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ADHERENS JUNCTIONS AND THE ACTOMYOSIN NETWORK REGULATE ORGAN GROWTH BY MODULATING HIPPO PATHWAY ACTIVITY IN DROSOPHILA

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Adherens junctions and the actomyosin network regulate organ growth by modulating Hippo pathway activity in *Drosophila*

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Adherens junctions (AJs) and basolateral modules are important for the establishment and maintenance of apico-basal polarity. Loss of AJs and basolateral module members lead to tumor formation, as well as poor prognosis for metastasis. Recently, in mammalian studies it has been shown that loss of either AJ or basolateral module members deregulate Yorkie activity, the downstream transcriptional effector of the Hippo pathway. Importantly, it is unclear if AJ and basolateral components act through the same or parallel mechanisms to regulate Yorkie activity.

Here, we dissect how loss of AJ and basolateral components affects Hippo signaling in *Drosophila*. Surprisingly, while *scrib* knock-down tissue displays increased reporter activity autonomously, *α-cat* knock-down tissue shows a cell autonomous decrease and a cell non-autonomous increase of Hippo reporter activity. We provided several lines of evidence to show the differential regulation in polarity protein localizations and oncogenic cooperative overgrowth by AJs and basolateral complexes. Finally, we show that Hippo pathway activity is induced in *α-cat* and *scrib* double knocked-down tissue. Taken

together, our results provide evidence to show that basolateral modules and AJs act in parallel to modulate Hippo pathway activity.

Non-muscle myosin II is an actomyosin component that interacts with the actin. Non-muscle myosin II also interacts with lgl, though the function of this interaction is not clear. Our lab demonstrated that modulating F-actin regulates Hippo pathway activity, and *lgl* also has been described as a Hippo pathway regulator. Therefore we suspect that myosin II is also involved in Hippo pathway regulation.

We first characterized non-muscle Myosin II as a novel tumor suppressor gene by affecting Hippo pathway activity. Upstream regulators of Myosin II, members in the Rho signaling pathway, also displayed similar phenotypes as the Myosin II knock-down tissues. Apoptosis is also induced in myosin II knock-down tissues, however, blocking cell death does not affect myosin II knock-down induced Hippo activation. Our data suggested hyperactivating myosin II induced F-actin accumulation so therefore induces Hippo target activation. Unexpectedly, we also observed that reducing F-actin activity induced Hippo target activation in vivo. These controversial data indicated that actomyosin may regulate the Hippo pathway through multiple mechanisms.

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Introduction

1.1 Growth control

All multi-cellular organisms arise from a single cell embryo and develop to achieve their final size with multiple types of cells and tissues. Although largely varied, animals mostly grow to certain sizes and then stop growth. For instance, the differences in size between different species such as a fruit fly and an elephant are quite dramatic, yet variations within species are much smaller. To achieve similar sizes, tissues must be able to sense their global proportions and coordinate their growth. If we ablated cells in early developmental stages, a tissue can sense the loss of cell numbers and induce extra proliferation to compensate for the missing cells. How cells know they reach the limit and stop growth is still a mystery. Many efforts have been made to understand how tissue size is regulated, and several models are proposed to facilitate understanding of mechanisms for growth control.

One of the theories to describe tissue growth is through the action of gradients of signaling molecules (Dekanty and Milan, 2011). Many signaling pathways receive extracellular inputs, and usually these extracellular cues will bind to ligands for the signal and transduce input into cells. Some molecules are secreted from cells and diffuse into the extracellular space and are therefore capable of long distance traveling and achieving gradient distribution. Others might present at the membrane as a ligand and interact with extrinsic signals. Cells can detect and measure the amount of these molecules between neighboring cells and change their growth profiles. Also, the receptors may be

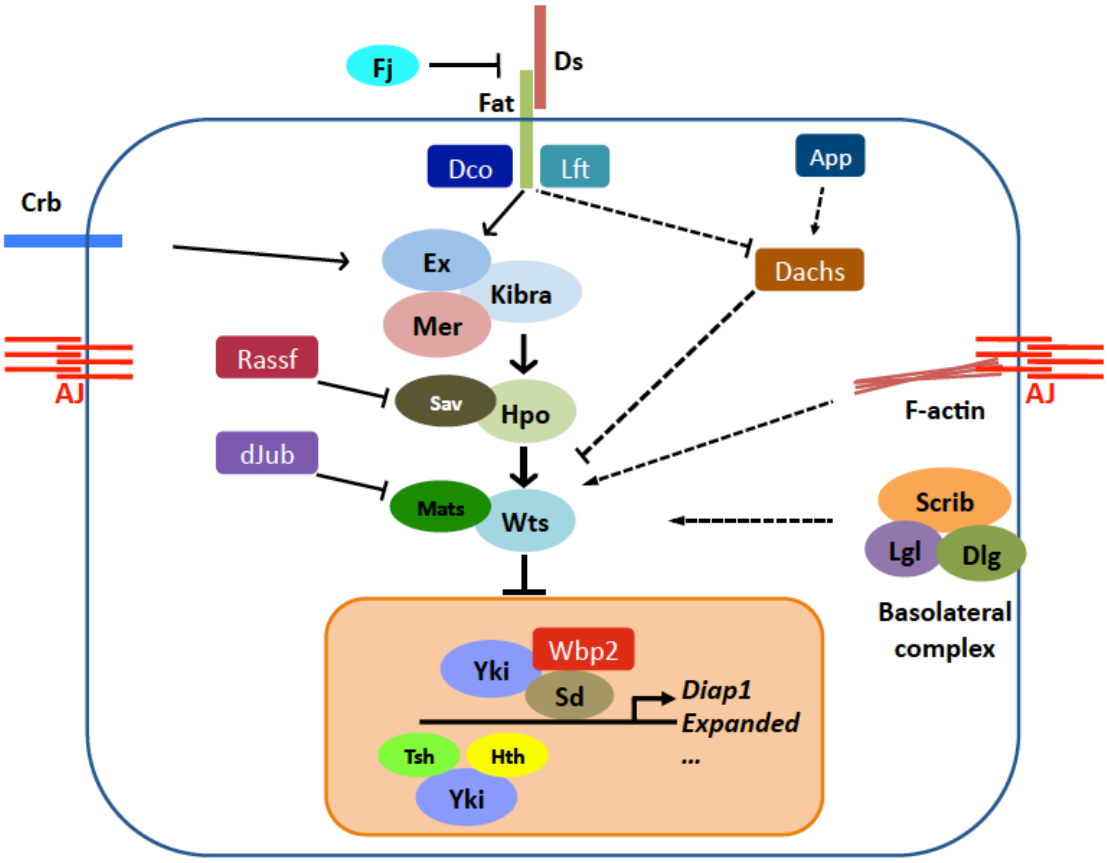
distributed in complementary gradients. Two famous examples are Decapendaplegic (Dpp) with its inhibitor Brinker (*brk*) (Campbell and Tomlinson, 1999; Jazwinska et al., 1999; Minami et al., 1999), and Dachsous (Ds) and Four-jointed (Fj) in *Drosophila* (Halder and Johnson, 2011). In *Drosophila* developing wing tissues, Dpp is expressed in the middle line of the wing and as a gradient to both anterior and posterior parts of the tissue. The presence of Dpp suppresses the expression of Brinker, a negative transcription factor for the Dpp targets (Campbell and Tomlinson, 1999; Jazwinska et al., 1999; Minami et al., 1999). Proto-cadherin Ds also forms a gradient distribution and the kinase Fj is expressed in a reverse pattern to inhibit function of Ds in tissue growth (Brittle et al., 2010). Changes in the steepness of these gradients induced extra cell proliferation in order to form an evenly distributed gradient, suggesting an important role for gradient establishment of morphogens (Willecke et al., 2008).

Another model for growth control is through changes in mechanical force (Kumar and Weaver, 2009). Mechanical forces are a physical stimulus that can cause objects to change their shapes or otherwise respond to the effect of the force. Cells proliferate and grow to a certain stage where they sense the physical compression of the surrounding environment and send signals to inhibit cell cycle activity. This mechanism is mainly achieved by cell-cell interactions or interactions between cells and their surrounding environments, and we will address more details of this aspect throughout the introduction.

1.2 The *Drosophila* Hippo pathway

Our lab has long been interested in finding new factors for growth control. A few years ago, we identified a novel serine/threonine kinase in a genetic screen, and the mutant of this gene showed dramatic overgrowth phenotype. The mutant tissue of this kinase showed a dramatic overgrowth, increased cell proliferation, as well as inhibition of apoptosis (Pantalacci et al., 2003; Udan et al., 2003; Wu et al., 2003). Because of the oversized phenotype that imitates the features of real animals, we named it *hippo*. The Hippo pathway was first characterized as an essential regulator for growth in *Drosophila* (Halder and Johnson, 2011). Since the first component was reported, the Hippo pathway has been studied intensively in both fly and mammalian models, and numerous members of the pathway have been identified. The core of the Hippo pathway resides in a kinase cascade, which consists of the Hippo (Hpo) and Warts (Wts) kinases (Halder and Johnson, 2011). Hpo binds to its cofactor Salvador to phosphorylate Wts. Wts then acts with Mob as tumor suppressor (Mats) to phosphorylate the transcription coactivator Yorkie (Yki) (He et al., 2005; Lai et al., 2005). Rassf modulates the activity of Hpo kinase and a LIM domain protein dJub functions as a negative regulator for Wts activity (Das Thakur et al., 2010; Polesello and Tapon, 2007). 14-3-3 is a protein family that binds to various signaling molecules and modulates different signaling events (Oh and Irvine, 2008). Phosphorylated Yki interacts with both14-3-3ε and 14-3-3ζ proteins in the cytoplasm, thus separating it from the nucleus and keeping it inactive (Zhao et al., 2007). Non-phosphorylated Yki enters the nucleus and binds to transcription

factors, including Scalloped (Sd), Homothorax (Hth), or Teashirt (Tsh), to control downstream gene expression (Peng et al., 2009; Wu et al., 2008; Zhang et al., 2008b; Zhao et al., 2008b). Yki-Sd binding induces growth-related target gene expression in most tissues, whereas Yki binding to Hth and Tsh only turns on microRNA *bantam* expression in uncommitted eye progenitor cells (Peng et al., 2009). WW domain binding protein 2 (Wbp2) interacts with Yki through the protein-protein interaction WW domain to promote growth (Zhang et al., 2011). Upstream of Hpo kinase, Kibra and two FERM (*4.1 proteins, ezrin, radixin and moesin*) domain containing proteins -Expanded (Ex) and Merlin (Mer) - receive upstream signals to regulate Hpo pathway activity (Baumgartner et al., 2011; Buther et al., 2004; Genevet et al., 2011; Hamaratoglu et al., 2006; Xiao et al., 2011a). In *Drosophila*, several groups reported different upstream inputs that modulate the Hippo pathway, and many of them are affecting the atypical cadherin Fat (Ft) (Cho et al., 2006; Rogulja et al., 2008; Tyler and Baker, 2007; Willecke et al., 2006; Willecke et al., 2008). Ft works through the atypical myosin Dachs and Ex to transduce signals to regulate the Hpo kinase cascade (Rogulja et al., 2008; Willecke et al., 2006). The extracellular domain of Fat interacts with its ligand protocadherin Dachsous (Ds), and therefore signals into the cells to regulate growth (Willecke et al., 2008). The binding of Ft and Ds promote Casein Kinase 1ε Disc overgrown (Dco) to phosphorylate the intracellular domain of Ft (Sopko et al., 2009). Lowfat (lft) and a palmitoyl-transferase Approximated (App) are two components that interact with Ft and help its localization on the membrane at the subapical region (Mao et al., 2009; Matakatsu and Blair, 2008).


Fj
Ds
Fat
Dco
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App
Crb
Ex
Kibra
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Dachs
Rassf
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Lgl
Dlg
Basolateral
complex
Wbp2
Yki
Sd
Diap1
Expanded
...
Tsh
Hth
Yki

Fig. 1.1

Figure 1.1 Hippo pathway components: a schematic view

The atypical cadherin Fat transduces a signal to activate Hippo signaling. Two FERM-domain-containing proteins Ex and Mer form a complex with Kibra to activate Hpo kinase. Hpo works together with Sav and Rassf, to phosphorylate and activate Wts kinase. Wts, together with Mats, then phosphorylates and inhibits the transcriptional coactivator Yki. Unphosphorylated Yki tranlocates into the nucleus and forms complexes with the transcription factor Sd, Hth orTsh to express downstream target genes. Wbp2 also physically interacts Yki to promote its activity. Other molecules that have been reported to regulate the Hippo pathway include dJub, Fj, Ds, Lft, Dco App, and Dachs.

Four-jointed (Fj) phosphorylates Ft in the Golgi thereby modulating the interaction between Ft and Ds (Brittle et al., 2010; Willecke et al., 2008). While Ft signaling through Ex has been characterized by many groups, the upstream inputs to the Mer branch still remain an open question. Meanwhile, several groups indentified apico-basal polarity components and F-actin modulators as regulators for Hippo pathway activity, which will be addressed later in this study (Sansores-Garcia et al., 2011).

Downstream targets of the Hippo pathway are mainly involved in cell proliferation and cell survival. These genes include *cyclinE* (*cycE*), *diap1* (*Drosophila inhibitor of apoptosis protein-1*), *bantam* microRNA and *myc* (Neto-Silva et al., 2010; Stocker, 2011; Ziosi et al., 2010). CycE binds to Cdk2 and regulates the G1 to S phase transition during the cell cycle (Moroy and Geisen, 2004). Diap1 is an anti-apoptotic gene, and overexpression of *diap1* protects cells from death by inhibiting downstream activation of caspases (Steller, 2008). *bantam* is a microRNA that plays an important role in tissue growth and cell-survival (Nolo et al., 2006; Thompson and Cohen, 2006). Myc is a growth promoting transcription factor, and has been suggested to have an interdependent relationship with Yki in regulating growth (de la Cova et al., 2004; Neto-Silva et al., 2010). Other than these growth dependent regulators, several upstream components are also transcription targets of the Hippo pathway. *ex*, *mer*, *kibra* and *bantam* miRNA are transcriptionally increased in tissues lacking Hippo pathway activity (Baumgartner et al., 2011; Genevet et al., 2011;

Hamaratoglu et al., 2006; Yu et al., 2011), therefore providing a negative feedback loop to modulate pathway activity.

1.3 The Mammalian Hippo pathway

Most of the *Drosophila* Hippo pathway components have orthologs in mammals, and many studies also suggest these mammalian counterparts share the same regulatory mechanisms (Bao et al., 2011; Halder and Johnson, 2011; Zhao et al., 2008a). Many of their mammalian counterparts have several orthologs, and the redundancy of pathway components allows more complex regulation for the pathway activity. The core kinase cascade starts from hippo homologs Mst1 and 2 (mammalian Ste20 like kinase) to phosphorylate Wts homologs Lats1 and 2 (Large tumor suppressor) (Chan et al., 2005; Dong et al., 2007; Hao et al., 2008), and Lats1/2 then phosphorylates the transcription cofactors Yap and Taz (Yki homolog) (Halder and Johnson, 2011; Hao et al., 2008; Zhang et al., 2008a). Sav and Mobs play a similar role as their *Drosophila* counterparts (Sav and Mats) as adaptor proteins (Bichsel et al., 2004; Chow et al., 2010; Dong et al., 2007; Hirabayashi et al., 2008). Tead1-4 are transcription factors for the mammalian Hippo pathway that share homology with Sd (Wu et al., 2008; Zhang et al., 2008b). Mammalian Kibra and Mer also work upstream of Mst1/2 (Xiao et al., 2011b; Yu et al., 2011), but the Ex homolog appears to have lost the essential regulatory region needed to influence the Hippo pathway. Instead, the Angiomotin (AMOT) family took over the regulation ability of Ex. AMOT is not conserved in *Drosophila*, but studies showed that AMOT binds to

polarity protein PatJ and Yap (Zhao et al., 2011). Loss of AMOT leads to a reduction of tight junction formation and increased Yap/Taz nuclear localization, and as a result induces cell overproliferation (Chan et al., 2011). Another family member AMOTL2 interacts with Lats2 and increases its enzymatic efficiency (Paramasivam et al., 2011; Wang et al., 2011; Zhao et al., 2011), suggesting the AMOT family modulates the Hippo pathway activity through multiple mechanisms.

The entire core Hippo pathway components have been implicated as important regulators in tumorigenesis. Mst1/2 and Sav restrict liver growth in postnatal animals through regulating Yap phosphorylation (Lu et al.; Zhao et al., 2007). Lats 1/2 phosphorylates Yap at multiple sites with HXRXXS motifs, and have been implicated as tumor suppressor genes (Zhao et al., 2007). Hypermethylation of the Lats1/2 promoter results in inhibition of lats expression and facilitates the formation of sarcoma, astrocytoma, and breast cancer (Jiang et al., 2006). Yap and Taz are characterized as oncogenes since they have both been found to be overexpressed in several types of tumors (Avruch et al., 2012; Konsavage et al., 2012; Li et al., 2012a). All these studies provided the evidence that the Hippo pathway is conserved in mammalian systems and plays a pivotal role in growth regulation.

The upstream regulators of the mammalian Hippo pathway are still poorly understood. The merlin homolog NF2 (Neurofibromatosis 2) is reported as a

tumor suppressor since it is named after an inherited disease that causes non-malignant brain tumors (NF2) (Striedinger et al., 2008; Yi and Kissil, 2010; Zhang et al., 2010). NF2 mutant cells also display loss of contact inhibition, and re-expressing of NF2 in glioma cells activates MST1/2 and Lats1/2 and is followed by inactivation of Yap through phosphorylation (Morrison et al., 2001; Striedinger et al., 2008). Nevertheless, the other upstream components including Ft and Ex have not been shown to have a direct link to regulate the Hpo pathway. hEx lost the C-terminal domain that plays an important function in *Drosophila*. Although Fat4 showed the highest similarity with *Drosophila* Ft, recent mouse model research showed that a single mutant of both *fat4* and *dachs1* or double mutants combining both *fat4* and *dachs1* do not exhibit any growth phenotype (Mao et al., 2011). Rather, they only exhibited defects in planar cell polarity, which is another characteristic phenotype of Ft (Mao et al., 2011). Interestingly, another cell culture based study suggested Fat4 as a tumor suppressor gene in breast cancers (Qi et al., 2009). Knocking-down of *fat4* by shRNA increases YAP activity, and injection of *fat4* shRNA expressing cells into mammary glands induces tumorigenesis (Qi et al., 2009). These data implied the possibility of multiple inputs in pathway activity regulation.

1.4 Function of apico-basal polarity in cell homeostasis

The epithelium is one of the basic tissue types in animals. Epithelial cells line the body cavity and serve as the surfaces of organs throughout the whole animal. The epithelial cells form a barrier to separate the outside environment

and basement membranes. To interact within distinct contexts, epithelial cells developed highly delineated apico-basal polarity. Not surprisingly, apico-basal polarity must be tightly controlled for proper development. Numerous studies have shown loss of polarity causes severe epithelial defects, including changes in cell morphology and attachment (Dow and Humbert, 2007; Humbert et al., 2003). More importantly, defects in apico-basal polarity are tightly associated with cancer progression, including epithelial mesenchymal transition (EMT), tumor formation, and metastasis (Dow and Humbert, 2007; Humbert et al., 2003; Wodarz and Nathke, 2007).

1.4.1 Main components of the apico-basal polarity complexes

Multiple protein complexes define the apico-basal polarity of a cell. These include two apical protein complexes, the Crumbs (Crb) and the atypical protein kinase C (aPKC) complexes, as well as a basolateral module and adherens junctions (AJs) (Dow and Humbert, 2007; Genevet and Tapon, 2011; Humbert et al., 2003; Wodarz and Nathke, 2007). The Crb complex localizes to the apical domain of epithelial cells and is composed of Crb and the adaptor proteins Stardust and PatJ (Genevet and Tapon, 2011; Martin-Belmonte and Perez-Moreno, 2012). Another apical complex consists of aPKC, and the PDZ domain containing proteins Par6 and Bazooka (Humbert et al., 2006). Both apical complexes antagonize the function of the basolateral module, which is comprised of the proteins Scribble (Scrib), Discs large (Dlg) and Lethal giant larvae (Lgl),

though Lgl associates with Scrib and Dlg transiently (Humbert et al., 2003). Adherens junctions serve as the boundary for apical and basal domains, and are physically located between the apical complex and the basolateral module (Baum and Georgiou, 2011; St Johnston and Sanson, 2011).

Many studies have been done to understand the role of apical complexes and basolateral modules in polarity regulation. The asymmetrical localizations of these polarity protein complexes are important for establishment and maintenance of cell polarity. aPKC has been demonstrated to phosphorylate the apical protein Crb and the basolateral component Lgl (Hutterer et al., 2004; Sotillos et al., 2004). Physical binding and phosphorylation of Crb by aPKC is required and sufficient for Crb localization (Sotillos et al., 2004). Phosphorylation of Lgl by aPKC prevents Lgl from localizing to the apical membrane therefore achieving the opposing effect between apical and basolateral complexes (Tanentzapf and Tepass, 2003). Although the hierarchy of polarity genes in polarity establishment remains debatable, it has been shown that disrupting the apical complexes results in loss of apical markers as well as expansion of the basolateral complexes (Kaplan et al., 2009). On the other hand, disruption of basolateral modules also causes loss of basolateral markers and increased apical domains (Kaplan et al., 2009). These data further support the concept that they are functionally interdependent and antagonize each other.

Disruption of apico-basal polarity resulted in defects in developmental processes, as evidently shown in many studies in *Drosophila* and *C.elegans* (Humbert et al., 2008; Nance, 2005; Parisi and Vidal, 2011). Also, it has been found that human cancers often contain mutations in polarity components. While both are important for normal epithelial development, perturbations of the apical complex and the basolateral complex lead to very different effects. Cells with mutation in the apical determinant *crb* hyperproliferate and *crb* mutant tissues overgrow. Interestingly, overexpression of Crb also induced tissue proliferation with an expanded apical domain (Hamaratoglu et al., 2009; Lu and Bilder, 2005), suggesting a dominant negative effect caused by overloading the system. When mutants occur in any of the basolateral components, they exhibit a neoplastic tumor feature where tissues become largely overgrown with poorly recognizable differentiated features (Humbert et al., 2003). In addition to inducing overproliferation, many of the apical proteins mislocalize in cells, suggesting a strong disruption of polarity (Humbert et al., 2003). Taken together, the literature suggests that apico-basal polarity is important for maintenance of proper epithelial function.

