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WASHINGTON UNIVERSITY

Division of Biology and Biomedical Sciences

(Molecular Genetics and Genomics)

Dissertation Examination Committee:

Gregory D. Longmore, Chair

James B. Skeath

David C. Beebe

Raphael Kopan

Aaron Diantonio

Andrey Shaw

THE ROLE OF AJUBA LIM PROTEINS IN EPITHELIAL GROWTH REGULATION

by

Meghna Das Thakur

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of Washington University in
partial fulfillment of the
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Abstract of the Dissertation

The mammalian Ajuba LIM proteins (Ajuba, LIMD1, WTIP) are cytosolic adapter proteins recruited to nascent epithelial adherens junctions, where they are thought to contribute to junctional assembly and/or stability. They also shuttle into the nucleus acting as corepressors of the Snail family of transcriptional repressors, thereby contributing to epithelial mesenchymal transition. As such they have the potential to communicate cell adhesive events with nuclear responses to remodel epithelia. Determining their role(s) *in vivo*, however, has been challenging due to shared interacting proteins, overlapping tissue expression and functional redundancy in cells. Thus, we turned to the *Drosophila* model system where a single gene, *CG11063* or *djub*, exists. The generation and analysis of *Drosophila* containing *djub* mutant loss-of-function alleles or depleted of dJub by RNAi identify *djub* as an essential gene required for normal development and a novel regulator of epithelial organ growth as a component of the conserved Hippo pathway, which has been implicated in both tissue size control and cancer development. *djub*-deficient epithelial tissues were small due to decreased cell numbers resulting from increased apoptosis and decreased proliferation due to the downregulation of DIAP1 and cyclin E, phenocopying tissues deficient for Yorkie (Yki), the downstream target of the Hippo pathway. *djub* genetically interacts with the Hippo pathway, and genetic epistasis suggests that *djub* influences *wts* activity. In mammalian and *Drosophila* cells, Ajuba LIM proteins/dJub specifically interact with LATS/Wts and WW45/Sav to inhibit phosphorylation of YAP/Yki. This work describes a novel role for the Ajuba LIM proteins as negative regulators of the Hpo signaling pathway.

Chapter 1

Introduction

Growth Control and the Hippo Pathway

How animals, organs and tissue know when to stop growing is an unanswered question. Each organ in an animal has a specific architecture and pattern, and develops to a defined final size in proportion with the rest of the animal (Dong, Feldmann et al. 2007; Harvey and Tapon 2007; Saucedo and Edgar 2007). Several examples exist of experimental systems to dissect organ size control, one such being the regeneration of tissue after tissue injury or experimental procedures. For instance, after partial hepatectomy, the hepatocytes in the liver mobilize to proliferate rapidly and thereby increase the size of the regenerating liver. Once the liver reaches the original predetermined size, the cells stop dividing ensuring that the regenerating liver is not overgrown (Fausto, Campbell et al. 2006). The myth of Prometheus suggests Greeks were aware of the regeneration capabilities and growth regulating abilities of the liver.

As in the case of the regenerating liver, the development of a functional organ requires both regulated patterning mechanisms that allow its constituent cells to acquire proper identities, and growth-regulatory mechanisms that determine final organ size. Over the past two decades, developmental geneticists have identified most of the key signaling pathways that regulate cell fate decisions. Such as, Notch, Wnt, TGF- β , Hedgehog, receptor tyrosine kinase, nuclear receptor, and Jak/STAT pathways (Barolo and Posakony 2002). More recently, genetic approaches have identified two signaling cascades that govern organ growth. One is the tuberous sclerosis tumor suppressor complex (TSC)–target of the rapamycin (TOR) pathway. This pathway controls organ size by regulating cell growth and size (Pan, Dong et al. 2004). The other is the Hippo

(Hpo) pathway, which controls organ size by regulating cell growth, proliferation, and apoptosis.

Genetic studies in *Drosophila* have been at the forefront of the identification of the pathways that control organ size. The *Drosophila* imaginal discs (small sacs of cells within *Drosophila* larvae that give rise to the adult structures of the adult fly) provide an ideal system to study growth control. Imaginal discs are first set aside during late embryogenesis after which they proliferate exponentially during the larval stages, thereby increasing their mass almost one-thousand fold. During metamorphosis they differentiate into their respective adult structures like the eye, wing or leg (Cohen 1993). A key advance that enabled identification of genes that govern growth control in flies was the application of FRT/FLP- based site recombination events towards the ability to generate mosaics. This is especially relevant for genes that regulate growth control as animals that lack the function of one such gene die early in embryogenesis preventing the analysis of imaginal disc development. Thus, the generation of small patches of cells homozygous mutant for these genes in a heterozygous background in an imaginal disc was essential to be able to follow the effect of these genes on growth control (Figure 1).

In the past fifteen years, many groups have carried out genetic screens in *Drosophila* to identify mutants that specifically affect growth and not patterning (St Johnston 2002). Through these screens the first three members, *hippo* (*hpo*), *salvador* (*Sav*), and *warts* (*wts*), that later came to be known as the Hippo pathway were identified due to their overgrowth phenotype. *Warts* (also known as *lats*) was one of the very first genes isolated using the FLP/FRT based mosaic screen (Figure 1). *Wts* is a tumor

suppressor, and encodes a kinase of the Nuclear Dbf-2-related (NDR) family (Xu and Rubin 1993; Justice, Zilian et al. 1995). Loss of *wts* leads to severe cell-autonomous overgrowth in epithelial structures, but has no effect on cell fate determination. Next, the binding partner of *wts*, *salvador* (*sav* or *shar-pei*), was identified (Figure 3A). This tumor suppressor gene encodes a WW domain-containing protein and mutations in *sav* lead to cell-autonomous overgrowth as well (Kango-Singh, Nolo et al. 2002; Tapon, Harvey et al. 2002). Importantly, it was found that loss of *wts* or *sav* coordinately regulates both the increase in cell proliferation and a reduction in apoptosis. Future experiments revealed that the increase in proliferation and decrease in apoptosis is brought about by the misregulation of the pathway's downstream transcriptional targets CyclinE, *Drosophila* inhibitor of apoptosis protein DIAP1 respectively (Nolo, Morrison et al. 2006; Willecke, Hamaratoglu et al. 2006).

The *hpo* tumor suppressor gene, which encodes a Ste-20 family protein kinase (Harvey, Pflieger et al. 2003; Pantalacci, Tapon et al. 2003; Udan, Kango-Singh et al. 2003; Wu, Huang et al. 2003) and phenocopies the *wts* and *sav* loss of function overgrowth phenotypes (Figure 2), was the next critical member of the pathway identified. Interestingly, Hpo phosphorylates and activates Wts, with Sav further potentiating this phosphorylation reaction (Figure 3A) (Wu, Huang et al. 2003). Another NDR family regulatory protein, Mob1-related protein, termed Mats in *Drosophila*, was isolated as a binding partner of Wts, and found to potentiate the intrinsic kinase activity of Wts (Lai, Wei et al. 2005). Mutations in *mats* yield loss of function phenotypes essentially identical to those observed for *hpo*, *sav* and *wts*. Thus, within the Hippo pathway kinase cascade the Hpo and Wts kinases both associate with and are stimulated

by regulatory proteins, Sav and Mats, respectively. In total, the Hpo kinase cascade inhibits proliferation by downregulating CyclinE levels, and promotes cell death by downregulating DIAP1 levels. The Hippo pathway mediates its effects on CycE and DIAP1 via its negative effect on the function of the transcriptional coactivator Yorkie (yki). Yki is the critical substrate and directly inhibited by the kinase activity of Wts (Huang, Wu et al. 2005). Wts phosphorylates Yki on residue S168, which inactivates Yki by promoting its cytoplasmic retention through an interaction with 14-3-3 binding proteins (Huang, Wu et al. 2005; Dong, Feldmann et al. 2007). Overexpression of Yki phenocopies the *wts* loss of function phenotype with respect to both the morphological overgrowth phenotypes and the downregulation of *diap1* and *cyclinE* transcription. In contrast, *yki* loss of function leads to tissue atrophy (Figure 3).

Diap1 and *cyclinE* are two of the target genes regulated by Yki/Hippo pathway with additional targets being identified recently. The microRNA *bantam*, is a positive regulator of imaginal disc growth that regulates both cell proliferation as well as apoptosis, (Brennecke, Hipfner et al. 2003) is one of these as the levels of *bantam* increase in tissue overexpressing Yki (Nolo, Morrison et al. 2006). *Bantam* binds the IAP inhibitor head involution defective (*hid*) as one of its targets, (Brennecke, Hipfner et al. 2003) however loss of *bantam* only partially reverts the *yki* overgrowth phenotype suggesting that the overgrowth phenotype results from a combination of other Yki targets such as DIAP1, CyclinE and others.

