fixation.

The following antibodies were used (source and dilutions in parentheses): guinea-pig anti-Mer (R. Fehon, 1:4,000), guinea-pig anti-Ex (R. Fehon, 1:2,000), rat anti-Fat (M.A. Simon, 1:2000), mouse anti-BrdU (Becton-Dickinson, 1:50), mouse anti-Dlg (Developmental Studies Hybridoma Bank, 1:300), mouse anti- $\beta$ -Gal (Promega, 1:2000), rabbit anti- $\beta$ -Gal (Cappel, 1:600), mouse anti-Crb (K. Choi, 1:200), rat anti-Crb (H. Bellen, 1:500), mouse anti-Patj (H. Bellen, 1:500), mouse anti-V5 (Invitrogen, 1:200), rat anti-Ci (R. Holmgren, 1:150), mouse anti-CyclinE (H. Richardson 1:40), mouse anti-DIAP1 (B. Hay 1:200), rabbit anti-aPKC (1:500, Santa Cruz), rabbit anti-cleaved Caspase3 (Cell Signaling, 1:50), rat anti-Elav (Developmental Studies Hybridoma Bank, 1:60), rabbit anti-Yki (D. Pan, 1:500), and rabbit anti-Yki (K. Irvine, 1:500). Secondary antibodies were from Jackson ImmunoResearch (West Grove, Pennsylvania), except Cy3 anti-goat from Sigma-Aldrich (St. Louis, MO).

### **3. 2. Genetic techniques in *Drosophila***

#### **Overexpression**

The UAS/Gal4 system (Brand and Perrimon, 1993) together with Gal80 (Matsumoto et al., 1978), and the flip-out technique (Neufeld et al., 1998) were used to ectopically express genes of interest at specific stages in desired tissues. In the UAS/Gal4 system, the yeast transcription factor Gal4, which is expressed under the control of a *Drosophila* promoter, binds to UAS (upstream-activating-sequence) to drive expression of the gene. Gal80 represses Gal4 transcriptional activity by binding to the Gal4 activation domain (Matsumoto et al., 1978). The temperature-sensitive version of GAL80 (Gal80<sup>ts</sup>) represses GAL4 at permissive temperatures (McGuire et al., 2004; Zeidler et al., 2004). Thus, genes of interest can be overexpressed with temporal and spatial control by using different promoters and different temperatures. Alternatively, overexpression clones were induced by using the flip-out technique in which a FRT (Flippase-Recombination-Target) cassette is placed between a promoter and Gal4 to stop gene expression when flippase expression is not induced.

#### Mosaic analysis of mutant clones

The Flp/*FRT* (Flippase/ Flippase-Recombination-Target) system (Golic and Lindquist, 1989; Xu and Rubin, 1993) is well established in *Drosophila* to generate homozygous mutant clones in an otherwise heterozygous animal. In this system, the Flp recombinase catalyzes site-specific mitotic recombination between *FRT* sites. During mitosis in a heterozygous mutant animal with *FRT* sites at corresponding positions, chromosome segregation after recombination

between FRT sites can yield a homozygous mutant clone and a homozygous wild-type twin spot. When the wild-type chromosome carries marker genes, mutant clones can be marked by the absence of marker gene expression, such as *white*<sup>+</sup> or *GFP* expression. To positively mark mutant clones or to overexpress a gene of interest in the mutant clone, mosaic analysis with a repressible cell marker (MARCM) system (Lee and Luo, 1999) was used. In the MARCM system, expression of genes of interest and GFP can be globally induced by the UAS/Gal4 system and suppressed by Gal80. By flipping a mutant chromosome against the corresponding chromosome that carries Gal80, GFP expression positively marks the mutant clones. In combination with tissue specific Flp or heat shock induction of Flp recombinase (*hs-Flp*), mosaic clones of cells can be generated with temporal and spatial control.

### RNA interference in flies

To knock down the expression of a specific gene, the UAS/Gal4 system is used to drive the expression of a hairpin RNA (hpRNAs) to induce RNA interference (RNAi), a phenomenon where double-stranded RNAs (dsRNAs) initiate post-transcriptional gene silencing (Ueda, 2001). To generate RNAi constructs, multiple copies of UAS sites are followed by inverted repeats (IRs) in the antisense-sense orientation (Dietzl, 2007). By crossing to Gal4 lines, the dsRNAs are ectopically induced to be processed by Dicer into small

interference RNAs (siRNAs) which direct sequence-specific degradation of the target mRNA.

### 3. 3. *Drosophila* stocks

The following tissue specific Gal4 lines were used for overexpression studies:

*GMR-Gal4* (Freeman, 1996), *hedgehog-Gal4* (Tanimoto et al., 2000), *decapentaplegic-Gal4* (Takaesu et al., 2002), *engrailed-Gal4* (Harrison et al., 1995), *tubulin-Gal4* (Lee and Luo, 1999), *C765-Gal4* (Brand and Perrimon, 1993), *nubbin-Gal4* (Azpiazu and Morata, 2000), and flip-out Gal4 (*act<y+<Gal4*) (Neufeld et al., 1998).

The following UAS-transgenic lines were used in my studies:

*UAS-p35* (Hay et al., 1995), *UAS-DIAP1* (Hay et al., 1995), *UAS-Crb<sup>FL</sup>* (Wodarz et al., 1995), *UAS-Crb<sup>intra</sup>* (Wodarz et al., 1995), *UAS-Crb<sup>intraΔJM</sup>* (Wodarz et al., 1995), *UAS-Crb<sup>intraΔPBM</sup>* (Wodarz et al., 1995), *UAS-Crb<sup>intraΔJM/ΔPBM</sup>*, *UAS-Dachs* (Mao et al., 2006), *UAS-crb<sup>RNAi</sup>* (VDRC and NIG), *UAS-mer<sup>RNAi</sup>* (VDRC and NIG), *UAS-Yki* (Huang et al., 2005), *UAS-Ex* (Boedigheimer et al., 1997), *UAS-Hpo* (Udan et al., 2003), *UAS-Wts* (Lai et al., 2005), *UAS-GFP<sup>nls</sup>*, *UAS-bsk<sup>DN</sup>* (Igaki et al., 2002), *UAS-Ras<sup>V12</sup>* (Karim and Rubin, 1998), *UAS-dMyc* (Johnston et al., 1999).

For generating mutant clones, the following alleles were used to flip against the corresponding chromosomes: *ex<sup>BQ</sup>* (null) (Hamaratoglu et al., 2006), *hpo<sup>42-47</sup>* (Wu et al., 2003), *d<sup>GC13</sup>* (Mao et al., 2006), *ft<sup>422</sup>* (null) (Rawls and Wolff, 2003), *mer<sup>4</sup>* (null) (LaJeunesse et al., 1998), *wts<sup>x1</sup>* (Justice et al., 1995), *crb<sup>11A22</sup>* (Tepass et al., 1990), and *scrib<sup>2</sup>* (null) (Bilder et al., 2000).

The following reporter transgenes were used in my studies:

*ex<sup>e1</sup>* (*ex-lacZ*) (Boedigheimer and Laughon, 1993), *ex<sup>697</sup>* (*ex-lacZ*) (Boedigheimer and Laughon, 1993), and *diap1-3.5-GFP* (Zhang et al., 2008b).

Other stocks used: *yki<sup>B5</sup>* (Huang et al., 2005), *egr<sup>1</sup>* (Igaki, et al., 2002).

The detailed *Drosophila* genotypes used in the results section are listed in the Appendix section. Mitotic clones were generated by *ey-Flp*, *ubx-Flp* (Newsome, 2000) or *hs-Flp* (Xu and Rubin, 1993). Heat shocks were performed at 37°C for 30 minutes during the first or second larval instar stages. To generate *crb* mutant heads and wings nearly entirely mutant for *crb*, we induced mitotic recombination by flipping against *Minute* chromosomes using *ey-FLP* and *ubx-FLP* respectively.

All crosses were kept at 25°C unless otherwise noted. *nub-Gal4* driven *Crb<sup>intra</sup>* overexpression causes strong effects which lead to pupal lethality at

temperatures above 18°C. To bypass the early lethality caused by *nub-Gal4* driven *Crb*<sup>intra</sup> overexpression, crosses were kept at 18°C. Crosses included Gal80<sup>ts</sup> and were kept at 18 °C until they were shifted to 30°C for 5 to 24 hours, as noted, before dissection. Larvae (40-50 hours after egg laying) were heat shocked for 20-45 minutes at 34°C or 37°C to induce a proper amount of clones.

### **3. 4. Scanning electron microscopy**

Scanning Electron Microscopy (SEM) of adult flies was processed following the Hexamethyldisilazane (HMDS) method (Braet et al., 1997), with modifications. Flies were fixed in 70% acetone for 1 day, and washed twice in 100% acetone for 4 hours each. Acetone was exchanged with HMDS through two washes in 1:1 acetone:HMDS and two washes in 100% HMDS over 2 days. Samples were air dried for 1 day prior to sputter coating with 25 nm platinum alloy and examined in a JSM-5910 scanning microscope at an accelerating voltage of 5kV.

### **3. 5. Statistical Analysis**

The quantification of the mutant phenotypes was done by using ImageJ software (National Institute of Health). The areas of interest were outlined with the 'threshold' function and measured with the 'analyze particle' function.



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## Chapter 4:

### ***Crumbs* acts through the Hippo pathway to regulate organ growth**

All of the figures in this chapter have been published in:

**Chen, C.L.**, Gajewski, K., Hamaratoglu, F., Bossuyt, W., Sansores-Garcia, L., Tao, C., Halder, G. (2010) The apical-basal cell polarity determinant *Crumbs* regulates Hippo signaling in *Drosophila*. *Proc. Natl. Acad. Sci. USA* 107(36):15810-5.

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## Introduction

Proper establishment and maintenance of apical-basal polarity is critical for normal development. Alterations of apical-basal polarity are often associated with cancer in vertebrates. In *Drosophila*, abnormal expression of apical-basal determinants, such as overexpression of Crumbs (Crb) or loss of Scrib, can lead to loss of cell polarity and proliferation control, which are two hallmarks of cancer. Several models have been proposed to explain the overgrowth phenotypes. For example, expansion of the apical domain may cause the accumulation of receptors that deregulate many growth controlling pathways and thus lead to the overgrowth phenotype. Alternatively, the polarity complex proteins may specifically modulate one or more growth control pathways (Hariharan and Bilder, 2006; Vaccari and Bilder, 2005). However, the pathways through which apical-basal polarity determinants affect growth remain unclear.

I specifically investigated how the apical determinant Crb regulates growth. Crb is a transmembrane domain protein that localizes apically with Patj and Stardust (Sdt) to establish and maintain cell polarity. I found that Crb acts through the Hippo pathway to regulate growth. The genetic data presented below indicate Crb regulates growth and cell polarity acting through different motifs in its intracellular domain and identify a pathway through which Crb affects growth.

## Results

### 4. 1. Crumbs gain of function causes overgrowth and induces Hippo target genes expression

Overexpression of full length Crumbs ( $\text{Crb}^{\text{FL}}$ ), or a truncated version of Crb that does not contain the extracellular domain ( $\text{Crb}^{\text{intra}}$ ) during wing development by using *C765-gal4* results in overgrown adult wings (Figure 4.1A-C, and data not shown). Similarly, overexpression of  $\text{Crb}^{\text{FL}}$  or  $\text{Crb}^{\text{intra}}$  along the anterior-posterior compartment boundary by using *decapentaplegic-Gal4* (*dpp-Gal4*) causes dramatic enlargement of the overexpression domain in wing discs (Figure 4.1D-G). The expansion of the overexpression domain is seen with extra cell proliferation that is revealed by higher levels of bromodeoxyuridine (BrdU) incorporation. BrdU incorporation labels cells in S-phase of the cell cycle (Figure 4.1D,E). In contrast, cell size remains unaffected in the overexpression region. Therefore, we conclude that overexpression of Crb promotes cell proliferation in wing discs.

To gain insight into the pathway through which Crb induces overgrowth, we tested for effects on the Hippo pathway, a conserved growth control pathway that specifically regulates cell number but not cell size (Harvey and Tapon, 2007; Pan, 2007; Reddy and Irvine, 2008). We assayed the expression

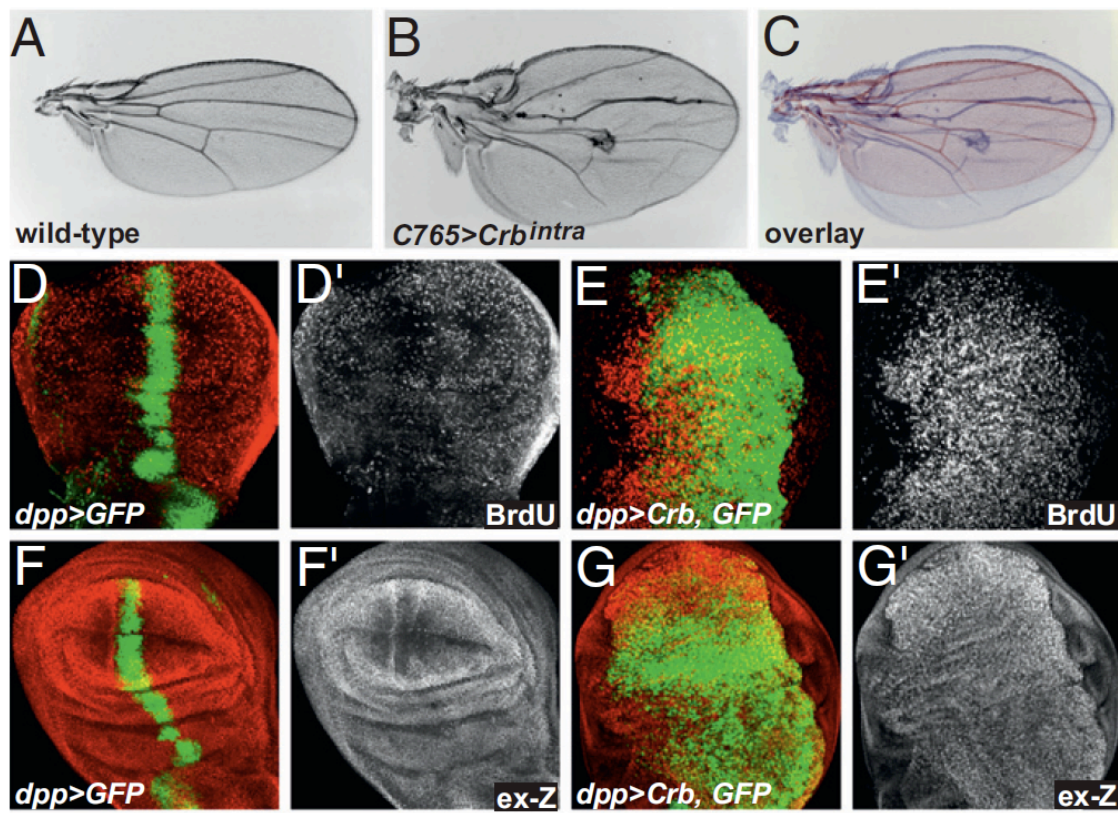


Figure 4.1.

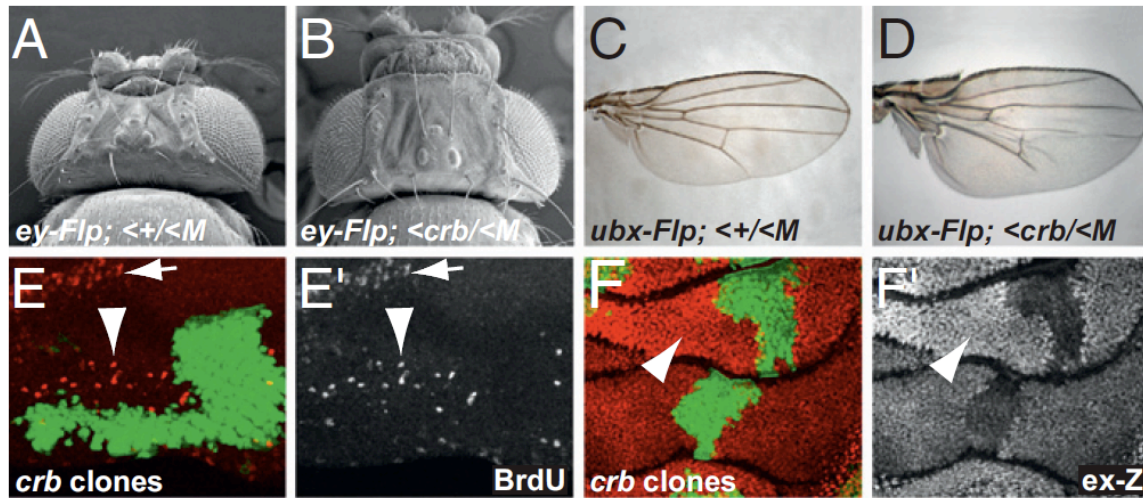
**Figure 4.1. Crb overexpression causes overgrowth, overproliferation and induction of Hippo target gene expression.**

**(A)** WT wing. **(B)** Wing ectopically expressing Crb<sup>intra</sup> during development under the control of *C765-Gal4*. **(C)** Overlay of the images in A (red) and B (blue) shows that the Crb-expressing wing is overgrown. **(D-G)** Confocal images of wing imaginal discs of third instar larvae expressing GFP which is driven by *dpp-Gal4* (D,F) and larvae overexpressing Crb in addition to GFP (E,G). (D,E) Imaginal discs stained for BrdU incorporation to mark cells in S-phase (red in D,E, gray in D',E'). (F,G) Imaginal discs stained for  $\beta$ -gal to reveal the expression of the Hippo pathway reporter *ex-lacZ* (red in F,G, gray in F',G'). For disc panels, ventral is up and anterior is to the left.

of the Hippo pathway component *ex* using a *lacZ* enhancer trap insertion into the *ex* locus (*ex-lacZ*) (Boedigheimer and Laughon, 1993). *ex* is regulated by the Hippo pathway in a negative feedback loop in multiple imaginal discs and is a widely used *lacZ* reporter to reveal the activity of the Hippo pathway (Hamaratoglu et al., 2006). We found that overexpression of *Crb*<sup>FL</sup> or *Crb*<sup>intra</sup> caused strong upregulation of *ex-lacZ* (Figures 4.1F,G and 4.5A), similar to the effects seen with defects in Hippo signaling and Yki overexpression (Hamaratoglu et al., 2006; Willecke et al., 2006). We thus conclude that *Crb* overexpression upregulates Hippo target gene expression.