1.4.2 Apico-basal polarity regulates growth through the Hippo pathway

Recent studies indicate that apico-basal polarity module components also regulate the Hippo growth control pathway activity. As mentioned earlier, the apical protein Crb interacts with Ex to regulate growth through the Hippo pathway

in *Drosophila* (Chen et al., 2010; Ling et al., 2010; Robinson et al., 2010) (Chen et al., 2010 Richardson, Morberg, Pan). Crb physically binds to Ex, and loss of Crb resulted in Ex being mislocalized from the apical membrane and accumulating at a more basal and intracellular region (Chen et al., 2010; Ling et al., 2010). In mammalian cells, Crb has been demonstrated to interact with YAP/TAZ (Varelas et al., 2010b). Knock-down of *crb3* disrupts the interaction of Crb complex components with Crb (Chartier et al., 2011). When cultured cells reach their confluency, the density of cells is high and those cells stop proliferating. Knocking-down Crb3 in those confluent cells resulted in increasing nuclear fraction of Yap, suggesting Crb is important for sequestering a stabilized fraction of Yap/Taz (Varelas et al., 2010b). Overexpression of aPKC is sufficient to induce cell proliferation and survival (Grzeschik et al., 2010a). Such cells showed mislocalized Hpo and Rassf proteins as well as increased nuclear localization of Yki (Grzeschik et al., 2010a). These effects are not only restricted to the apical proteins. Basolateral complex members also regulate tissue growth through the Hippo pathway. In *Drosophila*, homozygous mutant animals of *scrib* show a massive overgrowth phenotype with highly elevated Hippo pathway reporter activity (Chen et al, 2011). The hyperproliferation feature can be suppressed by halving the dosage of Yki, suggesting pathway specificity (Grzeschik et al., 2010b). *dlg* and *lgl* mutants also displayed similar phenotypes as *scrib* (Yamanaka and Ohno, 2008). Consistently, knock-down of *dlg* and *lgl* in *Drosophila* also showed significant increase of Hippo reporter activity (Grzeschik et al., 2010b; Sun and Irvine, 2010). *lgl* mutant clones have increased levels of

nuclear Yki, and Hpo and Rassf are mislocalzed in *lgl* mutants (Grzeschik et al., 2010a; Grzeschik et al., 2010b).

Notably, many studies showed that the subapical area is the most active region for Hippo pathway activity. The apical region defined by these complexes may be the key to the function of the Hippo pathway by providing a hub where pathway components are localized and can interact with one another (Genevet and Tapon, 2011). Four transmembrane proteins that regulate the Hippo pathway are apically localized: Ft, Ds, Crb and Ed (Chen et al., 2010; Genevet and Tapon, 2011; Robinson et al., 2010; Willecke et al., 2006; Willecke et al., 2008; Yue et al., 2012). Meanwhile, Ex, Mer and Kibra colocalized at the apical membrane as well as Dachs (Genevet et al., 2011; Halder and Johnson, 2011; Hamaratoglu et al., 2006; Mao et al., 2006; Yu et al., 2011). Yki binds to Ex, therefore might localize to the apical membrane and is kept from entering the nucleus (Badouel et al., 2009). Apically localized Hpo and Rassf also have been observed (Grzeschik et al., 2010a). In mammals, Amot is also apically localized (Zhao et al., 2011). Additionally, several studies suggested that the apical fraction of MST1 contains the highest enzymatic activity, emphasizing the physiologically significant role of the subapical region (Hergovich et al., 2006; Ho et al., 2010; Praskova et al., 2004; Praskova et al., 2008). Interestingly, a recent study suggested human Scrib physically interacts with MST2, Lats1, and TAZ to form a protein complex (Cordenonsi et al., 2011). Knocking down *scrib* resulted

in MST2 dissociating from the complex and TAZ moved into the nucleus to activate downstream targets and drive EMT (Cordenonsi et al., 2011). Further characterization is needed to understand if this complex interacts with other apically localized components.

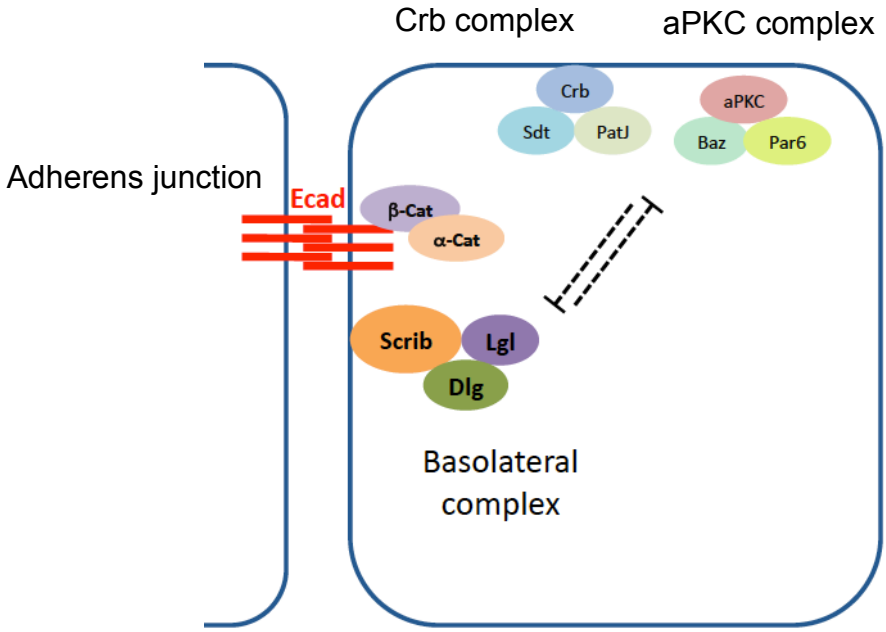

Fig 1.2 Schematic view of apico-basal polarity complexes

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 basolateral complex localizes basolaterally and inhibits the formation of the apical domain. Adherens junction is composed of E-cad, β-Cat and α-Cat (Adapted from Genevet and Tapon, 2011).

1.4.3 Components of the adherens junction

Adherens junctions serve as major adhesion units between cells. The main components of AJs are E-cadherin (E-cad), β-Catenin (β-Cat), and α-Catenin (α-Cat).

E-cadherin stands for Epithelial calcium ion dependent adhesion protein. It is a transmembrane protein, and the extracellular domain of E-cad contains Calcium ion binding domains (Koch et al., 1997). The formation of the AJ requires homophilic interaction of extracellular domain of E-cad in two neighboring cells, and the presence of Ca^{2+} helps to maintain the protein-protein interaction and stabilizes the junction (Koch et al., 1997; Pertz et al., 1999). The intracellular domain interacts with several proteins, including β-cat and p120 (Aghib and McCrea, 1995; Oda et al., 1994).

β-catenin encodes one of the armadillo family proteins that contains multiple armadillo (arm) repeat domains, which is important for protein-protein binding (Funayama et al., 1995). β-Catenin interacts with E-cad and many other proteins through Arm repeats, and binds to α-Cat through a distinct interaction domain (Oda et al., 1993; Tabibzadeh et al., 1995; Tian et al., 2011). It works as a bridge between α-Cat and the E-cad to form an intact AJ. When not binding to other AJ components, *β-cat* has been characterized as an important regulator of the Wnt pathway, which may enter the nucleus and turn on the expression of

downstream targets (Aberle et al., 1996; Burgess et al., 2011; Kikuchi et al., 2011).

The third component of AJ is α-Cat. Although named catenin, *α-catenin* does not share homology with *β-catenin* (Kemler, 1993). Instead, it shows strong structural and functional similarity with another membrane cytoskeletal protein Vinculin (Kemler, 1993). There are three *vinculin* homology (VH) domains-VH1, VH2, and VH3- present in the α-Cat protein (Kobielak and Fuchs, 2004; Rudiger, 1998; Shapiro and Weis, 2009; Weiss et al., 1998). α-Cat physically interacts with β-Cat and F-actin, and therefore has been thought to be a direct link between the cell adhesion complex and the actin cytoskeleton (Kobielak and Fuchs, 2004; Shapiro and Weis, 2009). Two important studies discovered that α-cat can only interact with β-Cat as a monomer, and binds to F-actin when α-Cat forms a homo-dimer (Drees et al., 2005; Yamada et al., 2005). Peptide interaction analysis and structure analysis suggested the VH1 domain of α-Cat is responsible for both dimerization of α-cat and interacting with β-cat, and therefore established the current model for the function of α-cat (Shapiro and Weis, 2009). However, since α-Cat can only interact with F-actin or β-Cat, how the actin cytoskeleton links to the cell junctions is still unknown and yet to be discovered.

1.4.4 Adherens junctions modulate the Hippo pathway in mammals

In mammals, components of AJs have been shown to regulate Hippo pathway activity, and E-Cad has been reported as a key regulator of contact inhibition (Kim et al., 2011). In dense-cell culture, homophilic interaction of E-cadherin decreases cell proliferation and facilitates the translocation of Yap from the nucleus to the cytoplasm (Kim et al., 2011). When Hippo pathway components are knocked-down or Yap is ectopically expressed in dense culture, the proliferation rate is restored and more Yap stays in the nucleus. α-Cat has also recently been shown to be a key regulator of Hippo activity (Schlegelmilch et al., 2011; Silvis et al., 2011). In the keratinocytes of the *α-cat* knock-out mouse, Yap is largely observed in the nucleus (Schlegelmilch et al., 2011; Silvis et al., 2011). Additionally, the nuclear localization of Yap is tightly associated with loss of *α-cat*, suggesting a pivotal role of α-Cat in regulating the Hippo pathway (Schlegelmilch et al., 2011; Silvis et al., 2011).The proposed mechanism for this regulation is that α-Cat interacts with 14-3-3 scaffolding protein and Yap, therefore retaining Yap in the cytoplasm. Together, these findings provide evidence that cell-cell contacts influence tissue growth through the Hippo pathway. While nicely demonstrated in mammals, the evidence for AJs to regulate the Hippo pathway in *Drosophila* is still very poor.

1.5 Cytoskeleton and signaling

The cytoskeleton is considered to play a major role in organizing the cellular architecture. The cytoskeleton provides the mechanical structure and sets up the framework for maintaining cell shape. More importantly, through dynamic networking the cytoskeleton facilitates cell homeostasis, cell mobility and provides mechanical stability (Fletcher and Mullins, 2010). The main components of cytoskeleton are actin and microtubules in *Drosophila* (Fyrberg and Goldstein, 1990). Intermediate filaments serve as another member in mammals to help orchestrate cell structure (Steinert and Roop, 1988). All these components form a meshwork and interconnect the cytoskeleton with cell junctions and therefore contact with neighboring tissues (Yonemura, 2011b). The role of cytoskeleton in growth control has not really been addressed. Many consider cytoskeleton components to be housekeeping genes and thought that changes of the cytoskeleton are a downstream effect of signaling events. However, this bias has recently changed and more studies have been focused on examining the signaling role of the cytoskeleton.

First of all, mechanosensing in tissue homeostasis has become a popular research topic. The cytoskeleton is important to transduce those mechanical inputs from the environment into chemical cues in the cells in order to respond to the changes (Fletcher and Mullins, 2010; Maruthamuthu et al., 2011). The links between cell junctions and the cytoskeleton provide a perfect source to execute the mechanical sensing. Moreover, recent research has identified that cytoskeleton is important in signaling pathway inputs. Modulating F-actin caused

growth phenotypes in both mammals and *Drosophila* (Bras-Pereira et al., 2011; Sansores-Garcia et al., 2011; Wada et al., 2011). Tao-1, a negative regulator of microtubule plus-end growth, also has been characterized as a growth regulator in *Drosophila* (Boggiano et al., 2011; Poon et al., 2011). In brief, the cytoskeleton is now considered as important signaling molecules instead of only pure scaffolding proteins.

1.5.1 Actomyosin cytoskeleton

The name actomyosin is derived from the names of its two major components: actin and myosin (Gorfinkiel and Blanchard, 2011). These two components often interact with each other in different cellular events and have been considered to be important force generating partners (Gomez et al., 2011; Lecuit et al., 2011). Although actin and myosin are important motor proteins in muscles as well, here we only discuss the non-muscle function of actomyosin since non-muscle actomyosin has many physiological functions in all kinds of cells. Actomyosin has been shown to interact with the junctional complex (Gomez et al., 2011; Smutny and Yap, 2011). In particular, it has been found that the adherens junction protein α-Cat interacts with F-actin (Drees et al., 2005; Yamada et al., 2005; Yonemura, 2011). Through this interaction the actomyosin cyctoskeleton and adherens juctions orchestrate the network for cell-cell interaction, and may play a central role in mechanosensing.

Actin

Actin is a globular molecule and is highly conserved through all organisms. Monomeric actin, also called globular actin (G-actin), is one of the most abundant proteins in cells. G-actin can be polymerized and become filamentous actin (F-actin), which is the main structure that is present in the cytoskeleton (Carlier et al., 1994). Several proteins are involved in actin polymerization. Profilin facilitates conjugating of ATP to G-actin, and this is a critical step to prepare G-actin for polymerization (Bugyi and Carlier, 2010; Yarmola and Bubb, 2009). Formins will dimerize through the FH2 domain interactions and form a ring structure to promote unbranched actin growth on the plus end (Goode and Eck, 2007; Pollard, 2007). Meanwhile, another type of nucleation is directed by Arp2/3 complex, which allows the formation of branched F-actin and composes different structures (Pollard, 2007). At the minus end, cofilin will depolymerize F-actin to monomeric G-actin and relase the inorganic phosphate (P_i) from G-actin-ADP (Hawkins et al., 1993; Poukkula et al., 2011). Dissociated G-actin can be recycled to form new F-actin (Yarmola and Bubb, 2009). In mammalian cells, F-actin is found to form a bundle structure called a focal adhesion, and these adhesions serve as attachment sites for the cell and many signaling events occur at focal adhesions (Albiges-Rizo et al., 2009). Focal adhesions are important not only for cell attachment but also serve to regulate cell movement (Quadri, 2012; Rottner and Stradal, 2011). Cells extend their filopodia to actively move from one site to another, and the dynamics of focal adhesions are the key for cell motility (Albiges-Rizo et al., 2009; Quadri, 2012).

Non-muscle myosin II

Non-muscle myosin II consists of non-muscle Myosin II heavy chain, non-muscle myosin regulatory light chain and non-muscle myosin essential light chain (Wang et al., 2011a). Two myosin II heavy chains dimerize and myosin II light chains subsequently bind to both heavy chain molecules (Rottner and Stradal, 2011). The head region of myosin II heavy chain interacts with F-actin, and studies have shown that the myosin II can "walk" on the actin filament to generate directional movement (Wang et al., 2011a). In *Drosophila*, Zipper (Zip) is the non-muscle myosin II heavy chain and Spaghetti-squash (Sqh) is the regulatory light chain (Morgan, 1995).

Non-muscle myosin II is an important regulator for many cellular processes. First of all, it is important for the actions of the contractile ring in mitosis (Straight et al., 2005; Urven et al., 2006). During mitosis, two daughter cells need to be divided and the contractile ring is necessary for this function. At the end of mitosis, the cells start to form a contractile ring with highly concentrated F-actin filaments. Non-muscle myosin II then appears and gets activated at the contractile ring close to the dividing point and cuts the mother cell into two daughter cells (Straight et al., 2005; Urven et al., 2006). In *Drosophila*, the functions of myosin II have been extensively studied in dorsal closure of the embryo (Bloor and Kiehart, 2002; Franke et al., 2005; Tan et al., 2003; Young et al., 1993). During embryonic development, there is a gap caused by the

presence of the amnioserosa. Somatic cells surrounding the amnioserosa change their shape and extend their protrusions to close the gap and form an intact epithelial sheet. Myosin II is concentrated at the edges of the body segments where both ends of the soma are met. Also, Myosin II activity is required for antagonizing the Baz/Par complex during embryonic axis elongation (Fernandez-Gonzalez et al., 2009; Simoes Sde et al., 2010). In *Drosophila* imaginal discs, Myosin II regulates cell rearrangement and higher-order architecture during eye development (Baumann, 2004; Lee and Treisman, 2004). It has also been suggested that Myosin II plays a pivotal role in cell proliferation, cell sheet adhesion, and also affects wing hair structure during wing morphogenesis (Franke et al., 2010; Urven et al., 2006; Vicente-Manzanares et al., 2009; Wang et al., 2011a).

Myosin II has generally been considered to be a motor protein (Bond et al., 2011). Due to its function as a motor protein, studies suggested non-muscle myosin II affects intracellular trafficking (Neto et al., 2011; Stow et al., 1998). In mammalian cells, non-muscle myosin II regulates intracellular trafficking during membrane repair (Togo and Steinhardt, 2004). Deficiency in non-muscle myosin II results in membrane repair machinery targeting failure (Togo and Steinhardt, 2004). Meanwhile, studies found that when mixing hyperactivated Ras expressing cells with normal cells, those Ras-hyperactivated cells change their morphology with increased height and also have elevated levels of phospho-myosin light chain compared to their wild type neighbors (Hogan et al., 2009).

Those cells also extend their pseudopodia and display a metastasis like phenotype (Hogan et al., 2009). Taken together, these data clearly demonstrated Myosin II is a multifunctional protein complex.

1.5.2 Regulation of myosin II activity

The regulatory light chain is the key regulator of myosin II function (Ikebe, 2008). Phosphoylation of regulatory light chain results in activation of myosin II (Ikebe, 2008; Vicente-Manzanares et al., 2009). This modification is the indicator of myosin activity. In *Drosophila*, two serine residues on myosin II regulatory light chain are responsible for the activation of myosin II. The regulatory light chain can be phosphorylated by two kinases, Rho kinase (Rock, Rok in flies) and myosin light chain kinase (MLCK) (Matsumura et al., 2001). Since Rok is one of the downstream effectors of Rho, it is not surprising that the Rho signaling pathway regulates myosin activity. Rho GTPase has been found to be involved in multiple cellular events, including cytoskeleton dynamics (Narumiya et al., 2009). Meanwhile, another downstream effector of Rho signaling is Diaphanous (*dia*), the *Drosophila* ortholog of formin that regulates actin assembly (Mulinari et al., 2008; Narumiya et al., 2009; Warner and Longmore, 2009b). By affecting actin and myosin, Rho signaling apparently serves as an essential regulator of actomyosin.

1.5.3 Cytoskeleton and mechanical force regulate Hippo signaling

A cell junction serves as a contact point between neighboring cells, therefore it is a great candidate to act as a sensor of mechanical inputs from outside environments. The actomyosin cytoskeleton connects to cell junctions and organizes a network inside the cells, which potentially can be the receiver to transduce the mechanical cues into the cell. Several studies have shown that disrupting the apical domain of a cell triggers changes in apical tension and induces defects in apical constriction (Franke et al., 2005; Warner and Longmore, 2009a; Warner and Longmore, 2009b). In many cases, F-actin structures are also altered in apical domain defects (Warner and Longmore, 2009a; Warner and Longmore, 2009b).

Our lab conducted a cell culture based genetic screen to identify important novel regulators for the Hippo pathway. In that screen, we identified several proteins involved in F-actin regulation. These candidate genes are *Drosophila* profilin twinstar (*tsr*), capping protein a (*cpa*), capping protein b (*cpb*), and actin (Sansores-Garcia et al., 2011). After careful examination, we confirmed these actin modifiers indeed are able to regulate Hippo pathway activity (Sansores-Garcia et al., 2011). Knocking down of *cpa* and *cpb* induced ectopic tissue growth and elevated levels of Hippo pathway reporters. This effect can also be achieved by overexpressing a constitutively active form of *Drosophila* formin Dia (Sansores-Garcia et al., 2011). We investigated the pathway specificity and found it did not affect other signaling pathways. Epistasis experiments place F-

actin downstream of Hpo and upstream of Wts in the pathway. Mammalian cell culture also demonstrated that activated mDia induces Yap nuclear localization and disruption of F-actin reduces nuclear YAP staining (Sansores-Garcia et al., 2011; Wada et al., 2011). Taken together, our results as well as observations from the Janody lab identified F-actin as a signal input for the Hippo pathway (Fernandez et al., 2011; Sansores-Garcia et al., 2011).

Zyxin (Zyx) is a LIM-domain protein, which has been shown to mediate cell-cell interaction and cytoskeleton organization (Beckerle, 1997; Hirata et al., 2008). Zyxin regulates actin dynamics in response to mechanical inputs, and therefore has been suggested to be a mechanical force sensor. In *Drosophila*, Zyxin is required for normal wing development, and genetic analysis reveals that it functions between Ft and Wts (Rauskolb et al., 2011). A proposed model suggested binding of Zyx to Wts reduces Wts activity and leads to Wts degradation. In mechanosensing, Zyx accumulated at the edge of wound sites, suggesting a leading role in response to changes in cell tension (Smith et al., 2010).

In mammalian cells, several groups utilize different methods to dissect the role of mechanical manipulations in Hippo pathway regulation (Cordenonsi et al., 2011). These mechanical manipulations include modulating the stiffness of extracellular matrix, changes in cell morphology, and disrupting cell attachment.

The following three paragraphs will address the Hippo pathway regulation from these mechanical cues.

The extracellular matrix (ECM) is the non-cellular part of tissues that surrounds and interacts with cells (Noguera et al., 2012). Cells change their behaviors in response to the modulation of the ECM (Sun et al., 2012). Researchers found YAP/TAZ changes its localization upon facing materials or surfaces of different stiffness (Dupont et al., 2011). In high stiffness environments, YAP/TAZ are mainly localized in the nucleus, whereas in soft stiffness environments there is more cytoplasmic YAP/TAZ. Inhibition of Rho signaling or disruption of the actomyosin cytoskeleton inhibited YAP/TAZ activation in high stiffness environments, suggesting an important role of YAP/TAZ in mechanical sensing. Further experiments have also demonstrated that ECM stiffness regulates Hippo signaling independent of MST and LATS (Dupont et al., 2011). In brief, cellular microenvironments send mechanical signals to regulate YAP/TAZ activity in cells.