Yki is a transcriptional coactivator. Thus, Yki-interacting transcription factors must provide Yki with its promoter selectivity. Scalloped (Sd), a TEAD-domain

containing transcription factor, has been reported to mediate Yki-induced gene expression as well as the overgrowth phenotype. Sd is a crucial regulator of cell proliferation and survival in wing imaginal disc cells (Halder, Polaczyk et al. 1998; Goulev, Fauny et al. 2008; Wu, Liu et al. 2008). Although Yki and the Hippo pathway function ubiquitously throughout the *Drosophila* tissue, Sd is expressed in narrower spectrum of cells (Campbell, Inamdar et al. 1992). Also *yki* mutants have a much more severe growth defect as compared to *sd* mutant clones and in fact Yki mutants that are unable to bind Sd still induce an overgrowth defect (Huang, Wu et al. 2005; Wu, Liu et al. 2008; Zhao, Ye et al. 2008). This suggests that other transcription factors exist that mediates the function of Yki and the Hippo pathway in tissues other than the wing and/or in a partially redundant manner with Sd.

Upstream signaling to the Hippo pathway

Merlin (Mer) and its related protein Expanded (Ex) have been identified as potential upstream regulators of Hpo (Figure 3A). Both Mer and Ex are FERM, 4.1, Ezrin, Radixin, Moesin, domain containing proteins. This family of proteins typically function as adapter proteins that link transmembrane proteins to the cytoskeleton or to cytoskeleton-associated proteins (Hamaratoglu, Willecke et al. 2006). Mer has a human ortholog, neurofibromatosis 2 (NF2), a tumor suppressor gene that has been found to be mutated in central nervous system tumors (McClatchey and Giovannini 2005). Both Mer and Ex localize adherens junctions *Drosophila*. Furthermore, Mer and Ex also heterodimerize with each other and seem to function redundantly to promote signaling through the Hippo pathway to inhibit *yki* function, as tissue doubly mutant for *ex* and *mer* yield overgrowth phenotypes that are virtually indistinguishable from *hpo*, *wts* or *sav*

mutant tissue based on morphology and molecular criteria (McCartney, Kulikauskas et al. 2000). Genetic epistasis tests place *mer* and *ex* upstream of *hpo*, with the overexpression of Mer and Ex leading to an increase in Wts phosphorylation and a downregulation of Yki activity (Willecke, Hamaratoglu et al. 2006; Feng and Irvine 2007). How Mer and Ex activate the Hippo kinase cascade is still unknown since neither bind Hpo directly (Hamaratoglu, Willecke et al. 2006).

Most of the Hippo pathway components in *Drosophila* are ubiquitously expressed throughout imaginal disc development. The pathway may be regulated by a specific signal given out in a spatial or temporal fashion, triggering cell death or cell cycle exit at the appropriate time during development. Another view with respect to the mode of regulation of the Hippo pathway is that the pathway is typically constitutively active however its signaling capacity is modulated by other pathways. Data suggest that such cues may come from morphogens such as Decapentaplegic (DPP) and wingless or other growth regulatory pathways such as JAK-STAT (Janus kinase -signal transducer and activator of transcription), epidermal growth factor receptor (EGFR) and Delta-Notch (Hariharan and Bilder 2006). For instance, membrane proteins such as EGF receptor, Notch, E-cadherin and the Hedgehog receptor Patched, are found to be upregulated in the *mer;ex* double mutants suggesting that transmembrane receptor signaling is altered in these cells (Maitra, Kulikauskas et al. 2006). Mer and Ex might therefore regulate the activity of the Hippo pathway by regulating the abundance of some of these receptor proteins at cell surfaces.

The transmembrane protein Fat (Ft), an atypical cadherin, may be the upstream receptor that activates the Hippo pathway (Bennett and Harvey 2006; Silva, Tsatskis et al. 2006; Willecke, Hamaratoglu et al. 2006). *Ft* is a tumor suppressor gene that acts linearly upstream of Ex and Hpo (Figure 3A). *Ft* mutant cells have a phenotype similar to that of *hpo* or *wts*, including the upregulation of *cycE* and *diap1* along with increased cell proliferation and decreased cell death. Fat has been shown to regulate Hippo pathway activity by promoting the stability and/or localization of Ex to the apical membranes of cells (Bennett and Harvey 2006; Silva, Tsatskis et al. 2006; Willecke, Hamaratoglu et al. 2006). The regulation of Ex is crucial since its expression is in fact stimulated when the Hippo pathway activity drops via a negative feedback loop. In fact both Ex and Mer are upregulated in clones lacking Hippo pathway genes, *hpo*, *sav* and *wts*. The upregulation is a result of derepressed transcription, since the levels of *ex* transcripts is elevated in the mutant clones. Ex and Mer were both found in higher levels in *hpo*, *sav* and *wts* mutant clones. This regulation is independent of the developmental stage of the tissue or the position of the clone. Thus, the expression of the Hippo signaling pathway components regulates itself via the feedback loop. The regulation of *mer* and *ex* expression levels by Hippo signaling suggests that feedback mechanism might be an important system in place to keep Hippo signaling in a steady state.

Ft is capable of regulating the Hippo pathway independent of Ex and Hpo in a parallel pathway. *ft* mutant clones show decreased Wts protein levels. Ft controls the abundance of the Wts protein by negatively regulating Dachs, an unconventional myosin (Cho, Feng et al. 2006). Dachs directly binds Wts acting as a scaffold to bring Wts to proteins that will promote Wts proteolysis. Both Fat and Dachs localize to apical cell

junctions, however so far no direct interaction between them has been established. Furthermore, the overexpression of Wts can rescue *ft* mutants to viability (Cho, Feng et al. 2006; Mao, Rauskolb et al. 2006; Feng and Irvine 2007). Thus the Ft-Dachs and the Hpo-Sav pathways act in parallel to control the levels of Wts or the activity of Wts respectively.

In summary, the *Drosophila* Hippo pathway model as it stands now involves a series of events that leads to the activation of the Hippo pathway that leads to phosphorylation of Warts, phosphorylating Yki. This phosphorylation of Yki on Ser168 in turn introduces a 14-3-3 binding site sequestering Yki in the cytoplasm. In its unphosphorylated state, Yki is not inhibited by the Hippo pathway and can bind to the cofactor Scalloped (Sd) and enter the nucleus where it is able to induce the transcription of genes, CyclinE and DIAP1, required to promote cell growth and inhibit apoptosis. Finally, Ex, Merlin and Fat can activate the Hippo pathway and the activation of the Hippo pathway can also be affected by Dachs, which binds to Wts and promotes its degradation (Figure 3A).

Hippo signaling in mammals

The Hippo pathway is highly conserved throughout evolution and the function of the fly proteins and their mammalian counterparts are conserved as well. Mst1/2 are the mammalian homologs of Hpo, WW45 the homolog of Sav, Lats1/2 are the mammalian homologs of Wts, Mob1 is Mats homolog, YAP is the Yki homolog, NF2 the Mer homolog and to lesser extent FRMD6 is the mammalian homolog of Ex homolog and Fat4 the Fat homolog (Figure 3B). Expression of the mammalian proteins in several of

the loss of function mutant flies have been rescued with their respective human counterparts; namely YAP, Lats1, Mst1, and Mob1 in flies mutant for the orthologous fly gene (Tao, Zhang et al. 1999; Wu, Huang et al. 2003; Lai, Wei et al. 2005). The strong rescue of the mutant phenotype indicates that these proteins are functionally conserved from *Drosophila* to mammals. The Hippo pathway function is conserved in mammalian cells or models with respect to organ size control since the overexpression of YAP in a mouse liver results in a striking increase in liver size and leads to tumor metastasis (Camargo, Gokhale et al. 2007; Dong, Feldmann et al. 2007).

The conservation of the pathway for the upstream components Fat and Ex is less clear in mammalian cells. However, similar to Hpo in *Drosophila*, Mst is crucial in the mammalian Hippo pathway and functions to phosphorylate the core components of the pathway. Mst1/2 phosphorylates Lats1/2 on its activation loop and in addition, Lats is capable of autophosphorylation (Chan, Nousiainen et al. 2005). Further, WW45 interacts with Mst, following which Mst phosphorylates WW45 (Callus, Verhagen et al. 2006). Mst1/2 also phosphorylates Mob1 which in turn strengthens the Mst-Lats interaction (Praskova, Xia et al. 2008). Similar to their *Drosophila* counterparts, Mst, WW45, Lats and Mob induce the phosphorylation, cytoplasmic translocation and inhibition of YAP (Overholtzer, Zhang et al. 2006; Zhang, Smolen et al. 2008). Also TEAD family transcription factors which are mammalian homologs of *Drosophila* Sd have been found to mediate YAP function in mammalian cells (Zhao, Ye et al. 2008). In fact, Lats directly phosphorylates YAP on serine residues in five conserved motifs, one of them being S127 (Hao, Chun et al. 2008). As in the case of Yki, the S127 phosphorylation on YAP by Lats generates a 14-3-3 binding site which sequesters YAP in the cytoplasm leading to its

nucleus to cytoplasm translocation (Figure 3) (Lee, Kim et al. 2008). In fact using mutant S168 YkiA or S127A YAP transgenes in *Drosophila*, where the serine residues are changed to Alanine and cannot be phosphorylated, leads to overgrowth. This function of YAP being negatively regulated by the Hippo pathway is evolutionarily conserved since YAP's role in promoting cell proliferation and oncogenic transformation are both inhibited by co-expressing Lats1 and Mst1 (Zhang, Smolen et al. 2008).