#### **4. 2. Mutations in *crumbs* cause overgrowth and inhibition of Hippo pathway activity**

To determine whether loss of *crb* also regulates growth, I characterized the phenotypes of *crb* mutant cells in imaginal discs and in adult tissues. In order to generate tissues nearly wholly mutant for *crb*, we flipped chromosomes carrying *crb*<sup>11A22</sup> (the null allele) against chromosomes carrying a *Minute* mutation with GFP or *white*<sup>+</sup> pigmented marker by using either *ey-FLP* or *ubx-FLP*. We found that *crb* mutant tissues, such as heads and wings, are enlarged (Figure 4.2A-D) with venation defects in the wing as was previously observed (Richardson and Pichaud, 2010). To assay the effect of loss of *Crb* function in the regulation of cell proliferation, we analyzed the pattern of BrdU incorporation in the posterior of the eye discs. In wild-type discs, cells posterior of the



**Figure 4.2. Crb is required for proper organ size determination, cell-cycle arrest, and Hippo target gene expression.**

(A,B) Adult heads of wild-type and *crb* mutant flies imaged by SEM. The *crb* mutant head is composed nearly entirely of mutant tissue and is overgrown. (C) Wild-type wing. (D) *crb* mutant wing containing mostly mutant tissues is overgrown. (E) Eye imaginal disc of a third instar larva labeled for BrdU incorporation (red in E, gray in E'). *crb*<sup>11A22</sup> mutant clones are marked by the absence of GFP expression (green). Cell proliferation is normally arrested posterior to the second mitotic wave (arrows) in wild-type cells. *crb*<sup>11A22</sup> mutant cells show ectopic cell proliferation (arrowheads). Anterior is up. (F) Hinge region of a third instar wing disc stained for  $\beta$ -gal to reveal the expression of the Hippo pathway reporter *ex-lacZ* (red in F, gray in F'). *crb*<sup>11A22</sup> mutant clones are marked by the absence of GFP expression (green). The *ex-lacZ* expression is up-regulated in mutant non-GFP cells (arrowheads point to a mutant area). For disc panels, anterior is to the left and ventral is up.

morphogenetic furrow undergo an additional round of cell division, known as the second mitotic wave. After the second mitotic wave, cells cease proliferation and start to differentiate into photoreceptors. In contrast to wild-type eye discs, *crb* mutant cells showed ectopic incorporation of BrdU. (Figure 4.2E, arrowhead). This result suggests that Crb is required to arrest cell cycle progression in the region posterior to the morphogenetic furrow. We thus conclude that Crb is required to restrict cell proliferation and maintain appropriate organ size.

The observation that Crb overexpression induces Hippo target gene expression raises the question of whether loss of *crb* also affects Hippo signaling. To answer this question, we monitored Hippo pathway activities by using the *ex-lacZ* reporter. We found that expression of the *ex-lacZ* reporter is autonomously upregulated in *crb* mutant clones. This effect was especially prominent in the hinge region of wing discs (Figure 4.2F, arrowhead). My results indicate that Crb is required for appropriate regulation of Hippo target genes.

Notably, the phenotypes of Crb overexpression on growth and Hippo signaling are similar but not stronger than those of *crb* loss of function. The similarity between the loss and gain of function phenotypes of Crb indicates that



wild-type levels of Crb are essential for normal functioning of the Hippo pathway.

#### **4. 3. Crumbs genetically interacts with Hippo pathway components**

As described previously, the overgrowth phenotypes of *crb* mutants resemble those seen in loss of Hippo signaling. However, the *crb* mutant phenotypes are not as drastic as those of *hpo* mutant clones. The difference is most evident in the pupal retina. *hpo* mutant retinæ show a large excess of interommatidial cells (Udan et al., 2003) whereas *crb* mutant retinæ showed no extra interommatidial cells (Figure 4.3A). The weak phenotype of *crb* in pupal retinæ is very similar to that of *ft*, *ex*, and *mer* (Bennett and Harvey, 2006; Hamaratoglu et al., 2006; Silva et al., 2006; Willecke et al., 2006), components of two upstream branches of the Hippo signaling pathway. Abolishing both branches causes a stronger phenotype than depleting either single one alone. *mer;fat* and *mer;ex* double mutants show synergistic phenotypes, such as many extra interommatidial cells (Hamaratoglu et al., 2006; Silva et al., 2006; Willecke et al., 2006) which was not observed in the single mutants. To test whether Crb acts upstream in the Hippo pathway in parallel to Mer or Fat, we examined the *crb* mutant pupal retinæ in either Mer or Fat knocked down background. Similarly, we found that the *crb* mutant pupal retinæ in a Mer knock down background showed extra interommatidial cells while knocking down Mer by GMR-Gal4 driven *UAS-mer<sup>RNAi</sup>* in retinæ did not result in extra interommatidial

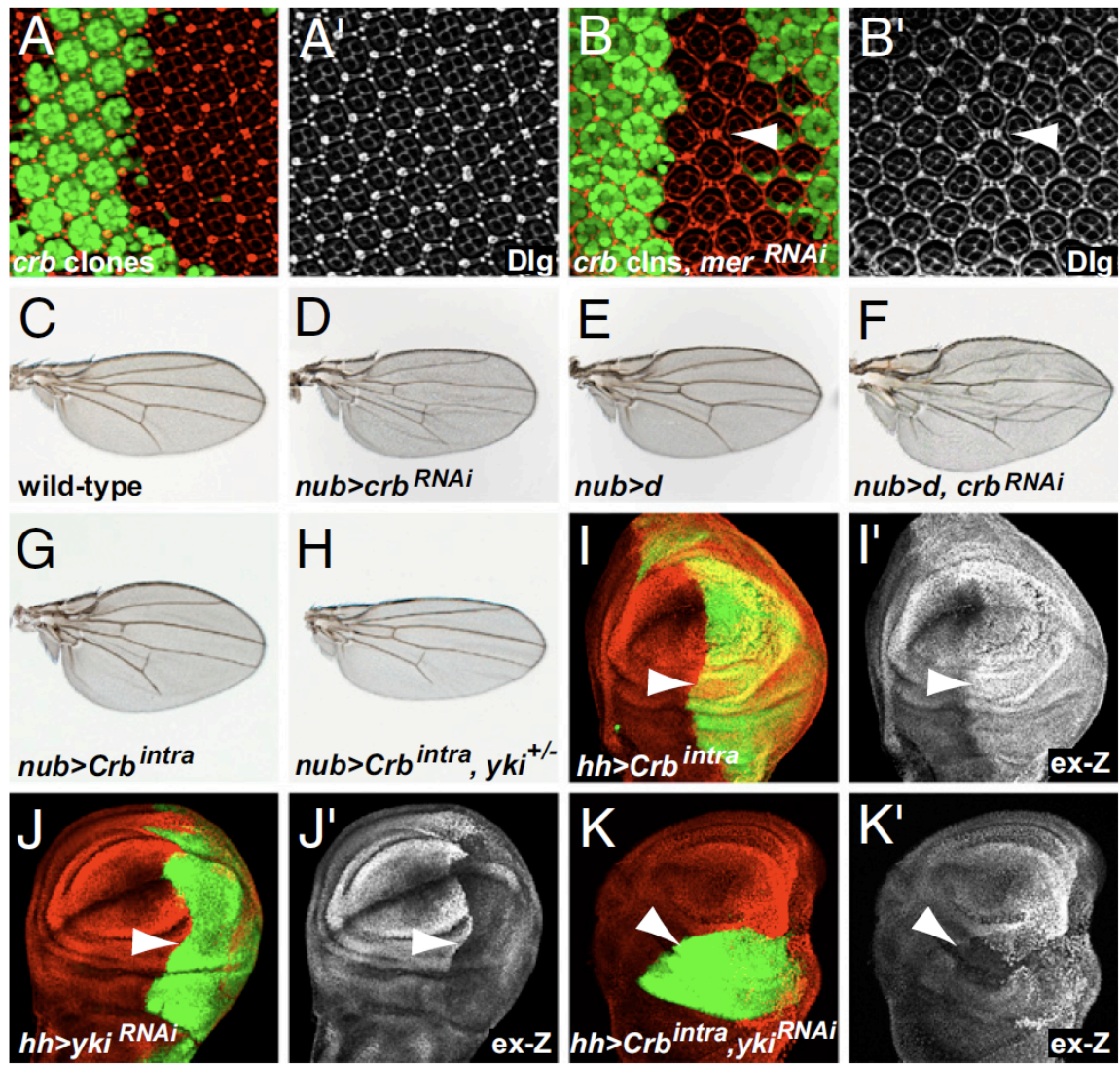


Figure 4.3.

**Figure 4.3. Crb genetically interacts with Hippo pathway components and regulates growth and Hippo target genes through Yki. (A,B)** Confocal images of pupal retina stained for Dlg to mark cell outlines (red in A and B, gray in A' and B'). *crb*<sup>11A22</sup> mutant clones marked by the absence of GFP expression (green). The *crb* mutant clones are normal in a wild-type background (A) but show extra interommatidial cells in a Mer knockdown background (B, arrowheads) which is generated by using *GMR-Gal4* to drive UAS-mer<sup>RNAi</sup> construct expression. **(C–H)** Adult wings of the indicated genotypes. Overexpression of **(D)** UAS-*crb*-RNAi construct or **(E)** D alone in wings using nub-Gal4 did not cause obvious overgrowth. **(F)** Coexpression UAS-*crb*<sup>RNAi</sup> construct with D caused synergistic overgrowth effects. **(G,H)** Overexpression of Crb<sup>intra</sup> by nub-Gal4 caused overgrowth phenotype, which was suppressed by heterozygosity of *yki*. **(I–K)** Confocal images of third instar wing discs stained for β-Gal to reveal the levels of the Hippo reporter *ex-lacZ*. **(I)** Crb<sup>intra</sup>, **(J)** UAS-*yki*<sup>RNAi</sup> construct, and **(K)** both Crb<sup>intra</sup> and *yki*<sup>RNAi</sup> constructs are overexpressed in the posterior compartment using *hh-Gal4*. The expression regions are marked by the coexpression of GFP. The overgrowth and induction of *ex-lacZ* caused by Crb<sup>intra</sup> overexpression can be suppressed by knocking down Yki. Arrowheads point to the compartment boundaries.

cells (Figure 4.3B). In contrast, the *crb* mutant pupal retinæ in a Fat knocked down background do not have synergistic effects. Thus, we conclude that Crb can synergize with Mer to regulate cell number in the pupal retina.

In addition, loss of *crb* interacts genetically with D, an unconventional myosin that functions downstream of Fat (Mao et al., 2006). Knocking down *crb* in the wing by *nubbin-Gal4* (*nub-Gal4*) driven *UAS-crb<sup>RNAi</sup>* resulted in slightly larger wings compared to wild-type wings (Figure 4.3C,D). Overexpression of D in the developing wing caused weak overgrowth phenotypes (Figure 4.3E). Interestingly, overexpression of D in addition to knock down of *crb* resulted in synergistic effects and significantly overgrown wings (Figure 4.3F). We conclude that Crb genetically interacts with components of the Hippo pathway.

#### **4. 4. Yorkie is required for Crumbs induced phenotypes**

To further test the hypothesis that Crb functions through the Hippo pathway, we investigated whether the deregulation of Hippo signaling is necessary for the growth control function of Crb. We tested whether Yki is required for the overgrowth phenotype caused by Crb overexpression. Overexpressing Crb<sup>intra</sup> in the wing by *nub-Gal4* causes lethality when the crosses are incubated at 25°C. We found that heterozygosity for *yki* rescued the lethality induced by overexpressing Crb. Similarly, when the crosses are incubated at 18°C, heterozygosity for *yki* suppressed the overgrowth phenotype

induced by overexpressing Crb in the wing (Figure 4.3G,H). Additionally, the overgrowth phenotype and induction of *ex-lacZ* caused by *hedgehog-Gal4* (*hh-Gal4*) driven Crb<sup>intra</sup> overexpression in the wing discs can be reversed by knocking down Yki via RNAi (Figure 4.3I-K). Therefore, we conclude that Yki is required for the overgrowth and Hippo pathway target gene induction caused by Crb overexpression. Thus, Crb acts upstream of Yki in the Hippo pathway to regulate growth.

#### **4. 5. Crumbs regulates growth and cell polarity through different domains**

Crb is a single-pass transmembrane protein with a relatively short intracellular domain of only 37 a.a. The extracellular domain of Crb contains 29 epidermal growth factor like repeats and 4 laminin-A globular domain-like repeats. The intracellular domain of Crb is conserved and contains two conserved protein binding motifs (Figure 4.4). The juxtamembrane motif (JM) is a FERM domain binding motif, that has been reported to bind to the FERM-domain of Yurt (Laprise et al., 2006) and forms complexes with  $\beta$ -spectrin and Moesin. The C-terminal PDZ domain binding motif (PBM) has been shown to bind to Sdt and thus form a complex with Patj to regulate apical-basal polarity in various tissues, including embryonic epithelial cells, follicle cells, and pupal retina (Bachmann et al., 2001; Hong et al., 2001; Izaddoost et al., 2002; Klebes and Knust, 2000). Crb overexpressed in the pupal retina was mislocalized throughout the cell and was sufficient to recruit Patj to the basolateral

	JM	PBM
Dm Crb	RNKRATRGTYSPSAQEYCNPRLEMDNVLKPPPEERLI	
Hs Crb1	SNKRATQGTYSRQEKEGSRVEMWNLMPPPAMERLI	
Hs Crb2	RKRQSEGTYSQEVAGARLEMDSVLKVPPEERLI	
Hs Crb3	REKRQTEGTYRPSSEEQVGARVPPTPNLKLPPPEERLI	
Consensus	R KR T GTYSPS QE R EM LK PPEERLI	

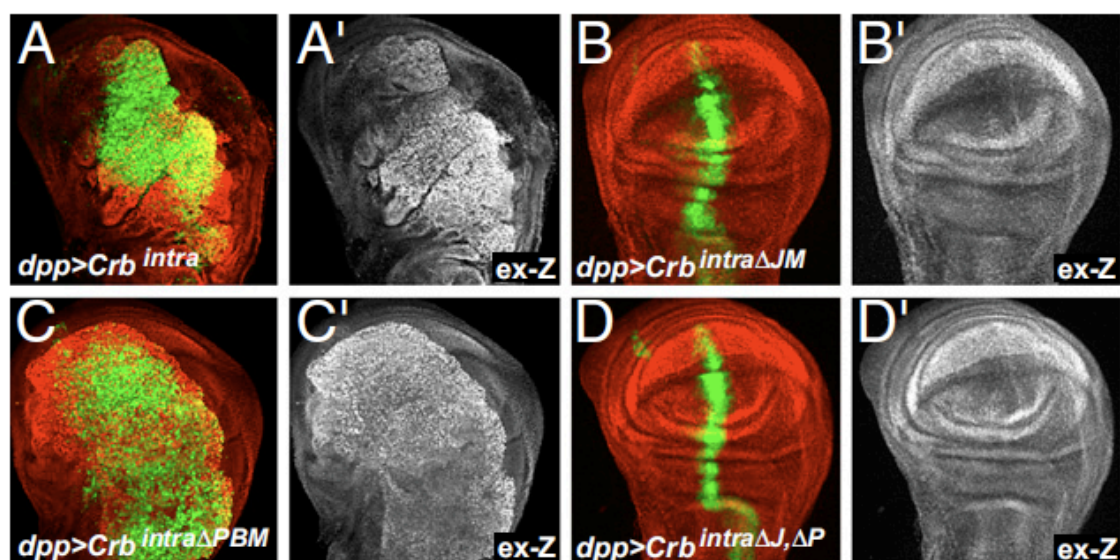
**Figure 4.4. Sequence alignment of the of *Drosophila* Crb (Dm) intracellular domain with that of its human Crb homologs (Hs Crb1–3).**

Conserved residues are in red. JM and PBM are indicated with blue bars.

Consensus sequence is indicated below.

membrane. Similarly, overexpression of Crb in the embryo also caused redistribution of Sdt throughout the cell. The effects on Patj and Sdt specifically require the PBM but not the JM (Klebes and Knust, 2000). To test whether Crb utilizes the same motif and the same mechanism to regulate growth and cell polarity, we quantified the overgrowth phenotypes by using ImageJ and monitored Hippo signaling activity when overexpressing Crb with the different motifs by using *dpp-Gal4* (Figure 4.5A-D). The relative size of different genotypes is calculated by comparing the ratio of the expression domains area marked by GFP expression to the overall size of the discs. By statistical analysis, we found that the overexpression regions of full length Crb and Crb<sup>intra</sup> are about three fold larger than that of the corresponding area in wild-type discs (Figure 4.5E). Interestingly, mutation of the JM or removal of both motifs abrogated the growth effects, while deletion of the PBM still allowed for growth effects similar to those of intact Crb<sup>intra</sup> (Figure 4.5E).

As mentioned previously, overexpression of Crb<sup>intra</sup> caused overgrowth phenotypes and the induction of the Hippo reporter *ex-lacZ* (Figure 4.1G, 4.5A). Consistent with the quantification results, mutation of the JM or removal of both motifs completely abolished these effects (Figure 4.5B,D). In contrast, overexpressing Crb without the PBM still resulted in the induction of *ex-lacZ* and the overgrowth phenotype (Figure 4.5C).



E

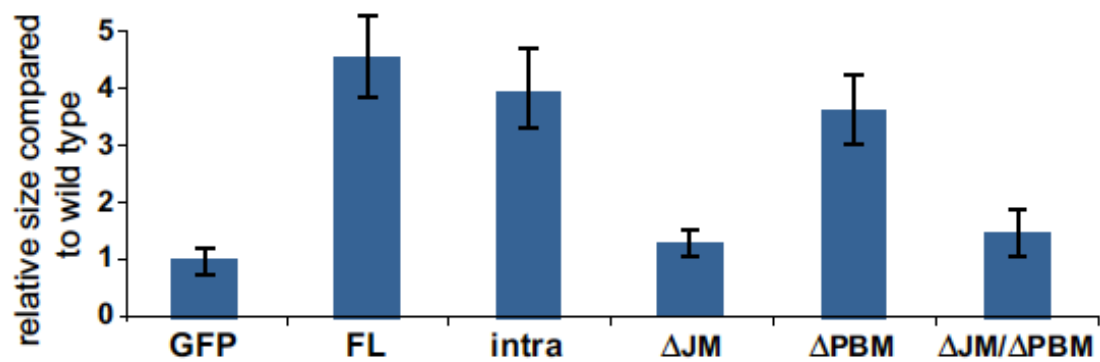


Figure 4.5.