It is known that cells behave differently in high-density and low-density cultures (Li et al., 2012b). One of the differences is that cells have different geometry, namely in surface area and the height of the cells. Researchers were wondering if simply manipulating the morphology of a cell affects the signaling activity, especially the Hippo pathway. Indeed when single cells are cultured in a largely open area, cells become flat and have high YAP nuclear localization

(Dupont et al., 2011). On the other hand, growing a cell in a compact space, the cell becomes more columnar and YAP is translocated into the cytoplasm (Dupont et al., 2011). The Piccolo group further provided evidence that the change seen in YAP activity is not because of the total area contacting with ECM but is due to the change of cell geometry.

The effects of cell attachment to either extracellular matrix or neighboring cells have been addressed earlier. However, is the attachment itself important for Hippo pathway activity? Using a cell culture system, the Guan group showed that Yap will be dephosphorylated and downstream targets of YAP expression is increased upon cell attachment (Zhao et al., 2012). They also demonstrated that the actin cytoskeleton modulated LATS1/2 activity to regulate YAP activity. Anoikis is a specific form of cell death where cells detach from the surrounding matrix and execute apoptosis. Interestingly, cell detachment induced anoikis can be suppressed by increasing Yap activity (Zhao et al., 2012). The evidence that detached cells could survive with elevated Hippo target activity provides an excellent explanation for understanding metastasis events in cancer development.

In summary, all the above studies demonstrated that mechanical inputs regulate tissue growth through multiple mechanisms. In my dissertation, I will focus on how cell-cell interaction (adherens junctions) and intracellular cytoskeleton (the actomyosin network) mediate organ growth through the Hippo pathway.

Chapter 2:

Rationale and dissertation aims

2.1 Rationale for studying AJs and actomyosin in Hippo signaling regulation

Cancer is one of the most common diseases in the world, and each year millions of people are diagnosed with it. Tremendous efforts have been made to understand how cancers progress and how to stop them, however, there are still many knowledge gaps. Currently, cancers are defined by eight hallmarks: (1) sustaining proliferative signaling; (2) evading growth suppressors; (3) activating invasion and metastasis; (4) enabling replicative immortality; (5) inducing angiogenesis; (6) resisting cell death; (7) reprogramming of energy metabolism; (8) evading immune destruction (Hanahan and Weinberg, 2000; Hanahan and Weinberg, 2011). More than 85% of cancers arise from epithelia, and apico-basal polarity is the major regulator to maintain epithelial integrity. Disruption of apicobasal polarity causes cells to delaminate from the epithelia, and results in cell migration such as metastasis. In cancer development, one of the malignancy markers is that a cell leaves its place of origin and metastasizes to distal sites. Studies have shown cancer cells carry many mutations in their genomes, and these mutations resulted in misregulations of apico-basal polarity components (Martin-Belmonte and Perez-Moreno, 2012). Cell-cell contact dependent inhibition of cell proliferation, which is also called contact inhibition, is one of the major mechanisms for stopping activating invasion and metastasis (Liu and Dean, 2010). The adherens junction is one of the cell junctions that contact with neighboring cells. As mentioned earlier, AJ component E-cad is one of the regulators for contact inhibition (Kim et al., 2011). α*-cat* is another AJ molecule

and shares similar regulatory mechanisms for contact inhibition (Kim et al., 2011). Surprisingly, little is known about the function of *α-cat* and how it affects tissue growth. The first part of my thesis is to understand the role of Adherens junctions in growth regulation, particularly the effects of *α-cat*. In mammals both AJs and the baso-lateral complexes mediate the activity of the Hippo pathway, but the underlying differences between AJ and baso-lateral complexes in Hippo pathway regulation are still largely unknown. Here we performed experiments to distinguish the differences between AJs and basolateral complex in Hippo signaling regulation.

Cell shape and morphology are mainly controlled by cortical force that is generated by cytoskeleton underlying the apical plasma membranes of epithelial cells (Warner and Longmore, 2009b). Actin filaments and non-muscle Myosin II are enriched in this area, and their distribution is spatially and temporally controlled (Gorfinkiel and Blanchard, 2011). The actomyosin networks generate force to actively induce cell contraction (Gorfinkiel and Blanchard, 2011). The most well characterized feature for actomyosin action is the formation of the contractile ring at the end of mitosis (Gorfinkiel and Blanchard, 2011; Monier et al., 2011; Mulinari et al., 2008; Straight et al., 2005). The activated myosin generates force on the peripheral actin ring to close the opening between two daughter cells and eventually cleaves them into two separate cells (Gorfinkiel and Blanchard, 2011; Mulinari et al., 2008; Straight et al., 2005). Meanwhile, the cortically localized actomyosin is important to maintain tissue morphology and to

coordinate tissue homeostasis (Vicente-Manzanares et al., 2009). Currently, there is no study directly suggesting myosin's function in growth. However, myosin interacts with F-actin, and F-actin organization has recently been characterized as a novel regulator for growth control, mainly through mediating the Hippo pathway. Since Actomyosin cooperatively controls many cellular events, we are interested in understanding if non-muscle myosin II also plays a role in growth regulation. Also, Zip physically interacts with *lgl*, one of the baso-lateral module proteins that also affects the Hippo pathway (Strand et al., 1995). Although the interaction of Zip and Lgl is prominent, the physiological basis for their interaction is still unclear. In the *Drosophila* neuroblast, the asymmetric localization of Zip and Lgl helps define proper localization of cell fate determinants in daughter cells (Betschinger et al., 2003). Therefore, we are interested in finding out if myosin II plays a role in Hippo signaling regulation. In this part we examined how myosin II regulates growth and how this growth regulation compares to modulating F-actin induced growth phenotypes.

2.2 Dissertation research aims

To understand the role of α-cat in *Drosophila*, we characterized two p-element insertion mutants and verified these alleles are functionally null mutants (Schuldiner et al., 2008). The early lethal phenotype mimics the phenotype in mutants of other AJ components (Orsulic and Peifer, 1996; Tepass et al., 1996). Disrupting *α-cat* resulted in decreased tissue size, and the size changes in *α-cat* knocked-down tissues are correlated with an increase in apoptosis and decrease

in cell proliferation. Since it has been shown that α-cat regulates Hippo pathway activity in mammalian keratinocytes, we then tested if the expression of the Hippo pathway reporters has been affected in α-cat knocked-down tissue. Surprisingly, *ex-lacZ* is down-regulated in α-cat knock-down tissue. However, a dramatic increase of *ex-lacZ* has been observed in the neighboring tissue. While we checked the effect on *ex-lacZ* in scrib knocked-down tissue, we only observed the autonomous induction of *ex-lacZ*. These results implied AJs and basolateral complexes regulate Hippo activity through different mechanisms.

We analyzed the differences in phenotypes when knocking-down of *α-cat* or *scrib*. Knocking-down of α*-cat* and *scrib* has very different effects on cell proliferation and apoptosis. Also, we found that disrupting *α-cat* or *scrib* do not affect the localization of each other. Meanwhile, while disrupting *α-cat* and *scrib* both induced autonomous JNK activity, we provided evidence to demonstrate α-cat does not present synergistic overgrowth with dominant negative JNK like *scrib* does. Finally, we performed *α-cat* and *scrib* double knock-down experiments to show that the autonomous induction of Hippo pathway reporters is dependent on basolateral complexes. This finding defines the hierarchy for AJs and basolateral complexes in regulating the Hippo pathway.

In chapter 5 we address the role of myosin II in Hippo pathway regulation. We first demonstrated that disrupting myosin II caused tissue expansion and

induced ectopic cell proliferation, suggesting myosin II regulates tissue growth. We then examined the growth related signaling pathway and found that Hippo pathway activity is greatly induced in myosin II knocked-down tissue. Likewise, upstream regulators of myosin II phenocopied the phenotypes we observed in myosin knock-down tissues. These results implied a novel function for myosin II as a tumor suppressor. Although inducing extra cellular proliferation, we also observed a significant amount of apoptotic cells. Interestingly, blocking cell death did not affect Hippo reporter induction in myosin II knock-down tissue, indicating that Hippo activation is not caused by apoptosis-induced tissue regeneration. We also examined the effect of increasing myosin II activity. Surprisingly, we also observed an increase in *ex-lacZ*. Hyperactivated myosin II induced F-actin accumulation, indicating that activated myosin II regulates the Hippo pathway through modulating F-actin. Intriguingly, disrupting actin polymerization also induced *ex-lacZ*. While contradictory to our expectation, this result together with the Myosin II data strongly suggested the existence of a complicated regulatory mechanism in regulating the Hippo pathway.

Chapter 3.

Materials and methods

3.1 *Drosophila* as a model organism

We chose *Drosophila* as our model organism to investigate the molecular mechanisms of growth control. Many essential players in various signaling pathways are functionally conserved between *Drosophila* and mammals (Brumby and Richardson, 2005). However, mammals tend to have multiple orthologs of important genes. This feature of the mammalian genome makes it more difficult to investigate the function of a gene because of the redundancy. Therefore, the simplicity of the fly genome makes them more accessible as a genetic model system. Also, more than 70% of essential genes in *Drosophila* are conserved in vertebrates, including many oncogenes and tumor suppressor genes (Brumby and Richardson, 2005; Oldham and Hafen, 2003; Pan et al., 2004; Prober and Edgar, 2001). Thus, understanding growth regulation in *Drosophila* helps us identify pivotal mechanisms in organ size regulation and disease, such as tumorigenesis.

The developing eye and wing of *Drosophila* have been used to study many developmental processes (Figure 3.1), including growth control. The *Drosophila* life cycle consists of four stages: embryo, larva, pupa, and adult. Most of the adult organs are derived from primordial epithelial tissues called imaginal discs. During embryogenesis, 20-50 cells cluster together and form the precursors of imaginal discs (Baker, 2001; Diaz-Benjumea and Cohen, 1993). During the larval stage, the disc cells undergo several rounds of cell proliferation to achieve an exponential increase of cell number and tissue size. At the third

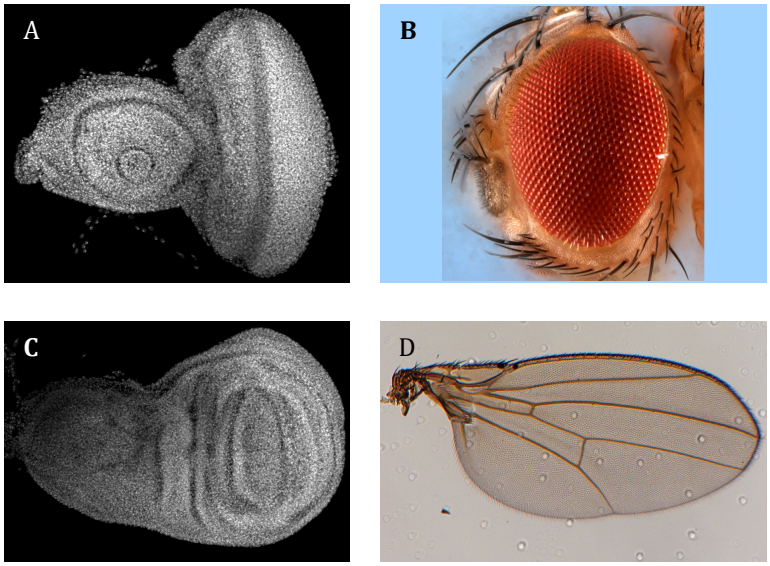

Fig 3.1 Developmental tissues of *Drosophila melanogaster*

The most frequent used tissues in *Drosophila* are the eye imaginal disc (A), which will give rise to adult compound eye (B), and also the wing imaginal disc (C), which will become the adult wing.

instar larval and pupal stages, cells in imaginal discs start to differentiate into their corresponding adult tissues. Finally, flies finish their metamorphosis in the pupal case and eclose to reach the final adult stage. The whole development of a fly takes around 10 days at 25°C, and this short life cycle feature exemplifies another advantage of *Drosophila* as an excellent model for genetic research. Additionally, many sophisticated tools and techniques for experimental analysis in *Drosophila* have been well established. These features make flies amenable to functional studies and for use in elegant genetic screens.

3.2 Gal4-UAS system

Originally found in yeast, the UAS-Gal4 system is now one of the most useful genetic tools developed in *Drosophila* for biological studies (Brand and Perrimon, 1993). Gal4 is a yeast transcription factor, and it binds to promoters that contain upstream activating sequence (UAS) to transcribe the following gene. Since it does not exist in the *Drosophila* genome, only the transgene with the UAS promoter will be expressed upon the presence of *Gal4*. Many tissue specific Gal4 lines have been generated to allow spatial-temporal control of ectopic gene expression. The *Gal4* drivers we used in this study are listed in Table 3.1, and we express a *UAS-GFP* construct with all these drivers to mark their expression patterns in third instar larval discs.

Gal4 line	Disc observed	expression region
ptc-Gal4	wing	AP boundary
en-Gal4	wing	posterior compartment
hh-Gal4	wing	posterior compartment
ey-Gal4	eye	eye disc
nub-Gal4	wing	wing pouch
dpp-Gal4	wing	AP boundary

Table 3.1 *Gal4* drivers used in this study.

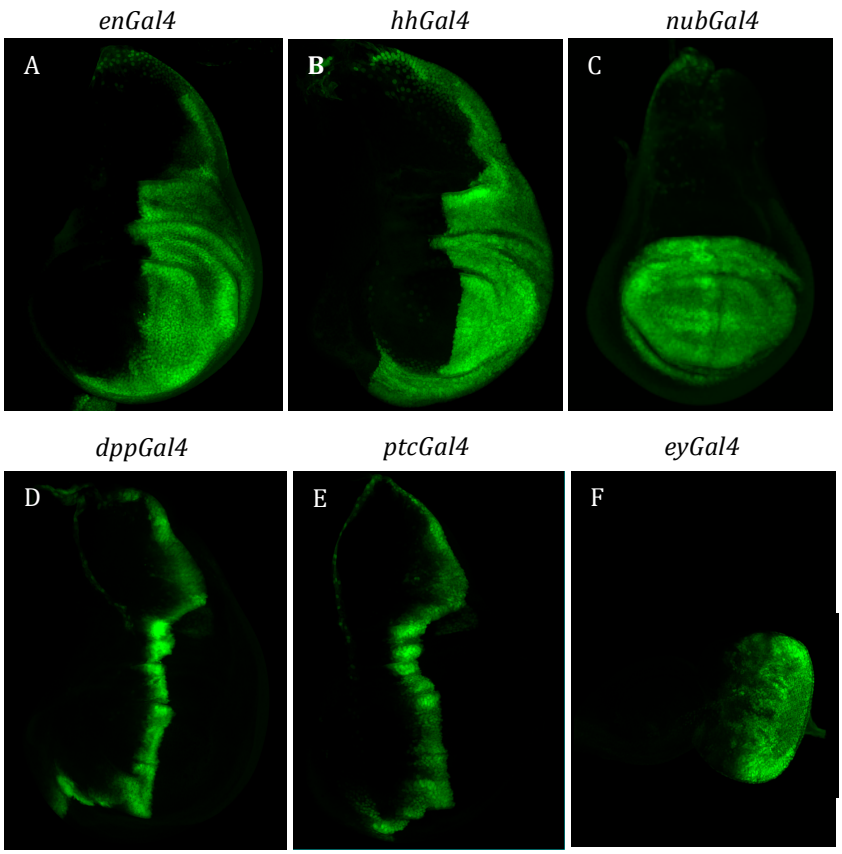

Fig 3.2 The expression patterns of Gal4 drivers used in this study

(A-E) Wing imaginal discs; (F) eye imaginal disc.

A *UAS-GFP* construct is expressed with different *Gal4* lines.

(A) *enGal4*; (B) *hhGal4*; (C) *nubGal4*; (D) *dppGal4*; (E) *ptcGal4*;

(F) *eyGal4*.

UAS-RNAi stocks

RNA interference (RNAi) is a process that cells use to modulate gene function (Seyhan, 2011). First identified in *C. elegans*, RNAi has become a common tool to knock-down gene expression (Fire, 2007). Transcribed non-coding RNA forms double strand RNA and can be processed by DICER complexes to become small double strand siRNA. The siRNA then finds its target mRNA and inhibits its expression through post-transcriptional gene silencing and mRNA degradation. Several groups systematically generated UAS-RNAi transgenic flies for the fly community (Dietzl et al., 2007; Ni et al., 2009). The UAS-RNAi lines we used in this study are listed in Table 3.2.

Drosophila stocks and culture:

All crosses are maintained at 25°C. The UAS-Gal4 system (Brand and Perrimon, 1993) was used for overexpressing genes of interest or expressing RNAi constructs to knock-down specific genes. The complete list of UAS-lines and other stocks used in this study are also presented in Table 3.2.

Sample preparation and immunostainings

Drosophila third instar larvae are selected and dissected in PBS. After dissection, we fixed tissues with 3.7% formaldehylde in PBT (PBS with 0.3% Triton X) for 30 minutes. Antibody stainings of imaginal discs and BrdU incorporations were performed as previously described (Hamaratoglu et al.,

2006), with the exception of Crb staining, where tissues are incubated in ice-cold acetone for 10 minutes after formaldehyde fixation.

The following antibodies were used (source and dilutions in parentheses): mouse anti-BrdU (Becton-Dickinson, 1:50), mouse anti-βGal (Promega, 1:2000), rat anti-Elav (DSHB, 1:60), rat anti-DECad (DSHB 1:30), rat anti-α-Cat (DSHB, 1:30), mouse anti-DLG (1:300); guinea pig anti-DLG (1:1000), guinea pig anti-Mer (R. Fehon, 1:4000), mouse anti-Arm (1:20), mouse anti-Patj (H. Bellen, 1:500), rabbit anti-aPKC (1:500), rabbit anti-cleaved caspase 3 (Cell Signaling, 1:50), rabbit anti-myosin (E. Wieshaus, 1:100) rabbit anti-phospho-myosin II (Cell signaling, 1:50), Alexa647-conjugated phalloidin (Invitrogen, 1:50). Images were taken on an Olympus FV1000 confocal microscope.

Adult tissue images

The adult flies are frozen in -20°C prior to image. Images are taken with a Zeiss Axioplan microscope and processed by Apotome software program.

UAS construct	**Source**
UAS-α-catRNAi	VDRC#107298
UAS-α-catRNAi	TRiP # 33430
UAS-scribRNAi	TRiP #29552
UAS-scribRNAi	TRiP #35778
UAS-scribRNAi	VDRC #105412
UAS-DEcadRNAi	VDRC #27081
UAS-dlgRNAi	TRiP #31520
UAS-dlgRNAi	VDRC #41136
UAS-dlgRNAi	VDRC #41134
UAS-lglRNAi	TRiP #31089
UAS-lglRNAi	VDRC #51249
UAS- zipRNAi	VDRC #7819
UAS-sqhRNAi	VDRC #7917
UAS-sqhRNAi	TRiP #31542
UAS-rhoRNAi	VDRC #12734
UAS-rokRNAi	VDRC #104675
UAS-diaRNAi	TRiP #28541
UAS-chicRNAi	NIG Japan
UAS-sqh^{DD}	Nishida, Y
UAS-RasV12 (II)	Bloomington
UAS-Ras12 (III)	Bloomington
UAS-bsk^{DN} (X)	Bloomington
UAS-bsk^{DN} (III)	Bloomington
UAS-α-cat-GFP (X)	Kyoto stock center
UAS-α-cat-GFP (II)	Kyoto stock center
Fly stock	**Source**
H99	Bergmann lab
Dronc^{I29}	Bergmann lab
pBac[SAstopDsRed] 07736	Kyoto stock center
pBac[SAstopDsRed] 00441	Kyoto stock center

Table 3.2 *Drosophila* stocks used in this research

Chapter 4

Differential regulation of the Hippo pathway by adherens junctions and basolateral complexes in *Drosophila*

4.1 Introduction

Epithelial-mesenchymal transition (EMT) is a frequent marker of tumor malignancy, as well as being indicative of cancer that is more susceptible to metastasis (Zhao et al., 2008c). One of the important hallmarks of EMT is loss of cell polarity (Hanahan and Weinberg, 2000; Hanahan and Weinberg, 2011; Zhao et al., 2008c). Numerous studies have demonstrated that loss of adherens junctions (AJs) and baso-lateral complexes both induce EMT (Hugo et al., 2011; Zhao et al., 2008c), but the underlying differences in how these complexes regulate EMT are still poorly understood. Mutants in both AJs and basolateral components are frequently associated with cancers, and Hippo pathway activity appears to be mediated by both AJs and basolateral complexes (Cordenonsi et al., 2011; Lei et al., 2008). Although adherens junctions and basolateral complexes both regulate Hippo pathway activity, it is unknown if they act via the same mechanism to regulate growth. Therefore, we are interested in understanding the hierarchy of apico-basal polarity components in growth regulation. Also, there was no published report on AJ component α-*catenin* mutant phenotype in *Drosophila* when we started our research. Here, we first characterized mutant phenotypes of α-*catenin,* and then we examined roles of adherens junctions and basolateral complex components in regulation of organ growth in *Drosophila* imaginal discs. Through this study we propose a model where the basolateral complex and adherens juctions act in parallele to regulate Hippo pathway activity and growth.

4.2 Results:

4.2.1 Characterize the phenotypes of *α-catenin* mutant

α-catenin is one of the major players in AJ. Due to the reasons mentioned in the introduction, there is no report in *Drosophila* on *α-cat* mutants. To investigate the function of α-catenin in *Drosophila*, we searched all possible stock collections and found two p-element insertion lines that might affect or disrupt the function of *α-cat*. Both lines –LL07736 and LL00441- are *pBac[SAstopDsRed]* insertions generated by Liquin Lo's group (Schuldiner et al., 2008). The p-element contains splicing acceptor with all six frames of stop codon and a neuronal DsRed reporter that can be seen in larval brain. LL00441 is inserted 7 b.p. after the transcription start, and L00736 is located at the first intron. Both lines show embryonic lethality, which is consistent with DE-cad and Arm mutant phenotypes (Orsulic and Peifer, 1996; Tepass et al., 1996). To further confirm these two lines are *α-cat* mutants, we conducted a rescue experiment by overexpressing *UAS-α-catGFP*. We first checked if overexpressed *α-cat-GFP* caused any phenotypes. Using *ubiquitous-Gal4* to overexpress this ectopic *α-cat-GFP* in wild type animals, we found it localized to the apical membrane, which is similar to the expression of endogenous *α-cat* (Fig. 4.1F). The *α-cat* overexpressing animals survive to adult stage, suggesting that overexpression does not affect animal viability. When we overexpressed *α-cat* in potential *α-cat* mutants, the *UAS-α-cat-GFP* construct can rescue both alleles to adult stage,

and both of them are fertile (Fig. 4.1B). These data confirmed both alleles are genuine *α-cat* mutants.