Hippo pathway and cancer

The inactivation of the Hippo pathway promotes growth by promoting cell proliferation and inhibiting cell death, and thus may lead to cancer. In addition, several of the *Drosophila* Hippo pathway mammalian homologs have been implicated in tumorigenesis. The mammalian homolog of Hpo, Mst1/2, is known to be proapoptotic in cultured mammalian cells, (Cheung, Ajiro et al. 2003, Lehtinen, Yuan et al. 2006). Mice lacking the *wts* mammalian homolog *lats*, develop soft-tissue sarcomas and ovarian tumors (St John, Tao et al. 1999). Lats has also been implicated in the control of mitosis and cytokinesis in mammalian cells, and its loss can lead to multinucleation, centrosome amplification and genomic instability (McPherson, Tamblyn et al. 2004). Also, Lats1 and Lats2 have been found to have methylation dependant silencing that correlate with the aggressive phenotype in human breast cancers (Takahashi, Miyoshi et al. 2005) and Lats2 can be regulated by microRNAs miR-371 and miR-373 which are upregulated in testicular cancer lines and tumor samples (Aylon, Michael et al. 2006; Voorhoeve, le Sage et al. 2006). Therefore, the downregulation of Lats via several different mechanisms may promote tumor formation. WW45, the human orthologs of *sav* is deleted in several

renal cancer cell lines and in fact the WW45 knockout mouse displays hyperplasia and differentiation defects in mouse epithelial structures (Lee, Kim et al. 2008). MOB1K1B (*mats*) has been found to be deleted in cell lines derived from human melanoma samples and mouse mammary gland carcinomas (Tapon, Harvey et al. 2002; Lai, Wei et al. 2005). NF2 or *mer* is a known tumor suppressor gene mutations in which can lead to neurofibromatosis. Mutations in NF2 have been observed in sporadic tumors of the nervous system as well as other tumors like mesothelioma (McClatchey and Giovannini 2005). Recently YAP (Yki) has been implicated in mammalian cancers. The amplicon 11q22 in humans contains both *YAP* and *cIAP2* and has been found to be amplified in several human cancers including liver, lung, pancreatic, ovarian and oesophageal (Imoto, Yang et al. 2001; Dai, Zhu et al. 2003; Snijders, Schmidt et al. 2005). Besides the genomic amplification, YAP expression and its nuclear localization has also been reported to be elevated in multiple types of human cancers (Zender, Spector et al. 2006; Steinhardt, Gayyed et al. 2008). Also Yap when overexpressed is able to transform immortalized mammary epithelial cells *in vitro* including the ability to induce growth-factor and anchorage-independent growth, epithelial-mesenchymal transition and resistance to apoptosis (Dai, Zhu et al. 2003; Overholtzer, Zhang et al. 2006). Thus, both *yki* and *Yap* act as oncogenes.

LIM domain containing proteins

As a result of the studies conducted in this thesis, we have discovered a novel regulator of the Hippo pathway, namely the Ajuba subfamily of LIM proteins. The Ajuba LIM proteins are a subgroup of a larger group of LIM domain containing proteins that are known for their ability to carry out protein-protein interactions. LIM domains were

originally identified as novel cysteine-rich protein motifs, common to the *Caenorhabditis elegans* cell-lineage protein LIN-11, the rat insulin gene-enhancer-binding protein Is11, and *C. elegans* neuronal specification gene MEC-3 (Freyd, Kim et al. 1990; Karlsson, Thor et al. 1990). LIM domains are protein-protein interaction domains that act as key components of the regulatory machinery in a cell. These domains enable LIM proteins to recruit specific target proteins via protein-protein interactions to specific subcellular compartments, modulate the activity of their targets or help nucleate the assembly of multi-component complexes. Thus, through the specific binding of their targets LIM domain proteins are able to regulate a diverse array of cellular circuits.

Although LIM domains are absent in prokaryotes, they are found in almost every eukaryotic organism whose genome has been sequenced, such as yeast, slime moulds and plants to *Drosophila* and humans. In the human genome, 135 LIM domains have been identified within 58 proteins (Hobert and Westphal 2000). A LIM domain contains 2 tandem zinc-finger motifs, and LIM proteins can contain up to 5 LIM domains. Each LIM domain typically consists of approximately 55 amino acids. The LIM consensus sequence is $CX_2CX_{16-23}HX_2CX_2CX_{16-21}CX_2(C/H/D)$ (Schmeichel and Beckerle 1994), however the consensus sequence becomes more variable between species and also within the array of LIM sequences (Michelsen, Schmeichel et al. 1993).

LIM domains can be linked to many different proteins domains within LIM-containing proteins such as, SH or PDZ domains, homeodomains, catalytic domains, cytoskeletal-binding domains etc and finally the LIM domains can be C-terminus, N-terminus or internal. Structural studies have made it clear that the LIM domains are

multiple protein binding adapters and several can use their additional motifs to mediate interactions with a variety of proteins, both sequentially as well as at the time with multiple proteins (Kadmas and Beckerle 2004). The LIM domains can form dimers with other LIM domains or bind structurally varied protein motifs. LIM domain containing proteins are found in the nucleus as well as in the cytoplasm. The proteins are broadly categorized into actin associated LIM proteins, nuclear LIM proteins, LIM only proteins, and catalytic LIM proteins (Kadmas and Beckerle 2004).

The actin associated LIM proteins like the members of the zyxin, paxillin and enigma proteins are able to shuttle between the cytoplasmic and nuclear compartments therefore influencing gene expression (Breen, Agulnick et al. 1998). This group of LIM proteins interact with a wide variety of partners. These proteins also contain various other protein-protein interaction motifs such as PDZ, LD (leucine-aspartate repeat) and actin target domains (Figure 4). Several of these proteins localize to focal adhesions (Zyxin, Paxillin). Extracellular signals induce the translocation of these proteins into the nucleus potentiating the transcriptional regulation of target genes. Once in the nucleus these proteins also act as transcriptional co-activators and co-repressors (Wang and Gilmore 2003). The Ajuba subfamily of proteins are members of this group and will be the focus of the thesis in the following chapters.

Nuclear proteins like the LIM homeodomain proteins (LHX) and nuclear LIM only proteins (LMO) contain N terminal tandem LIM domains and are primarily involved in transcription during development (Figure 4) (Hobert and Westphal 2000). The LHX and LMO proteins also play a role in cell lineage determination and pattern formation

during development (Breen, Agulnick et al. 1998). The LMO proteins are transcriptional co-factors and form complexes with other transcription factors in order to regulate transcription. LHX proteins however are transcription factors that bind DNA through their homeodomain. (Jurata and Gill 1997; Matthews and Visvader 2003; Deane, Ryan et al. 2004).

The LIM only proteins include four-and-a-half LIM (FHL), cysteine rich proteins (CRP) and particularly interesting new cystein- and histidine-rich proteins (PINCH) as well as some nuclear LMOs (Figure 4). These are found both in the nucleus as well the cytoplasm and both associate with the cytoskeleton (Weiskirchen and Gunther 2003). FHL2 and FHL3 are examples of proteins that are components of the adhesion complexes (Li, Kotaka et al. 2001; Samson, Smyth et al. 2004).

The third group, the catalytic LIM protein group is similar to the actin-associated group with respect to containing other protein-protein interaction motifs but this group contains the mono-oxygenase or kinase catalytic motif (Figure 4), which distinguishes them from the other LIM proteins (Kadrmaz and Beckerle 2004). This group includes LIM-kinases and molecules interacting with CasL (MICALs), which are involved in cell cycle regulation and actin polymerization and depolymerization.

Ajuba/Zyxin family of LIM proteins

The Ajuba/Zyxin families of LIM proteins are actin binding proteins, and consist of six members: Zyxin family; Zyxin, lipoma preferred partner (LPP), thyroid hormone interacting protein 6 (Trip6), and Ajuba family; Ajuba, LIM domain-containing protein 1

(LIMD1), Wilms tumor 1 interacting protein (WTIP) (Figure 5) (Crawford, Michelsen et al. 1992; Petit, Mols et al. 1996; Beckerle 1997; Goyal, Lin et al. 1999; Kiss, Kedra et al. 1999; Wang and Gilmore 2003). These proteins are characterized by the presence of three tandem homologous LIM domains in the carboxyl terminus (LIM region) and unique proline-rich N-terminal PreLIM regions (Figure 5) (Schmeichel KL et al., 1997). The proteins all share high sequence homology within the LIM domain region.

These proteins are also components of the cell-cell junction adhesive complexes in epithelial cells and fibroblasts (Hoffman, L. M et al., 2003, Marie et al 2003). As such, they have been shown to regulate cell migration in fibroblasts and contribute to the establishment and/or maintenance of cell-cell junctions in epithelial cells (Marie, H.S. et al., 2003 Crawford and Beckerle, 1991). In addition, these proteins are capable of shuttling to and from the nucleus as each one contains a nuclear export signal (NES) in their individual PreLIM regions (Figure 5) (Nix and Beckerle 1997; Kanungo, Pratt et al. 2000). This characteristic renders them strong candidates to mediate signal transduction steps from the cell surface to the nucleus. The purpose of this thesis is to further examine the role of this family in signal transduction. In fact understanding how these proteins function at the level of junctions as well as in other compartments will shed light on the outside-in signaling that cells are able to orchestrate.

Ajuba/Zyxin LIM proteins have been implicated in cell motility regulation, localize to focal adhesion sites, and associate with the actin cytoskeleton (Crawford, Michelsen et al. 1992; Petit, Fradelizi et al. 2000; Yi, Kloeker et al. 2002). Zyxin family members have the ability to bind α -actinin and contribute to the bundling of actin fibers.