**Figure 4.5. JM of the Crb intracellular domain is required for the regulation of growth and the Hippo pathway.**

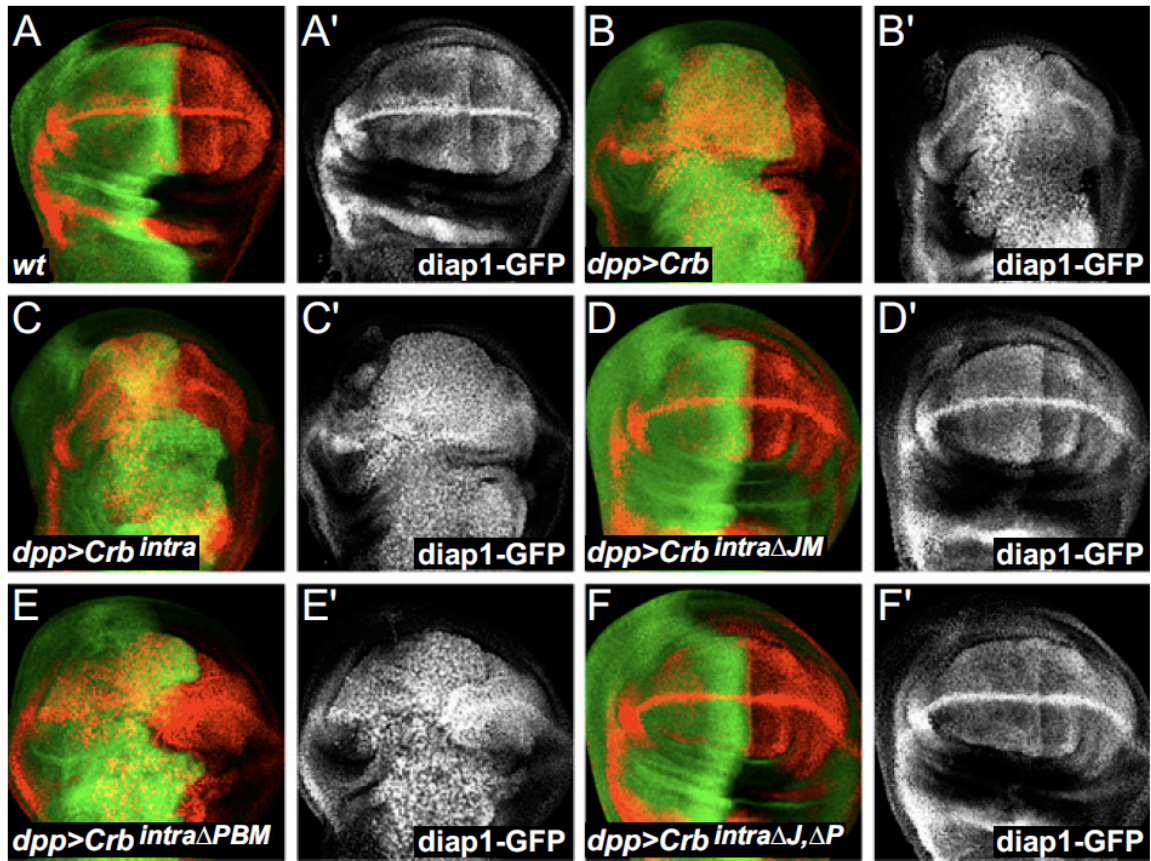
**(A–D)** Confocal images of third instar wing discs overexpressing different mutant versions of Crb<sup>intra</sup> driven by *dpp-Gal4*. Genotypes are as indicated. These discs are stained for  $\beta$ -Gal to reveal the expression of *ex-lacZ* (red in A–D, gray in A'–D'). The expression domain is marked by the coexpression of GFP (green). Overexpression of the wild-type version of Crb<sup>intra</sup> and the Crb<sup>intra $\Delta$ PBM</sup> mutant caused growth and induction of *ex-lacZ* expression, whereas overexpression of Crb<sup>intra</sup> with mutations in the JM domain (Crb<sup>intra $\Delta$ JM</sup>) did not cause these effects. **(E)** Quantification of the growth phenotypes of overexpressing different mutant forms of Crb that are shown in A–D. FL: full-length Crb. For disc panels, anterior is to the left and ventral is up.

In addition to *ex-lacZ*, we examined the transcriptional expression of a Hippo target gene, *Diap1*, by using a reporter transgene, *Diap1-GFP* while we overexpressed different Crb deletion constructs by *engrailed-Gal4* (*en-Gal4*) in the posterior compartment of the wing discs. We also assayed the effects of *en-Gal4* driven overexpression of different Crb deletion constructs on Wingless (Wg) expression, which is regulated by the Hippo pathway in the hinge region of the wing discs. The overexpression of full length Crb and Crb<sup>intra</sup> similarly induced *Diap1-GFP* and Wg expression (Figure 4.6A-C, 4.7A-C). However, while mutation of the JM or removal of both JM and PBM motifs abrogated the growth effects, deletion of the PBM only still retained the ability to affect growth (Figure 4.6D-F, 4.7D-F).

In summary, our data show that the effects of Crb on the Hippo pathway required the JM but not the PBM. Therefore, Crb regulates growth and cell polarity through different domains and thus through different mechanisms.

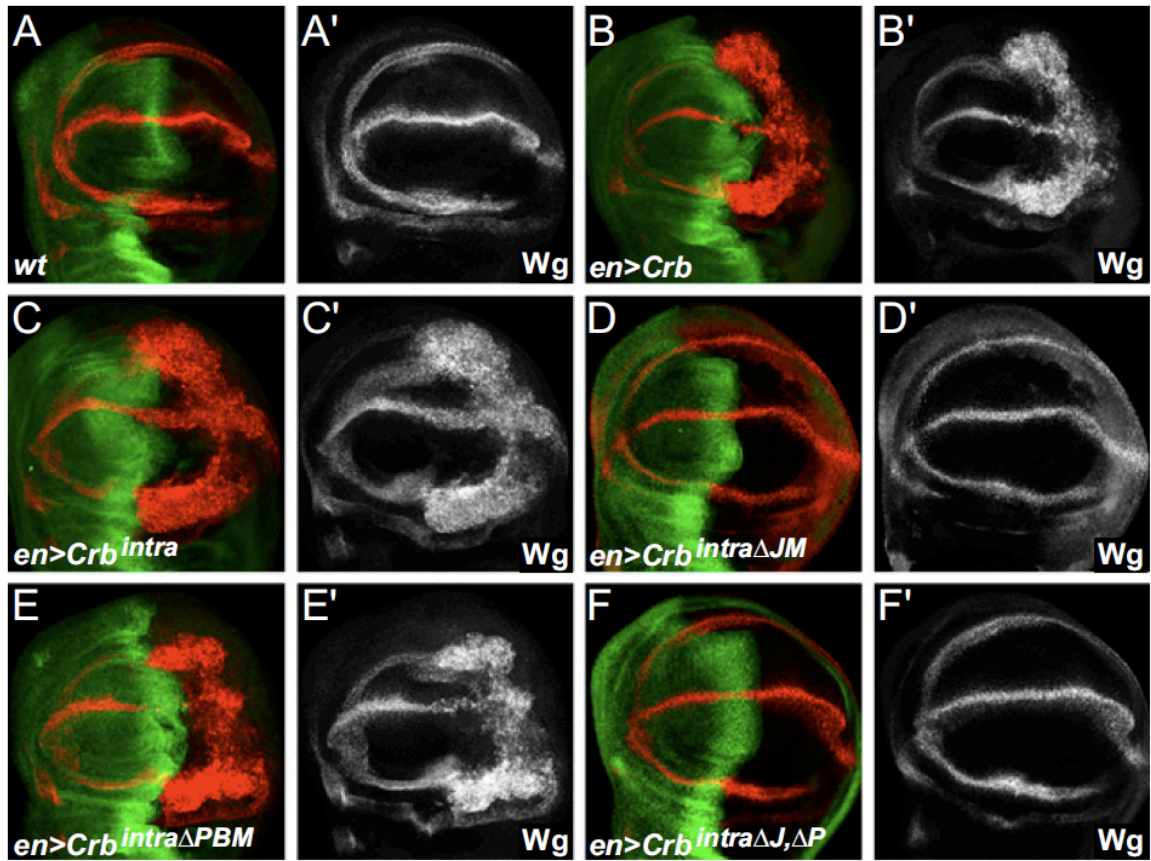
#### **4. 6. Crumbs is required for Expanded membrane localization**

Crb is localized to the apical membrane where Fat, Ex, and Mer localize. As a transmembrane protein, Crb may function as a receptor of Hippo signaling. These facts raise the question of whether Crb interacts with upstream components of Hpo signaling and thus acts upstream in the Hippo pathway.



**Figure 4.6. Crb regulates the Hippo target diap1-GFP through the JM.**

(A–F) Confocal images of third instar wing imaginal discs with expression of the Hippo pathway reporter *diap1-GFP* (red in A–F, gray in A'–F'). Discs are wild-type (A), overexpressing full-length Crb (B) or different mutant forms of Crb<sup>intra</sup> as indicated (C–F) along the anterior-posterior compartment boundary by *dpp-Gal4*. The anterior compartments are marked by Cubitus interruptus (Ci) stainings (green). For all disc panels, anterior is to the left and ventral is up.

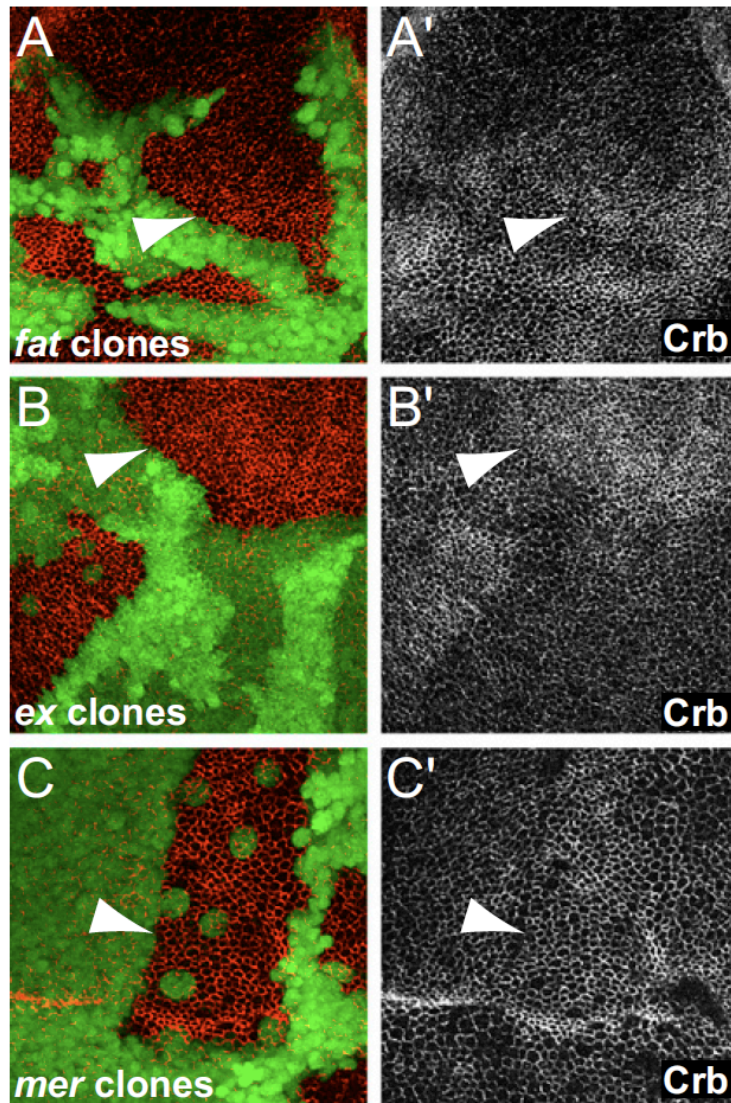


**Figure 4.7. Crb regulates the Hippo target Wg through the JM.**

(A–F) Confocal images of third instar wing imaginal discs that are stained for the expression of Wg, which is regulated by the Hippo pathway in the hinge region (red in A–F, gray in A'–F'). Discs are wild-type (A), overexpressing full-length Crb (B) or different mutant forms of Crb<sup>intra</sup> as indicated (C–F) in the posterior compartment using *en-Gal4*. The anterior compartments are marked by Cubitus interruptus (Ci) stainings (green). For all disc panels, anterior is to the left and ventral is up.

We first asked whether Fat, Ex, Mer, and Crb affect each other's localization. We found that the correct localization of Crb is not affected in *fat*, *ex*, and *mer* mutant cells (Figure 4.8A-C). Rather, it has been reported that *ex* or *fat* mutant cells had higher levels of Crb at the membrane (Genevet et al., 2009; Hamaratoglu et al., 2009) while *mer* mutant cells, similar to wild-type cells, had normal amounts of Crb. (Figure 4.8C, arrowhead). However, loss of Crb leads to Ex mislocalization. In *crb* mutant cells, Ex was largely absent from the apical membrane and diffused into the cytoplasm at the basal lateral region (Figure 4.9A,B). When *crb* mutant cells were produced in a *Minute* background, which grows slower during development, they often did not have cytoplasmic Ex (Figure 4.9C). This observation suggests that Ex may have been degraded. Our data indicated that Crb regulates the localization and/or stability of Ex. Interestingly, we observed that Ex is lost from the membranes of wild-type cells that are adjacent to *crb* mutant cells. The localization of Ex thus results in fork-like localization patterns at *crb* mutant clone borders (Figure 4.9C, arrowhead). Similarly, the localization of Crb as well as that of Patj, at *crb* mutant clone borders also formed fork-like localization patterns (Figure 4.9D,E, arrowheads). It indicates that Crb homophilically interacts with Crb molecules on neighboring cells through its extracellular domain and this interaction is required for its localization (Izaddoost et al., 2002; Tanentzapf et al., 2000). Crb may thus be required non-autonomously for Ex localization, as well as that of Patj, in neighboring cells.





**Figure 4.8. Crb localization is unaffected in *fat*, *ex*, and *mer* mutant clones.**

The *fat*<sup>422</sup> (A), *ex*<sup>e1</sup> (B), and *mer*<sup>4</sup> (C) mutant clones in wing imaginal discs are marked by the absence of GFP expression (green). Crb localization (red in A–C, gray in A'–C') is similar in mutant cells and in wild-type cells. Crb levels are slightly elevated in *fat*, *ex*, and *mer* mutant cells. Arrowheads point to clone borders.

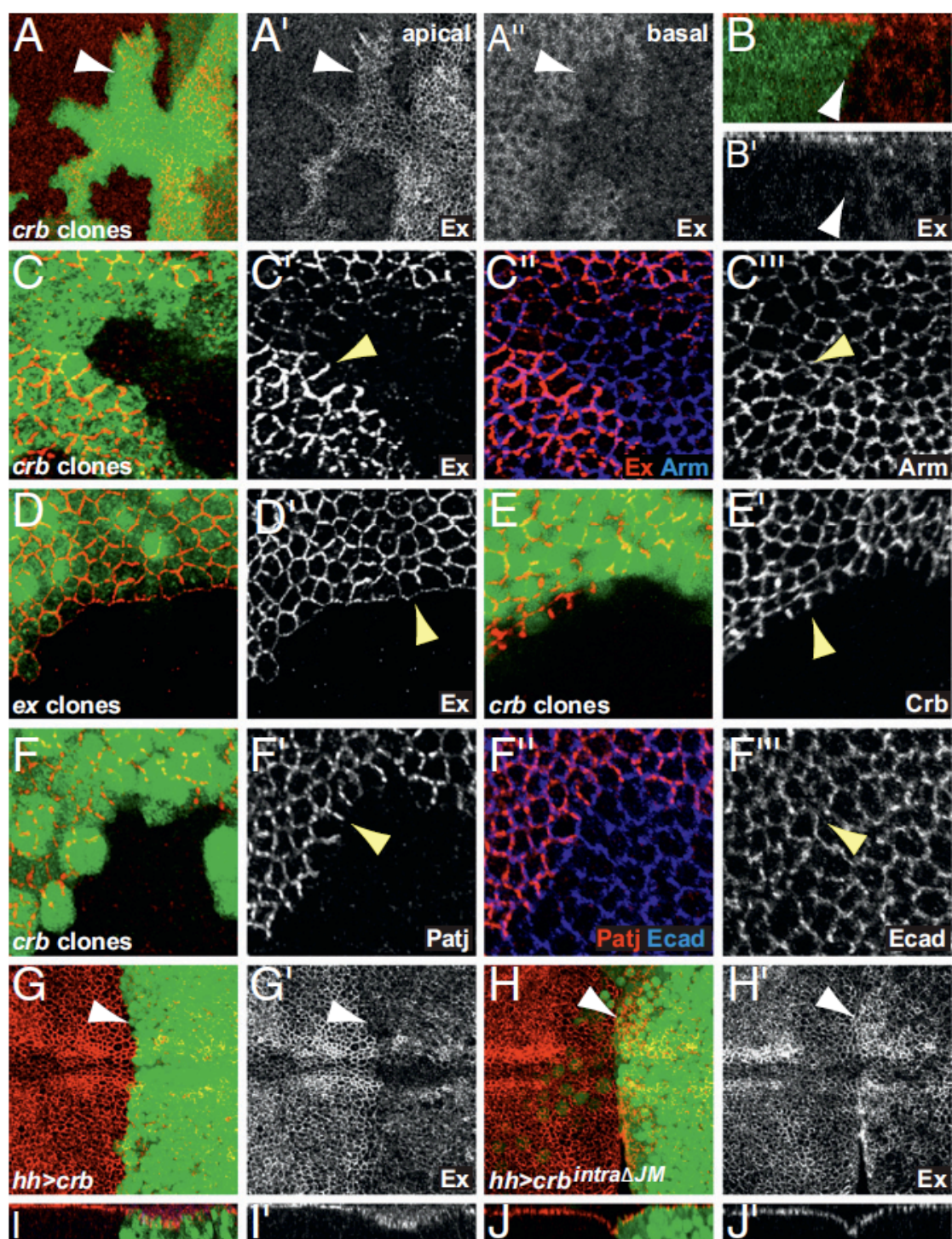


Figure 4.9.



**Figure 4.9. Crb is required for proper Ex membrane localization.**

**(A–C,E,F)** *crb*<sup>11A22</sup> mutant clones in wing imaginal discs are marked by the absence of GFP expression (green). **(A–A'')** Ex staining with *crb* mutant clones. **(A')** apical section. **(A'')** basal section. Ex is lost from the apical membrane in *crb* mutant cells and accumulated in more basal and intracellular regions. **(B)** Z-section through a *crb* mutant clone. Ex is mislocalized in *crb* mutant cells. **(C)** Higher magnification of Ex staining with *crb* mutant clone borders. Ex localization forms finger-like patterns at clone boundaries (arrowheads), which indicates that Ex is also lost from the corresponding membrane of neighboring wild-type cells. Armadillo staining marks adherens junctions, which are unaffected (blue in C'', gray in C'''). **(D)** Ex is lost from the apical membranes of *ex*<sup>et</sup> mutant clones but not in the wild-type neighboring cells (arrowhead). **(E,F)** Crb and Patj are lost from the apical membranes of *crb* mutant cells (red in E,F,F''; gray in E', F'). Crb and Patj localizations form similar finger-like patterns at clone boundaries (arrowheads). E-cad staining marks adherens junctions, which are unaffected (blue in F'', gray in F'''). **(G)** Overexpression of Crb<sup>intra</sup> in the posterior compartment by *hh-Gal4* causes redistribution of Ex: Ex at the apical membrane is reduced and basal localized Ex is increased. **(H)** Mutation of the JM abolishes the effects of Crb<sup>intra</sup> overexpression on Ex localization. **(I,J)** Z-sections through the discs shown in (G) and (H). Arrowheads point to clone borders or compartment boundaries.



In contrast, Fat and Mer are not significantly lost from the subapical membrane of *crb* mutant cells. Therefore, Crb is not required for the localization of Mer or Fat (Figures 4.10A,B, arrowheads). We conclude that Crb is specifically required for the localization of Ex to the membrane, but not other Hippo pathway components.