Since *α-cat* mutant cannot survive to larval stage, it is difficult to study its function in developmental processes. A classical way to overcome the obstacle is using FLP/FRT system to generate mutant clones and compare the phenotypes with the neighboring WT tissues. Unfortunately, the gene locus of α-cat is at 80F, closer to the centromere than the 3L FRT insertion site, therefore it does not allow us to perform the mutant clone analysis. Tepass lab generated a genetic rescue construct and performed clonal analysis. The rescue constructs are driven by ubiquitously expressing Gal4 and were combined with a FRT site. Combined with *α-cat* mutant in this circumstance, the mutant tissue can survive when the tissues have ectopically expressed *α-cat*. Consistent with our observation, they found the mutant clones that didn't contain the rescue DNA can not survive. This result leads us to think about other alternatives to decipher the function of *α-cat*.

The UAS-RNAi transgenic flies have been systematically generated and are available from the Vienna *Drosophila* RNAi Center and the TRiP collection from Harvard University (Dietzl et al., 2007; Ni et al., 2009). Studies have demonstrated that many of them have reasonable knock-down efficiency, so we decided to use *UAS-α-catRNAi* as a tool to study the function of *α-cat*. When we

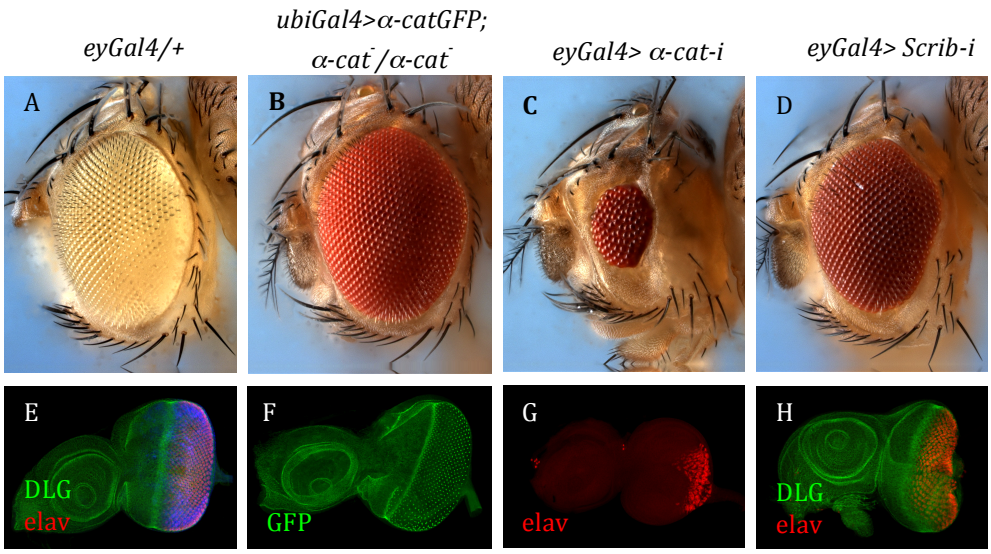

Fig 4.1 Disrupting *α-catenin* results in eye development defects.

(A-D) *Drosophila* adult eye images; (E-H) confocal images of eye imaginal discs. An *eyGal4* adult eye (A) and an eye-antenna disc (E) without any transgene serves as a control for our experiment. The DLG and Elav show a wild type pattern of developing eye discs. *α-cat* mutants are embryonic lethal, and *ubiGal4* drive a *UAS-α-catGFP* construct can rescue the animal to adult with a complete normal eye (B). An eye-antenna disc from the same rescue genotype is shown with normal morphology and properly localized *α-catGFP* (F). Using *ey-Gal4* to knock-down *α-cat* (C) or *scrib* (D) shows very different phenotypes. *α-cat* knock-down leads to small adult eye (C) and also smaller eye discs with only few differentiated ommatedia (G). On the other hand, knocking down of *scrib* has only mild effects on both adult eye and developing eye discs (H).

knock-down the expression of *α-cat* by *ey-Gal4*, we found a significant decrease of eye size with rough eye phenotype (Fig. 4.1C). Another independent *UAS-α-catRNAi* construct from a different collection has an even stronger phenotype which animals only developed into pharate adults with no head tissue and died in pupal cases. We looked at eye imaginal discs at the third instar larval stage and found those strong *α-cat* knocked-down animals did have much smaller eye discs with only few differentiated ommatedia (Fig. 4.1G), which further confirmed the lethal phenotype we mentioned earlier. In brief, our data suggested *α-cat* is an essential gene that contributes to cell viability and tissue growth.

4.2.2 Disruptions of adherens junctions and baso-lateral complexes affect the Hippo pathway differently

Several studies have shown apical proteins and the baso-lateral proteins regulate the Hippo pathway in *Drosophila*, but there is no report on the effect of *Drosophila* AJs in regulating the Hippo pathway. To understand how AJs affect Hippo signaling in *Drosophila*, we first examined the effect on disrupting AJs. We choose *patched-gal4 (ptc-gal4)* to express the *UAS-RNAi* in specific regions. Knocking-down of *α-cat* and *E-cad* both induced Hippo target expression, as shown by *expanded-lacZ* staining (Fig. 4.2A, C, and E). We also saw a significant induction of *ex-lacZ* in s*crib*, *lgl* and *dlg* knock down tissue. Surprisingly, when we look at the cross section, we found the effect on *ex-lacZ* by knocking-down *α-cat* and *scrib* are totally different. When we examined *α-cat*

and *E-cad* knock-down tissues, they shows an autonomous reduction of *ex-lacZ*. Interestingly, knock down of *α-cat* and *E-cad* also induced a very strong non-autonomous signal of *ex-lacZ* (Fig. 4.2B and D). In contrast, *scrib* knock-down tissue displayed an autonomous increase of *ex-lacZ* (Fig. 4.2F). We used different *UAS-RNAi* lines targeting different regions of *α-cat* and *scrib*, and all of them show similar phenotypes with various strengths. These results suggested that AJs and baso-lateral complexes may regulate Hippo targets through distinct inputs. To confirm the *RNAi* lines function properly, we performed antibody staining and showed that *α-catRNAi* efficiently knocked-down *α-cat* expression and *scribRNAi* disrupted the formation of basolateral modules, as showed by loss of DLG staining (Fig. 4.5). These results demonstrate that while initially the effects on Hippo pathway reporter appear to be similar, AJs and baso-lateral complexes have completely different inputs into the Hippo pathway.

4.2.3 Disruptions of adherens junctions and baso-lateral complexes have different effects on tissue size

The phenotypes of AJ components in *Drosophila* imaginal discs have not been documented well. Homozygous *E-cad* and β*-cat* mutants are embryonic lethal, and their mutant clones poorly survive. The genetic locus of α*-cat* has prevented mutant characterization for many years. A recent study addressed that α*-cat* mutant phenotype is similar to the weak *arm* and *shg* allele (Sarpal et al., 2012). Using rescue constructs to perform clonal analysis, they found that α*-cat*

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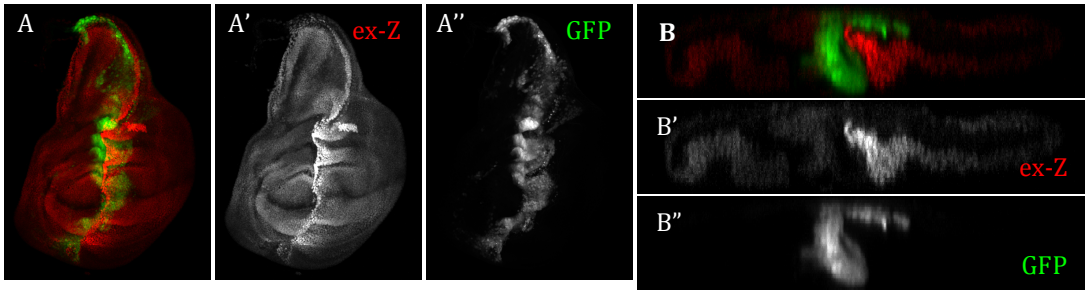
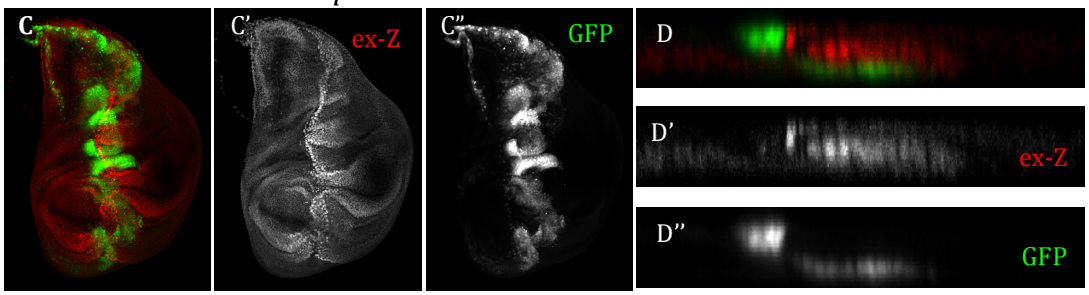


ptcGal4> UAS-DEcadRNAi UAS-GFP



ptcGal4> UAS-scribRNAi UAS-GFP

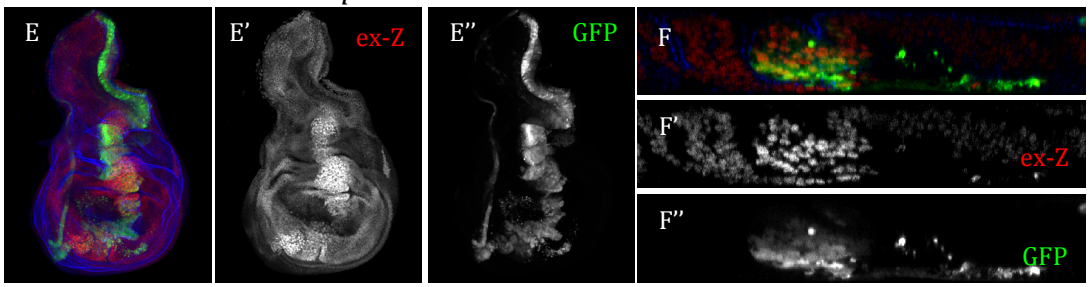


Fig 4.2

Figure 4.2 Knocking down of α-*cat* and *Ecad* show distinct inductions of Hippo pathway activity compared with knocking-down of *scrib*

Confocal images of wing imaginal discs. (A,C,E) *Drosophila* whole wing discs with various genetic background. (B,D,F) The Z-section of wing discs showing in A, C, and E respectively. Knocking-down of α-*cat* strongly induces *ex-lacZ* in wing discs (A), but strikingly, the cross section (B) reveals that α-*cat* causes cell autonomous decrease and non-autonomous increase of *ex-lacZ*. Similarily, knocking-down of *E-cad* also shows the same effect on *ex-lacZ* in the whole discs (C) and the cross section (D). Disruption of *scrib* induces mainly cell autonomous increase of *ex-lacZ*, as shown in whole discs (E) and Z-section (F).

mutant clones cannot survive. On the other hand, homozygous animals of *scrib*, *dlg* and *lgl* can survive to third instar larvae with giant larvae and massively large and poorly differentiated imaginal discs. These lines of evidence demonstrate the differences between AJs and baso-lateral complexes on growth regulation.

To examine if RNAi knock-down of AJs and baso-lateral complexes also behave like mutant situations, we use *nubbin-gal4* to disrupt their expression. Compared with the *UAS-GFP* control, knock-down of *α-catenin* in the wing pouch strongly reduced the pouch size (Fig 4.3B). On the other hand, knock-down of *scrib* did not have much effect on pouch size (Fig. 4.3C). Similar effects on tissue size can be observed when using *ey-Gal4* to knock-down *α-cat* and *scrib*, suggesting this effect is not tissue specific (Fig. 4.1C and D). Knocking-down of *α-cat* induced large amounts of apoptosis, as shown in cleaved caspase 3 staining (Fig. 4.3B). Nevertheless, *scrib* knock-down only shows a slight effect on apoptotic signals (Fig. 4.3C). We also performed BrdU incorporation in *α-cat* knock-down tissue. Compared with the evenly distributed signals in control tissue, the BrdU staining is reduced within the *α-cat* knock-down tissue but concentrated in the adjacent cells (Fig. 4.4). All together, these results suggested *α-cat* and *scrib* knock-down caused different effects on tissue size via regulating apoptosis and proliferation.

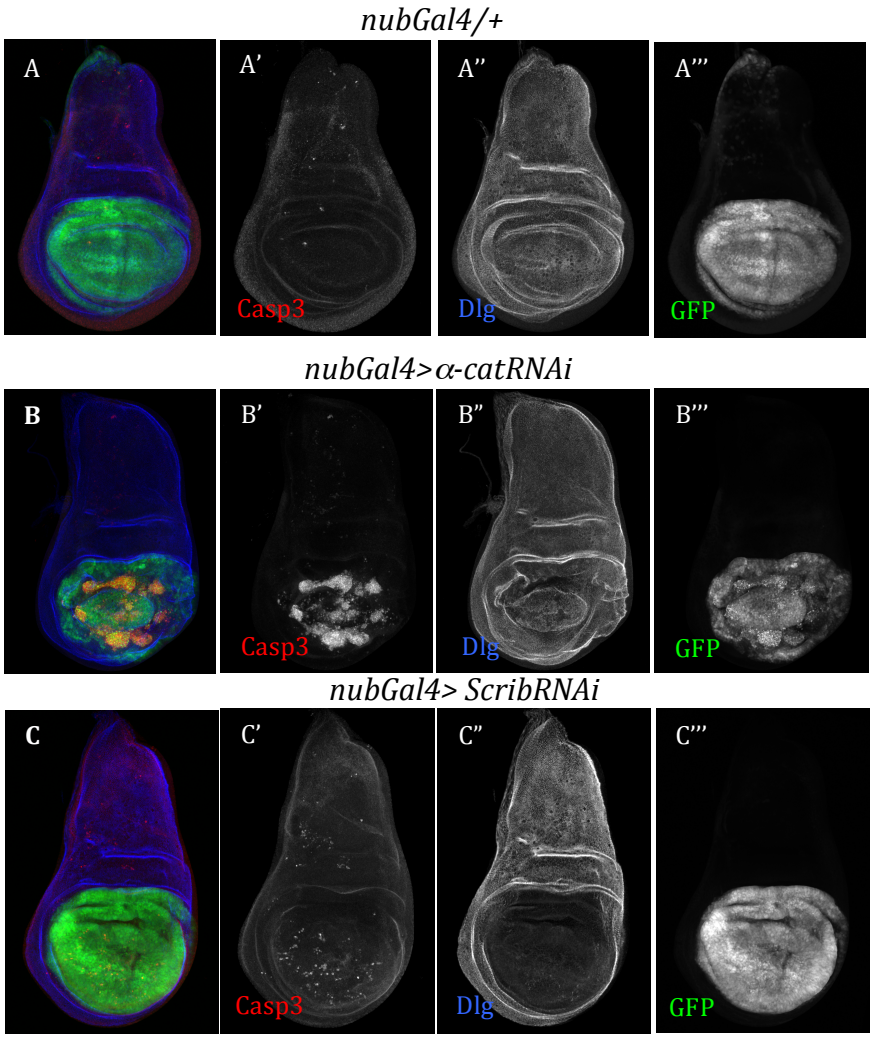

nubGal4/+
A
A'
A''
A'''
Casp3
Dlg
GFP
nubGal4>α-catRNAi
B
B'
B''
B'''
Casp3
Dlg
GFP
nubGal4> ScribRNAi
C
C'
C''
C'''
Casp3
Dlg
GFP

Fig 4.3

Figure 4.3 Knocking-downs of α-*cat* and *scrib* show differential induction of apoptotic signals in the wing pouch of *Drosophila*

Confocal images of wing imaginal discs. Using *nubGal4* wing discs expressing UAS-GFP only as a control (A), we compare the ability to induce apoptosis by α-*cat* (B) and *scrib* (C). Consistent with our previous result, knocking-down of α-*cat* induces apoptosis vigorously (B'), whereas knocking-down of *scrib* only show sparse signals of cleaved caspase 3 staining (C').

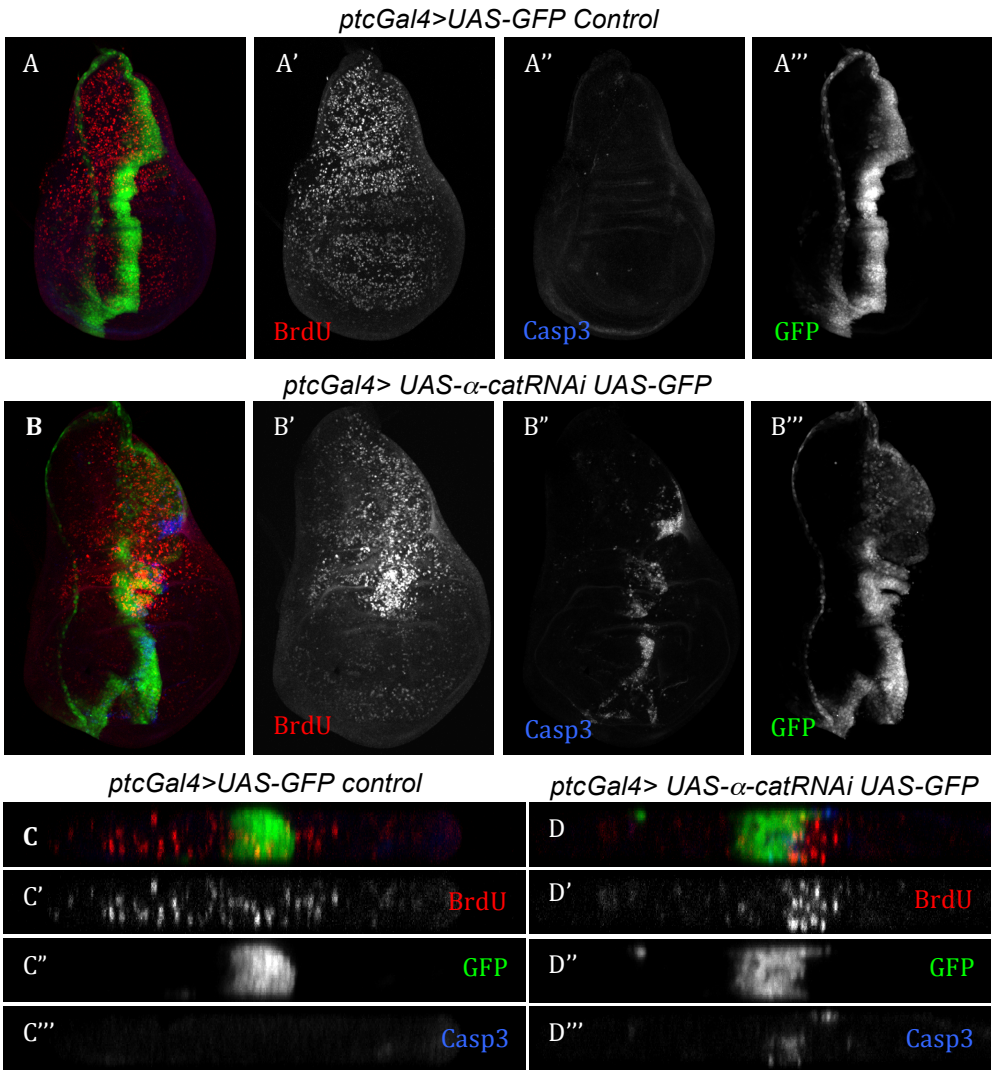

ptcGal4>UAS-GFP Control
A
A'
A''
A'''
BrdU
Casp3
GFP
ptcGal4> UAS-α-catRNAi UAS-GFP
B
B'
B''
B'''
BrdU
Casp3
GFP
ptcGal4>UAS-GFP control
ptcGal4> UAS-α-catRNAi UAS-GFP
C
D
C'
D'
BrdU
C''
D''
GFP
C'''
D'''
Casp3

Figure 4.4

Fig 4.4 Knocking-down of *α-cat* induces non-autonomous proliferation and autonomous cell death activation.

Confocal images of wing imaginal discs. (A and C) Control wing imaginal discs; (B and D) *α-cat* knock-down wing discs. Wild type tissues show uniform BrdU incorporation (A' and C') and very few cleaved caspase 3 signal (A''' and C'''). We observed an increase of BrdU incorporation in the neighboring tissues of *α-cat* knock-down (B' and D'), and a large amount of apoptosis is induced within the *α-cat* knock-down tissue (B''' and D''').

4.2.4 Disruption of AJs did not have a strong effect on baso-lateral protein localization, and vice versa

To gain more understanding about the possible explanation for the differences between AJs and baso-lateral complexes, we examined the effect on other apico-basal components when disrupting one of the complexes. We first evaluated α-*cat*. As we expected, knocking-down α-*cat* disrupted the junctional complexes, as shown in mislocalized DE-cad and α-Cat stainings (Fig. 4.5 C and E). At the same time, the localization of apical proteins, including aPKC, Crb, and Mer, are also missing from the apical membrane. Interestingly, baso-lateral protein Dlg localization is largely retained (Fig. 5 A), suggesting the baso-lateral membranes are still mostly intact. Knocking-down *scrib* clearly disrupted the baso-lateral complexes (Dlg staining in Fig. 4.5 B), however, AJ proteins DE-Cad and α-Cat are still largely localized properly (Fig. 4.5 D and F). It is thought the AJs, apical domain and baso-lateral junction are important for maintaining each other. Here our data suggested that in *Drosophila* knocking-down of AJs has a strong impact on apical domain maintenance but not on the baso-lateral membranes; similarly, knocking-down *scrib* also has only little effect on the AJ localization.