Abrogating the Zyxin- α -actinin interaction displaces Zyxin from its normal subcellular localization and the cells show inhibited migration and spreading (Drees, Andrews et al. 1999). The Zyxin family members, and not the Ajuba family members, are recruited to the leading edge of cells where they influence the actin assembly via an interaction with Ena/VASP proteins (Renfranz and Beckerle 2002).

With respect to the nuclear function of the Ajuba/Zyxin proteins, although the significance of Zyxin family members nuclear localization is not clear, we know that the accumulation of Ajuba in the nucleus plays a role in growth control and cell differentiation (Kanungo, Pratt et al. 2000). Overexpression of just the LIM domain region alone, which lacks the nuclear export sequence will accumulate in the nucleus and induce endodermal differentiation. Exactly how the Ajuba proteins are recruited to these different cellular is not well understood. As for the Zyxin family members, these proteins can localize to the nucleus but what their nuclear biological function and the regulation of their nuclear localization is not understood (Crawford and Beckerle 1991; Crawford, Michelsen et al. 1992; Nix and Beckerle 1997). Also both Ajuba and Zyxin have been implicated in mitotic cell cycle regulation. Both can associate with the tumor suppressor Lats1 and Ajuba can also interact with mitotic kinase Aurora A (Hirota, Morisaki et al. 2000; Abe, Ohsugi et al. 2006).

The zinc finger structures in the LIM domains are known to mediate DNA binding in transcription factors. Evidence suggests that the Ajuba/Zyxin family members may not directly bind DNA but may act in the nucleus to affect transcription. These proteins are able to bind several nuclear proteins as well as transcription factors and have

been shown to have transactivation ability as measured by reporter gene assays (Lee, Choi et al. 1995; Petit, Fradelizi et al. 2000; Yang, Guerrero et al. 2000; Wang and Gilmore 2003). Based on the present data it appears that the LIM domains mediate the interaction with the transcription factors and the N-terminal domains act to enhance the transcriptions. It is also possible that the domains may act as sites for nucleation for the recruitment of transcription factor co-activators.

The Ajuba/Zyxin LIM proteins have been shown to regulate other signaling pathways as well. For instance, the PreLIM region consists of a putative SH3 recognition motif and in fact Ajuba and Zyxin interact with the SH3 domains of Grb2 and Vav respectively (Hobert, Schilling et al. 1996; Goyal, Lin et al. 1999). The functional relevance of the Zyxin-Vav interaction is unclear but the Ajuba-Grb2 interaction leads to an increase in Ras-dependent serum stimulated extracellular signal-regulated kinase (ERK) activation. The ERK activity in turn results in increased fibroblast proliferation (Goyal, Lin et al. 1999).

In summary, the Ajuba/Zyxin families play a robust and sometimes overlapping role in signal transduction within the cellular system. However, the roles of the Ajuba subfamily start to diverge from that of the Zyxin subfamily in epithelial systems.

Cellular roles of the Ajuba subfamily of LIM proteins

Studies have shown that Ajuba LIM proteins influence cell migration without impacting cell adhesion and cell spreading. In these studies, Ajuba acts upstream of focal

adhesion protein p130Cas localizing it to nascent adhesive sites in migrating fibroblast cells. The p130Cas–Crk complex leads to activation of Rac1, via an interaction with the DOCK180–ELMO guanine nucleotide exchange factor (GEF). Previous studies from our lab have also shown that Ajuba null primary MEFs are defective in cell migration. In response to the migratory cues Rac activation was found to be defective in the null cells, in part through aberrant assembly and localization of the p130Cas–Crk–DOCK180–ELMO Rac GEF (Pratt SJ et al., 2005). These data show that Ajuba regulates cell motility by activating Rac through regulating the recruitment of p130Cas to nascent adhesion sites.

In experiments conducted to further understand the signal transducing role of the Ajuba LIM proteins, it was found that the LIM region of Ajuba interacts with the atypical protein kinase C (aPKC) scaffold protein, p62, to regulate IL-1 induced NF- κ B activation by impacting the assembly and activity of the aPKC/p62/Traf6 multiprotein signaling complex (Feng and Longmore 2005).

In the nucleus, the Ajuba family members were identified as interactors of the SNAG domain of Snail, a transcriptional repressor. Interestingly none of the Zyxin family members interacted with the SNAG domain. In both in vitro as well as in vivo studies Ajuba LIM proteins function as SNAIL co-repressors to repress the transcription of E-cadherin. The Ajuba LIM proteins are recruited to the endogenous E-cadherin promoter in a SNAIL dependent manner (Langer, Feng et al. 2008). In vivo studies showed that expression of the Ajuba family members is similar to the expression pattern of SNAIL and they cooperate with each other during neural crest development in

Xenopus. In another study with respect to the nuclear function of Ajuba LIM proteins, it was found that the gene transcription regulating protein arginine methyltransferase (PRMT5), is a component of the SNAIL-silencing complex and does so being bound to Ajuba (Krause, Yang et al. 2007).

The Ajuba subfamily of LIM proteins are most abundantly expressed in tissue with extensive epithelia such as skin, kidney, and lung. In primary human keratinocytes Ajuba LIM proteins co-localize with cadherin adhesive complexes at sites of cell-cell contacts. Ajuba is recruited to cadherin adhesive complexes at AJ, in response to calcium addition, and occurs via a direct interaction with α -catenin bound to cell surface E-cadherin. Ajuba null mice appear to be completely viable, healthy and fertile with no gross morphological defects. However, Ajuba null mice keratinocytes exhibit abnormal cell-cell junction formation and/or stability and function. The Ajuba LIM domains responsible for targeting Ajuba to epithelial cell junctions do so through a regulated interaction with α -catenin (Reinhard, Zumbunn et al. 1999; Kanungo, Pratt et al. 2000; Marie, Pratt et al. 2003), while the PreLIM region of Ajuba directs its interaction with filamentous actin (Marie et al 2003). These data suggest that Ajuba may contribute to the bridging of the cadherin adhesive complexes to the actin cytoskeleton (Marie H et al., 2003).

Although the molecular mechanism for many of these processes is not completely understood these data provide us with clues to the roles the Ajuba subfamily of LIM proteins may play within the context of cellular processes. Based on previous studies and the shuttling ability of the Ajuba LIM proteins, the family members make strong

candidates for signal transduction between cell-cell junctions, the cytosol and the nucleus. Further investigations are necessary to determine other signaling pathways that are targets of the Ajuba LIM proteins and also the impact of the subcellular localization of the LIM proteins on their function. One issue that has not yet been overcome is the potential for functional redundancy between Ajuba, LIMD1 and WTIP, which would make it difficult to dissect their roles. This thesis will focus on creating an epithelial system to assay for the function of the Ajuba LIM protein subfamily as a whole. Further, it will establish a novel growth regulatory role for the Ajuba subfamily of LIM proteins and also signaling mechanism of this regulation via the Hippo pathway.

Figure 1: **FLP FRT mediated clonal analysis** allows the induction of mitotic recombination in a heterozygous ($^{+/-}$) background of cell (light green) that leads to one homozygous mutant ($^{-/-}$) daughter cell (GFP negative) and another homozygous wild-type ($^{+/+}$) cell (GFP positive- dark green) following cell division. Ensuing rounds of cell proliferation of the two sister-cell populations is then assessed later in development. Cells that are homozygous null for a growth-promoting gene (yki or bantam) will form smaller clones as compared to the darker green wild type cells (top, right panel). Cells that become homozygous null for the mutation in a growth-restrictive gene (such as hippo or warts) form larger clones relative to their wild-type sisters (bottom, right panel). And if there is no growth defect then the GFP negative clone of cells remain comparable to the wild-type twin spot (middle, right panel).