The requirement of Crb for Ex localization prompted the question of whether Crb overexpression is sufficient to cause the redistribution of Ex. To answer this question, we further investigated the effect of Crb overexpression on Ex localization. Full length Crb and Crb<sup>intra</sup> that are ectopically expressed in various tissues localize throughout the cell (Izaddoost et al., 2002; Klebes and Knust, 2000). Because overexpressed Crb is very potent and often causes strong overgrowth phenotypes and morphological defects, it is difficult to assay protein localization of the genetically manipulated cells. To bypass this problem, we utilized temperature-sensitive Gal80 in combination with *hh-Gal4* to further fine-tune its expression temporally. Crosses were kept at 18°C and shifted to 30°C for either 5 hours or 1day before being assayed. We found that after 5 hour induction of Crb overexpression, the total amount of Ex in cells is reduced (Figure 4.9G), while the amount of basolaterally localized Ex is increased (Figure 4.9I). Crb overexpression in embryonic epithelial cells also results in similar effects on Sdt (Bachmann et al., 2001; Hong et al., 2001). We found that overexpression of Crb<sup>intraΔJM</sup> does not cause Ex relocalization (Figure 4.9H,J).

Consistent with the requirement for growth, the JM domain is necessary for the effect on Ex localization. We conclude that overexpressed Crb is sufficient to relocalize Ex. This supports our model that Crb is essential for apical localization of Ex. Our data indicate that Crb, in particular the JM domain, regulates Ex localization and/or stability.

It has been reported that the level of Ex is decreased in *fat* mutant clones in a D dependent manner (Cho et al., 2006). However, the deregulation of Ex is not observed in *ft*, *d* double mutant clones (Feng and Irvine, 2007). To test whether Crb regulates Ex independently of Fat, we examined the consequence of loss of Crb on Ex in a *d* mutant background. We found that removing D does not rescue the loss of Ex in *crb* mutant clones. Ex was still mislocalized in *crb* mutant cells in a *d* mutant background (Figure 4.10C, arrowhead). Our data indicate that Crb regulates Ex membrane localization in a D independent manner. Moreover, D localization, as well as Fat localization, remained intact in *crb* mutant clones (Figure 4.10D, arrowhead). Thus, our data support the idea that Crb regulates Ex membrane localization through a Fat and D independent mechanism.

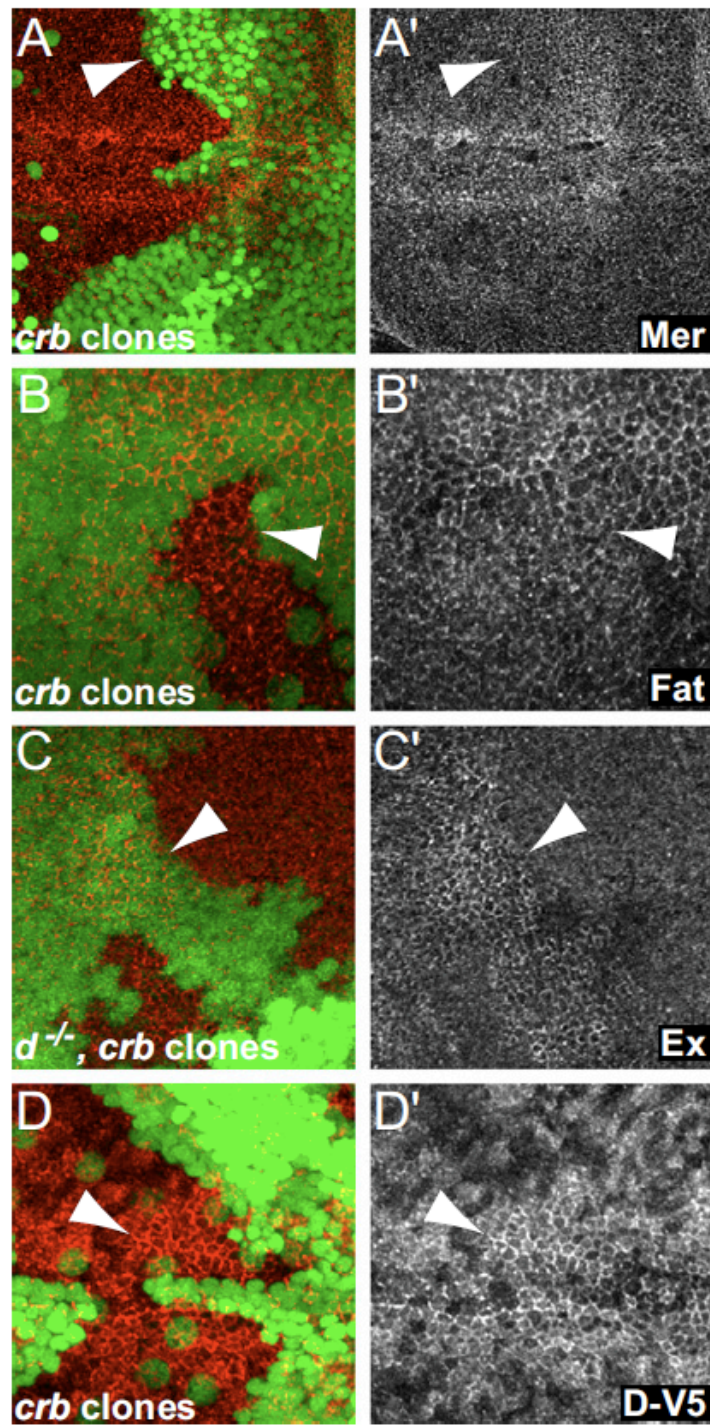


Figure 4.10.

**Figure 4.10. Crb is not required for Mer, Fat, and D localization.**

**(A-D)** Confocal images of third instar wing imaginal discs. *crb*<sup>11A22</sup> mutant clones are marked by the absence of GFP expression (green). Mer (red in A, gray in A') and Fat localization (red in B, gray in B') are not significantly affected in *crb*<sup>11A22</sup> mutant clones. **(C)** Ex (red in C, gray in C') was still lost from the membranes of *crb*<sup>11A22</sup> mutant clones in a *d*<sup>GC13</sup> homozygous mutant background. **(D)** Wing discs overexpressed a V5-tagged D by *nub-Gal4* and stained for V5 to visualize D localization (red in D, gray in D') which is not significantly affected in *crb*<sup>11A22</sup> mutant cells. Arrowheads point to clone borders in A–C and to mutant cells in D.

## Discussion

The studies described in this Chapter connect the growth regulatory activity of Crb with Hippo signaling and thus identify Crb as a novel component of the Hippo pathway. We showed that Crb gain and loss of function cause overgrowth, ectopic proliferation, and the upregulation of Hippo pathway target genes. The overgrowth phenotypes of *crb* and the induction of Hippo target genes require Yki, indicating that Yki is epistatic to Crb. Moreover, loss of Crb genetically interacts and synergizes with mutations of Hippo pathway components. Furthermore, the proper level of Crb is required for the correct localization of Ex. Taken together, our data indicate that Crb regulate tissue size through modulation of the Hippo pathway.

### 4. 7. Crb functions upstream in the Hippo pathway

To date, multiple inputs into the Hippo pathway have been identified, including the atypical cadherin Fat and Mer. Nevertheless, the mutant phenotypes of upstream components, such as *ft* and *mer*, are generally weaker than those of downstream components, such as *hpo* and *wt*s. Interestingly, *ft;mer* double mutants, which abolish signals from those two different upstream branches, display a stronger phenotype that resembles mutant phenotypes of downstream components. Similarly, loss of *crb* synergized with knock down of *mer* in the pupal retina indicating that Crb and Mer function in different upstream branches and cooperate to modulate Hippo pathway activity. This

also supports the idea that Crb functions in the Ex branch and specifically regulates the localization of Ex but not that of Mer.

Proper level and localization of Crb appear to be essential for the correct localization of Ex to the sub-apical region of the plasma membrane. Crb loss and gain of function had reduced protein levels of Ex at the sub-apical plasma membrane even though the transcription level of *ex* was increased. Crb is likely to regulate Ex post-transcriptionally and affects the localization of Ex to the apical membrane. This further supports the model that Crb functions upstream of Ex in the Hippo pathway.

Furthermore, Crb itself is controlled by a negative feedback loop through the Hippo pathway (Genevet et al., 2009; Hamaratoglu et al., 2009). Similar feedback mechanisms have been observed for several other Hippo pathway components (Genevet et al., 2010; Hamaratoglu et al., 2009; Hamaratoglu et al., 2006). Epithelial cells mutant for *hpo* and *wts* display elevated levels of Crb as well as Ex, Mer, Kibra, and Fat (Genevet et al., 2010; Hamaratoglu et al., 2006). At least for *ex* and *kibra*, the feedback depends on transcriptional regulation and is thus not simply a secondary consequence of the enlargement of the apical domain observed in Hippo pathway mutants. Rather, it constitutes a direct feedback loop in the Hippo pathway. Those feedback regulations may provide a homeostatic effect on the regulation of the Hippo pathway. Notably,

the feedback regulation of Crb is not dependent upon transcriptional regulation because *crb* mRNA level is not increased in Yki overexpressing tissue (Genevet et al., 2010). This indicates that more than one mechanism may contribute to achieve homeostasis of Hippo pathway activity.

#### **4. 8. Expanded stability and membrane localization**

Ex was largely absent from the apical membrane and diffused into the cytoplasm at the basal lateral region in *crb* mutant cells, while the Ex level at the apical domain is decreased and localized more basolaterally with ectopically expressed Crb. Interestingly, when we extended the duration of those genetic manipulations, such as by prolonged induction of Crb overexpression, not only is the Ex level at the apical domain decreased but also the basolaterally localized Ex is no longer observed. This implies that Ex may be degraded when not localized properly. Because Ex is recruited by overexpressed Crb to a more basolateral region and then degraded, it is unlikely that the interaction between Crb and Ex would stabilize Ex. Crb may act as a scaffold that is required to recruit Ex to the sub-apical membrane making it available for another unknown regulator to stabilize Ex. Alternatively, Crb may make Ex unavailable for proteins that degrade Ex and normally localize basolaterally. In either case, it appears that the localization of Ex is important for its stability, and the degradation of Ex may be a potential regulatory mechanism of the Hippo

pathway. Further understanding of how the presence of Crb affects Ex stability will offer insights into how Hippo signaling is regulated.

#### **4. 9. Crb regulates cell polarity and Hippo signaling through different mechanisms.**

It is interesting that Crb coordinately interacts with cell polarity determinants and components of the Hippo tumor suppressor pathway. However, our data indicate that Crb regulates these two pathways by different domains and thus through different mechanisms. Crb<sup>intra</sup>, which is lacking the extracellular domain, is sufficient to mediate the functions of full-length Crb in modulating Hippo signaling and cell polarity (Izaddoost et al., 2002; Wodarz et al., 1995). Specifically, the effects on Hippo signaling require the JM whereas the effects on cell polarity require the PBM, which binds to Sdt. These results indicate that the function of Crb in apical-basal polarity and growth control can be uncoupled.

The JM is a FERM-domain binding motif that can physically interact with the FERM domain protein Yurt during development (Laprise et al., 2006). It has been shown that Yurt can negatively regulate Crb to control cell polarity (Laprise et al., 2006). However, *yurt* mutants do not have growth defects, unlike *ex* mutants. Several lines of evidence imply that Crb may bind to Ex directly. First, the JM is a FERM-domain binding motif that is potentially capable of



interacting with the FERM domain of Ex. Second, the JM is required for the Crb-induced growth phenotypes that are similar to those with loss of Hippo activity. Third, a proper amount of Crb is required for correct Ex localization. Similar results are also reported recently by independent researches (Chen et al., 2010; Grzeschik et al., 2010; Ling et al., 2010; Robinson et al., 2010). Indeed, it has also been reported by Ling et al. that Crb can directly interact with the FERM domain of Ex through the JM. Consequently, the effects on the Hippo pathway by Crb loss and gain of function may be caused by loss of Ex which leads to decreased Hippo activity. Nevertheless, the phenotypes of Crb overexpression are stronger than ex mutants and thus cannot simply be explained by loss of Ex. Therefore, Crb is likely to interact with another FERM domain protein that cooperates with Ex to regulate Hippo signaling. The identification of novel interaction partners for Crb will certainly shed light on the molecular mechanism of Crb's action.

As discussed previously, Crb regulates apical-basal polarity and growth by using different domains and thus through different mechanisms. Crb potentially mediates the crosstalk between the apical-basal polarity pathway and growth control signaling through the Hippo pathway. Notably, Crb is required for proper Crb localization on neighboring cells and is thus non-autonomously required for the localization of Ex and Patj at the apical membrane. Crb may simply function as a scaffold protein that is required for

proper membrane localization of Ex and Patj. Alternatively, Crb may act as a receptor and transduce the extracellular cue to both the cell polarity pathway and the Hippo pathway. In this scenario, homophilic binding of Crb may coordinate growth and polarity information signal between cells. These results thus identify a cell-cell interaction dependent mechanism that is mediated by Crb and regulates Hippo pathway activity.

Do Crb homologs act through Hippo signaling in mammals? Three Crb homologs, Crb1-3, have been identified in mammals. However, it is not clear whether any of the vertebrate Crb homologs regulate growth. The intracellular domains of Crb1-3 are conserved and important for proper apical-basal polarity (Bazellieres et al., 2009). Notably, Crb3 has been reported to function as a tumor suppressor in immortalized mouse kidney epithelial cells (Karp et al., 2008). In the process of establishing tumorigenic cell lines, the expression of Crb3 is lost. Interestingly, overexpression of Crb3 can restore contact inhibition and cell polarity, and suppress tumor progression. In addition, the highly conserved Hippo signaling pathway has been implicated in tumor suppression in vertebrates (Harvey and Tapon, 2007; Pan, 2007; Reddy and Irvine, 2008; Zhao et al., 2008). Therefore, our study placing Crb within the Hippo signaling pathway may have important implications for the study of cancer development and treatment.

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## Chapter 5:

# Cell competition eliminates tumorigenic cells through modulation of Hippo signaling

Data in this chapter has been submitted to Developmental Cell:

**Chen, C.L.\***, Schroeder, M.\*, Kango-Singh, M., Tao, C., Halder, G., Tumor suppression by cell competition through regulation of the Hippo pathway.

\* These authors contributed equally to this work.

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## Introduction

Animals have evolved homeostatic mechanisms that help eliminate abnormal cells and prevent disease. However, how these processes occur is not well understood. The elimination of cells mutant for neoplastic tumor suppressor genes from *Drosophila* imaginal discs provides a prominent example of how an organism eliminates abnormal cells that have the potential to become tumorous (Igaki et al., 2009; Vidal, 2010). *Drosophila* larvae that are homozygous mutant for *scribble* (*scrib*), which encodes a conserved apical-basal polarity determinant, produce imaginal discs that grow into large and amorphous tumors capable of metastasis (Bilder et al., 2000). *Scrib* therefore acts as a neoplastic tumor suppressor gene in *Drosophila*. Interestingly, this phenomenon is context-dependent. *scrib* mutant cells that arise in wild-type discs do not hyperproliferate, in stark contrast to discs comprised wholly of *scrib* mutant cells (Brumby and Richardson, 2003; Igaki et al., 2009; Pagliarini and Xu, 2003). Rather, *scrib* mutant cells surrounded by wild-type neighbors are eliminated and therefore prevented from manifesting their tumorigenic potential (Brumby and Richardson, 2003; Igaki et al., 2009; Moreno, 2008; Vidal, 2010). Theories to explain how imaginal discs remove *scrib*<sup>-</sup> clones include cell competition (Brumby and Richardson, 2003), a process whereby less fit cells are removed from tissues with cells of varying fitness. However, the role of wild-type cells in preventing *scrib* mutant cells from forming tumors remains

controversial (Vidal, 2010).

Here we show that cell competition between *scrib* mutant cells and wild-type cells prevents tumor formation through modulation of the Hippo tumor suppressor pathway. This suppression can be circumvented by increasing the fitness of *scrib* mutant cell, which can be achieved by hyperactivating Ras signaling or overexpressing Myc. Given the oncogenic role of the Ras and Myc in mammals, acquiring mutations that prevent the elimination of tumorigenic cells by cell competition may be a fundamental event in the formation of tumors.

## Results

### **5. 1. Activation of JNK restrains the growth potential of *scrib* mutant cells in addition to inducing apoptosis**

Scrib was identified as a regulator of epithelial cell polarity in the *Drosophila* embryo (Bilder and Perrimon, 2000). Imaginal disc cells mutant for *scrib* display several hallmarks of carcinomas: they lose apical-basal cell polarity, have defects in differentiation, and can form neoplastic tumors. In contrast, patches of *scrib* mutant (*scrib*<sup>-</sup>) cells surrounded by wild-type cells (*scrib*<sup>-</sup> clones) in imaginal discs do not display a tumorous phenotype, instead they are eliminated from the tissue, consistent with previous reports (Figure 5.1A,B) (Brumby and Richardson, 2003; Igaki et al., 2009). *scrib*<sup>-</sup> clones activate JNK signaling and induce JNK-dependent apoptosis, which has been

proposed to explain how *scrib*<sup>-</sup> cells are eliminated (Igaki et al., 2009). To evaluate the growth potential of *scrib*<sup>-</sup> cells in different cellular contexts, we

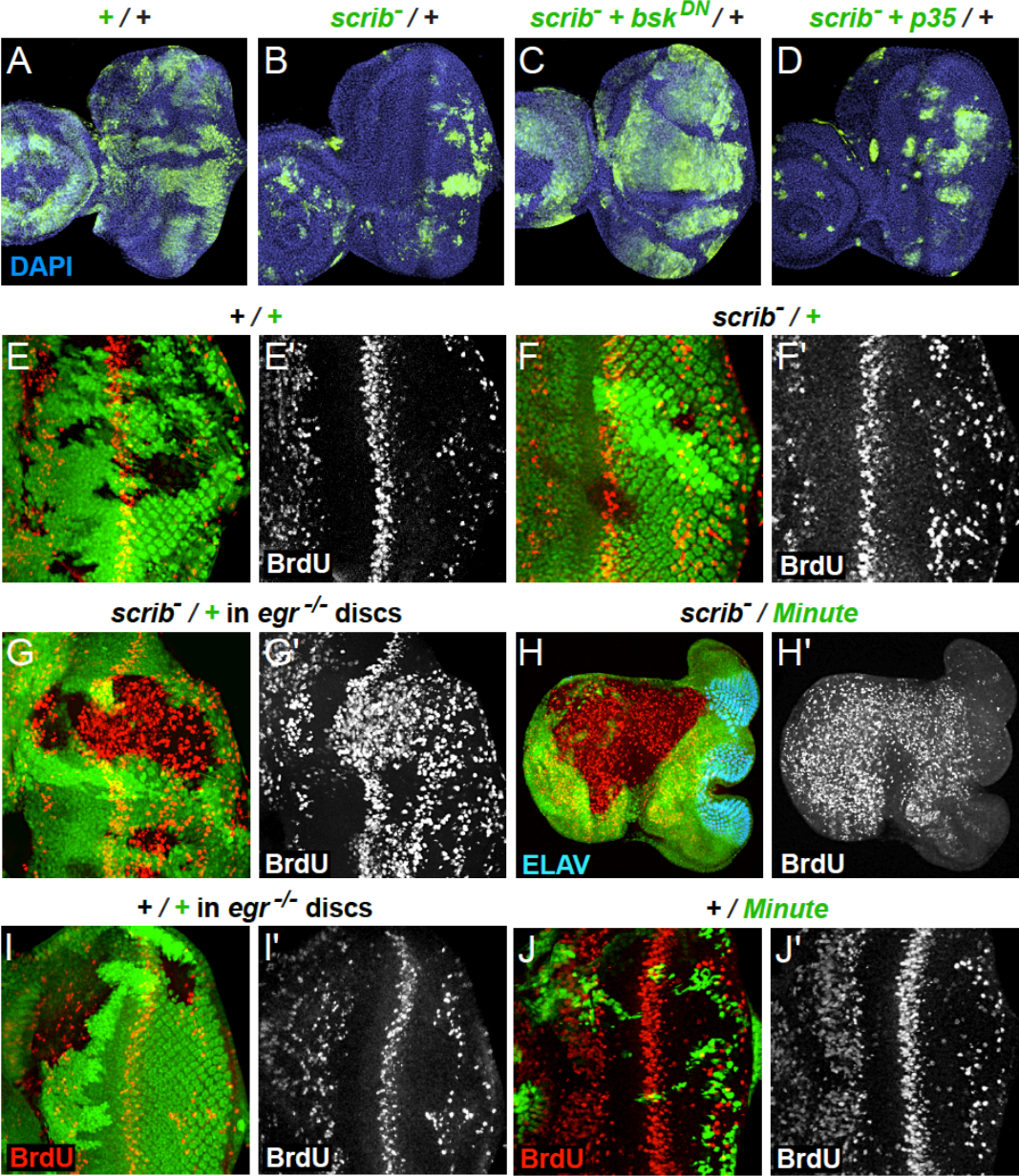


Figure 5.1.