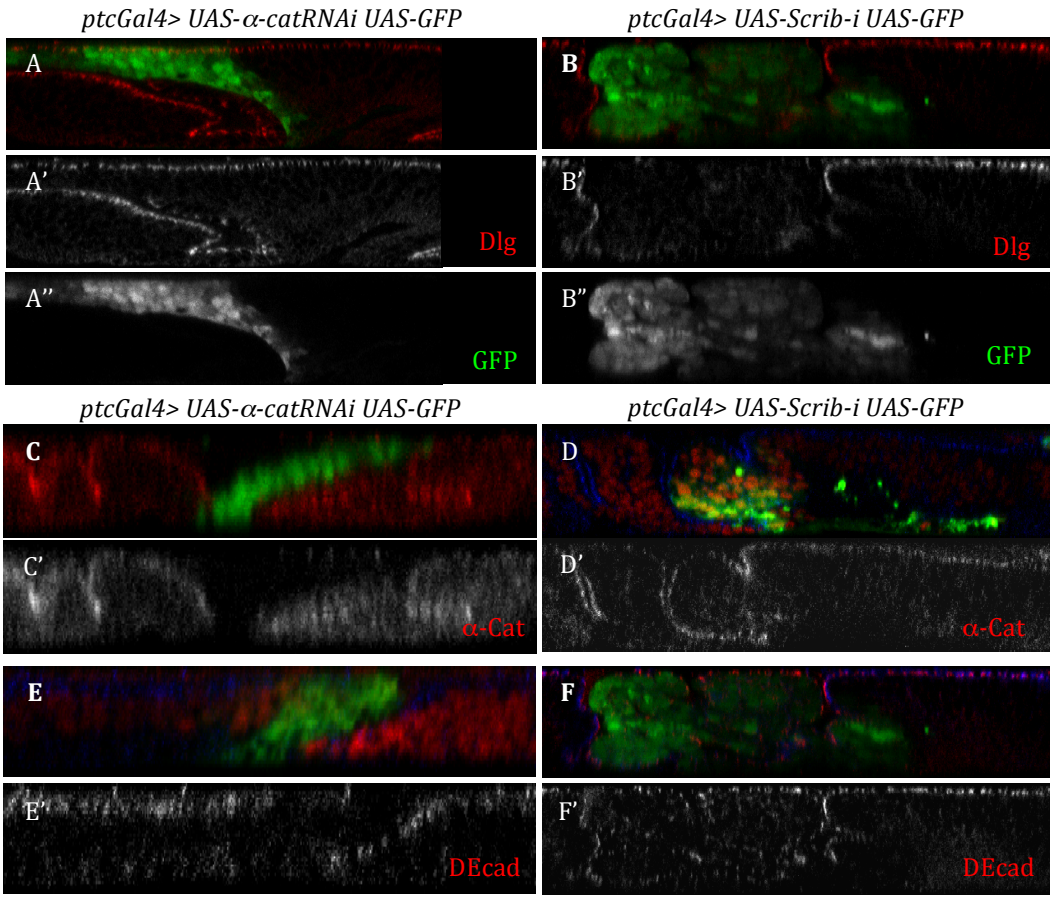

ptcGal4> UAS-α-catRNAi UAS-GFP
ptcGal4> UAS-Scrib-i UAS-GFP
A
B
A'
B'
Dlg
Dlg
A''
B''
GFP
GFP
ptcGal4> UAS-α-catRNAi UAS-GFP
ptcGal4> UAS-Scrib-i UAS-GFP
C
D
C'
D'
α-Cat
α-Cat
E
F
E'
F'
DEcad
DEcad

Figure 4.5

Figure 4.5 knocking-downs of α-*cat* or *scrib* does not affect the localizations of each other

Confocal images of wing imaginal discs. (A, C, and E) cross section images of α-*cat* knock-down wing discs; (B, D, and F) cross sections of *scrib* knock-down wing discs. In α-*cat* knock-down tissues, α-Cat and DE-Cad are lost as we expected (C' and E') but DLG is still largely maintain its normal localization. On the contrary, DLG is missing in *scrib* knock-down tissues (B') whereas α-Cat and DE-Cad are still presented at the plasma membrane (D' and F').

4.2.5 *α-cat* knock-down tissue does not exhibit the same synergistic effect with dominant negative JNK as with *scrib*$^{-}$

The c-Jun N-terminal kinase (JNK) is an important responding factor when cells face damage or any kind of stress. Once cells face stress, JNK signaling will be turned on and trigger the JNK signaling kinase cascade and respond quickly to the input. When apico-basal polarity changes, cells sense the defect and turn on JNK signaling. Once JNK signaling is activated, it will initiate apoptosis and eliminate the damaged cells. Since we observed significant cell death in *α-cat* knock-down tissues, we wondered if JNK signaling is activated in these tissues. Indeed, we found a significant increase of a JNK reporter in the *α-cat* knock-down region, shown as *puckered-lacZ* (*puc-lacZ*) staining (Fig. 4.6 A). These data suggested that *α-cat* knock-down tissue induced JNK signaling to eliminate itself. Interestingly, several studies reported that *scrib* mutant clones also induce JNK activation. As we expected, we also observed a significant induction of *puc-lacZ* in *scrib* knocked-down regions (Fig. 4.6 B). These results suggested whereas the phenotype on the Hippo pathway is different, knocking down of *α-cat* and *scrib* both show autonomous upregulation of JNK signaling.

Previous studies in our lab and many other groups have demonstrated there is a synergistic effect when blocking JNK activity in *scrib* mutant clones (Chen et al, 2012). When expressing dominant negative JNK in *scrib* mutant tissue, *scrib* mutant tissue overproliferated and induced excessive Hippo target

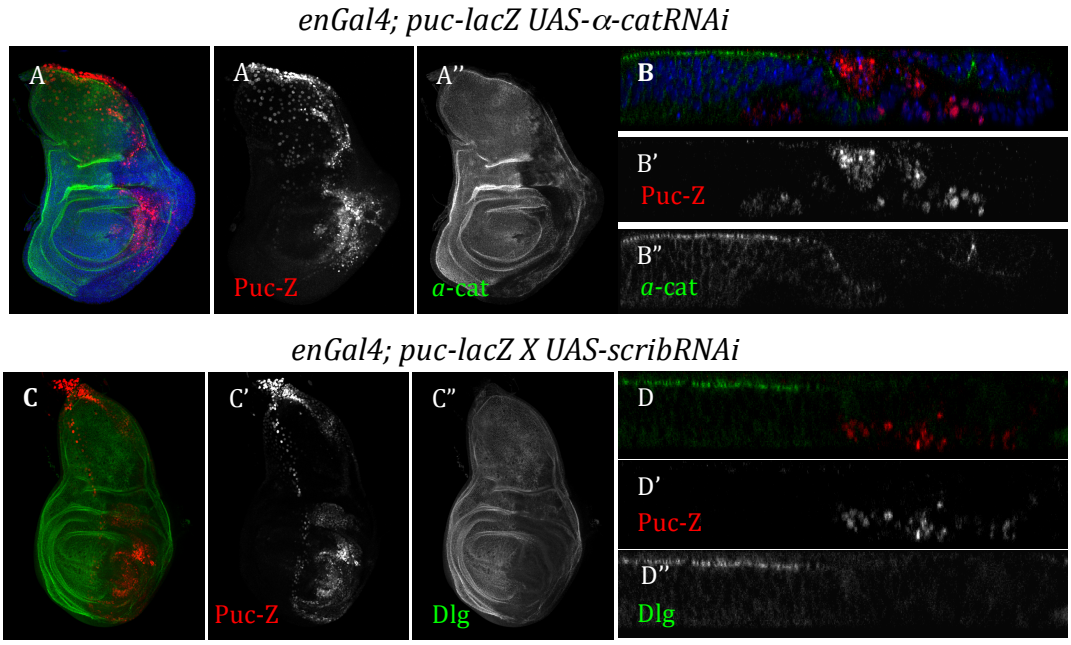


Figure 4.6 JNK activity is induced in both *α-cat* and *scrib* knock-down tissues

Confocal images of wing imaginal discs. (A and C), Whole wing discs; (B and D) corresponding z-sections through the discs in A an C. Knocking downs of *α-cat* (A' and B') and *scrib* (C' and D') both induce autonomous *puckered-lacZ* expression. Stainings of α-Cat in (B'') demonstrates that the *α-catRNAi* work properly and DLG in (D'') indicate that *scribRNAi* successfully disrupt the basolateral complex localization.

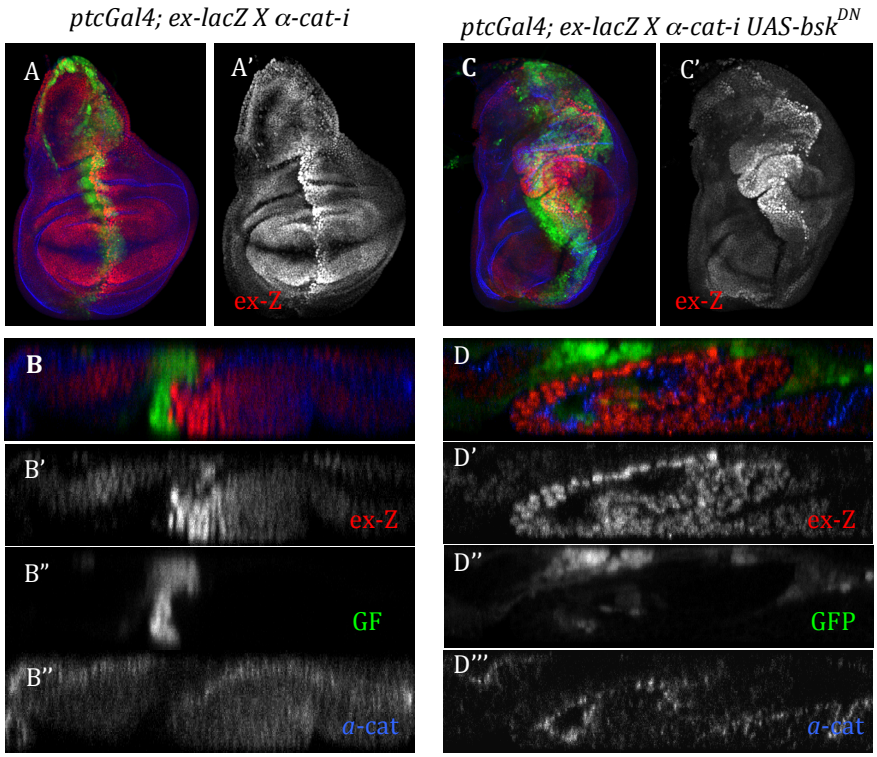


Figure 4.7 Blocking JNK activity do not induce cell autonomous Hippo target activation in *α-cat* knock-down cells

Confocal images of wing imaginal discs. (A and B) *α-cat* knock-down wing discs; (C and D) *α-cat* knock-down + *bsk*^{DN} overexpressing wing discs. When blocking JNK activity by *bsk*^{DN}, the ex-lacZ is still induced (C') compared with *α-cat* knock-down only tissues (A'). Nevertheless, the GFP positive *α-cat* knock-down cells still do not activate intrinsic expression of *ex-lacZ* (D').

expression (Chen et al., 2012; Leong et al., 2009; Ohsawa et al., 2011). To determine the roles of JNK activation in *α-cat* and *scrib* mutant tissues, we disrupted the JNK activity by ectopically expressing dominant negative *basket* (bsk^{DN}), the JNK ortholog in *Drosophila*, in *α-cat* and *scrib* tissues. When we blocked JNK activity in *α-cat* knock-down tissue, we observed an increase of GFP expressing region (Fig. 4.7 C and D). This effect should be the result of reduced apoptosis by blocking JNK activity. Surprisingly, the *ex-lacZ* level strongly increased in the adjacent tissue but not in cells co-expressing *α-catRNAi* and bsk^{DN}. This result implies that the regulation of the Hippo pathway in *α-cat* knock-down tissue is not JNK dependent. In summary, although both *α-cat* and *scrib* knock-down both induced JNK activity, they showed divergent effects in promoting growth with impaired JNK signaling.

4.2.6 Scribble and adherens junctions work in parallel to regulate the Hippo pathway

The effect of *α-cat* knock-down on Hippo pathway reporter expression is intriguing since it has been demonstrated that knocking-down *α-cat* in mammalian keratinocytes induces Yap activity (Schlegelmilch et al., 2011; Silvis et al., 2011), which contradicts our observation. Also, the fact that the *scrib* mutant tissue showed different effects on induction of the Hippo reporter could suggest different mechanisms in regulating the Hippo pathway. Interestingly, recent studies implied Scrib forms a protein complex with Lats1/2 and Mst1/2 in

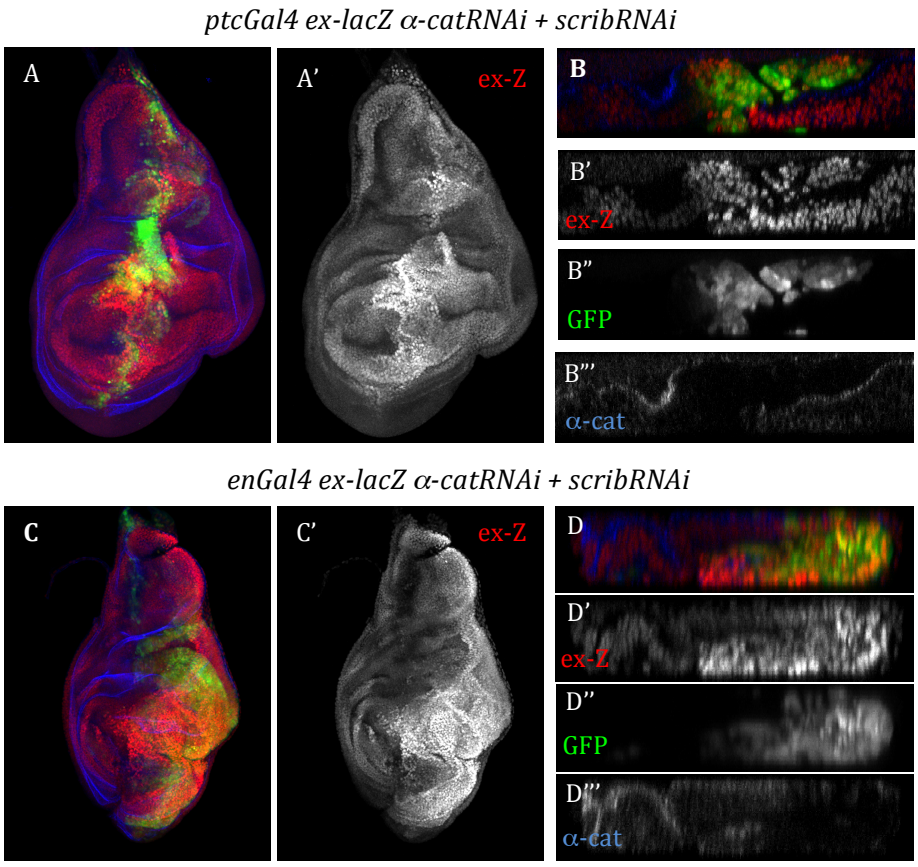


Figure 4.8 Combinatorial effects on *ex-lacZ* in *α-cat* and *scrib* double knock-down tissues

Confocal images of wing imaginal discs. *Drosophila* wing imaginal discs coexpress *α-catRNAi* and *scribRNAi* by *ptcGal4* (A and B) or enGal4 (C and D). When we coexpress *α-catRNAi* and *scribRNAi*, the *ex-lacZ* is induced both cell autonomously and non-autonomously (A' and C'). Cross sections clearly demonstrated the cell autonomous and non-autonomous induction of *ex-lacZ* in double knock-down tissues (B' and D').

mammals and delocalization of Scrib leads to deregulation of Hippo pathway activity (Cordenonsi et al., 2011). In *α-cat* knock-down tissue, we only observed fairly normal baso-lateral module localization. This result made us wonder if the properly localized basolateral module was able to suppress activation of Hippo targets in *α-cat* knock-down tissues. To test this hypothesis, we performed a double knock-down experiment where we overexpressed *α-catRNAi* and *scribRNAi* at the same time (Fig. 4.8). α-Cat and Dlg stainings suggest that both AJ and basolateral complexes were successfully disrupted (Fig. 4.8A). Indeed, we now observed the induction of *ex-lacZ* in the double knock down region (Fig. 4.8A' and B'). In addition, the non-autonomous induction of *ex-lacZ* in α*-cat* knock-down tissue is still present, suggesting *α-cat* might have a novel regulatory mechanism to regulate the Hippo pathway through interactions with neighboring tissues. In summary, our data suggest that *scrib* works in parallel with *α-cat* in regulating the Hippo pathway.

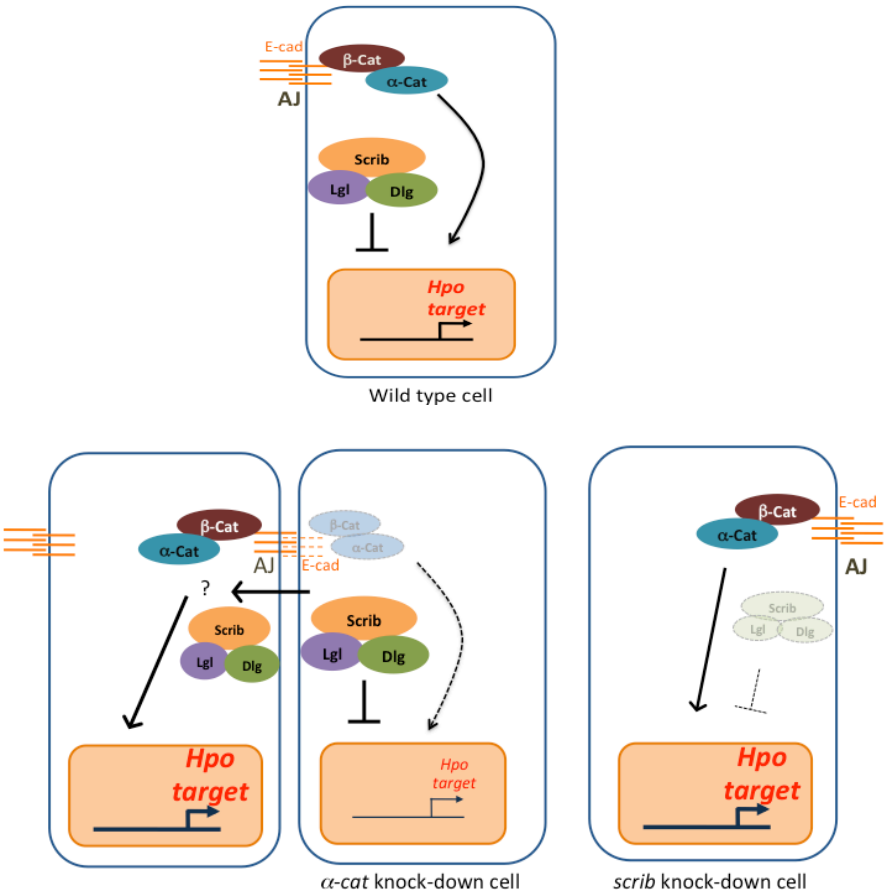


Figure 4.9 Model of AJs and basolateral complex in Hippo pathway regulation

Based on our study, we propose a model that in wild type cells, basolateral complexes inhibit Hippo target activation where AJs promote Hippo target activation. Disruption of *α-cat* does not induce cell autonomous activation of Hippo targets. Rather, it causes strong induction of the Hippo reporter in the neighboring cell. Knocking-down *scrib* results in the loss of the inhibitory signal from basolateral complexes and induction of cell-autonomous Hippo target activation.

4.3 Discussion:

In this study, we first characterize the function of α-cat in *Drosophila*. We found two genuine *α-cat* alleles and both of them can be rescued by ectopically expressing α-Cat. We then address the fundamental differences between AJs and basolateral complexes in cell-autonomous growth regulation. We find that knock-down of AJs and basolateral complexes both induce ectopic expression of Hippo reporters, though loss of AJs cause mainly non-autonomous induction of reporters, while loss of the basolateral module induces an autonomous increase in reporter activity. We also saw a differential regulation in apico-basal protein localization when knocking down *scrib* and *α-cat*. In addition to their different effects on Hippo signaling, *scrib* knock-down can cooperate with dominant negative JNK to cause oncogenic overgrowth, but *α-cat* knock-down cells do not have the same synergistic effect. Interestingly, when we co-expressed *α-catRNAi* and *scribRNAi*, we observed an increase of *ex-lacZ* expression in both the knock-down region and it adjacent tissues. These data suggested *scrib* and *α-cat* regulate the Hippo pathway through distinct inputs.

4.3.1 Characterization of *α-cat* mutant alleles

α-cat is a major component of adherens junctions and has been identified fdecades ago. While many studies addressed the function of the other two components- *E-cad* and *β-cat*, surprisingly, the mutant of *α-cat* was not reported

in *Drosophila*. The centromeric locus of α*-cat* resulted in hampering the progress on studying it since the location is more difficult to access, and the genetic tools that are commonly used for clonal analysis cannot work in this circumstance. We characterized two alleles that phenocopy mutants of other AJ components and can be rescued by ectopically expressing α*-cat*, suggesting they are real mutants for α*-cat.* Another research group acquired different p-element insertions and generated null mutants (Sarpal et al., 2012). The phenotypes from their null alleles and the two insertion alleles we used are similar, suggesting our two alleles are functionally null mutants. Therefore, these two alleles can be used for future studies as α*-cat* mutants. Meanwhile, although we cannot perform clonal analysis for α*-cat* mutants, genomic rescue constructs can be generated by inserting the duplicated chromosome into another site which allows us to make a genomic rescue construct. This construct will be created for future research.

4.3.2 Non-autonomous effect in α*-cat* mutant

The non-autonomous effect we observe in *α-cat* knock-down tissue is an intriguing phenomenon. Several groups have reported non-autonomous effects on Hippo pathway regulation in different mutant contexts. Studies from our lab have shown that disrupting the gradients of Dachsous and Four-jointed expression can induce non-autonomous activation of the Hippo pathway (Willecke et al., 2008). Furthermore, overexpression of the pro-apoptotic gene *reaper*, the JNK signaling activator *eiger*, or discs mosaic for mutations in *hyd* all

display non-autonomous activation of Yorkie signaling, which is speculated to be part of a regenerative response (Grusche et al., 2011). Though we chose to focus on the autonomous effects of loss of AJs or basolateral components in this study, we speculate that one possibility for the mechanism of this non-autonomous effect is that changes in mechanical force can transduce changes in Hippo signaling. Zyxin, a potential sensor for the mechanical force, has been reported as a regulator of the Hippo signaling (Rauskolb et al., 2011). Furthermore, Yap/Taz have been identified as sensors and mediators of mechanical cues from the cellular microenvironment. AJs are important for maintaining surface tension across epithelia, and this feature fits the possibility that disruption of AJs could lead to imbalance of apical tension and could induce distant cells to proliferate in order to regenerate tissue as a whole. Further studies will shed light on this hypothesis, as well as provide important insight into understanding how sick cells interact with their neighboring cells, which could have important implications for our understanding of tumor biology.