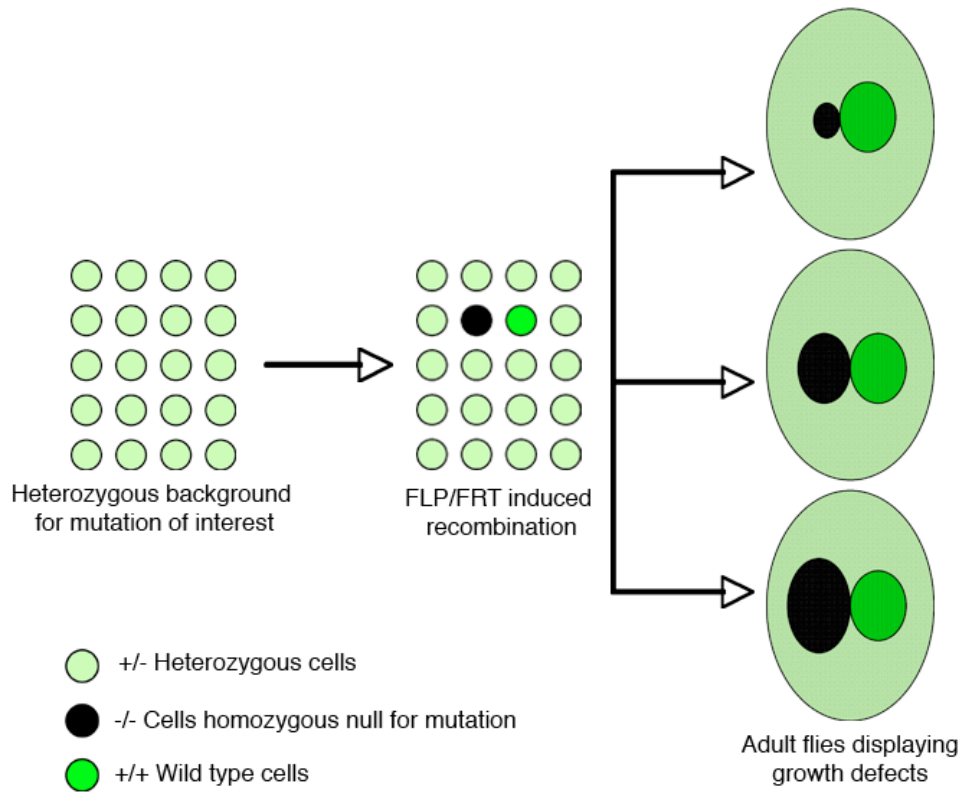
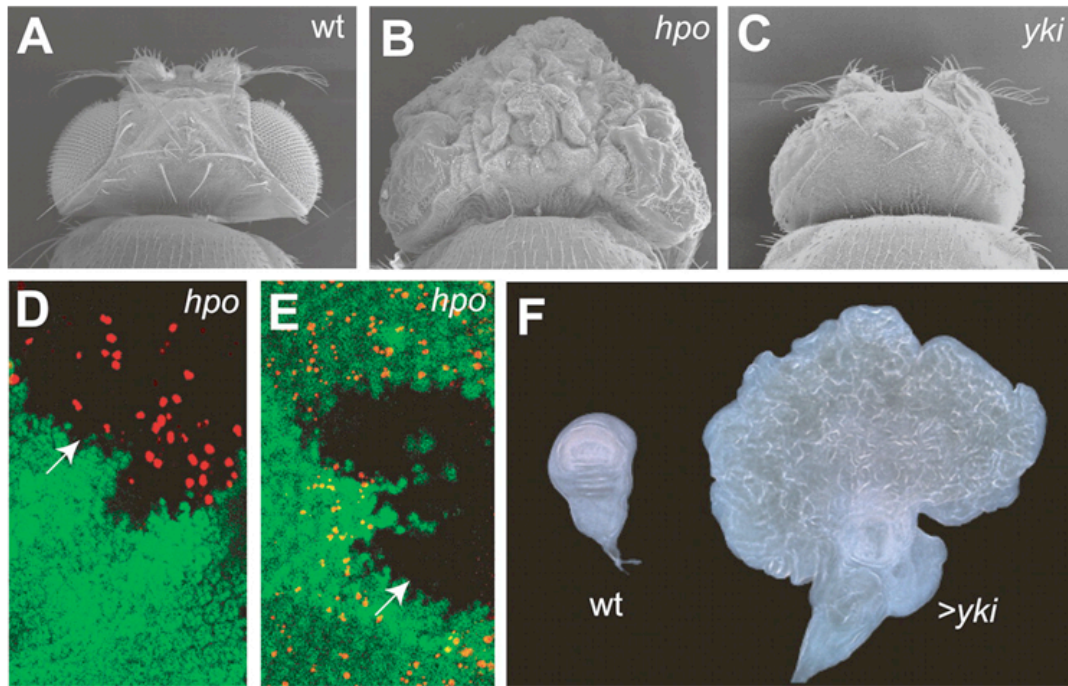


Figure 1

Figure 2: **Growth control by the hippo pathway.** (A-C) SEM images of adult *Drosophila* eyes the following genotypes: Wild type (A), Flies homozygous null for *hpo* displaying an overproliferation eye phenotype (B) and adult fly eye homozygous null for *yki* displaying severely reduced eye structure (C). BRDU staining in a *hpo* null clone (GFP negative cells) generated using eyeless-flp method. Increase in proliferation is reflected by the increase in BRDU (red) staining (D). TUNEL (red) staining in a *hpo* null clone (GFP negative cells) generated using Eyeless-flp method. There is a decrease in cell death (E). Overexpression of Yki in a larval wing disc (right) shows a dramatic increase in cell proliferation as seen by the overgrowth as compared to the wild-type wing disc on the left. The phenotype is similar to *hpo* null tissue (F).



Adapted from Wu et al. 2003 and uang et al. 2005

Figure 2

Figure 3: **The Hippo pathway in *Drosophila* and in mammals** as elucidated by genetic epistasis analysis and biochemical tests. Salvador/WW45 and Mats/MOB activate the kinase activity of Hippo/Mst and Warts/Lats, respectively. The membrane-associated proteins Merlin and Expanded promote the phosphorylation and activation of Warts/Lats by Hippo/Mst. Once activated, Warts phosphorylates and inactivates the transcriptional co-activator Yorkie/Yap. The green arrows represent the Hippo pathway in its ON state which leads to the phosphorylation of Yki/Yap and its sequestration in the cytoplasm. The red arrows represents the Hippo pathway in its OFF state which would allow Yki/Yap to enter the nucleus and act as a transcriptional coactivator to turn on growth genes.

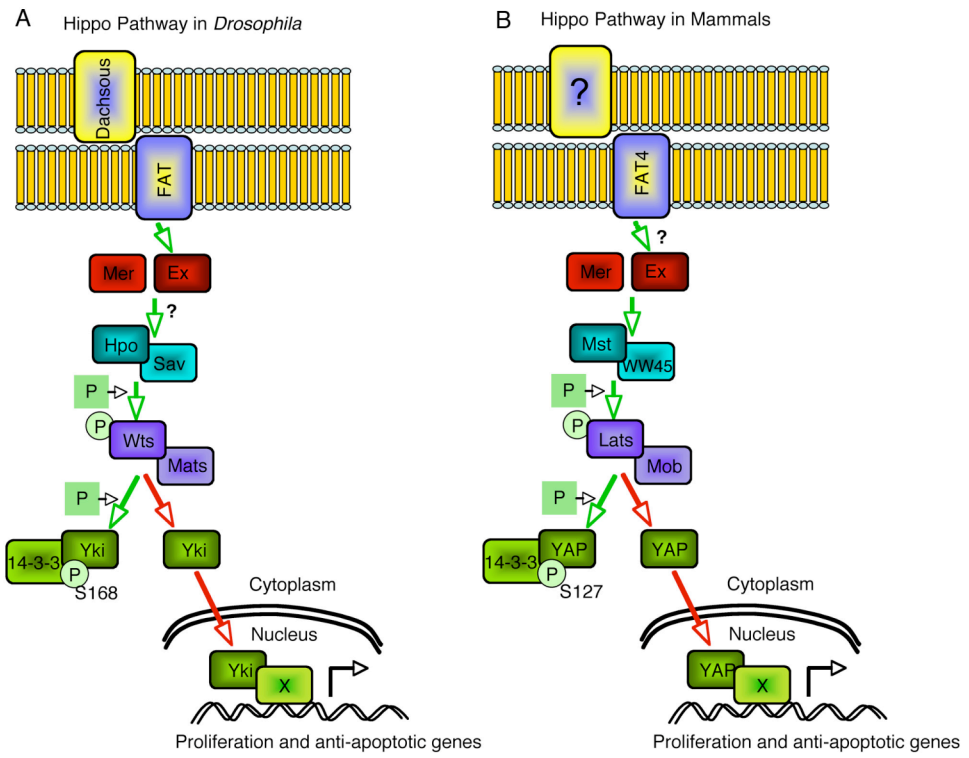
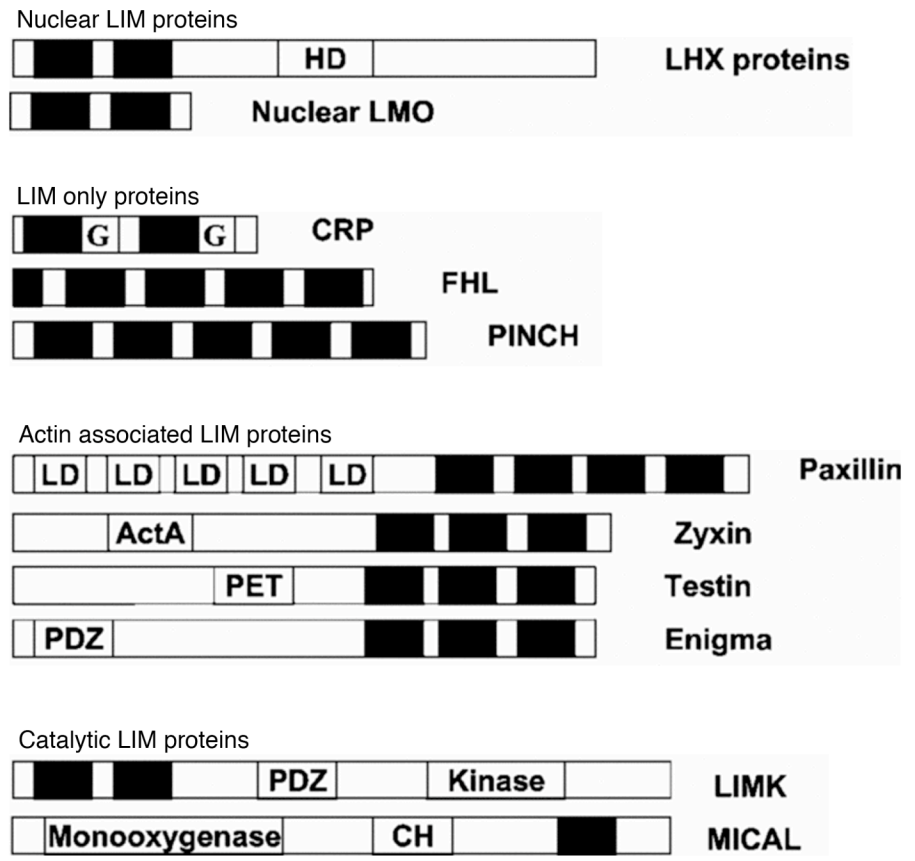


Figure 3

Figure 4: **Classification and the domain structures of LIM domain proteins.** LIM domain proteins are roughly classified into four groups according to the arrangement and position of LIM domains. The groups are: Nuclear LIM proteins, LIM only proteins, actin associated LIM proteins and catalytic LIM proteins. The individual LIM domains are shown as black boxes; other domains are shown as white boxes and indicated respectively on the Figure. HD: homeodomain; SH3, Src homology-3.