**Figure 5.1. Activation of JNK but not apoptosis is required to limit the growth potential of *scrib* mutant cells**

**(A-D)** Confocal images of eye imaginal discs containing clones of cells with different genotypes as indicated. Clones were generated using the MARCM system (Lee and Luo, 1999) to positively label mutant clones by GFP expression (yellow) and *ey-Flp* to induce recombination in eye discs. Cell nuclei are labeled with DAPI (blue). **(A)** Wild-type clones. **(B)** *scrib*<sup>-</sup> clones. **(C)** *scrib*<sup>-</sup>+*bsk*<sup>DN</sup> clones. **(D)** *scrib*<sup>-</sup>+*p35* clones. Compared to wild-type clones, *scrib*<sup>-</sup> clones lacking JNK activity overgrow while *scrib*<sup>-</sup> clones prevented from apoptosis grow poorly. **(E-H)** Confocal images of eye imaginal discs containing clones of the indicated genotypes marked by the absence of GFP expression (green) and stained for BrdU (red in E-H and gray in E'-H') to reveal cells in S-phase. **(E,E')** Wild-type clones. **(F,F')** *scrib*<sup>-</sup> clones. **(G,G')** *scrib*<sup>-</sup> clones in homozygous *egr*<sup>-</sup> discs. **(H,H')** *scrib*<sup>-</sup> cells surrounded by *Minute* mutant tissues. Compared to wild-type clones and *scrib*<sup>-</sup> clones in a competitive environment, *scrib*<sup>-</sup> clones hyperproliferate in a non-competitive environment. **(I-I')** Wild-type clones in an *egr*<sup>-</sup> disc. **(J,J')** *Minute* clones marked by GFP expression.

induced high levels of mitotic recombination in eye discs by using *ey-FLP*, which constitutively express flippase in the developing eye discs. In combination with the MARCM system (Lee and Luo, 1999), we can positively mark homozygous mutant cells by GFP expression. This system produces a similar amount of GFP marked cells in which recombination occurs and allows us to examine the contribution of this population to third-instar eye discs as an indicator of cell survival rate and cell proliferation ability. As previously reported, *scrib*<sup>-</sup> cells in which JNK signaling was blocked by expressing a dominant-negative form of the *Drosophila* JNK *basket* (*bsk*<sup>DN</sup>) were no longer eliminated (Figure 5.1C) (Igaki et al., 2009). Suppression of apoptosis by overexpression of the caspase inhibitor p35, however, did not rescue the small clone phenotype of *scrib*<sup>-</sup> clones (Figure 5.1D) (Igaki et al., 2009). Therefore, the induction of p35-dependent apoptosis is not sufficient to explain how *scrib*<sup>-</sup> clones are eliminated, and JNK may regulate processes in addition to apoptosis in *scrib*<sup>-</sup> cells.

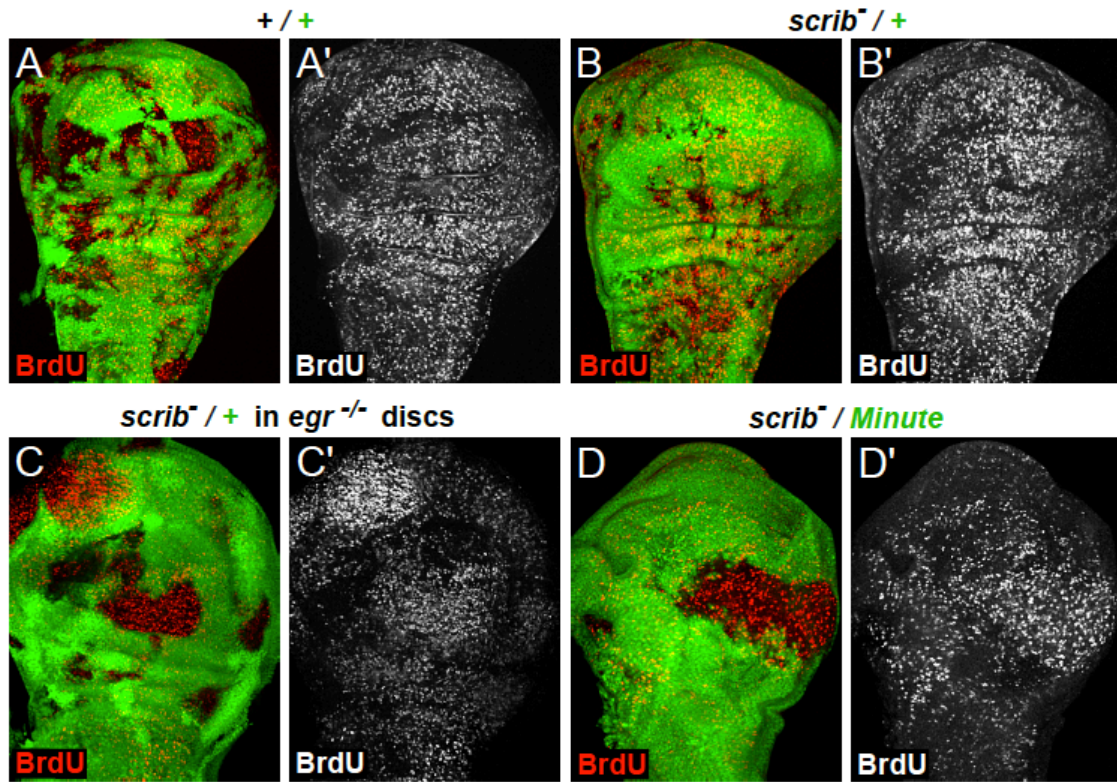
To investigate how JNK facilitates the removal of *scrib*<sup>-</sup> cells, we compared *scrib*<sup>-</sup> clones in wild-type animals with clones in animals that cannot activate JNK. In addition to expressing *bsk*<sup>DN</sup> in *scrib*<sup>-</sup> cells, we generated *scrib*<sup>-</sup> clones in animals mutant for *eiger* (*egr*), a secreted ligand that activates JNK signaling (Igaki et al., 2002). Similar to *scrib*<sup>-</sup> cells expressing *bsk*<sup>DN</sup> (*scrib*<sup>-</sup> + *bsk*<sup>DN</sup>), *scrib*<sup>-</sup> clones in *egr* mutant animals were not eliminated (Figure 5.1E-G



and 5.2A-C). If the only role of JNK in *scrib*<sup>-</sup> cells was initiation of apoptosis, then *scrib*<sup>-</sup> clones lacking JNK signaling would be expected to exhibit similar proliferation patterns to *scrib*<sup>-</sup> clones. However, *scrib*<sup>-</sup> clones in *egr* mutant animals overproliferated, as revealed by an excess of BrdU incorporating cells in mutant clones (Figures 5.1E,G,I and 5.2A,C). In contrast, *scrib*<sup>-</sup> cells in wild-type tissues did not overproliferate, were mostly eliminated, and occasionally formed small clones (Figures 5.1F and 5.2B) (Igaki et al., 2009). Thus, our data suggest that JNK signaling counteracts the overproliferation potential of *scrib*<sup>-</sup> cells by enforcing a growth control mechanism.

## **5. 2. Cell competition eliminates tumorigenic *scrib* mutant cells**

The observation that the proliferation of *scrib*<sup>-</sup> cells is restricted in the presence of wild-type neighbors posed the question of the role of neighboring cells. It has been proposed that removal of *scrib*<sup>-</sup> clones may depend on cell competition, on the presence of neighboring cells with normal apical-basal polarity, or on circulating hemocytes that attach to *scrib*<sup>-</sup> mutant cells and secrete Egr (Vidal, 2010). To determine whether *scrib*<sup>-</sup> cells are eliminated by cell competition, we decreased the fitness of the surrounding *scrib*<sup>+</sup> cells by making them heterozygous for a *Minute* mutation, dominant mutations in ribosomal components that cause cells to grow slowly and be poor competitors



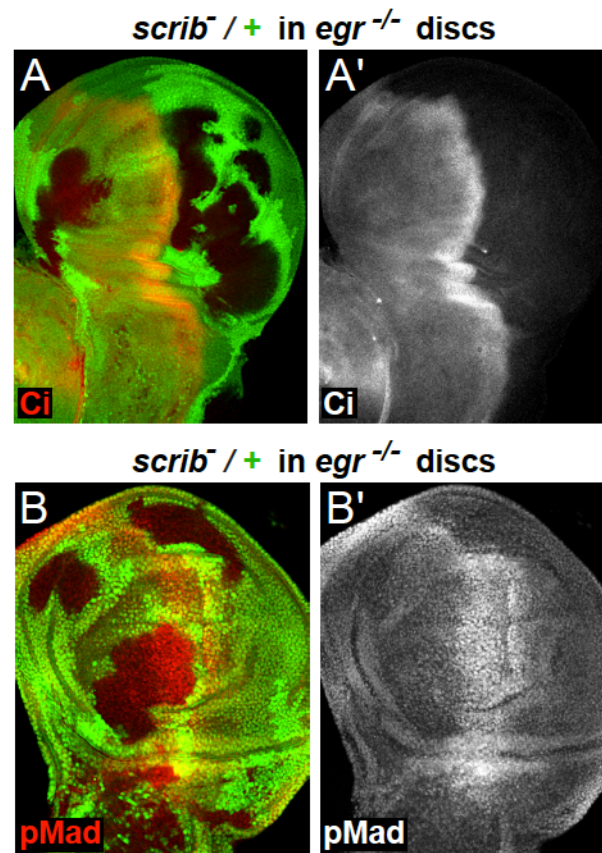
**Figure 5.2. *scrib* mutant clones rescued from cell competition hyperproliferate in wing discs.**

Confocal images of wing imaginal discs containing clones of cells with different genotypes as indicated. Clones of cells are marked by absence of GFP (green) and discs are stained for BrdU (red in A-D, grey in A'-D') to reveal cells in S-phase. **(A)** Wild-type clones. **(B)** *scrib*<sup>-</sup> clones. **(C)** *scrib*<sup>-</sup> clones surrounded by *Minute* mutant cells. **(D)** *scrib*<sup>-</sup> clones in *egr*<sup>-</sup> background. Compared to wild-type clones and *scrib*<sup>-</sup> clones in a competitive environment, *scrib*<sup>-</sup> clones hyperproliferate in a non-competitive environment.

(Morata and Ripoll, 1975). *scrib*<sup>-</sup> cells with *Minute* neighbors formed large clones with high levels of BrdU incorporation (Figures 5.1H,J and 5.2D) that often resulted in deformed and overgrown imaginal discs. This result demonstrates that the suppression of the tumorigenic potential of *scrib*<sup>-</sup> cells depends on the fitness of their neighbors.

### **5. 3. Hippo signaling is deregulated in *scrib* mutant cells protected from cell competition**

The overproliferation of *scrib*<sup>-</sup> cells protected from cell competition raised the question of which growth control pathways are misregulated. We surveyed the activity of pathways known to regulate growth and patterning in imaginal discs. However, readouts of the Hedgehog and TGF-beta pathways were not significantly affected in *scrib*<sup>-</sup> clones in *egr* mutant discs (Figure 5.3A,B). In contrast, *expanded-lacZ* (*ex-lacZ*) (Hamaratoglu et al., 2006), a reporter of the Hippo tumor suppressor and growth control pathway was dramatically upregulated in *scrib*<sup>-</sup> cells in *egr* mutant discs (Figure 5.4A,B,D). The Hippo pathway regulates cell proliferation and survival by suppressing the activity of Yorkie (Yki), a growth promoting transcriptional co-activator. Phosphorylation by the Warts (Wts) kinase results in cytoplasmic retention of Yki. Consistent with elevated Yki activity, we found that Yki was more concentrated in the nuclei of *scrib*<sup>-</sup> cells in *egr*<sup>-</sup> discs than in surrounding *scrib*<sup>+</sup> cells (Figure 5.4C). Remarkably, Hippo pathway reporters that were



**Figure 5.3. *scrib* mutant clones protected from cell competition do not display noticeable defects in Hh and Dpp signaling.**

Confocal images of wing imaginal discs containing *scrib*<sup>-</sup> clones in *egr*<sup>-</sup> animals. Clones of cells are marked by absence of GFP (green). **(A)** Discs are stained for anti-Ci (cubitus interruptus), a transcription factor that undergoes proteolytic cleavage in the absence of Hedgehog (Hh), to reveal the activity of Hh signaling. **(B)** Discs are stained for anti-phospho-Mad to reveal the activity of Dpp signaling. Upon Dpp activation, Mad (Mothers against Dpp) is phosphorylated.



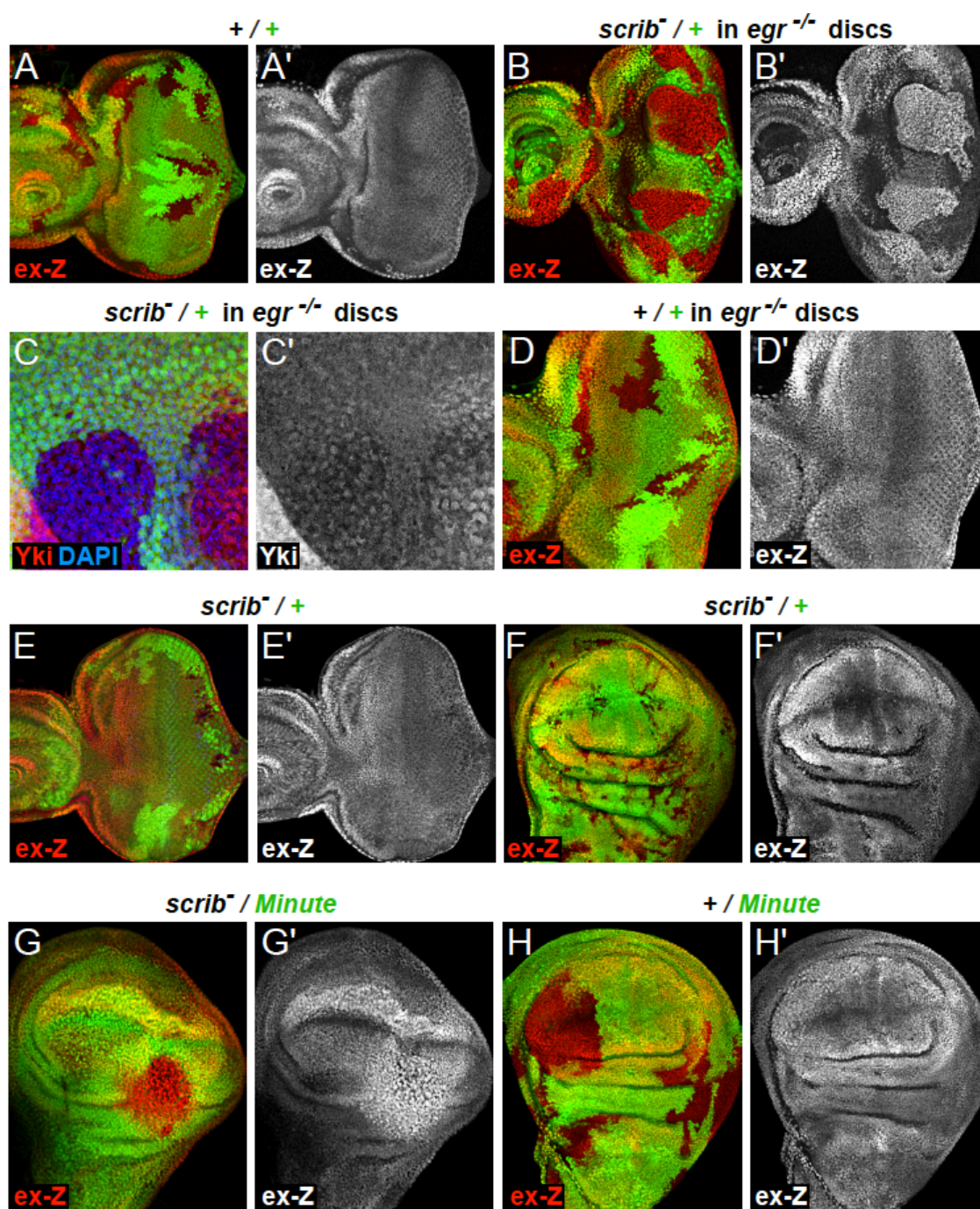


Figure 5.4.

**Figure 5.4. Hippo signaling is deregulated in overproliferating *scrib* mutant cells.**

**(A-H)** Confocal images of eye (A,B,D,E) and wing (C,F-H) imaginal discs containing clones of cells of the indicated genotypes marked by the absence of GFP expression (green). Discs are stained for  $\beta$ -Gal to show *ex-lacZ* expression (red). **(A,A')** Wild-type clones. **(B,B')** *scrib* mutant clones in an *egr* mutant disc have high levels of *ex-lacZ*. **(C,C')** Yki (red in C, gray in C') is concentrated in the nuclei of *scrib*<sup>-</sup> cells compared to *scrib*<sup>+</sup> cells in an *egr*<sup>-</sup> disc. DAPI is in blue. **(D)** Wild-type clones in an *egr*<sup>-</sup> disc. **(E,F)** *scrib*<sup>-</sup> clones in a wild-type disc. **(G,G')** *scrib* mutant clones in a *Minute* mutant disc. **(H,H')** Wild-type clones surrounded by *Minute* tissues do not have an effect on *ex-lacZ* expression. *scrib*<sup>-</sup> cells facing cell competition do not have increased Yki activity.