4.3.3 Relationship between AJs and other polarity complexes

The mutant phenotype of *α-cat* in *Drosophila* is surprising considering recent reports in mammalian cells (Schlegelmilch et al., 2011; Silvis et al., 2011). In *Drosophila*, both *E-cad* and *α-cat* mutants are embryonic lethal and mutant clones do not survive, while in mammals, knock-down of *E-cad* causes an increase in cell proliferation. Several groups have reported that conditional

knock-out of *α-cat* in mouse skin cells induce lesions and results in early lethality (Schlegelmilch et al., 2011; Silvis et al., 2011). The knock-out animals also developed tumors with elevated nuclear Yap staining in mutant tissues, suggesting a role for *α-cat* in Hippo pathway regulation (Schlegelmilch et al., 2011; Silvis et al., 2011). The effect on Yap nuclear localization can also be found in cancer patient samples. How then do we reconcile the results in *Drosophila* to the results in mammalian systems? Our genetic evidence suggests that Scrib works downstream of AJs in regulation of the Hippo pathway. This may indicate that in specific mammalian cell types, loss of AJ proteins also causes defects in basolateral protein localization or function, and therefore induces deregulation of the Hippo pathway. As mentioned previously, studies in breast cancer cell lines have demonstrated that hScrib binds to TAZ and therefore sequesters it in the cytoplasm, thus restraining its oncogenic capability (Cordenonsi et al., 2011). Another study found that knock-down of AJ component *E-cad* disrupted hScrib localization (Qin et al., 2005). These results imply that disruption of AJs in mammalian cells can trigger mislocalization of the basolateral module. Importantly, the effects of *α-cat* knock-out have only been shown in keratinocytes or tumors derived from those cells. Therefore, it is reasonable to speculate that the regulation of the Hippo pathway through AJs is cell-type or context dependent. Specifically, we would hypothesize that in situations where apico-basal polarity is disrupted, the loss of the basolateral complex would be the primary disruption that leads to deregulation of Hippo signaling. More studies need to be performed to provide further clarification of this hypothesis.

4.3.4 Differential effects on complex localizalization when disrupting polarity proteins and AJ components

Another interesting aspect of our study is the fact that loss of AJs does not lead to loss of the basolateral complex in imaginal disc cells. During apico-basal polarity establishment, apical protein complexes and the basolateral module mutually restrict each other's localization, while AJs demarcate the boundary between apical and basolateral domains. Numerous studies have described roles for apical and basolateral proteins in polarity regulation, but how AJs contribute to apico-basal polarity is still poorly understood. Gladden et al. reported that Mer binds to *α-cat* and thus links AJs to the Par-3 complex, demonstrating an important role for apical protein complexes in AJ maturation (Gladden et al., 2010). Consistently, our findings suggest that knock-down of AJs disrupts the proper localization of apical proteins, such as aPKC and Mer. However, there is only little effect on baso-lateral module localization. In contrast, we found that knock-down of the basolateral complex component *scrib* disturbs the formation of basolateral modules, but it does not affect AJ formation. Consistent with our study, Richardson's group showed that *lgl* mutant cells still have proper AJ formation, supporting our finding that basolateral polarity complexes and AJs can exist independently in imaginal disc cells (Grzeschik et al., 2010a). Interestingly, a study in mammalian cell lines implied Scrib is required for stabilizing AJs (Qin et al., 2005). While contradictory to our observations in imaginal disc cells,

together these data might suggest the existence of more distinct regulatory mechanisms between AJs and polarity components in different cell types.

In closing, several recent studies have described that Hippo pathway components are apically localized, and suggested that this localization is important for pathway activity (Genevet and Tapon, 2011; Grzeschik et al., 2010a; Halder and Johnson, 2011). Also, the apical proteins aPKC and Crb have been proven to modulate the activity of the Hippo pathway (Grzeschik et al., 2010a). Our data contributes a new point of view to these findings. Specifically, when AJs are disrupted, despite the fact that aPKC and Crb are mislocalized, there is a lack of Hippo reporter activation cell autonomously. Our result could potentially put AJs downstream of apical proteins in regulating the activity of the Hippo pathway. More investigation is needed to further prove this point.

Chapter 5:

The actomyosin cytoskeleton regulates tissue growth through modulating the Hippo pathway in *Drosophila*

5.1. Introduction

The Actomyosin cytoskeleton has been described in regulation of a large set of biological processes, including mitosis, vesicle trafficking, and cell mobility (Gorfinkiel and Blanchard, 2011; Samuel et al., 2011; Yonemura, 2011a). Two major components of actomyosin are the structural protein actin and the motor protein non-muscle myosin II. Although functions of actomyosin have been largely characterized, knowledge about their role in tissue growth is limited. Recent works from our lab and Janody lab indicate that modifying F-actin regulates tissue growth through the Hippo pathway (Fernandez et al., 2011; Sansores-Garcia et al., 2011). Additionally, non-muscle Myosin II also interacts with tumor suppressor gene *lethal giant larvae (lgl*) (Strand et al., 1995), which affects the Hippo pathway. Both lines of evidence suggest non-muscle Myosin II may affect growth, in particular through regulation of Hippo pathway activity. Therefore, we hypothesize that non-muscle Myosin II regulates tissue growth through modulating Hippo pathway activity.

We first knocked down the expression of non-muscle Myosin II heavy chain Zipper (Zip) and regulatory light chain Spaghetti-squash (Sqh) and found a prominent overproliferation phenotype. We next examined the expression of Hippo pathway reporters and found a significant increase of these reporters, indicating that Hippo pathway activity is suppressed when Myosin II is knocked-down. Also, upstream regulators of non-muscle myosin II mimic the effects of knocking down non-muscle myosin II. We also found hyperactivated myosin II

also regulates the Hippo pathway, possibly through increasing the amount of F-actin. Taken together, our research provides a link between the cytoskeleton and growth control regulation.

5.2 Results:

5.2.1 Knock-down of myosin II components induces cell proliferation

To understand if myosin II plays a role in growth regulation, we first tested if there is any growth phenotype in myosin II knock-down tissue. Using *ptc-Gal4* to knock-down myosin II heavy chain *zip* expression, we found a significant morphological expansion of the knock-down region (Fig. 5.1B"). We then checked if this tissue expansion is correlated with cell proliferation. BrdU incorporation clearly showed an increase in staining within *zip* knock-down tissues (Fig.5.1B'). Similar results also can be observed when knocking-down *zip* by *en-Gal4* (Fig 5.1E). This result is surprising since non-myosin II is considered an essential gene, therefore tissues should not be able to survive when disrupting its expression. Rather, we saw an increase of cell proliferation, suggesting a novel function for non-muscle myosin II. To confirm that this effect is caused by disruption of non-muscle myosin II, we also tested the non-muscle myosin II regulatory light chain *sqh*. As expected, knock-down *sqh* also showed an increase in BrdU incorporation (Fig. 5.1 C'). Together, our results demonstrate that disruption of myosin II induces cell proliferation.

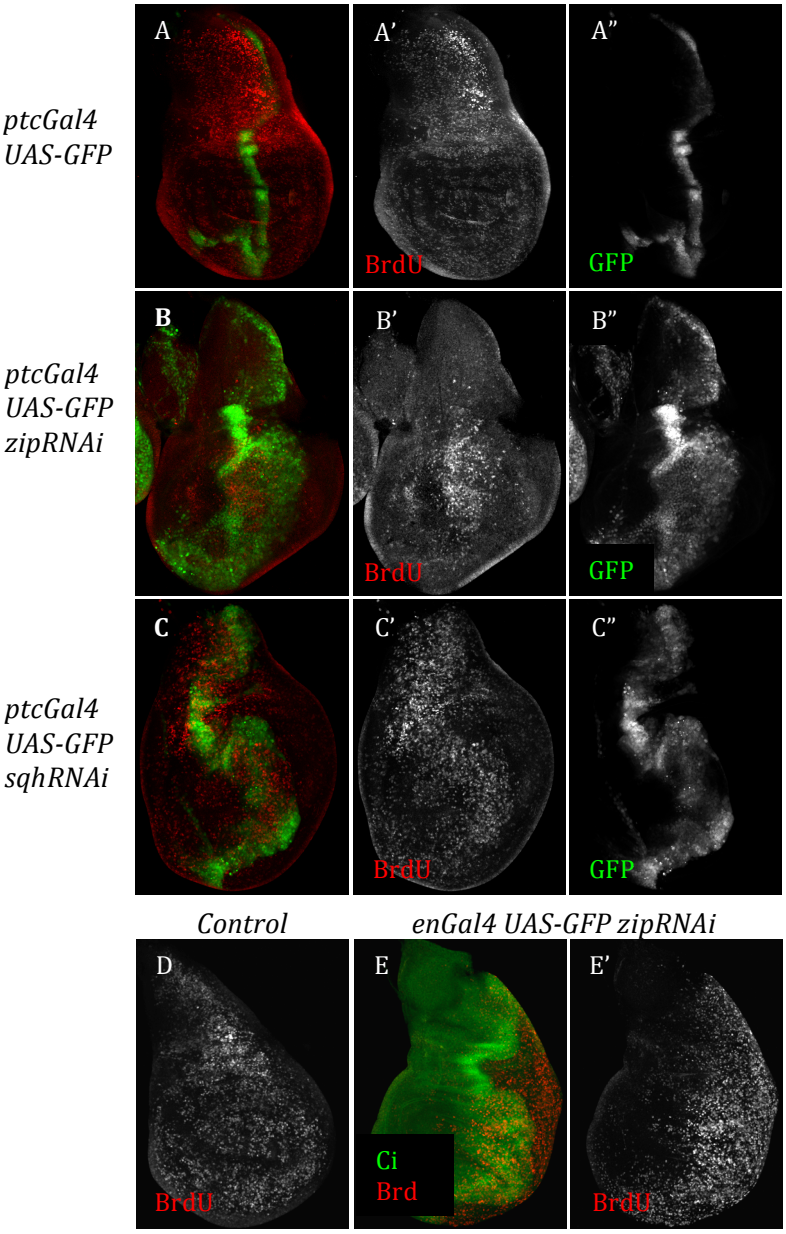

ptcGal4
UAS-GFP
A
A'
A"
BrdU
GFP
ptcGal4
UAS-GFP
zipRNAi
B
B'
B"
BrdU
GFP
ptcGal4
UAS-GFP
sqhRNAi
C
C'
C"
BrdU
GFP
Control
enGal4 UAS-GFP zipRNAi
D
E
E'
BrdU
Ci
Brd
BrdU

Fig 5.1

Figure 5.1 Disruption of non-muscle Myosin II induces cell proliferation

Confocal images of *Drosophila* wing imaginal discs. (A-C) BrdU incorporations in control and myosin II knock-down tissues with *ptcGal4* driver; (D) a wild type control for *enGal4>zipRNAi* tissue (E). Compared with Control (A' and A''), knocking-downs of zip and *sqh* by *ptcGal4* both cause GFP region expansion (B'' and C'') and show more proliferation within the ptcGal4 expression domains (B' and C'). We saw a very similar phenotype when using enGal4 to express *zipRNAi*.

5.2.2 Myosin II regulates Hippo pathway activity

Since knock-down of non-muscle myosin II components induced cell proliferation, we wondered which signaling pathway might be affected and contributed to this phenotype. As mentioned earlier, myosin II interacts with *lgl* and the actin cytoskeleton, and both of them have been shown to regulate Hippo pathway activity (Fig. 5.2). Therefore, we assayed expression of the Hippo pathway reporter *ex-lacZ*. Indeed, knocking-down *zip* and *sqh* both showed clear elevation of *ex-lacZ* staining. To further confirm that myosin modulates Hippo pathway activity, we also examined other reporters: Wingless (Wg) protein, *Diap1-GFP*, and Yki localization. As shown is Figure 5.3, *Diap-1GFP* expression is also strongly increased in both *zip* and *sqh* knock-down tissue (Fig. 5.3B and C). Wg is normally expressed as a ring structure at the outside region of the wing pouch and at the dorsal-ventral boundary of the wing pouch. When we used *ptc-gal4* to knock-down myosin expression, we clearly observed an ectopic increase of Wg expression along the anterior-posterior boundary (Fig. 5.3B and C). Finally, Yki nuclear localization is the most stringent assay of all of the Hippo pathway reporters, and we were able to observe Yki translocated into the nuclei when *sqh* is knocked-down (Fig 5.3G). Together, these results strongly suggest that myosin regulates Hippo pathway activity.

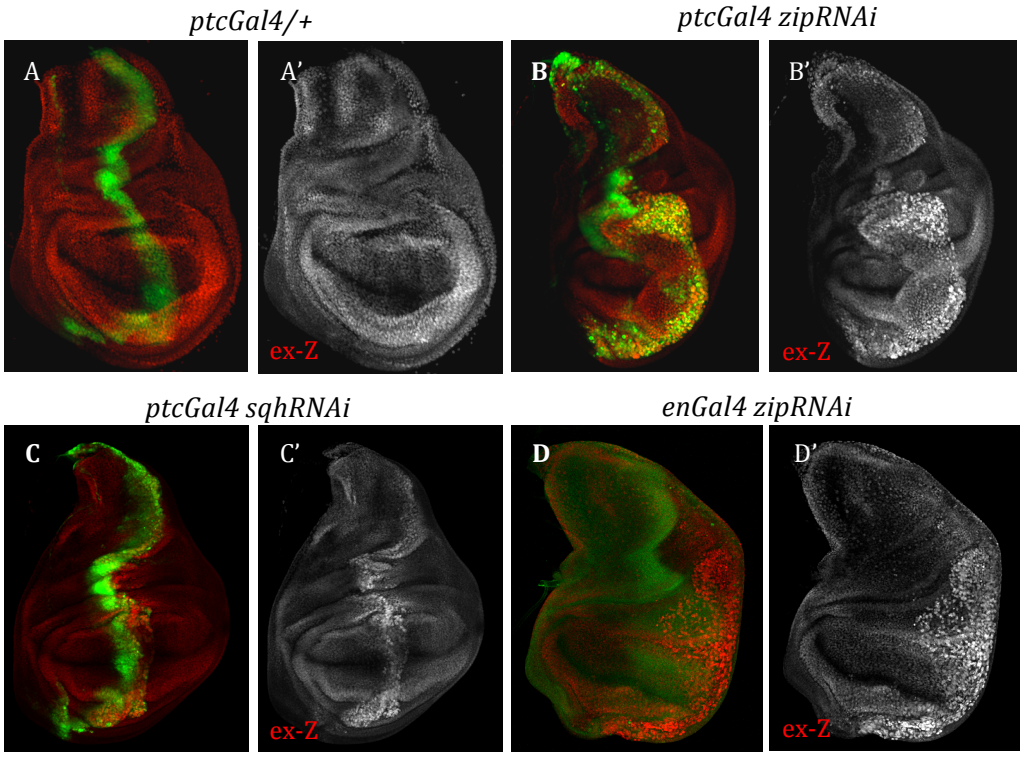


Figure 5.2 Knocking down of myosin II induces *ex-lacZ*

Confocal images of wing imaginal discs. Discs are stained with *b-gal* to reveal *ex-lacZ* expression, which is a Hippo pathway reporter. (A) wild-type disc shows the *ex-lacZ* expression pattern. Knocking-down of *zip* (B) and *sq*h (C) both show induction of *ex-lacZ*. Another driver enGal4 is used to knock down zip (D), and we observed similar induction of *ex-lacZ* in this situation.

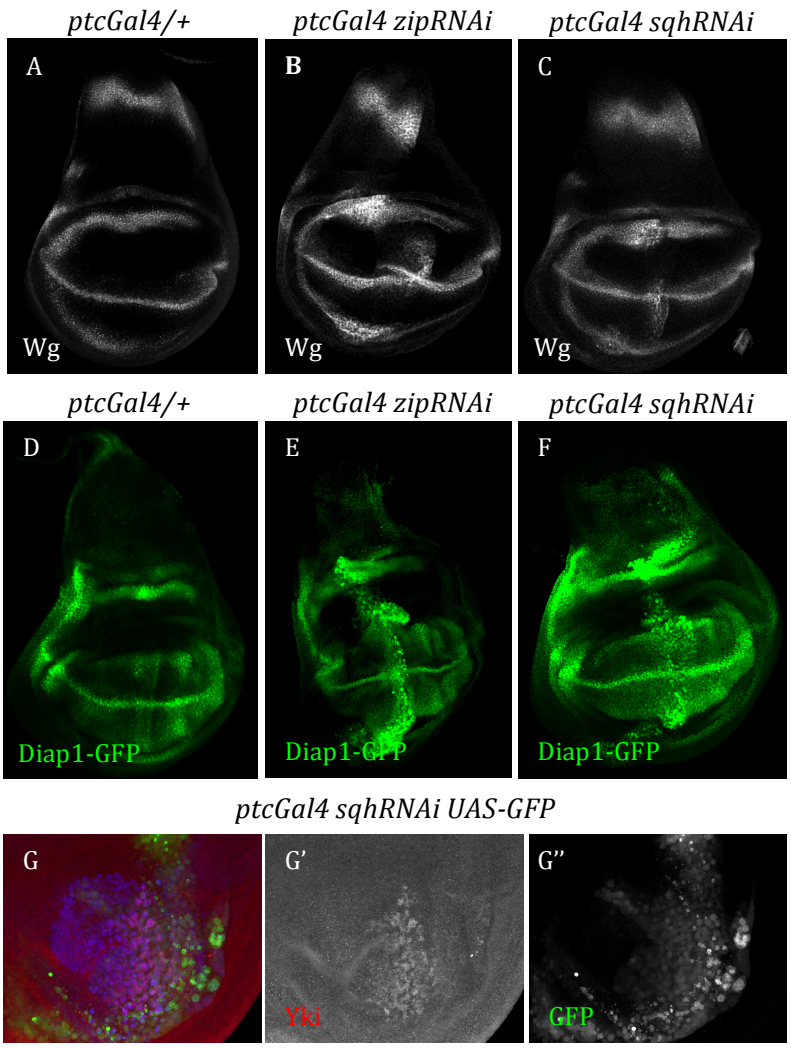


Fig 5.3 Knocking down of myosin II induces the expression of several Hippo targets

Confocal images of *Drosophila* wing imaginal discs. (A-C) Wingless (Wg) staining; (D-F) *Diap1-GFP* reporter; (G-G'') Yki staining. Compared with control discs (A and D) knocking downs of zip and sqh show increase signals of Wg (B and C) or *Diap1-GFP* (E-F) reporter. G shows the Yki translocates into nucleus in sqh knock-down tissue (G').

5.2.3 Myosin II does not alter other growth pathway activities

We found that the disruption of Myosin II strongly induced the Hippo pathway. To understand if myosin knock-down only affects Hippo pathway activity, we examined the activities of several different growth related pathways. The Notch pathway is an essential regulator of cell-type specification events such as lateral inhibition, as well as cell proliferation. One Notch downstream target gene is *cut*, a homeodomain protein that regulates wing margin development under the control of Notch. Cut is expressed in a few rows of cells at the Dorsal-ventral (D-V) boundary in the wing pouch. When we examined Cut staining, we noticed the cells have enlarged size within the *zip* knock-down region (Fig. 5.4 F'). The numbers of cut expressing cells are still the same, and we did not observe significant changes of the amount of total protein in *zip* knock-down cells (Fig. 5.4F'). These data suggest Notch signaling is not affected when disrupting myosin II. We also checked the Hedgehog (Hh) signaling pathway, which is important for *Drosophila* segment polarity at both the embryonic stages and later in development. Hh protein is secreted from the posterior part of the wing to the anterior compartment and stabilizes Cubitus interuptus (Ci) protein. Since ptc-gal4 is expressed at the border of the anterior and posterior (A-P) compartment, overlapping with the boundary of Ci staining, it is difficult to judge the change in expression of Ci. To solve this problem, we used nub-gal4 to knock-down *zip* in the whole wing pouch and compare the levels of Ci protein. Again, we did not see any obvious change in Ci pattern, suggesting that Hh signaling is not altered (Fig. 5.4B). Finally, we looked at the Dpp pathway. Dpp is a morphogen that

participates in patterning and controlling growth and size of tissues. Phosphorylation of Mothers against Dpp (pMad) is considered a reliable reporter for Dpp activity. The staining pattern of pMad is largely along the A-P boundary, and there is no apparent difference in *zip* knock-down tissues compared to wild type animals (Fig 5.4C and D). In summary, knock-down of *zip* specifically induces Hippo pathway activity, but does not appear to alter the activity of other growth control pathways.

5.2.4 Myosin II works downstream of Wts to regulate Hippo pathway activity

Our data suggested disruption of Myosin II modulates Hippo pathway activity. To understand where myosin II genetically acts to regulate the Hippo pathway, we conducted an epistasis experiment. Overexpression of Ex, Mer, Hpo, and Wts have been shown to activate the Hippo pathway and results in suppression of *ex-lacZ* and reduction of tissue size. We expressed each of them together with *zipRNAi* and looked at the effect on *ex-lacZ*. Interestingly, we found *ex-lacZ* levels in all of them are still highly elevated compared with non-overexpressing regions. These data suggest myosin II is epistatic to Ex, Mer, Hpo and Wts, and therefore places it downstream of Wts in regulating the Hippo pathway.