Adapted from Zheng et al 2007

Figure 4

Figure 5: **Schematic diagram of the Ajuba/Zyxin family of LIM proteins.** This subgroup of LIM proteins is characterized by three homologous C-terminal LIM domains and a non-homologous N-terminal PreLIM region. Phylogenetically, the family can be split into two subfamilies as shown, one being the Ajuba subfamily consisting of Ajuba, LIMD1 and WTIP and the other being the Zyxin subfamily comprising of Zyxin, LPP and TRIP6.

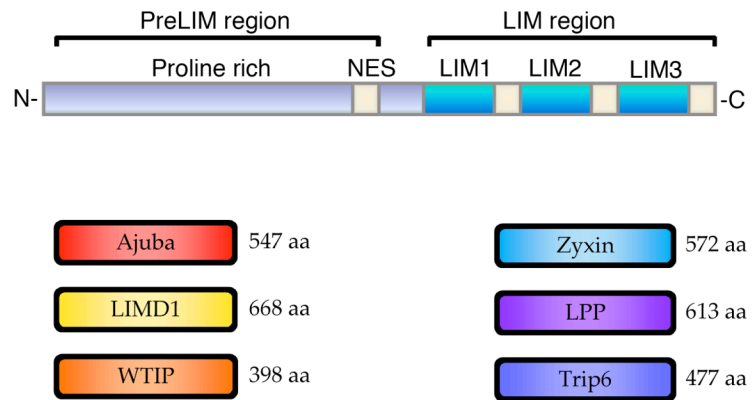


Figure 5

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Chapter 2

The role of Ajuba in cell-cell adhesion and epithelial polarity

Introduction:

In multicellular organisms epithelia constitute the boundary that separates the individual from the environment. Epithelial cells organize tissue architecture by acting as physiological and mechanical barriers by providing sites of exchange for ions and molecules (Rodriguez-Boulan and Nelson, 1989). The establishment of cell-cell adhesion contact sites or junctions is required for epithelia function. Once the junctions are formed, their integrity is preserved by the segregation and maintenance of specific proteins and lipids in distinct plasma membrane domains (i.e., epithelial polarity).

The link between epithelial cells is made possible by complexes such as desmosomes, gap junctions, adherens junctions (AJs) and tight junctions (TJ's) that together constitute the Intercellular Junctional Complex (Figure 1). The three main functions achieved by cell-cell junctions are: (1) adhesion, or mechanically attaching cells to one another, (2) communication between cells, which allows passage of chemical or electrical signals, and (3) establishment of epithelia apico-basal polarity. The junctional complexes contain transmembrane receptors, usually glycoproteins that mediate binding at the extracellular surface. The associated cytoplasmic proteins of these receptors structurally link them to the cytoskeleton. This connection helps establish molecular lines of communication to other cell-cell junctions and to cell-substratum junctions. The link between cell-cell junctions and the cytoskeleton allows single cells of an epithelial sheet to function as a coordinated tissue (Figure 1). Thus, intercellular junctions function to integrate a number of cellular processes ranging from cytoskeletal dynamics to proliferation, transcription, and differentiation (Kowalczyk et al. 1999 and Kowalczyk et al. 1999).

Recent evidence has uncovered a key role for AJs not only in directing coordinated cellular organization and movements within epithelia, but also in conveying information from the environment to the interior of cells. AJs are cadherin-dependent adhesive structures that are intricately linked to the actin microfilament network (Figure 1). The establishment and stability of AJs is tightly regulated and essential for processes such as wound healing, epithelial mesenchymal transition during normal development and in cancer metastasis, tissue morphogenesis and development. AJs consist of calcium independent (nectins) and calcium dependent (cadherins) transmembrane cell adhesion molecules (Perez-Moreno et al. 2003; Takai et al. 2003; Takai & Nakanishi 2003). Associated with the cytoplasmic tails of cadherins are the linker proteins, catenins that, in part, facilitate an interaction with the actin cytoskeleton. Precisely how cadherin engagement triggers AJ formation, epithelial polarity, and epithelial integrity is an area of active investigation. In addition to a structural role, AJ also generate local signals or cues that influence cell shape and motility (Gumbiner 1990). The activation of junctional complexes is thought to initiate various events such as epithelial polarization, assembly of other junctional components, such as desmosomes and TJs (Marrs et al 1995) and also initiating signaling cascades to trigger nuclear events leading to growth (proliferation via cell cycle genes) or cell death. Although the molecular and regulatory mechanisms are not fully understood, novel signaling events at AJ-cytoskeletal intersections are yet to be discovered.

The Ajuba LIM proteins are most abundantly expressed in tissue with extensive epithelia such as skin, kidney, and lung. As compared the Zyxin family of LIM proteins, the Ajuba subfamily are more highly expressed in epithelial tissue as compared to

fibroblasts (Figure 2A). In primary human keratinocytes Ajuba co-localizes with cadherin adhesive complexes at sites of cell-cell contacts. Ajuba is recruited to cadherin adhesive complexes at AJ, in response to calcium addition (Figure 2B), and occurs via a direct interaction with α -catenin bound to cell surface E-cadherin. Ajuba null mice appear to be completely viable, healthy and fertile with no gross morphological defects. However, Ajuba null mice keratinocytes exhibit abnormal cell-cell junction formation and/or stability (Figure 3) and function. The Ajuba LIM domains responsible for targeting Ajuba to epithelial cell junctions through a regulated interaction with α -catenin (Marie et al., 2003), while the PreLIM region of Ajuba directs its interaction with filamentous actin (Marie et al 2003). These data suggest that Ajuba may contribute to the bridging of the cadherin adhesive complexes to the actin cytoskeleton (Marie H et al., 2003). Although the exact role of Ajuba at cellular junctions and its downstream consequences are unclear, the LIM proteins may play a part in the assembly of protein complexes involved in processes leading up to stable junction formation between cells. We also know that Ajuba is recruited to cell-cell contacts at early stage of assembly of junctional complexes making it a good candidate for regulating the assembly of junctions. Many of the structural proteins at TJs and AJs act not only as structural components but also as signal transducers that may be involved in junctional biogenesis. However the molecular mechanisms by which Ajuba may be involved in cell adhesion as well as cell polarity are far from clear. Here we find that the depletion of Ajuba LIM proteins results in disrupted cell junctions and loss of cell polarity leading to a functional defect in both AJs and TJs in PDV and MDCK cells.

Results:

Establishing a cell based model to investigate the role of Ajuba LIM proteins in epithelia.

All previous studies regarding the epithelial role of Ajuba LIM proteins were done in primary keratinocytes from Ajuba null mice which although illustrative of the importance of Ajuba LIM proteins are not an ideal model system. The precise molecular mechanisms whereby Ajuba regulates junction formation and, or function are still not clear. The observed defects from the absence of Ajuba LIM proteins may arise from the inability to initiate the formation of proper junctions or the inability of Ajuba null cells to maintain proper junctions. To answer these questions the use of primary keratinocytes are not ideal owing to their inability to polarize precluding an analysis of epithelial polarity response and also for biochemical assays, since they do not proliferate.