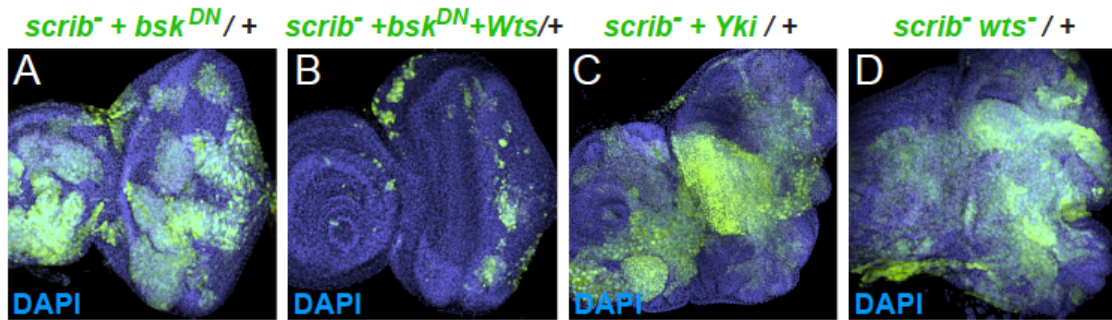
elevated in *scrib*<sup>-</sup> clones surrounded by *Minute* tissues were not induced in *scrib*<sup>-</sup> clones surrounded by wild-type neighbors and thus facing cell competition. (Figure 5.4E,F) Therefore, *scrib*<sup>-</sup> cells not facing cell competition have abnormally high levels of Yki activity and cell competition restricts this activation. To test whether these elevated levels of Yki activity are required for the hyperproliferation phenotype of *scrib*<sup>-</sup> cells not facing cell competition, we artificially decreased Yki activity in *scrib*<sup>-</sup>+*bsk*<sup>DN</sup> cells by co-expressing Wts (Halder and Johnson, 2011). (Figure 5.5A,B) We found that such cells only formed small clones. Thus, *scrib*<sup>-</sup> cells protected from cell competition have high levels of Yki activity and Yki is required for them to hyperproliferate.

#### **5. 4. Deregulation of Hippo signaling is essential for the overproliferation of *scrib* mutant cells.**

Previous work has shown that high levels of Yki activity can make cells super-competitors capable of eliminating wild-type cells and rescuing *Minute* cells from elimination (Tyler et al., 2007) (Menendez et al., 2010; Ziosi et al., 2010). Our data show that *scrib*<sup>-</sup> cells that are protected from cell competition have elevated Yki activity. This prompted the question of how *scrib*<sup>-</sup> cells in wild-type backgrounds are outcompeted if they have high levels of Yki activity. To investigate this paradox, we examined Yki reporters in *scrib*<sup>-</sup> clones with wild-type neighbors. Interestingly, the Yki reporter *ex-lacZ*, which was upregulated in *scrib*<sup>-</sup> clones surrounded by *Minute* cells, was not upregulated in

*scrib*<sup>-</sup> clones surrounded by wild-type neighbors in most regions of eye and wing discs (Figure 5.4G,H). Clones in the dorsal hinge region of wing discs frequently displayed increased levels of *ex-lacZ* as previously reported (Grusche et al., 2010). However, *scrib*<sup>-</sup>+*bsk*<sup>DN</sup> clones were larger and had higher levels of *ex-lacZ* expression in all regions of eye and wing discs. These results suggest that cell competition and JNK activation prevents the elevation of Yki activity in *scrib*<sup>-</sup> cells. To test whether suppression of Yki in *scrib*<sup>-</sup> cells facing competition is required for their elimination, we artificially elevated levels of Yki in *scrib*<sup>-</sup> cells. Overexpression of Yki or loss of *wts* in *scrib*<sup>-</sup> cells is sufficient to rescue them from being outcompeted and results in the formation of big clones (Figure 5.5C,D). These data further support the model that Yki activity is not high in *scrib*<sup>-</sup> cells surrounded by wild-type cells. If Yki activity is significantly elevated in *scrib*<sup>-</sup> cells facing cell competition, overexpression of Yki or removal of Wts in *scrib*<sup>-</sup> cells are not expected to alter the growth phenotype of *scrib*<sup>-</sup> cells. These data indicate that *scrib*<sup>-</sup> clones are not simply comprised of dying cells with high Yki activity but that cell competition regulates the levels of Hippo signaling in *scrib*<sup>-</sup> cells. Our data indicate that *scrib*<sup>-</sup> cells facing competition fail to elevate Yki activity and that this is key to their elimination. We thus conclude that cell competition acts as a tumor suppressive mechanism by preventing Yki activation in *scrib*<sup>-</sup> cells.





**Figure 5.5. Deregulation of Hippo signaling is required and sufficient for the tumorigenic overproliferation of *scrib* mutant cells.**

**(A-D)** Confocal images of eye imaginal discs containing clones of cells with different genotypes as indicated. Mutant clones are positively marked by GFP expression (yellow) and cell nuclei are labeled with DAPI (blue). **(A)** *scrib*<sup>-</sup> + *bsk*<sup>DN</sup> clones grow large. **(B)** *scrib*<sup>-</sup> + *bsk*<sup>DN</sup> clones overexpressing *Wts*. **(C)** *scrib*<sup>-</sup> clones overexpressing *Yki*. **(D)** *scrib*<sup>-</sup> *wts*<sup>-</sup> double mutant clones. *Yki* activity is required for the growth of *scrib*<sup>-</sup> cells. Anterior is to the left in all panels.

### **5. 5. Expressing Ras<sup>V12</sup> protects *scrib* mutant cells from cell competition.**

Hyperactivation of Ras is known to rescue *scrib*<sup>-</sup> cells from being outcompeted and act synergistically with loss of *scrib* to form tumors (Wu et al., 2010) (Brumby and Richardson, 2003; Pagliarini and Xu, 2003) (Menendez et al., 2010). To test whether this is through regulation of Yki activity, we examined *ex-lacZ* in *scrib*<sup>-</sup> cells expressing Ras<sup>V12</sup>, an oncogenic form of Ras. *ex-lacZ* was sometimes affected in clones expressing Ras<sup>V12</sup> alone but was consistently elevated in *scrib*<sup>-</sup>+Ras<sup>V12</sup> clones (Figure 5.6A-D and 5.7A). Therefore Ras<sup>V12</sup> rescues *scrib*<sup>-</sup> cells from cell competition and thus prevents the suppression of Yki activity. These data point to an additional oncogenic role for Ras as a factor that can determine the fate of tumorigenic cells by conferring increased competitive fitness.

### **5. 6. *scrib* mutant clones that are not eliminated induce non-autonomous misregulation of Hippo signaling.**

While the above results illustrate a critical role for cell competition in counteracting the tumorigenic potential of *scrib*<sup>-</sup> cells, the hyperproliferation of these mutant cells is only a portion of the threat they pose to the organism. In addition to the cell-autonomous upregulation of *ex-lacZ*, *scrib*<sup>-</sup>+Ras<sup>V12</sup> clones exhibit a non-autonomous upregulation of *ex-lacZ* in neighboring wild-type cells

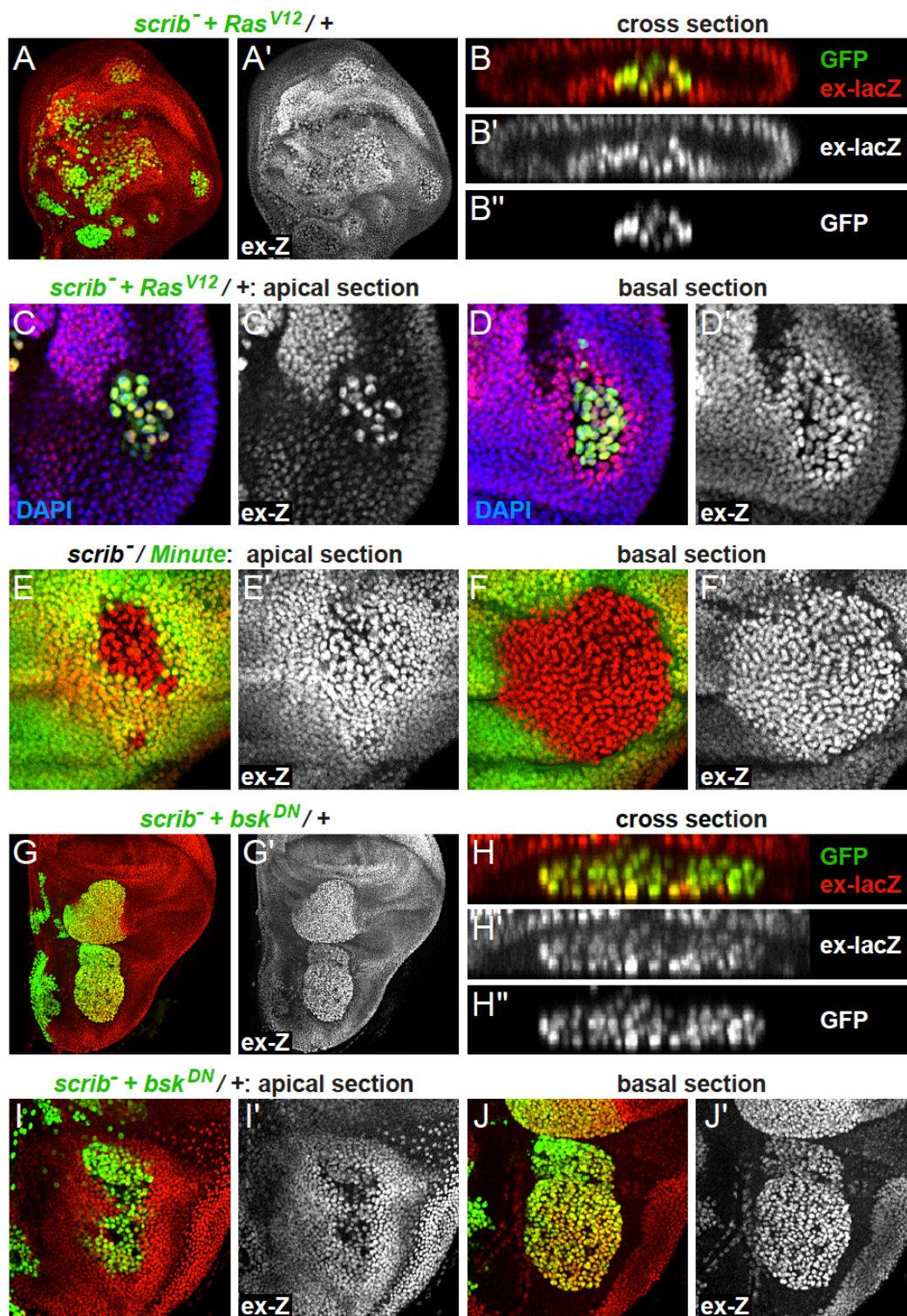


Figure 5.6.

**Figure 5.6. *scrib* mutant cells that escape elimination show non-autonomous effects on Hippo signaling.**

Confocal images of wing imaginal discs containing clones of cells with different genotypes as indicated. *ex-lacZ* is shown in red (A-J) or grey (A'-J'), DAPI in blue (A-I). **(A,A')** *scrib+Ras<sup>V12</sup>* clones marked by GFP expression (green). **(B-B'')** Optical cross section of a *scrib+Ras<sup>V12</sup>* clone showing that *ex-lacZ* expression is upregulated both inside and outside the clone. **(C,D)** Apical and basal sections of the disc in (A) at the higher magnification. **(E,F)** Apical and basal sections of *scrib<sup>-</sup>* clones surrounded by *Minute* mutant cells, marked by the absence of GFP. **(G,G')** *scrib+bsk<sup>DN</sup>* clones marked by GFP **(H-H'')** Optical cross section of a *scrib+bsk<sup>DN</sup>* clone. *scrib<sup>-</sup>* cells induce *ex-lacZ* expression non-autonomously in different genetic backgrounds. **(I,J)** Apical and basal sections of the disc in **(G)** at higher magnification.

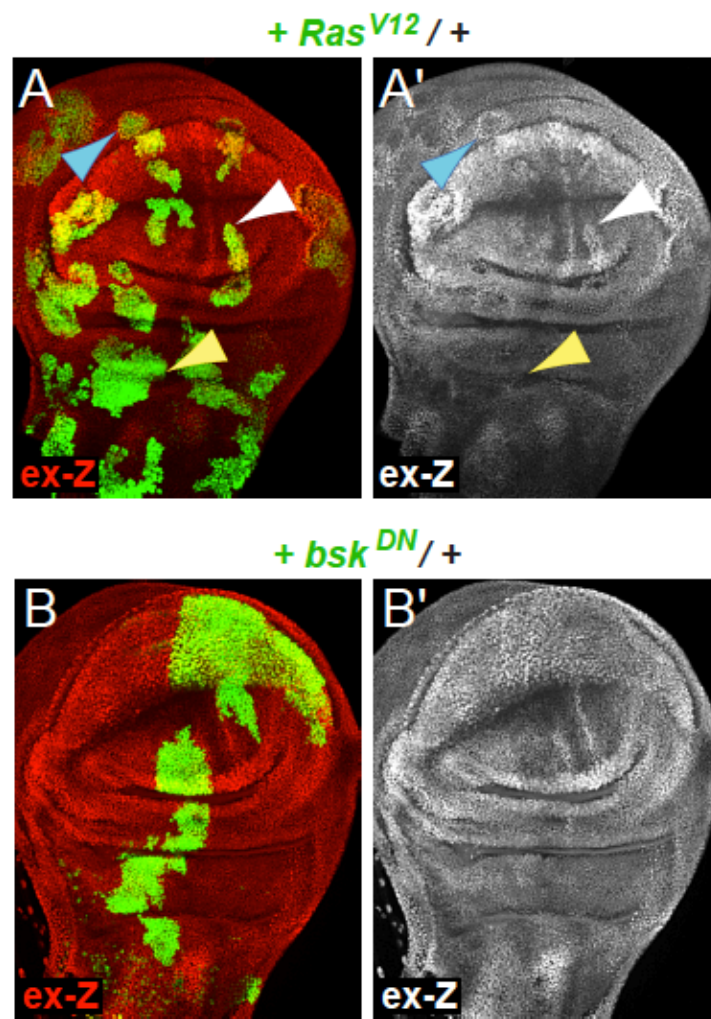


Figure 5.7.

**Figure 5.7. Effects of Ras<sup>V12</sup> overexpression and JNK removal on Hippo signaling in wild-type discs**

Confocal images of wing imaginal discs containing clones marked by expression of GFP. Discs stained for  $\beta$ -Gal to reveal the level of *ex-lacZ* expression. (red in A,B and grey in A'B') **(A)** Ras<sup>V12</sup> overexpressing clones have various effects on Hippo signaling in a wild-type background. Autonomous induction of *ex-lacZ* is indicated by a white arrowhead. No significant effect is indicated by a yellow arrowhead. Non-autonomous induction of *ex-lacZ* is indicated by a blue arrowhead. **(B)** *bsk*<sup>DN</sup> overexpressing clones do not exhibit any significant effect on *ex-lacZ* expression in a wild-type disc.

(Figure 5.6B-D). Therefore *scrib*<sup>-</sup> cells rescued from competition can cause sustained suppression of Hippo signaling in adjacent normal cells. *scrib*<sup>-</sup> clones that do not succumb to cell competition take on an unusual morphology. Cells grow into a multilayered mass that protrudes from the disc. (Figure 5.6B-D). This non-autonomous effect on *ex-lacZ* was also observed around *scrib* mutant cells rescued from elimination by other means. *scrib*<sup>-</sup> clones surrounded by *Minute* tissues and *scrib*<sup>-</sup>+*bsk*<sup>DN</sup> all showed similar non-autonomous effects and cell extrusion morphology (Figures 5.6E-J) whereas overexpressing *bsk*<sup>DN</sup> alone does not exhibit any defects in growth, cell morphology, or *ex-lacZ* expression (Figures 5.7B). The strength of the non-autonomous effects varied depending on timing and location of clone induction. The non-autonomous induction of *ex-lacZ* can be observed only when wild-type cells are juxtaposed (Figure 5.6B-F,H-J). We conclude that *scrib* mutant clones that evade competition not only display cell-autonomous defects in Hippo signaling but can also induce Yki activity in neighboring wild-type tissue.

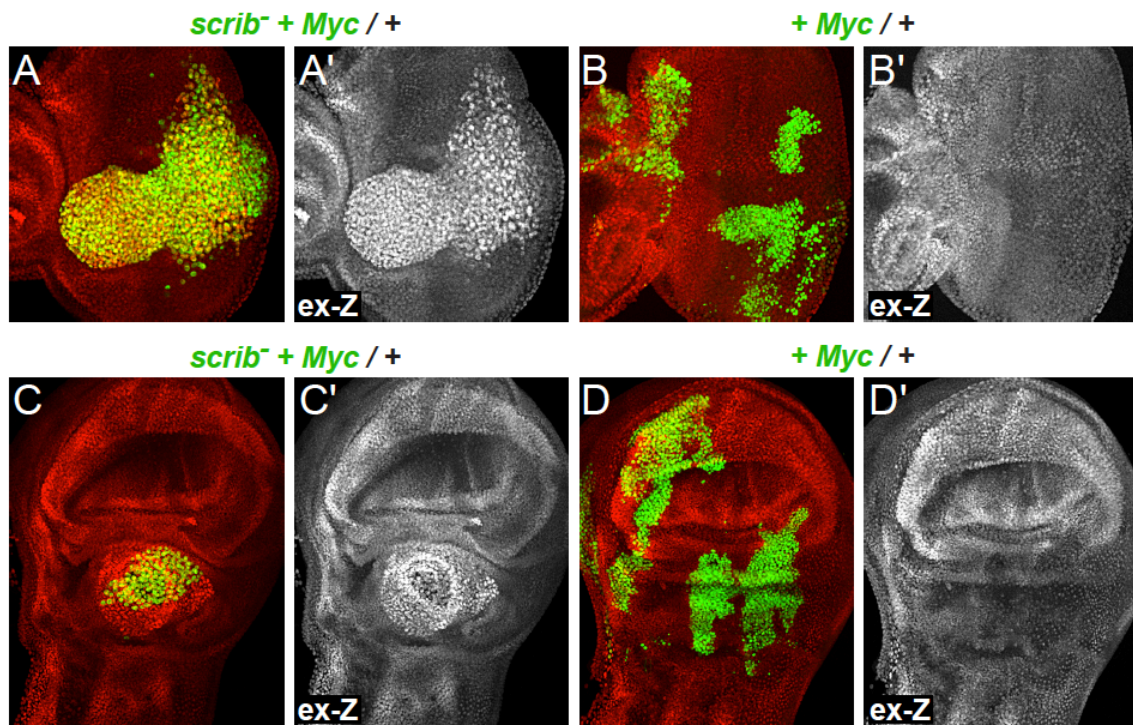
## **5. 7. Increasing relative fitness of *scrib* mutant cells by Myc overexpressing unleashes their tumorigenic potential.**

To further test the importance of cell competition in the elimination of *scrib*<sup>-</sup> cells we artificially increased their fitness by overexpressing Myc, a factor that transforms cells into supercompetitors and is a mammalian oncogene (Boxer and Dang, 2001; Froldi et al., 2010; Pelengaris et al., 2002). We found



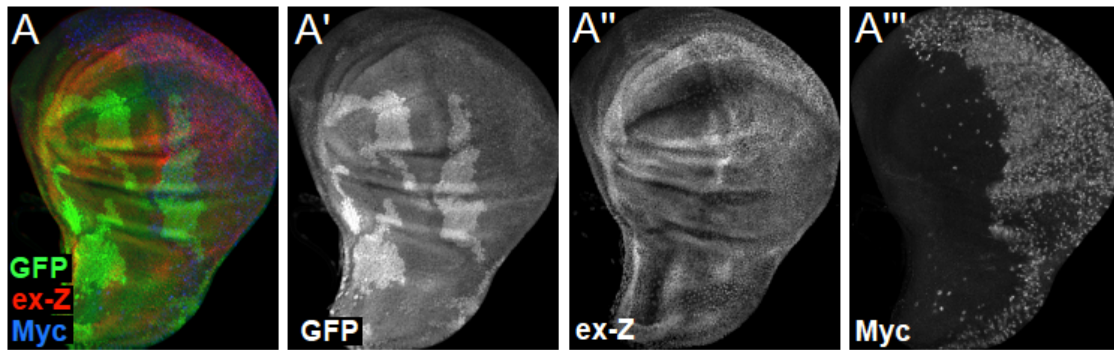
that overexpression of Myc in *scrib*<sup>-</sup> cells rescued their poor growth and increased expression of *ex-lacZ* (Figure 5.8A-D). This result is striking because overexpression of Myc in wild-type discs does not increase Yki activity, in fact, it slightly suppressed *ex-lacZ* expression (Figure 5.8B,D and (Neto-Silva et al., 2010)). Therefore Myc has differential effects on Hippo signaling in *scrib*<sup>-</sup> and wild-type cells. Consequently the oncogenic potential of Myc is more dramatically realized in *scrib*<sup>-</sup> cells. This suggests that Myc may most potently influence the proliferation of cells by counteracting the growth suppressing effects of cell competition faced by abnormal cells. To further exclude the possibility that Myc simply contributes to the growth ability of *scrib*<sup>-</sup> clones instead of acting through cell competition, we overexpressed Myc in both *scrib*<sup>-</sup> clones mutant cells and their neighboring cells. If Myc contributes to the absolute growth ability of *scrib* mutant clones rather than to relative growth ability, we would expect that *scrib*<sup>-</sup> clones would not be eliminated and would be able to grow when Myc is overexpressed in the background. Interestingly, *scrib* mutant cells are eliminated when Myc is overexpressed in both *scrib*<sup>-</sup> clones mutant cells and their neighboring cells (Figure 5.9). Therefore, we conclude that Myc increases the relative fitness of *scrib*<sup>-</sup> cells and thus acts as a proto-oncogene.