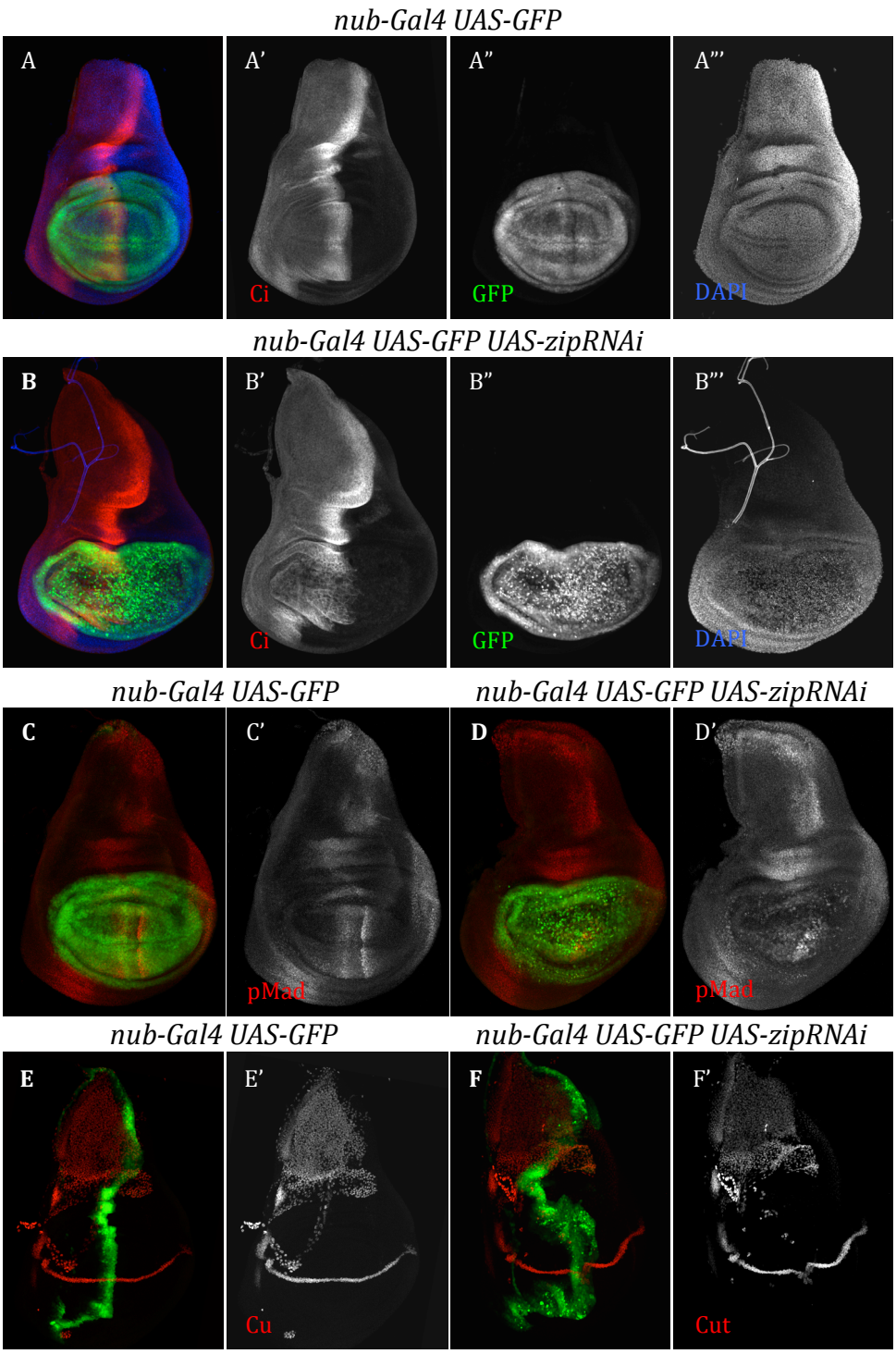

nub-Gal4 UAS-GFP
A
A'
A''
A'''
Ci
GFP
DAPI
nub-Gal4 UAS-GFP UAS-zipRNAi
B
B'
B''
B'''
Ci
GFP
DAPI
nub-Gal4 UAS-GFP
nub-Gal4 UAS-GFP UAS-zipRNAi
C
C'
D
D'
pMad
pMad
nub-Gal4 UAS-GFP
nub-Gal4 UAS-GFP UAS-zipRNAi
E
E'
F
F'
Cu
Cut

Fig 5.4

Figure 5.4 Knocking down of *zip* does not affect other signaling pathway activity

Confocal images of *Drosophila* wing imaginal discs. (A-D) Control or RNAi knock-down under the control of *nub-Gal4*. Compared with the control (A' and C'), *hh* pathway target Ci and *dpp* pathway reporter pMad do not show obvious effect in *zip* knock-down tissue. (E and F) Control tissue and *zip* knock-down tissue under the *ptcGal4* driver. In *zip* knock-down tissue, the Notch reporter Cut protein does not exhibit obvious change (F').

5.2.5 Upstream regulators of myosin II also regulate Hippo pathway activity

The activity of Myosin is dependent on the phosphorylation status of its regulatory light chain. One of the regulatory signals for myosin II activity is through the Rho signaling pathway. Rho affects myosin activity through phosphorylation of myosin light chain by its downstream effector Rok. Since we found myosin II as a new regulator for the Hippo pathway, we sought to understand if these upstream regulators were also able to signal to the Hippo pathway. Consistent with the myosin II knock-down results, we also see a significant induction of *ex-lacZ* and *Diap1-GFP* when Rho is knocked-down, suggesting upstream regulators of myosin might also regulate the Hippo pathway (Fig. 5.5B and E). Also, *ex-lacZ* and *Diap1-GFP* both showed increased expression when Rok is disrupted (Fig. 5.5C and F). The effects of Rok knock-down on Hippo reporters are relatively mild but reproducible, and it showed the same results when we used two independent RNAi lines. Thus, we identified Rho signaling as an upstream input to regulate the Hippo pathway through modifying Myosin II activity.

5.2.6 Disruption of Myosin and its upstream regulators strongly induces apoptosis

Knock-down of Myosin II and its upstream regulators showed increased BrdU incorporation and elevated *Diap-1 GFP* levels. Since studies have shown

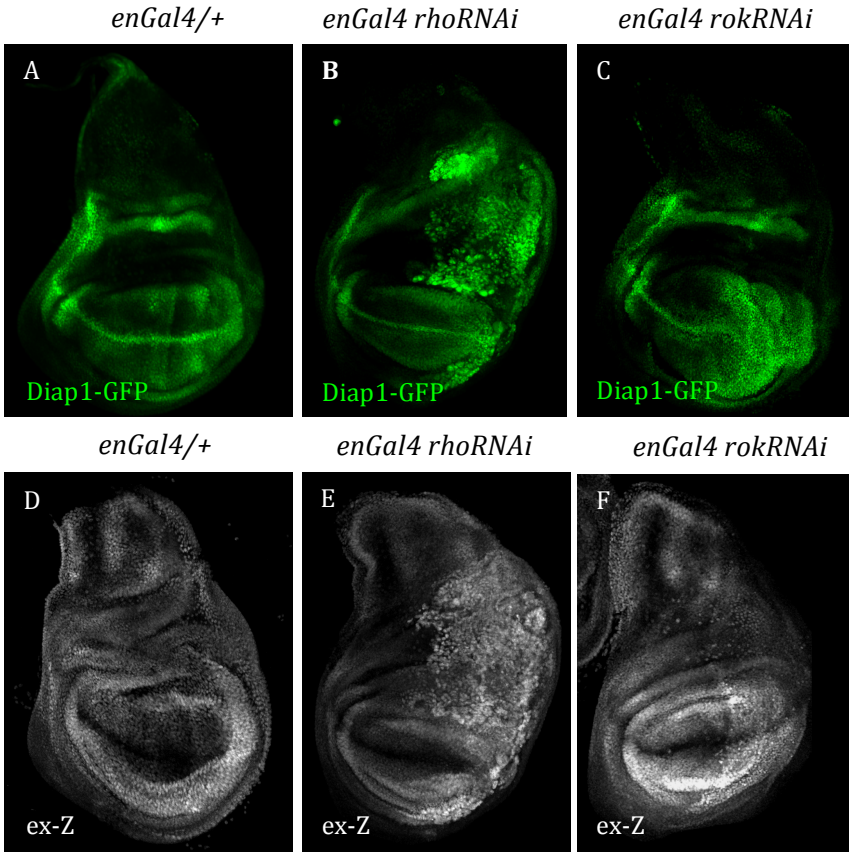


Figure 5.5 Upstream regulators of myosin II also affect Hippo signaling

Confocal images of *Drosophila* imaginal discs. (A-C) *diap1-GFP*; (D-F) *ex-lacZ* staining. Rho singaling regulate myosin through its downstream Rok. Knocking-down of rho strongly induces both *diap1-GFP* and *ex-lacZ* expression. Knocking-down of rok consistently shows mild effect yet noticeable on *diap1-GFP* and *ex-lacZ*.

that Hippo pathway activation suppresses cell death through Diap1 expression, we speculated that there should be less apoptosis signals in myosin II knock-down tissues. Caspase 3 is one of the downstream kinases of the apoptosis pathway. The cleavage of caspase 3 by intiating caspase will release its activity and trigger apoptosis. When we performed the cleaved caspase 3 staining, surprisingly, we observed a dramatic increase of caspase 3 activity in *zip* and *sqh* knock-down tissues (Fig. 5.6B and C). To further confirm this result, we also performed cleaved caspase 3 staining and found drastically increased cell death in *rho* knock-down tissue (Fig. 5.6 D). Therefore, our data implied disrupting myosin II induced both cell proliferation and cell death.

5.2.7 Blocking cell death signaling does not affect the induction of Hippo signaling by myosin II knock-down

The dual effects achieved by disrupting myosin II activity are puzzling, and could suggest that the activation of Hippo reporters is caused by apoptosis induced compensatory proliferation or regeneration. To understand if this was the case, we blocked cell death by various inputs for the apoptosis pathway. Dronc is a Nedd2-like caspase which acts upstream of caspase3 activation. H99 is a deficiency stock whose deleted region covers three important cell death regulating genes- *hid*, *reaper* and *grim*. Knocking-down *zip* in *dronc* homozygous mutant background or removing one copy of H99 showed a significant decrease in cleaved caspase 3 staining, suggesting that cell death has been successfully

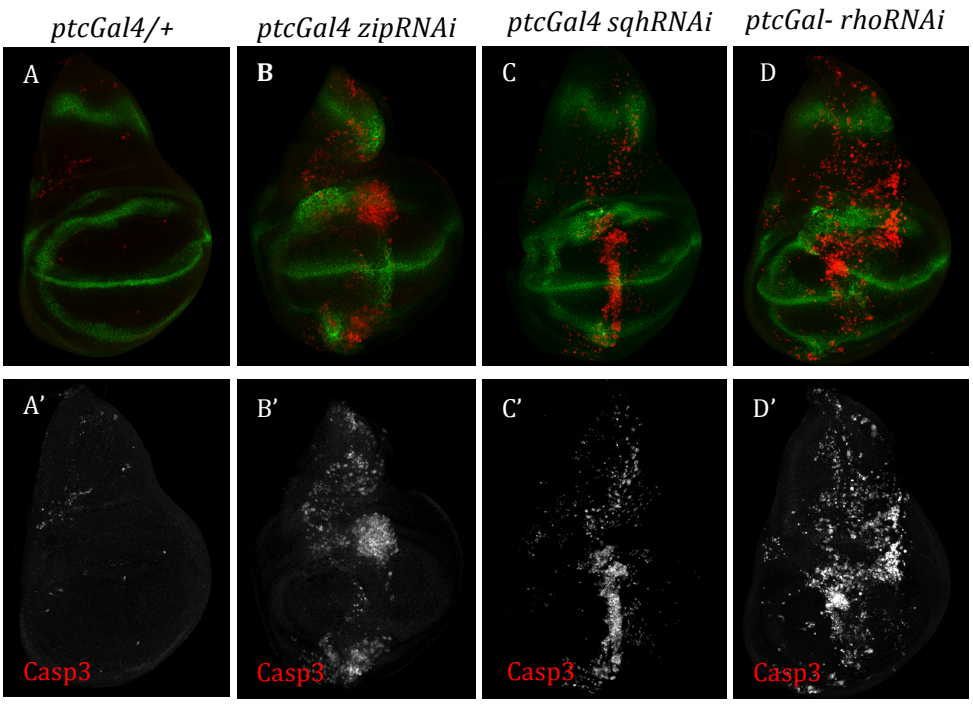


Fig 5.6 Disrupting myosin II and upstream regulators induce apoptosis

Confocal images of *Drosophila* wing imaginal discs. (A) control; (B) zip knock-down; (C) *sqh* knock-down, (4) *rho* knock-down. Cleaved caspase 3 staining is shown in red and gray in all pictures. Knocking-down of *zip, sqh*, and *rho* all show dramatic increase of caspase3 staining (B', C' and D').

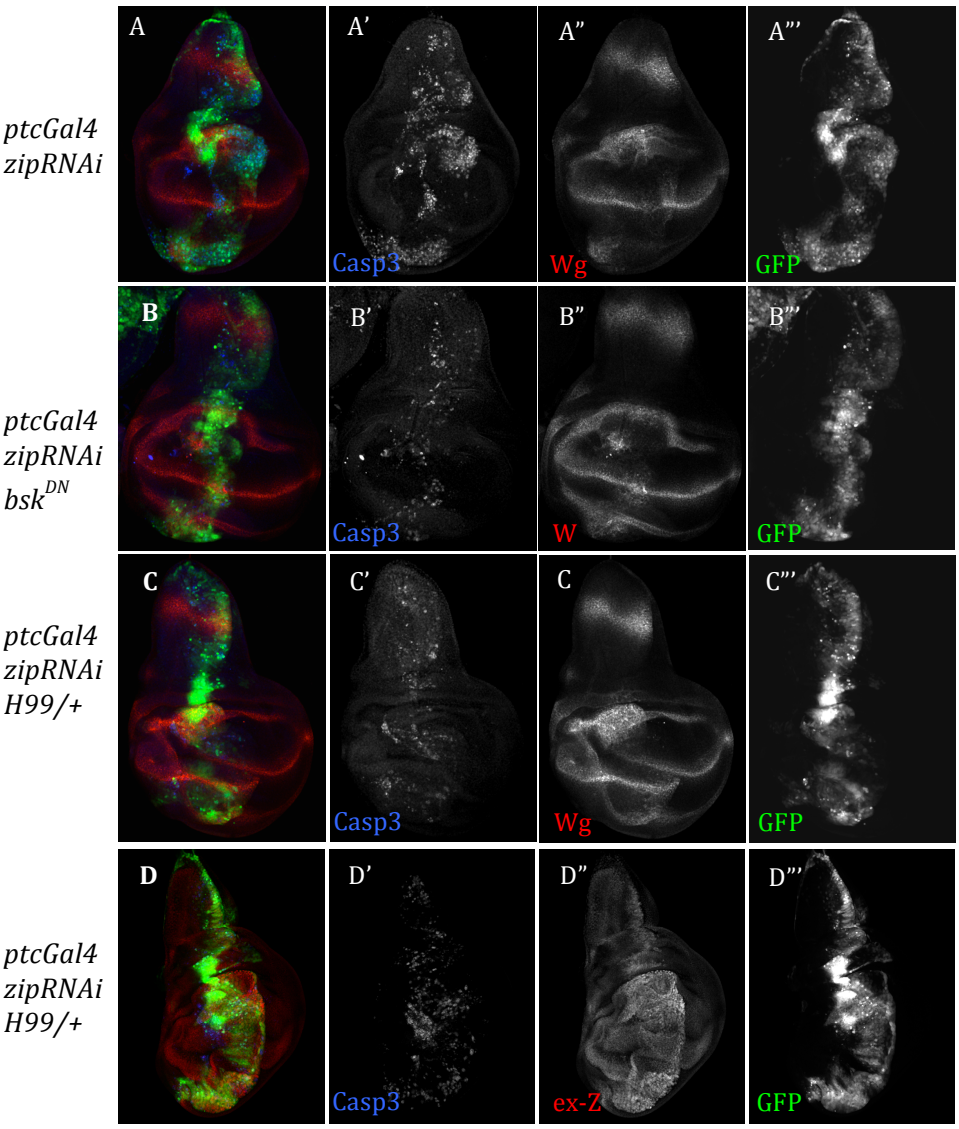

ptcGal4
zipRNAi
A
A'
Casp3
A"
Wg
A"'
GFP
ptcGal4
zipRNAi
bsk^DN
B
B'
Casp3
B"
W
B"'
GFP
ptcGal4
zipRNAi
H99/+
C
C'
Casp3
C
Wg
C"'
GFP
ptcGal4
zipRNAi
H99/+
D
D'
Casp3
D"
ex-Z
D"'
GFP

Figure 5.7

Figure 5.7 Blocking cell death does not affect myosin II knock-down induced activation of the Hippo pathway

Confocal images of *Drosophila* wing imaginal discs. Blocking apoptosis by various methods and assay for Wg protein and cleaved caspase 3 staining (A-C) or *ex-lacZ* together with casp3 (D). Compared with *zipRNAi* only, blocking cell death by overexpressing bskDN and in H99 heterozygous background show clear reduction of Casp3 staining (A', B', C', D'). However, the ectopic expression of Hippo reporters Wg (A'', B'', C'') and *ex-lacZ* (D'') are still present in all cases.

blocked (Fig. 5.7C' and D'). However, the ectopic expression of Wg and *ex-lacZ* are still present in those tissues (Fig. 5.7C'' and D''). These results argue that the effect of induction of Hippo signaling is not because of compensatory proliferation or regeneration events induced by excess apoptosis.

5.2.8 Blocking JNK activity does not affect induction of Hpo reporters in *zip* knock-down tissues

As mentioned in Chapter 4, JNK activity is also important for apoptosis induction. To understand if the JNK pathway is involved in regulating myosin II-mediated Hpo target activation, we performed similar experiments as in Chapter 4 by blocking JNK activity and observing the effect on Hpo target activation. As shown in Figure 5.7B, we clearly observed the ectopic Wg expression is still present while the cleaved caspase 3 staining is largely reduced in the *zip* knocked-down region. Therefore, JNK signaling is not required for Myosin II-regulated activation of Hpo signaling.

5.2.9 Increasing Myosin II activity also induces Hippo target activation

To gain more insight into how myosin II regulates growth through the Hippo pathway, we examined if there was a possible gain of function effect on Hippo activity by increasing myosin II activity. Since the effect on Hpo target activation upon knocking-down of myosin II is really strong, we predicted that the

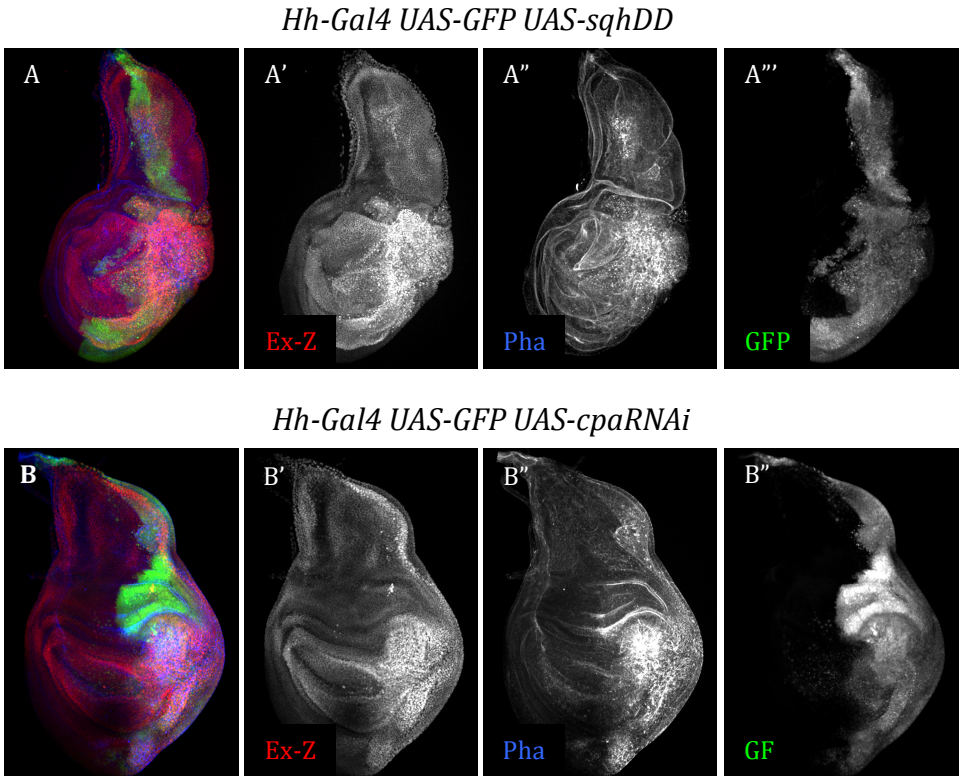


Figure 5.8 Hyperactivated Myosin II causes F-actin accumulation and activates Hippo target expression

Wing imaginal discs of *Drosophila*. (A) *hhGal4>sqhDD*; (B) *hhGal4> cpaRNAi*. Overexpressing a hyperactive form of myosin II light chain shows an increase of *ex-lacZ* (A'). The overexpressing tissue also contains high level of F-actin (A''). Knocking-down of *cpa* also shows increase in F-actin (B'') and induces *ex-lacZ* expression (B').

reporter activity will be suppressed upon myosin II activation. To induce ectopic activation of myosin II, we overexpressed a phosphomimietic form of *sqh* (*sqh*^{DD}). Strikingly, however, we also observed a significant increase of *ex-lacZ* (Fig. 5.8A). Notably, the pattern of *ex-lacZ* induction by increasing myosin II activity is different from those inductions from knocking down myosin II. Hyperactivation of myosin II induced *ex-lacZ* in both knock-down tissue and surrounding tissue, whereas reducing myosin II caused mainly autonomous induction of Hippo pathway activity. While it is possible to speculate that ectopic activation of one protein may cause a dominant negative effect therefore resulting in an effect similar to knock-down the same protein, this dominant negative effect on myosin II activity has not been reported. Also, since we observed different patterns in *ex-lacZ* regulation, this result suggested a possibility that hyperactivation of myosin II and reducing myosin activity regulate the Hippo pathway through distinct mechanisms.

5.2.10 Increasing myosin II activity induces F-actin accumulation

The fact that both increasing myosin II activity and knocking-down myosin II showed dramatic changes in Hippo pathway activity puzzled us, so we began investigating possible mechanistic differences between these two circumstances. Since actin and myosin II are the main members of the actomyosin cytoskeleton, and F-actin is one of the upstream regulators for the Hippo pathway, we decided to investigate if there was any change in F-actin organization upon modulating

myosin II. Interestingly, we found knocking-down or increasing myosin II activity had very distinct effects on F-actin. In sqh^{DD} overexpressing tissues, we observed an accumulation of F-actin, as shown by phalloidin staining (Fig. 5.8A''). This result suggests that activation of myosin II leads to F-actin accumulation and therefore induces Hippo target activation. On the other hand, we do not see a significant change in F-actin organization on myosin II knock-down tissues, further supporting the idea that regulation of the Hippo pathway by activating and reducing myosin II are through different mechanisms.