Thus to address these questions we developed a stable cell based system by making clones of cultured kidney epithelial cells (Madin-Darby canine kidney; MDCK) and mouse epithelial cells (PDV) deficient in Ajuba LIM proteins. MDCK cells are ideal for imaging epithelial formation because of their ability to polarize in both 2- and 3-dimensional culture systems and undergo nascent cell-cell junction formation in response to added calcium. First we established the localization of Ajuba in PDV cells as well MDCK cells. Ajuba localized to the cell-cell junctions in PDV cells. In MDCK cells using filters we were able to distinguish the adherens junctions from the tight junctions and found that Ajuba specifically colocalized with E-cadherin to the adherens junctions (Figure 4A-B). To modulate Ajuba protein levels in PDV cells we used a lentivirus

system expressing shRNAs against mouse Ajuba. This FLR lentivirus system allowed for controls against off-target effects of RNAi and structure-function analyses of genes of interest by allowing for concurrent endogenous gene product knock down and exogenous rescue with GFP-tagged, RNAi-resistant forms of the gene under study, in the same cell (Figure 5). The presence of a puromycin selection cassette permit selection of transduced cells. The lentiviral vector system provided high efficiency, stable integration, and thus, expression of the shRNA (Figure 5). Using this stable lentivirus system, we knocked down Ajuba in mouse PDVs and MDCK cells. Ajuba protein levels were reduced by 85 %, as detected by Western blots of cell lysates from the cells infected with the lentivirus system (Figure 5B). Moreover, the blots also show the reintroduction of RNAi resistant YFP-Ajuba protein in the knocked down cells (Figure 5B). This enabled us to confirm that any defective phenotype from the knock down is specific to Ajuba and not an artifact of RNAi off-target affects. Also this knock down was specific to Ajuba as there were no observable change in protein levels of other junctional proteins such as PAR-3 and E-cadherin (Figure 6B) or other Ajuba family members LIMD1 (Figure 6B), between knock down and control lysates. Ajuba RNAi knock-down was also confirmed by immunofluorescence (Figure 6A). We also used a transient transfection method with double stranded RNAi oligos (Ambion), to knock down Ajuba in MDCK cells (Figure 6C, top panel).

Cells depleted for Ajuba LIM proteins display delayed nascent junction formation

To manipulate the process of cell-cell junction assembly in the MDCK cells we used the calcium switch method (Gao et al., 2002). The experiment involves the withdrawal of calcium from the cell media causing the rapid loss of cell-cell adhesion and endocytosis of surface E-cadherins. This process is then reversed by the re-addition of calcium (a calcium switch), which allows for the rapid reformation of nascent epithelial junctions (Figure 7A-C and 8A-A''). Removal of calcium from the media results in the disassociation of cell-cell junctions and the re-distribution of E-cadherin and Occludin. In wt and Ajuba $-/-$ cells when calcium was removed from the media, the intracellular staining of E-cadherin and Occludin increased and the plasma membrane labeling decreased at cell-cell contacts (Figure 8A, B, C, D). As a result cells lose their cell-cell junctions. Following calcium addition, recruitment of Occludin and E-cadherin back to the cell surface takes place within two hours in wild type cells (Figure 8A-A''), however this relocalization of the junctional proteins is significantly delayed in the Ajuba kd cells (Figure 8B- B'' and F-F''). These results show that the absence of Ajuba results in a kinetic delay of the recruitment of E-cadherin and Occludin to the lateral membrane during junctional complex assembly. Interestingly when we knocked down LIMD1 we observed a similar kinetic delay in the relocalization of Occludin and E-cadherin (Figure 8C-C'', G-G''). In MDCK cells deficient for both Ajuba and LIMD1 (Figure 6C) the recruitment of Occludin and E-cadherin to nascent junctions was more severely affected than when only the individual proteins were knocked down (Figure 8D-D'' and H-H'').

Cells depleted for the LIM proteins display defective cellular morphology

In addition to carrying out a calcium switch we also looked at the morphology of cells when they were knocked down for the LIM proteins. PDV cells infected with control virus maintained a flat monolayer of cells, while cells infected with Ajuba siRNA appeared to be mounded or taller than control cells (Figure 9A-B). We further were able to confirm this morphology defect in MDCK cells with Z-stack analysis of confocal images. MDCK cells were infected with lentiviral vectors encoding Ajuba siRNA, resulting in a loss of about 90% of Ajuba. The lentiviral vectors used also encoded either YFP alone or YFP-tagged murine Ajuba to rescue the Ajuba siRNA. Expression of YFP-mAjuba was also confirmed by western blot (Figure 5). Immunofluorescence was then performed on control, Ajuba knock down and rescue cells for E-cadherin. In MDCK cells with Ajuba knockdown, E-cadherin levels were not altered by knock down of Ajuba (Figure 9, E as compared to F, E-cadherin in red). Z-stack analysis using confocal microscopy revealed that the Ajuba knockdown cells are taller and have E-cadherin expressed on the apical surface, suggesting a possible effect on cell polarity as well (Figure 9E-F). This phenotype was rescued by the reintroduction of murine Ajuba, showing specificity of the siRNA (Figure 9G). Another phenotype observed in cells depleted for Ajuba in MDCK cells was that the cells appeared to form projections that either were trying to form stable junctions with the adjacent cells or were unable to maintain or preserve the junctions. This was seen by nucleofecting MDCK cells with Ajuba and LIMD1 siRNA and comparing them to the control pool of cells nucleofected with LucsiRNA and then stained with E-cadherin (Figure 9 C-D).

Ajuba LIM proteins are necessary the normal function of epithelial cells measured by cell aggregate formation and functional tight junctions.

The immunofluorescence data suggested that the Ajuba LIM proteins may play a role in cell-cell adhesion and junction formation. However, the calcium switch assays did not provide any functional assessment on the stability of the junctions in the absence of Ajuba LIM proteins. To determine whether the Ajuba LIM proteins affect junctional stability we performed the hanging drop assay (Figure 10A) that measure initial junction formation and the stability of formed junctions. Wild type cells formed large cell aggregates faster than the knock down and also acquire resistance to the trituration forces (strengthening of junctions) at a higher rate than the knock down cells (Figure 10B-I). This suggested that Ajuba contributes to cell-cell junction formation and/ or the stabilization of newly formed junctions. The results indicated that Ajuba depletion delays or inhibits the formation of large cell clusters.

The delay in the recruitment of Occludin to the tight junctions during calcium switch (by immnuofluorescence) suggested that Ajuba might play a role in the establishment of tight junctions. We next sought to determine if Ajuba depletion affected the establishment of apical-basal polarity. Although the tight junctions appear intact at the end of 6 hours of a calcium switch in the knockdown cells, do they function properly? To answer this question, we measured the pericellular permeability of control and knockdown cells by trans-epithelial resistance (TER) during a calcium switch at various time points. Tight junctions serve as a selective permeability barrier for paracellular ion flow and generating a resistance (the TER) between the apical and basolateral media

(Figure 11A). Thus, the TER measurements can be used to monitor the tightness of the seal between neighboring cells serving as a functional measure of tight junction integrity. Control and rescued Ajuba kd cells showed a rapid increase in TER after the re-addition of calcium, while in contrast, Ajuba kd cells exhibited a significant delay in TER development (Figure 11B). These results suggested that Ajuba contributes to the assembly of tight junctions between epithelial cells.

Establishing an Ajuba family null system in epithelial cells

The above results confirmed that creating a family null would be critical so as to understand the role of this protein family. The results from Figure 8 make it clear that the depletion of multiple family members (phenotype of Ajuba and LIMD1 double knock down is worse than the two individual knockdowns) worsens the delay in nascent junction formation during a calcium switch. To achieve this, we next established a way to knock down the third Ajuba subfamily member, WTIP. This would allow us to perform junctional formation and maintenance assays in the absence of all three proteins to dissect the role of this family in epithelia. As with knocking down Ajuba in MDCK cells using synthetic oligos custom made by Ambion, we used the same technique to knock down canine WTIP. First, we established the ability to carry out immunofluorescence against the WTIP antibody in MDCK cells. Like Ajuba and LIMD1, WTIP localizes to cell-cell junctions (Figure 12A-A''). Next we subjected MDCK cells to nucleofection using siRNA made to WTIP. For images taken with the same exposure we were successfully able to reduce the levels of WTIP in the cells (Figure 12B compared to A). After

establishing that we can knock down WTIP we next wanted carry out triple knock down experiments in MDCK cells to achieve a family null. We accomplished this by first generating a stable MDCK line depleted of Ajuba using lentiviruses expressing canine Ajuba shRNA, and then transiently depleting Limd1 and WTIP using RNAi oligos. The Ajuba and LIMD1 double null cells were viable, but when these cells were depleted of WTIP to generate Ajuba/LIMD1/WTIP triple depleted MDCK cells, we found significant amounts of cell death, precluding planned cell biologic analyses.

Discussion

The Ajuba family of LIM proteins, comprising of Ajuba, LIMD1 and WTIP are proteins that are predominantly found at cell-cell E-cadherin junctions in normal epithelia. Ajuba plays an important role in the formation of nascent epithelial junctions. In fact if the LIM proteins are depleted in epithelia, cells display a disruption of E-cadherin staining and cell morphology is altered. A similar phenotype is observed with knocking down LIMD1 or WTIP.

In this chapter, we established cell lines, namely PDV and MDCK cells, to circumvent the drawbacks of using primary keratinocytes for our analysis of the LIM proteins. The MDCK cells proved to be ideal for imaging the formation of epithelial sheets given their ability to polarize on filters undergo nascent cell-cell junction formation in response to added calcium and grow in large numbers for biochemical experiments.

To assay for the role the Ajuba LIM proteins play in nascent junction formation, cells depleted for the LIM proteins were subject to calcium switch experiments. Removal of calcium from the media resulted in the disassociation of cell-cell junctions and the redistribution of E-cadherin and Occludin. In wt as well as in cells depleted for Ajuba, LIMD1 and Ajuba/LIMD1 double knock down, the intracellular staining of E-cadherin and Occludin increased and the plasma membrane labeling decreased at cell-cell contacts. However, following calcium addition, recruitment of Occludin and E-cadherin back to the cell surface was delayed in the Ajuba LIM protein kd cells. The immunofluorescence data suggested a role for Ajuba LIM proteins in cell-cell adhesion and junction formation.