**Figure 5.8. Myc overexpression promotes tumorigenesis in *scrib* mutant clones.**

Confocal images of eye and wing imaginal discs containing clones of cells with different genotypes as indicated. Clones of cells are marked by GFP (green) and discs are stained for  $\beta$ -Gal to reveal the levels of *ex-lacZ* expression (red in A-D and gray in A'-D'). **(A)** *scrib*<sup>-</sup>+*Myc* clone in an eye disc. **(B)** *Myc* overexpressing clones in an eye disc. **(C)** *scrib*<sup>-</sup>+*Myc* clones in a wing disc. **(D)** *Myc* overexpressing clones in a wing disc.



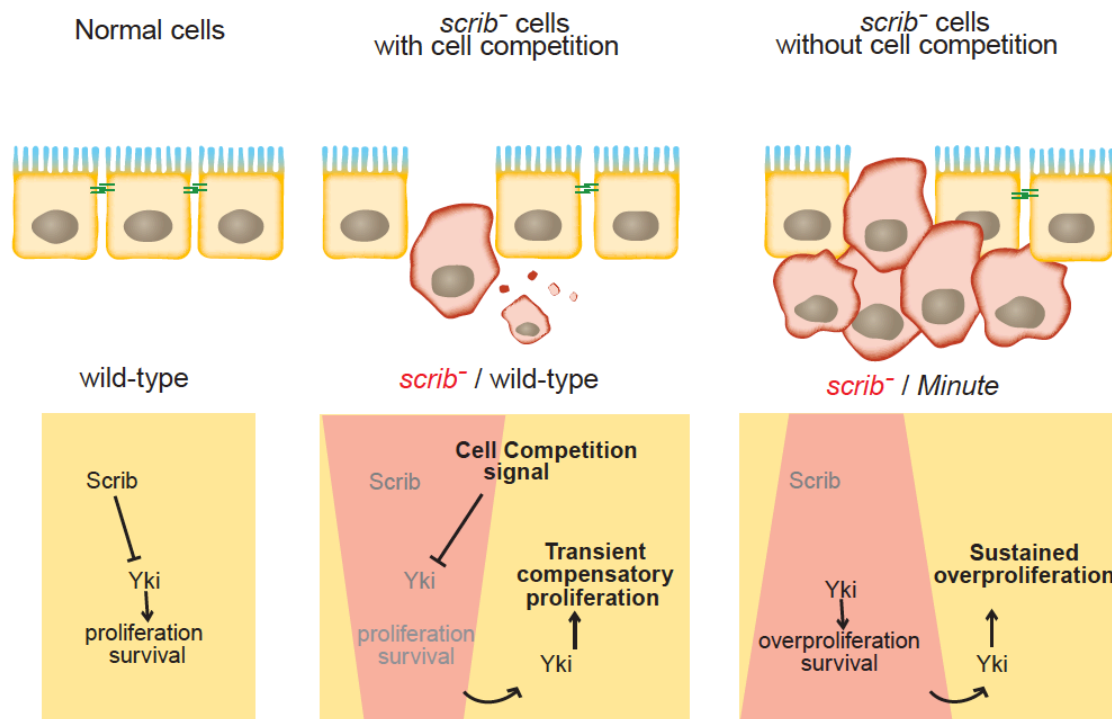
**Figure 5.9. *scrib* mutant cells are eliminated when Myc is overexpressed in both mutant cells and their neighboring wild-type cells.**

(A-A''') Confocal images of mutant clones in a wing disc. *scrib* mutant clones are marked by the absence of GFP and their corresponding twin-spot are labeled with 2X GFP expression (green in A and gray in A'). Discs are stained for  $\beta$ -Gal to show *ex-lacZ* expression (red in A and gray in A'). Myc is overexpressed by *en-Gal4* in the posterior compartment (shown in A'''). The lack of GFP negative cells in both anterior and posterior compartments indicates that *scrib* mutant clones are eliminated while their corresponding twin-spot can survive and overexpressing Myc in the background does not prevent the elimination of *scrib* mutant clones.

## Discussion

Our data show that cell competition between *scrib*<sup>-</sup> and wild-type cells prevents tumor formation through two cell-to-cell signaling events that each regulate the Hippo tumor suppressor pathway, which restrains proliferation and promotes apoptosis by antagonizing Yki, through autonomous and non-autonomous mechanisms (Figure 5.10). First, cell competition prevents the activation of Yki in *scrib*<sup>-</sup> cells. Second, *scrib*<sup>-</sup> cells that are not eliminated suppress Hippo signaling in neighboring cells, leading to hyperproliferation of surrounding cells. Thus, normal cells effectively suppress the *scrib*<sup>-</sup> cells from hyperproliferating via activation of the Hippo pathway. This suppression can be circumvented when *scrib*<sup>-</sup> mutant cells hyperactivate Ras signaling or overexpress Myc. Given the highly conserved functions of Ras and Myc in mammals, acquiring mutations that prevent elimination by cell competition may be a fundamental event in the formation of tumors. Moreover, the non-cell-autonomous mechanisms of the Hippo tumor suppressor pathway that we have unveiled have important implications for tumor-stromal interactions in human cancers.

*scrib*<sup>-</sup> clones surrounded by wild-type neighbors frequently displayed increased levels of *ex-lacZ* in the dorsal hinge region of wing discs as previously reported (Grusche et al., 2010), while *ex-lacZ* was not significantly affected in other regions of eye and wing discs. The hinge region may be a less competitive



**Figure 5.10. Model of how cell competition acts as a tumor suppressor mechanism.**

**(Left)** In wild-type cells, cells have normal polarity, and Scrib limits the amount of Yki activity. **(Center)** When *scrib*<sup>-</sup> cells (red) arise in a disc, they face cell competition, which leads to their elimination. In such tissues, the normal cells outcompete *scrib*<sup>-</sup> cells in a JNK dependent manner. A non-cell-autonomous signal is sent to neighboring wild-type cells to elevate Yki activity and promote compensatory proliferation. **(Right)** *scrib*<sup>-</sup> cells surrounded by *Minute* cells do not suppress the high levels of active Yki caused by loss of Scrib. They are not eliminated and send a sustained proliferation signal to neighboring cells through the Hippo pathway.

environment than the wing pouch because this region expresses lower levels of Myc, which induces cell competition (Johnston et al., 1999; Moreno and Basler, 2004; de la Cova et al., 2004; Froidi et al., 2010). *scrib*<sup>-</sup> clones with high *ex-lacZ* levels were relatively large and we hypothesize that they did not face enough cell competition to engage the tumor-suppression mechanism. Regional differences in the wing disc's ability to remove tumorous clones has been previously reported (Froidi et al., 2010). It will be interesting to test whether artificially increasing cell competition in the hinge region by overexpressing Myc can facilitate the elimination of *scrib*<sup>-</sup> mutant cells.

A non-cell-autonomous effect on *ex-lacZ* was observed around *scrib* mutant cells rescued from elimination. These results demonstrate that tumorigenic *scrib* mutant cells can emit oncogenic signals that change the growth properties of their neighbors if they are not efficiently removed by cell competition. Thus, the role of cell competition in limiting the cell-autonomous growth capacity of *scrib* mutant cells is only a portion of its tumor-suppressing function. Interestingly, *scrib* mutant cells with depleted JNK signaling still activate Yki in neighboring wild-type tissue while a recent study demonstrates that activation of JNK signaling can suppress Yki activity non-autonomously (Sun and Irvine, 2010). Our data suggest that *scrib* mutant clones induce Yki activity non-autonomously in a JNK independent manner. Further understanding of the mechanisms that regulate the Hippo pathway non-

autonomously and identification of the oncogenic signals emitted by tumorigenic *scrib* mutant cells to cause sustained proliferation in neighboring cells will provide insight into the contribution of cellular environments to tumor formation.

In summary, we conclude that cell competition is crucial in suppressing the tumorigenic capacity of *scrib* mutant cells and does so by regulating their Yki activity. Loss of cell competition results in overproliferation of these tumorigenic cells and the production of a JNK-independent signal that suppresses Hippo pathway activity in normal cells of the affected tissue. Efficient elimination of tumorigenic *scrib* mutant cells by cell competition prevents Yki-fueled overgrowth of mutant cells and prevents them from disrupting proliferation control throughout the tissue. Thus, we have identified a novel tumor-suppression mechanism that depends on signaling between normal and tumorigenic cells. These data identify evasion of competition as a critical step toward malignancy and illustrate a role for wild-type tissue in preventing the formation of cancers.

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# **Chapter 6:**

## **Summary, Significance and Future Directions**

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## 6. 1. Conclusions

My results presented in Chapter 4 identify Crb as a new component of the Hippo pathway. We demonstrated that Crb regulates apical-basal polarity and growth by using distinct domains of Crb, and thus through different mechanisms. Both Crb gain and loss of function cause overgrowth, excess proliferation, the induction of Hippo pathway target genes, and interact genetically with mutations in known Hippo pathway components. Moreover, Crb is required for the localization of Ex to the plasma membrane and is sufficient to redistribute Ex through the JM (juxtamembrane motif) of the Crb intracellular domain. Taken together, our data place Crb upstream of Ex to regulate the activity of Yki and thereby organ growth. This is one of the first demonstrations of the regulation of Hippo by apical-basal determinants.

The data presented in Chapter 5 demonstrated that cell competition suppresses the tumorigenic capacity of *scrib* mutant cells by regulating their Yki activity. Loss of Yki regulation by cell competition results in overproliferation of the tumorigenic cells and the production of a signal that suppresses Hippo pathway activity in nearby normal cells of the affected tissue, resulting in non-autonomous as well as autonomous growth. In the normal context, cell competition efficiently eliminates *scrib* mutant cells, thus preventing a tumorigenic cascade, and disrupting proliferation caused by Yki-fueled overgrowth throughout the tissue. Thus, we have identified a novel tumor-



suppression mechanism that depends on signaling between normal and tumorigenic cells.

## **6. 2. Biological significance**

My work in Chapter 4 indicates that Crb regulates growth through a specific mechanism rather than as a secondary consequence of defects in cell polarity. Manipulation of the expression of genes involved in the regulation of apical-basal polarity often causes neoplastic tumors in *Drosophila* imaginal discs (Hariharan and Bilder, 2006). For example, overexpression of the apical determinant Crb leads to overproliferation in addition to causing defects in cell polarity and expansion of apical domain markers to the basolateral domain (Humbert et al., 2003; Humbert et al., 2008; Vaccari and Bilder, 2005). Imaginal discs that are homozygous mutant for Scrib, Dlg, or Lgl show phenotypes similar to discs overexpressing Crb. All of these situations lead to an expansion of the apical domain. It has been speculated that an expansion of the apical region can cause accumulation and/or mis-trafficking of receptors and consequently induce deregulation of many growth controlling signaling pathways (Hariharan and Bilder, 2006; Vaccari and Bilder, 2005). Contrary to this model, we report that Crb is specifically required to localize Ex to the membrane, which in turn regulates Hippo signaling. Moreover, *crb* mutant cells, which have reduced apical membrane size (Hamaratoglu et al., 2009; Izaddoost et al., 2002; Pellikka et al., 2002), overproliferate and have deregulation of

Hippo signaling. These results demonstrated that the size of the apical membrane is not the only cause that accounts for the growth defects in tissues altering Crb levels. In summary, our data support a model in which Crb plays a direct role in the regulation of growth.

Our data in Chapter 5 identify competitive cell-cell interaction as a tumor suppressor mechanism and illustrate a role for wild-type tissue in preventing the formation of cancers. This work broadens our understanding of the early steps in oncogenesis and the interaction between wild-type cells and mutant cells. My results demonstrated that the presence of cell competition regulates a growth control pathway to limit neoplastic tumor growth. Thus, I identified novel tumor prevention machinery mediated by cell-cell interaction. My data support the multiple hit theory of tumor formation (Ashley, 1969) and identify evasion of competition as a critical step toward malignancy.

### **6. 3. Remaining questions and future directions**

The strong phenotype induced by Crb overexpression cannot simply be explained by the loss of Ex. For example, ex mutants do not exhibit many extra interommatidial cells in pupal retina while Crb overexpressing tissues do (my unpublished data and Robinson et al., 2010). Identifying the binding proteins of Crb will provide insights regarding this observation. Given that the JM, a FERM domain interacting motif, is important for the growth phenotypes induced by Crb

overexpression, a FERM-domain containing protein other than Ex and Mer may be required for Crb mediated growth regulation. To investigate this, we can utilize the lethality induced by overexpressing high level of Crb as a screening phenotype. Because heterozygosity for *yki* rescued the lethality caused by overgrowth, reducing the expression level of FERM domain-containing proteins that are functionally downstream of Crb to cause overproliferation may also rescue the lethality caused by Crb overexpression. In addition to mutant alleles, UAS-RNAi lines and Exelixis deficiency lines can be used to reduce the expression level of FERM domain-containing proteins and test their ability to alleviate the overgrowth phenotypes of Crb overexpression. Upon testing 37 UAS-RNAi lines that are targeting 22 different FERM domain-containing proteins in *Drosophila* (Tepass, 2009), I discovered that coexpressing 2 UAS-RNAi lines that target Pez, a protein tyrosine phosphatase, can rescue Crb induced lethality. In addition, two Exelixis deficiency lines that have disrupted regions containing Pez can also rescue Crb induced lethality. Given that the center of the Hippo pathway is a kinase cascade, it is likely that a phosphatase plays a critical role to inactivate the pathway. Further characterization of *Pez* mutant phenotypes will be required to define the mechanisms by which it influences Crb signaling and whether or not it is a Hippo pathway member.

Alternatively, a genetic screen for dominant modifiers of Crb overexpression would be useful and complementary, especially to identify

negative regulators of the pathway. Mutations in negative regulators of the Hippo pathway are likely to result in reduced organ size, but this phenotype could also be caused by mutations in any genes that are required for cell viability and unrelated to growth control. Since the common phenotypes of those negative regulators are shared by many other genes, it would make them difficult to be discovered by phenotype driven screens. Presumably, this is one of the main reasons why more positive regulators in the Hippo pathway have been identified than negative ones and most of the known negative regulators of Hippo signaling have been found by chance or by biochemical approaches. Therefore, a genome-wide dominant modifier screen of Crb overexpression provides an efficient strategy for the identification of novel negative regulatory inputs into the Hippo pathway.

In Chapter 5, we have shown that cell competition is able to act as a tumor suppressor mechanism and functions as a quality control process to remove abnormal cells. This suggests the following questions:

- What is the cell competition signal?
- How is the cell competition signal initiated in response to the *scrib* mutant cells?
- How does the cell competition signal function to eliminate the tumorigenic *scrib* mutant cells?

An active surveillance process may exist in normal tissues to recognize and to remove any dysfunctional cells. Alternatively, a signal could be generated in abnormal cells, such as *scrib* mutant cells, and trigger cell competition. To investigate how the cell competition signal is generated can be challenging, because this competitive cell-cell interaction is an action mutually dependent on two cell populations. The fact that cell competition alters cell growth ability makes it difficult to distinguish between causes and consequences of cell competition. It would be useful to have a cell competition marker or read-out to monitor Lose/Win status and allow for further analysis of different genetic manipulations or conditions.

Recently, Moreno's group has shown that upon cell competition induced by Myc overexpression, the expression levels of *flower* and *sparc* are elevated transcriptionally and post-transcriptionally in loser wild-type cells (Portela et al., 2010; Rhiner et al., 2010). Flower is a transmembrane protein that mediates the Lose/Win decision during cell competition (Rhiner et al., 2010; Yao et al., 2009), while Sparc is a secreted glycoprotein that protects losers from being eliminated (Portela et al., 2010). It has been proposed that Sparc is induced in loser cells generated in different competitive cell-cell interactions and thus can be a marker for cell competition (Portela et al., 2010). However, it is not known whether Sparc is also upregulated in *scrib* mutant clones. Investigating whether the induction of *flower* and *sparc* are associated and/or required for *scrib*

dependent cell competition will shed light on the cell competition mechanism and provide valuable information to determine their potential as reliable markers of cell competition for further studies.

Engulfment has also been shown to play a role in cell competition. Cells with mutations in engulfment genes fail to eliminate *Minute* cells. To test whether the elimination of *scrib* mutant cells relies on the engulfment mechanism, we can test whether *scrib* mutant cells are engulfed by neighboring cells and whether diminished engulfment ability of neighboring cells can prevent *scrib* mutant cells from elimination. Moreover, it will be interesting to investigate whether cell competition uses the same mechanism to remove different types of abnormal cells and whether the growth potential of *scrib* mutant cells and the deregulation of Hippo signaling in those cells also depends on engulfment.