5.2.11 Reducing F-actin organization also induces Hippo target activation

Since we observed that increasing activity and knocking-down myosin II both activate Hippo targets, we wondered if the same dual regulation happens with F-actin organization. Chickadee (Chic) is the *Drosophila profilin* that promotes F-actin formation, and the *Drosophila* formin Dia is directly involved in F-actin polymerization. When we knocked-down Chic or Dia by RNAi constructs, we clearly saw that F-actin is disrupted (Fig. 5.9A'' and B''). *chic* and *dia* knock-down tissues also showed enlarged cell nuclei, likely as a result of the mitotic defects from the disruption of F-actin (Fig. 5.9A''' and B'''). Nevertheless, *ex-lacZ* is highly induced in knocked-down tissues (Fig. 5.9A' and B'). This result together with our previous observation suggests knocking down actomyosin has profound effects on Hippo signaling. This also implies the regulation of the Hippo pathway through actomyosin is very complex.

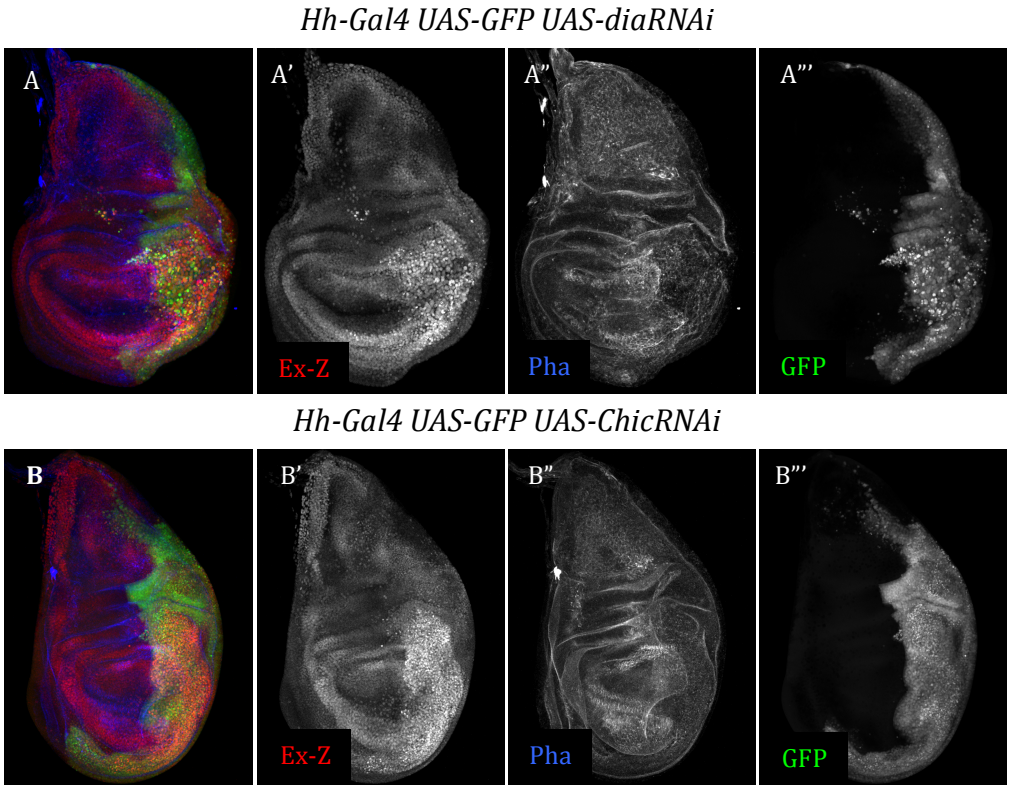


Figure 5.9 Disruption of actin polymerization affects the Hippo signaling

Disruption of actin polymerization in Drosophila wing discs by knocking-down *dia* (A) or *chic* (B). Phalloidin stainings show decrease signals of F-actin in both *dia* (A'') and *chic* (B'') knock-down tissues. Interestingly, the ex-lacZ still increased in Both *dia* (A') and *chic* (B') knock-down tissues.

5.3 Discussion

In this chapter, we characterized the function of myosin II in growth regulation. Knocking-down the components of myosin II induced cell proliferation, suggesting a potential tumor suppressing function for myosin II. We found that disrupting myosin II specifically affects the Hippo pathway, as shown by the lack of effects on other growth related pathways within knock-down tissue. Specifically, Myosin II works genetically downstream of Wts in regulating Hippo activity. We also observed a strong induction on Hpo targets when knocking down Rho signaling components, upstream regulators of myosin II, implying the activity of myosin II is also important for growth regulation through Hippo signaling. Meanwhile, knocking-down myosin II also induced significant levels of apoptosis. We provided evidence that myosin II induced cell death does not lead to activation of Hippo targets. Blocking JNK activity reduced cell death but not Hpo target activation upon myosin II knock-down. Intriguingly, we also observed a strong induction of ex-lacZ when hyperactivating myosin II. We provided evidence that hyperactivated myosin II possibly regulates the Hippo pathway through inducing the accumulation of F-actin. We also showed that decreasing F-actin induced Hippo pathway activity. Together, our data suggested there are multiple inputs that myosin II contributes to in regulating growth through the Hippo pathway.

5.3.1 Myosin II as a novel tumor suppressor

Myosin II is one of the genes that people recognize as an essential gene, yet do not really attempt to understand how it might contribute to different cellular processes. Also, attempts were made to understand the roles of non-muscle myosin II by generating mutant clones, but poorly developed clones made it difficult to study their function. With the development of the UAS-RNAi system, now we can get a better picture of myosin II function in development. Here, we reported a novel function for Myosin II as a tumor suppressor, as we showed that knocking-down of myosin induced ectopic proliferation. Several studies have addressed the levels of phospho-myosin staining and revealed there are increased levels of activated myosin II. Since actomyosin is important for cell mobility and phosphorylated myosin II is thought to be a marker of myosin II activity, it is believed that metastatic cells have increased myosin II activity and therefore promote malignant tumor cell migration. In this study we reported a novel tumor suppressing function for myosin II in growth regulation, which might lead to important new discoveries in tumor development.

5.3.2 Myosin II in Hippo regulation

Here we described a role for myosin II in regulating Hippo pathway activity. Disruption of myosin components and its upstream regulators induced Hippo targets, however, activation of Myosin II also induced Hippo pathway activity. The induction of ex-lacZ by disrupting myosin II suggested a novel mechanism for Hippo pathway regulation. Our epistasis experiment showed

Myosin II affects the Hippo pathway downstream of Wts, whereas F-actin works upstream of Wts in regulating the Hippo pathway. This result further supported the idea of a new role for Myosin II as a new input in regulating the Hippo pathway.

Several possibilities could lead to the alteration of Hippo signaling when disrupting Myosin II. Myosin II is a main member of the actomyosin cytoskeleton, and actomyosin is tightly associated with cell-cell junctions at the apical membranes. It is possible that disrupting myosin II interferes with the proper functions of AJs or apico-basal proteins, and therefore induces deregulation of the Hippo pathway. Further experimentation is needed to understand the relationship between actomyosin and polarity components.

Myosin II is also involved in cellular trafficking events. Studies have shown myosin II is required for both endocytosis and exocytosis of synaptic vesicles in neurons (Takagishi et al., 2005). Also, Myosin II is required for initiation of E-cadherin endocytosis (Levayar et al., 2011). Defects in endosomal trafficking are also commonly associated with cancers (Torres and Stupack, 2011). Interestingly, Vps25, a protein in the endosomal trafficking ESCRTII complex, has been demonstrated to regulate Hippo pathway activity (Herz et al., 2006). *vps25* mutant clones induced very strong non-autonomous effects on Hippo targets, and knocking-down *vps25* displayed both autonomous and non-autonomous increases of *ex-lacZ* (Graves et al., 2012). We will perform more

detailed analysis to gain additional understanding of the relationship between endosomal trafficking and myosin II in regulating the Hippo pathway.

5.3.3 Rho signaling in Hippo pathway activation

We provided evidence that Rho signaling, potentially via modulating myosin II, regulates the Hippo pathway. When we knocked-down the expression of Rok, we observed a relatively mild yet consistent effect on Hippo target activation. The main function of Rok in myosin II activity regulation is to phosphorylate Sqh, and therefore activate Myosin II (Narumiya et al., 2009). Since Sqh can be phosphorylated by other kinases, we speculated that the function of myosin II activation might be largely dependent on another kinase. Myosin II light chain kinase (MLCK) is the other kinase that phosphorylates Sqh (Matsumura et al., 2001). We will test if knocking-down MLCK shows a similar effect on Hippo signaling regulation. Also, another downstream effector of Rho is Dia, the essential protein for F-actin polymerization (Mulinari et al., 2008). Our lab demonstrated that overexpressing constitutively active Dia induced overgrowth through modulating Hippo activity (Sansores-Garcia et al., 2011). Here we showed disruption of Dia also induced Hippo target activation, suggesting that there are multiple inputs to regulate Hippo pathway activity through modulating F-actin.

Rho GTPase belongs to a small GTPase subfamily. The other two family members are Cdc42, and Rac1. Also, Rho is one of the Ras superfamily proteins. A hyperactivated *Ras*, *RasV12*, is commonly associated with cancer and has been utilized as a metastasis model for many cancer studies. *RasV12* expressing cells contained elevated level of phospho-myosin II and are more likely to migrate to other sites, suggesting myosin II is important for mobility for *RasV12* cells (Hogan et al., 2009). Interestingly, our lab together with several other groups demonstrated that *scrib* mutant cells when combined with *RasV12* are largely overgrown, and those cells induced high levels of Hippo targets (Chen et al., 2012; Doggett et al., 2011; Igaki et al., 2006). It will be interesting to further study the effects on actomyosin in *scrib* mutant with *RasV12* cells.

Cross talk between the Hippo pathway and other signaling pathways has been reported. During oogenesis, the Hippo pathway controls polar cell fate specification through repressing Notch activity (Chen et al., 2011). Also, the Hippo pathway is important for follicle cell proliferation, differentiation, and oocyte polarity establishment through Notch pathway activity (Yu et al., 2008). In *Drosophila* optic neuroepithelia, Hippo signaling modulates Notch signaling by changing *Delta* amounts through cell proliferation (Reddy et al., 2011). Meanwhile, the Hippo pathway suppressed the Wingless pathway in *Drosophila*, and overexpression of Yki suppresses the expression of a Wingless reporter. In mammals, phosphorylated Taz binds to Dishevelled therefore inhibiting the activation of the Wnt pathway (Varelas et al., 2010a). Another group reported

Yap/Taz binds to β-Cat and therefore suppresses the Wnt pathway (Hergovich and Hemmings, 2010). Here we reported Rho signaling as a new inputs that also mediates the function of the Hippo pathway, and further experiments for understanding the cross interaction between these two pathways could lead to more understanding of their biological functions.

Chapter 6.

Conclusions, Biological significance and Future directions

6.1 Conclusions

In Chapter 4, we first characterized *α-cat* mutant phenotypes in *Drosophila*. We found that *α-cat* mutants lead to reduced cell size, excessive apoptosis, and resulted in an autonomous decrease and non-autonomous increase of Hippo targets. These results contradict the reports from mammalian research. After a series of characterizations and a comparison with basolateral complexes, we provided several lines of evidence to show the differential regulation in polarity protein localizations and oncogenic cooperative overgrowth by AJs and basolateral complexes. Most importantly, our data indicated the intrinsic activation of Hippo signaling is largely dependent on appropriate localization of the basolateral complex. Our results suggested the basolateral complex works in parallel of adherens junctions in regulating Hippo pathway activation, providing further insight in understanding the mechanism of growth regulation by apico-basal polarity complexes.

In Chapter 5, we first characterized non-muscle Myosin II as a novel tumor suppressor gene by affecting Hippo pathway activity, as shown by RNAi knock-down results. We also identified upstream regulators of Myosin II, members in the Rho signaling pathway, that displayed similar phenotypes as the Myosin II knock-down. Apoptosis is also induced in myosin II knock-down tissues, however, blocking cell death does not affect *zipRNAi* induced Hippo activation. Our data suggested hyperactivating myosin II induces F-actin accumulation and therefore induces Hippo target activation. Unexpectedly, we also observed that

reducing F-actin activity also induced Hippo target activation *in vivo*. These controversial data indicated that actomyosin may regulate the Hippo pathway through multiple inputs.

6.2 Biological significance

In several types of cancer, such as breast and colon cancers, loss of polarity marks the first sign of transformation. Therefore, maintenance of proper apico-basal polarity has substantial influence on epithelial integrity. It is known that apical proteins antagonize the basolateral complexes, but the hierarchy for apico-basal polarity complexes in growth regulation is still largely debated.

Disruption of *α-cat* resulted in mislocalization of apical proteins, including Mer, aPKC, and Crb. Our lab previously demonstrated that Crb regulates Hippo pathway activity. Crb mutant clones induced high levels of Hippo pathway reporters. Nevertheless, we do not see an increase of Hippo reporters in *α-cat* knocked-down tissue, suggesting adherens junctions act epistatic to apical protein Crb in regulating Hippo signaling. Knocking-down *α-cat* does not alter the basolateral complex, and interfering with *scrib* does not affect AJ distribution. Meanwhile, the *α-cat* and *scrib* double knock-down experiment placed *scrib* downstream of *α-cat* in Hippo activation. Taken together, our results help define the hierarchy between apical proteins, AJs, and basolateral complexes in Hippo pathway regulation. These data help gain more advanced knowledge in polarity-dependent regulation, particularly in cancer progression.

Actin and non-muscle myosin II often function together in regulating many cellular processes. Here we describe a novel function for non-muscle Myosin II as a growth regulator. We found that activated Myosin II functions through F-actin modulation to regulate Hippo signaling. Hyperactivated Myosin II is able to "grab" more actin filament, and therefore cause the activation of Hippo signaling. On the other hand, reducing myosin II activity also activates Hippo targets through unknown mechanisms. Interestingly, disrupting actin polymerization also induced Hippo target activation. Although it is still unclear if the induction of the Hippo reporter by increase myosin II activity is through the same mechanism as disruption of actin polymerization, we clearly demonstrate that Myosin II has more diverse functions in cells.

6.3 Remaining questions and Future experiments

The conflict of *α-cat* mutant phenotypes between mammalian and *Drosophila* systems is the first priority for us to study. We notice the normally localized basolateral complexes in *α-cat* knock-down tissues, and the autonomous *ex-lacZ* levels only increase when we disrupted *scrib*. These results lead us to suspect that in mammals *α-cat* knock-down tissues also lose proper distribution of their basolateral complexes, and therefore induce Yap activity. To address our hypothesis, we will collaborate with other groups to conduct experiments and examine if hScrib is mis-localized in *α-cat* knocked-down cells. Also, We will overexpress a membrane-tethered form of *scrib* to artificially restore

normal *scrib* localization and investigate if Yap activity can be suppressed. These experiments will further strengthen our conclusion that the basolateral complex works downstream of AJs in Hippo pathway regulation.

The non-autonomous effect in *α-cat* knock-down tissue is really striking. In fact, it is the strongest non-autonomous effect we've ever observed. Studies from our lab discovered that non-autonomous activation of Hippo targets is important for cell competition. *scrib* mutant clones will face elimination by surrounding normal tissue, which is an example of cell competition. Our lab found that the surrounding wild type tissues contain high Yki activity, so are therefore able to regenerate and compensate for the eliminated tissues. When we block JNK activity in *scrib* mutant, we prevent the elimination of *scrib* mutant cells and cause both autonomous and non-autonomous overproliferation of *scrib* mutant cells. These results largely represent the real situation that cancers cause not only autonomous overgrowth but also induce neighboring cells to proliferate excessively. Therefore, identifying the non-autonomous regulators is a great benefit to the understanding of cancer signaling. We will use the *α-catRNAi* system to conduct a screen to identify these potential targets. Since *ptc-Gal4* showed prominent and consistent induction of non-autonomous expression of *ex-lacZ*, we will build a stock that contains *ex-lacZ*, *ptc-Gal4* and *UAS-α-cat-RNAi*. We will use two different ways to identify important effectors for the non-autonomous effect. First, we will utilize EMS to perform mutagenesis and assay how the mutants affect the non-autonomous induction of *ex-lacZ* in disruption of

α-cat background. Meanwhile, we can also cross the *ex-lacZ, ptcGal4, UAS-α-catRNAi* stocks with all *UAS-RNAi* collections and identify possible candidates that are important for non-autonomous Hippo activation.

In my dissertation, we separately addressed the functions of adherens junctions and the actomyosin cytoskeleton in Hippo pathway regulation. However, AJs interact with actomyosin through the direct binding of *α-cat* and F-actin. Since our data suggested both actomyosin and AJs are regulators for the Hippo pathway, we are interested in understanding if they regulate the Hippo pathway through similar or distinct mechanisms. In mammals, two substantial studies nicely elucidated the interactions between β-Cat, α-Cat and F-actin (Drees et al., 2005; Yamada et al., 2005). Their results show that monomeric α-Cat associates with β-Cat to form proper AJs, and dimerized α-Cat dissociated from the AJs then binds to F-actin. Interestingly, the recent publication of *α-cat* in *Drosophila* argued against this model (Sarpal et al., 2012). The Tepass group generated a chimera protein that fused *E-cad* and *α-cat* in the same construct and overexpressed this protein in *α-cat* null mutant background. This chimera protein localized normally at AJs and fully rescued the *α-cat* mutant, suggesting the AJ association is the most defining characteristic for *α-cat* in cells (Sarpal et al., 2012). Although the data are convincing, this study failed to show if the chimera protein interacts with F-actin or not. We will also be interested in testing if the chimera *E-cad-α-cat* protein performs similar or distinct regulation mechanisms for Hippo signaling.

Adherens junctions are intercellular contact sites and actomyosin is the intracellular force generator. Both of them are important for apical tension maintenance. All of these mechanical inputs need a sensor to receive and respond to these signals. Several proteins have been described as the mechanical sensors. Zyxin has been reported as a mechanical sensor that mediates the signals from *ft* to regulate the Hippo pathway (Rauskolb et al., 2011). Also, Vinculin has been identified as a linker between cell adhesion and the actin cytoskeleton (Gomez et al., 2011). It is also known that α-Cat interacts with Vinculin (Rudiger, 1998; Yonemura, 2011a). In mammalian cells, an engineered vinculin sensor construct has been reported, which allows researchers to study cell force alteration through Fluorescence resonance energy transfer (FRET) based live imaging (Grashoff et al., 2010). We are interested in understanding how these mechanosensors work in Hippo signaling, and more importantly if they modulate AJ and actomyosin mediated Hippo target regulation. The vinculin biosensor also can be used for understanding how many forces cells generate or receive compared with Hippo reporter activation when disrupting AJs and actomyosin. This study will provide quantifiable data to access how many forces actually changed upon changing mechanical cues.

We performed studies to understand how mechanical inputs from cell-cell interaction and cell intrinsic signals affect growth, yet there is another type of interaction that is also important for growth. The extracellular matrix (ECM)

directly contacts with cells and provides the environment for cells to live. Studies suggested that the change of the ECM largely influences the behavior of cells (Noguera et al., 2012; Schwartz, 2010; Srivastava et al., 2007; Sun et al., 2012). Also, the change of the ECM might be the signal for possible disease occurrence. For example, touch-based breast self-exam is often the first step for breast cancer diagnosis, and it clearly demonstrated that the environmental changes of stroma alter the stiffness of tissues. Yap/Taz has been described as a mechanosensor and mediators for the extracellular signals (Dupont et al., 2011). We are interested to learn if there is a relationship between AJs, actomyosin, and ECMs. We will examine if changing the stiffness of ECMs alter the actomyosin and AJ, and also if the changes correlate to Hippo pathway regulation.

We also identified that Rho signaling affects the Hippo pathway through modulating myosin II activity. Rho belongs to a small GTPase subfamily that contains three main members: Rho, Cdc42 and Rac1. All of the subfamily members are important for cell migration, cell cycle progression, and cell morphology (Rathinam et al., 2011). The small GTPases need to bind with GTP to achieve their activating status. Three classes of regulators regulate the activity of small GTPase. Guanine nucleotide exchange factors (GEFs) help removing the inactivated GDP and add a GTP to the G protein, therefore activating the small G protein. GTPase-activating proteins (GAPs) facilitate the function of GTPase in catalyzing GTP to GDP, and subsequently become inactivated.

Guanine nucleotide dissociation inhibitors (GDIs) bind to GTPase-GDP complex, therefore prevent the GTP binding to small GTPases (Ridley, 2006). Rho GTPase has been shown to affect apical tension in tissues (Warner and Longmore, 2009b). A recent study revealed the central role of RhoGEF/Rho family in Ras-cooperative tumor growth in *Drosophila* (Brumby et al., 2011). Since all Rho GTPase subfamily members are important for modulating the actomyosin cytoskeleton, we highly suspect that members in the Rho GTPase subfamily and their regulators might contribute to modulating the Hippo pathway. To expand to a broader picture, the Rho subfamily is one branch in the Ras GTPase superfamily. Ras superfamily members participate in various cell events, including cell proliferation, cell adhersion, cell mobility, and signaling pathway regulation (Cheng et al., 2009; Mizuno-Yamasaki et al., 2012; Vigil et al., 2010). Oncogenic mutations on Ras have a tremendous influence on human cancer formation, and the regulators of Ras are also important in tumorigenesis and have been intensively studied (Cheng et al., 2009; Vigil et al., 2010). Our previous results together with those from other groups showed the oncogenic form of Ras turned the low fitness *scrib* mutant cells into tumorigenic cells by regulating the Hippo pathway (Chen et al., 2012), and a recent study also revealed a role for Ras and its regulator Raf in coorperative overgrowth with *scrib* mutant (Brumby et al., 2011). These data enlighten a new territory for possible growth signal cross talk between Rho/Ras signaling and Hippo pathway regulation. In collaboration with Richardson's group, we will examine the effects

of various small GTPases and their regulators to understand their functions in growth regulation and Hippo pathway activation.

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