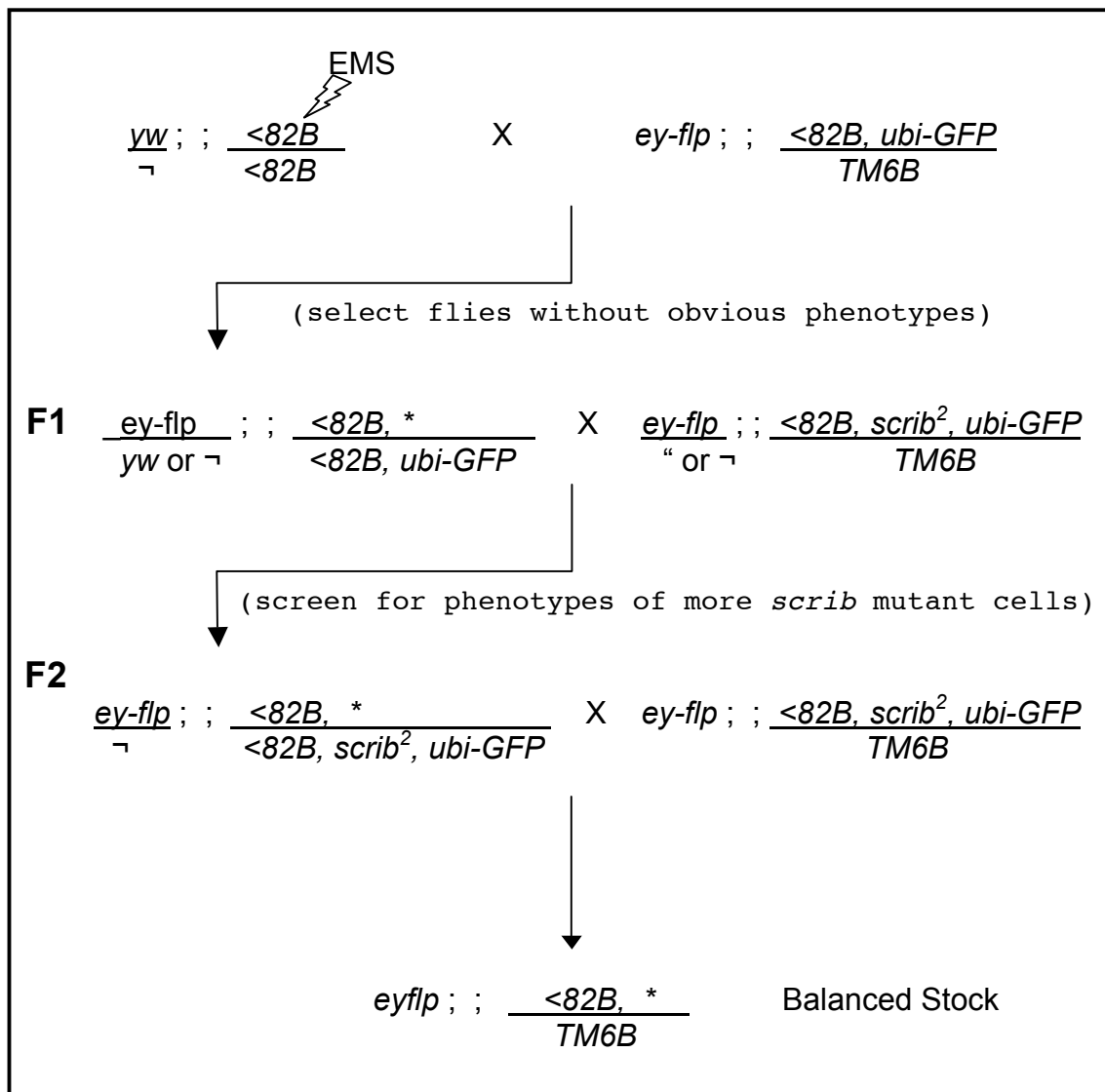
Another biological process that is altered in *scrib* mutant cells surrounded by wild-type cells is endocytosis. It has been shown that *scrib* mutant cells surrounded by normal cells have enhanced endocytosis while *scrib* mutant cells in a homotypic situation may have endocytic activity that is lower or similar to wild-type cells (Igaki et al., 2009). The enhanced endocytosis defect in loser *scrib* mutant cells leads to the accumulation of JNK ligand, Egr, in endosomes (Igaki et al., 2009). Blocking endocytosis by overexpressing the dominant negative form of Rab5 (Rab5<sup>DN</sup>) results in phenotypes resembling

those of blocking JNK in *scrib* mutant cells (Igaki et al., 2009). Interestingly, promoting endocytosis by overexpressing full length Rab5 is able to rescue the loser phenotype of *tkv* mutant cells (Moreno et al., 2002). Also, mutations that cause defects in endocytosis are known to induce non-autonomous proliferation in neighboring tissue and have been implicated in growth regulation (Herz et al., 2006; Lu and Bilder, 2005; Moberg et al., 2005; Thompson et al., 2005; Vaccari and Bilder, 2005; Wucherpfennig et al., 2003). It will be interesting to explore the role of endocytic trafficking in cell competition and the regulation of the Hippo pathway. Specifically, it will be interesting to test whether the endocytosis defects in *scrib* mutant cells are responsible for the non-autonomous induction of Hippo target gene expression. For example, increasing or decreasing endocytosis by overexpression or knock down of endocytic genes in *scrib* mutant cells could be used to test whether manipulation of endocytic trafficking can prevent the non-cell-autonomous effects on Hippo target gene expression.

A more detailed study of how cell competition is initiated and executed to ensure proper growth regulation and eliminate tumorigenic cells will doubtless be of great importance. A molecular understanding of the mechanism by which tumorigenic cells are eliminated may require the identification of genes that provide a critical tumor suppressing function without displaying a phenotype as a single mutant. One possible avenue of research would be to screen for mutations that are required for the elimination of *scrib* mutant cells, but do not

affect the viability of normal cells. The amenability and availability of sophisticated clonal analysis tools in *Drosophila* would enable elegant and direct methods to screen for these genes. The crossing scheme of a potential EMS screen for the identification of these genes is shown in Figure 6.1. This screen will allow us to identify genes that fail to eliminate *scrib* mutant cells without causing cell death in neighboring cells. These genes will be likely targets for mutation during the early stages of oncogenesis and our work provides a window into identifying and understanding a new class of tumor suppressors. The identification and characterization of such genes would further our knowledge of tumor formation mechanisms and present additional opportunity to advance our study of cancer prevention.





**Figure 6.1.**

**Figure 6.1 Crossing scheme to identify genes potentially required for initiating or executing cell competition**

Male flies bearing *FRT82B* are mutagenized with EMS and crossed to females containing *ey-flp*, *FRT82B*, and *ubi-GFP*. The F1 progeny without any obvious abnormal phenotypes are selected and crossed to flies containing *ey-flp*, *FRT82B*, *ubi-GFP* and *scrib*<sup>2</sup>. In the F2 generation, progeny are screened for phenotypes that may represent the failure of elimination of *scrib* mutant cells. The selected flies are backcrossed to confirm the phenotypes and establish stocks. Mutations that cause no obvious phenotypes in F1 indicate they are not required for cell viability and important developmental processes, so therefore the next generation (F2) animals that contain a mutation exhibit phenotypes that are dependent on the presence of *scrib*<sup>2</sup> mutant.

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## Appendix

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### ***Drosophila* Genotypes**

#### **Figure 4.1.**

A: *y w*

B: *C765-Gal4/ UAS-Crb<sup>intra</sup>*

D: *dpp-Gal4, UAS-GFP/ +*

E: *UAS-Crb<sup>FL</sup>/ + ; dpp-Gal4, UAS-GFP/ +*

F: *ex<sup>697</sup>/ + ; dpp-Gal4, UAS-GFP/ +*

G: *UAS-Crb<sup>FL</sup>/ ex<sup>697</sup> ; dpp-Gal4, UAS-GFP/ +*

#### **Figure 4.2.**

A: *y w, ey-Flp/ +; FRT82B, Minute(3), ubi-GFP/ FRT82B*

B: *y w, ey-Flp/ +; FRT82B, Minute(3), ubi-GFP/ FRT82B, crb<sup>11A22</sup>*

C: *y w, ubx-Flp/ +; FRT82B, Minute(3), ubi-GFP/ FRT82B*

D: *y w, ubx-Flp/ +; FRT82B, Minute(3), ubi-GFP/ FRT82B, crb<sup>11A22</sup>*

E: *y w, hs-Flp; FRT82B, ubi-GFP/ FRT82B, crb<sup>11A22</sup>*

F: *y w, hs-Flp; ex<sup>697</sup>/ +; FRT82B, Minute(3), ubi-GFP/ FRT82B, crb<sup>11A22</sup>*

**Figure 4.3.**

A: *y w, hs-Flp; ey-Gal4, GMR-Gal4/ +; FRT82B, ubi-GFP/ FRT82B, crb<sup>11A22</sup>*

B: *y w, hs-Flp; ey-Gal4, GMR-Gal4/ UAS-mer<sup>RNAi</sup>; FRT82B, ubi-GFP/ FRT82B, crb<sup>11A22</sup>*

C: *w*

D: *nub-Gal4/ UAS-Crb<sup>RNAi</sup>*

E: *nub-Gal4/ UAS-D*

F: *nub-Gal4/ UAS-D, UAS-Crb<sup>RNAi</sup>*

G: *nub-Gal4/ UAS-Crb<sup>intra</sup>*

H: *yki<sup>B5</sup>/ +; nub-Gal4/ UAS-Crb<sup>intra</sup>*

I: *UAS-Crb<sup>intra</sup>/ ex<sup>697</sup>; hh-Gal4, UAS-GFP/ +*

J: *ex<sup>697</sup>/ +; hh-Gal4, UAS-GFP/ UAS-yki<sup>RNAi</sup>*

K: *UAS-Crb<sup>intra</sup>/ ex<sup>697</sup>; hh-Gal4, UAS-GFP/ UAS-yki<sup>RNAi</sup>*

**Figure 4.5.**

A: *UAS-Crb<sup>intra</sup>/ ex<sup>697</sup>; dpp-Gal4, UAS-GFP/ +*

B: *UAS-Crb<sup>intraDJM</sup>/ ex<sup>697</sup>; dpp-Gal4, UAS-GFP/ +*

C: *UAS-Crb<sup>intraDPBM</sup>/ ex<sup>697</sup>; dpp-Gal4, UAS-GFP/ +*

D: *UAS-Crb<sup>intraDJ,DP</sup>/ ex<sup>697</sup>; dpp-Gal4, UAS-GFP/ +*

**Figure 4.6.**

- A: *diap1-GFP/+ ; dpp-Gal4/+*
- B: *diap1-GFP/ UAS-Crb<sup>FL</sup> ; dpp-Gal4/+*
- C: *diap1-GFP/ UAS-Crb<sup>intra</sup>; dpp-Gal4/+*
- D: *diap1-GFP/ UAS-Crb<sup>intraDJM</sup>; dpp-Gal4/+*
- E: *diap1-GFP/ Crb<sup>intraDPBM</sup>; dpp-Gal4/+*
- F: *diap1-GFP/ UAS-Crb<sup>intraDJ,DP</sup>; dpp-Gal4/+*

**Figure 4.7.**

- A: *en-Gal4/+ ; Gal80<sup>ts</sup>/+*
- B: *en-Gal4/ UAS-Crb<sup>FL</sup> ; Gal80<sup>ts</sup>/+*
- C: *en-Gal4/ UAS-Crb<sup>intra</sup>; Gal80<sup>ts</sup>/+*
- D: *en-Gal4/ UAS-Crb<sup>intraDJM</sup>; Gal80<sup>ts</sup>/+*
- E: *en-Gal4/ Crb<sup>intraDPBM</sup>; Gal80<sup>ts</sup>/+*
- F: *en-Gal4/ UAS-Crb<sup>intraDJ,DP</sup>; Gal80<sup>ts</sup>/+*

**Figure 4.8.**

- A: *y w, hs-Flp/+; FRT40A, ubi-GFP/ FRT40A, fat<sup>422</sup>*
- B: *y w, hs-Flp/+; FRT40A, ubi-GFP/ FRT40A, ex<sup>e1</sup>*
- C: *FRT19A, ubi-GFP/ FRT19A, mer<sup>4</sup>; ; hs-Flp/+*

**Figure 4.9.**

- A,B: *y w, hs-Flp; ; FRT82B, ubi-GFP/ FRT82B, crb<sup>11A22</sup>*
- C: *y w, hs-Flp; ; FRT82B, , Minute(3), ubi-GFP/ FRT82B, crb<sup>11A22</sup>*

D: *y w, hs-Flp/ +; FRT40A, ubi-GFP/ FRT40A, ex<sup>e1</sup>*  
 E,F: *y w, hs-Flp; ; FRT82B, ubi-GFP/ FRT82B, crb<sup>11A22</sup>*  
 G,I: *Gal80<sup>ts</sup>/ UAS-Crb<sup>intra</sup>, hh-Gal4/ +*  
 H,J: *Gal80<sup>ts</sup>/ UAS-Crb<sup>intraDJM</sup>, hh-Gal4/ +*

**Figure 4.10.**

A,B: *y w, hs-Flp; ; FRT82B, ubi-GFP/ FRT82B, crb<sup>11A22</sup>*  
 C: *y w, hs-Flp; d<sup>GC13</sup>/ d<sup>GC13</sup> ; FRT82B, ubi-GFP/ FRT82B, crb<sup>11A22</sup>*  
 D: *y w, hs-Flp; nub-Gal4/ UAS-D; FRT82B, ubi-GFP/ FRT82B, crb<sup>11A22</sup>*

**Figure 5.1.**

A: *y w, ey-Flp/ +; act>y+>GAL4, UAS-GFP/ +; FRT82B, tub-GAL80/ FRT82B*  
 B: *y w, ey-Flp/ +; act>y+>GAL4, UAS-GFP / +; FRT82B, tub-GAL80/ FRT82B, scrib<sup>2</sup>*  
 C: *y w, ey-Flp/ w, UAS-bsk<sup>DN</sup>; act>y+>GAL4, UAS-GFP/ +; FRT82B, tub-GAL80/ FRT82B, scrib<sup>2</sup>*  
 D: *y w, ey-Flp/ +; act>y+>GAL4, UAS-GFP/ UAS-p35; FRT82B, tub-GAL80/ FRT82B, scrib<sup>2</sup>*  
 E: *y w, hs-Flp; FRT82B, ubi-GFP/ FRT82B*  
 F: *y w, hs-Flp; FRT82B, ubi-GFP/ FRT82B, scrib<sup>2</sup>*  
 G: *y w, hs-Flp; egr<sup>1</sup>/ egr<sup>1</sup>, ex<sup>697</sup>; FRT82B, ubi-GFP/ FRT82B, scrib<sup>2</sup>*

H: *y w, hs-Flp; FRT82B, Minute(3), ubi-GFP/ FRT82B, scrib<sup>2</sup>*

I: *y w, hs-Flp; egr<sup>1</sup>/ egr<sup>1</sup>, ex<sup>697</sup>; FRT82B, ubi-GFP/ FRT82B*

J: *y w, hs-Flp; FRT82B, Minute(3), ubi-GFP/ FRT82B*

**Figure 5.2.**

A: *y w, hs-Flp; FRT82B, ubi-GFP/ FRT82B*

B: *y w, hs-Flp; FRT82B, ubi-GFP/ FRT82B, scrib<sup>2</sup>*

C: *y w, hs-Flp; egr<sup>1</sup>/ egr<sup>1</sup>, ex<sup>697</sup>; FRT82B, ubi-GFP/ FRT82B, scrib<sup>2</sup>*

D: *y w, hs-Flp; FRT82B, Minute(3), ubi-GFP/ FRT82B, scrib<sup>2</sup>*

**Figure 5.3.**

A,B: *y w, hs-Flp; egr<sup>1</sup>/ egr<sup>1</sup>, ex<sup>697</sup>; FRT82B, ubi-GFP/ FRT82B, scrib<sup>2</sup>*

**Figure 5.4.**

A: *y w, hs-Flp; ex<sup>697</sup>/ +; FRT82B, ubi-GFP/ FRT82B*

B,C: *y w, hs-Flp; egr<sup>1</sup>/ egr<sup>1</sup>, ex<sup>697</sup>; FRT82B, ubi-GFP/ FRT82B, scrib<sup>2</sup>*

D: *y w, hs-Flp; egr<sup>1</sup>/ egr<sup>1</sup>, ex<sup>697</sup>; FRT82B, ubi-GFP/ FRT82B*

E,F: *y w, hs-Flp; ex<sup>697</sup>/ +; FRT82B, ubi-GFP/ FRT82B, scrib<sup>2</sup>*

G: *y w, hs-Flp; ex<sup>697</sup>/ +; FRT82B, Minute(3), ubi-GFP/ FRT82B, scrib<sup>2</sup>*

H: *y w, hs-Flp; ex<sup>697</sup>/ +; FRT82B, Minute(3), ubi-GFP/ FRT82B*

**Figure 5.5.**

A: *y w, ey-Flp/ w, UAS-bsk<sup>DN</sup>; act>y+>GAL4, UAS-GFP/ +; FRT82B, tub-GAL80/ FRT82B, scrib<sup>2</sup>*

B: *y w, ey-Flp/ w, UAS-bsk<sup>DN</sup>; act>y+>GAL4, UAS-GFP/ UAS-wts; FRT82B, tub-GAL80/ FRT82B, scrib<sup>2</sup>*

C: *y w, ey-Flp/ +; act>y+>GAL4, UAS-GFP/ UAS-Yki; FRT82B, tub-GAL80/ FRT82B, scrib<sup>2</sup>*

D: *y w, ey-Flp/ +; act>y+>GAL4, UAS-GFP/ +; FRT82B, tub-GAL80/ FRT82B, scrib<sup>2</sup>, wts<sup>x1</sup>*

**Figure 5.6.**

A-D: *y w, hs-Flp, tub-GAL4, UAS-GFP/ +; ex<sup>697</sup>/ UAS-Ras<sup>V12</sup>; FRT82B, tub-GAL80/ FRT82B, scrib<sup>2</sup>*

E,F: *y w, hs-Flp; ex<sup>697</sup>/ +; FRT82B, Minute(3), ubi-GFP/ FRT82B, scrib<sup>2</sup>*

G-J: *y w, hs-Flp, tub-GAL4, UAS-GFP/ w, UAS-bsk<sup>DN</sup>; ex<sup>697</sup>/ +; FRT82B, tub-GAL80/ FRT82B, scrib<sup>2</sup>*

**Figure 5.7.**

A: *y w, hs-Flp, tub-GAL4, UAS-GFP/ +; ex<sup>697</sup>/ UAS-Ras<sup>V12</sup>; FRT82B, tub-GAL80/ FRT82B*

B: *y w, hs-Flp, tub-GAL4, UAS-GFP/ w, UAS-bsk<sup>DN</sup>; ex<sup>697</sup>/ +; FRT82B, tub-GAL80/ FRT82B*



**Figure 5.8.**

A,C: *y w, hs-Flp, tub-GAL4, UAS-GFP/+; ex<sup>697</sup>/+; FRT82B, tub-GAL80/ UAS-Myc, FRT82B, scrib<sup>2</sup>*

B,D: *y w, hs-Flp, tub-GAL4, UAS-GFP/+; ex<sup>697</sup>/+; FRT82B, tub-GAL80/ UAS-Myc, FRT82B*

**Figure 5.9.**

A: *y w, hs-Flp; ex<sup>697</sup>, en-Gal4/+; FRT82B, ubi-GFP/ UAS-Myc, FRT82B, scrib<sup>2</sup>*

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## **Vita**

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