To assay for the junctional stability of cells depleted for the Ajuba LIM proteins, we carried out cell-cell aggregation assays that measure initial junction formation and the stability of formed junctions. We found that in case of wild type cells, they formed large cell aggregates faster than the knock down and also acquire resistance to the trituration forces (strengthening of junctions) at a higher rate than the knock down cells. This suggested that Ajuba contributes to cell-cell junction formation and/ or the stabilization of newly formed junctions. These results indicated that Ajuba depletion delays or inhibits the formation of large cell clusters. The delay in the recruitment of Occludin to the tight junctions during calcium switch (by immunofluorescence) suggested that Ajuba might play a role in the establishment of tight junctions as well. So to measure the function of the tight junctions we measured the paracellular permeability of control and knockdown cells by trans-epithelial resistance (TER). TER measurements are a functional measure of tight junction integrity. The lowered resistance of the knock down cells indicates that the tightness of the seal between neighboring cells is weakened. These results suggested that Ajuba LIM proteins contribute to the assembly of tight junctions between epithelial cells.

From all the assays carried out to understand the role of the Ajuba subfamily of LIM proteins, it appeared that the defect was a kinetic delay. The results shed light on a possible mechanism, however they also suggested that each of the family members compensate for any defect brought out by the depletion of the other. This was brought out by the analysis of cells knocked down for LIMD1 in MDCK cells in addition to Ajuba to look at the effect of the double knock down on cell-cell junction formation integrity. In MDCK cells deficient for both Ajuba and LIMD1 the recruitment of E-cadherin

(adherens junctions protein) and Occludin (tight junction protein) to nascent junctions was more severely affected than when only the individual proteins were knocked down. Given the possibility of functional redundancy, the next obvious direction was to create a family null. However although we were able to successfully knock down WTIP, we ran into technical difficulties with respect to the triple knock down or depletion of Ajuba, LIMD1 and WTIP. These cells showed extensive cell death making analysis of their epithelial integrity very difficult. Thus, future experiments will involve methods to circumvent this limitation by moving to different model system. The main issue within the mammalian system is the problem with redundancy of the three Ajuba LIM proteins. Future experiments were designed in a simpler model organism namely, *Drosophila melanogaster* where only a single gene for this family exists.

Methods:

Lentivirus experiment

To modulate Ajuba protein levels in MDCK cells we used a lentivirus system expressing shRNAs against canine Ajuba. This FLR lentivirus system allows for controls against off-target effects of RNAi and structure-function analyses of genes of interest by allowing for concurrent endogenous gene product knock down and exogenous rescue with GFP-tagged, RNAi-resistant forms of the gene under study, in the same cell. The presence of a puromycin selection cassette permits selection of transduced cells. The lentiviral vector system provides high efficiency, stable integration, and thus, expression of the shRNA.

Calcium Switch assay

To manipulate the junction assembly in the MDCK cells we used a calcium switch method (Gao et al., 2002). The experiment involves the withdrawal of calcium from the medium causing the rapid loss of cell-cell adhesion and endocytosis of surface E-Cadherins. This process is then reversed by the re-addition of calcium (a calcium switch), which allows for the rapid reformation of nascent epithelial junctions. MDCK cells are plated on Transwell collagen filters in 1XMEM media with 1.8mM calcium (normal calcium medium HCM). After 40-44 hours once a confluent monolayer of cells has formed the cells are washed with PBS and incubated in media containing no calcium (LCM) for 16-18 hours. The following day the media was switched back to HCM for indicated times, after which cells are washed, fixed, and stained with antibodies to Ajuba, E-cadherin (AJ), and Occludin (TJ) and visualized via confocal microscopy (Fig 4).

Hanging Drop Assay

To examine how Ajuba functions in cell-cell adhesion we used a cell aggregation assay called the hanging drop assay (Elbert M et al., 2006, Redfield *et al.*, 1997). In this assay the cells are placed in suspension culture and allowed to aggregate for various times. The cells are then counted and binned into four groups 0-5, 5-10, 11-50 or >50 cell aggregates. Over time cells incorporate into larger aggregates reflecting the rate of cell-cell junction assembly or the ability of cells to form intercellular connections with one another. Next the cells are triturated to break weak cell-cell interactions while strong cell-cell junctions allow cells to remain as aggregates. So the ability of the cells to remain in an aggregate after trituration with a micropipette tip relative to the number of cells that were initially in the aggregate before trituration gives the rate at which the cell-cell junctions are strengthened (Fig 5).

Transepithelial Resistance Assay

The assay requires a confluent layer of MDCK cells grown on the Transwell collagen filters and subject to a calcium switch. The TER is then measured using voltage and current clamps (EVC4000 Precision V/I Clamp, World Precision Instruments) over a 10 hour time period (Fig 6). The same numbers of cells are plated per filter allowing the TER from Ajuba knocked down to be compared with wild type or cells expressing the rescue construct.

MDCK cell culture and transfection

MDCK canine kidney cells were cultured in 1X MEM (Gibco) containing 10% FBS

(Invitrogen) and 50 µg/mL penicillin/streptomycin. If the cells were grown on filters media was applied above the cells on the filter as well below the cells in the well in which the filter was placed. Nucleofections (Amaxa cell line Nucleofection Kit L) was used to transfect MDCK cells with siRNAi directed against Ajuba and LIMD1, according to the manufacturer's instructions. Equal numbers of cells were immediately plated on three different sized dishes so as to have cells plated at low, medium and high densities. Forty-eight hours after nucleofection the cells were harvested for immunoblot analysis.

MDCK and PDV Immunofluorescence on Cover slips and filters:

MDCK cells as well as PDV cells were plated at desired confluency on cover slips. If using filters for MDCK cells (Transwell, 6.5 mm diameter [3495 Cat #] or 12 mm diameter, 0.4micron pore size by Corning) cells must be a monolayer. When ready, the cover slips (or filters) were rinsed once with PBS (1X) and then fixed in 4% Paraformaldehyde. If using filters, cells were fixed in Borisy Stabilization Buffer- 10mM Pipes, pH6.5; 127mM NaCl; 5mM KCl; 1.1mM NaH₂PO₄; 0.4mM KH₂PO₄; 2mM MgCl₂; 5.5mM glucose; 1mM EGTA; 4% Paraformaldehyde). The Borisy Stabilization Buffer was made as a 2X stock and stored at 4C. After fixation, the process can be stopped by washing the cover slips/filters 3X times in PBS (5min each) and storing the cells in the last PBS wash at 4C. Next the cells were permeabilized by washing them 3X times, 5min each wash, in PBST (PBS + 0.2% Triton X-100). If the LIMD1 or WTIP antibody was used the cells were further subjected to Guanadine Hydrochloride treatment (GHCl). Cells were treated with 6N GHCl for 10min at Room Temp. Following this, the cells (cover slips and filters) were washed thoroughly with PBS. For these washes 3 beakers with PBS were set up and each cover slip or filter was dipped 10-15 times in each

beaker to make sure all the GHCl has come off). After the GHCl step, the cells were blocked for 1hr in PBST + 3% BSA (IgG free). After blocking, the cells were incubated in primary antibody for 1hr at 37C (Antibodies were diluted in PBST +3% BSA and then spun down 20min at 4C before using). After the primary antibody incubation, the cells were wash 3X times for 5min each in PBST. Next, the cells were incubated with secondary antibody. Once again, the antibody was diluted in PBST +3% BSA and spun down for 20min at 4C before using. The cells were incubated in secondary antibody for 30min at 37C or Room Temp. The cells were then washed 3Xtimes in PBS. If using DAPI to stain nuclei, incubate cells for 5 min in DAPI (1:1000 in PBS) at Room temp and wash 3X for 5 min each with PBS. If using cover slips, mount cover slips on glass slides and seal with nail polish. Vectasheild mounting media was used (with or without DAPI). If using filters, at this point, filters were cut out using sharp scalpels and placed facing up (cells on top and filter towards the glass slide) on the slides. Mounting media was applied and a cover slip was placed on top before sealing with nail polish.

Antibodies for immunofluorescence were used at: Rab anti-Ajuba: 1:250, Rab anti-LIMD1 (Affinity purified from Wistar): 1:250, WTIP: 1:250 and Mouse anti-E-cadherin: 1:500. Secondary antibodies were used at 1:250. In case of cover slips, the top of a 12 well dish was covered in parafilm and then 30 microliters of the antibody (primary or secondary) was applied. The cover slip was then placed (cells down toward the antibody drop) to incubate. If using filters between 100-300 microliters of antibody was applied to the filters. In both cases, the 12 well dishes were placed in a tupper ware (either with a lid or with parafilm) box lined with water-soaked tissue to prevent the antibody from drying off.

Immunoblotting and Antisera used

Cells were lysed in RIPA buffer [150 mM NaCl, 10 mM Tris-HCl (pH 7.5), 1mM EDTA, 1% Triton-X100, 0.35% DOC, 0.25% NP-40, 0.1%SDS], PMSF and protease inhibitor cocktail (Sigma). Lysates were then cleared by centrifugation and boiled for 5 minutes in SDS sample buffer and resolved by 10% SDSPAGE, under reducing conditions. Proteins were transferred to nitrocellulose in transfer buffer (48mM Tris, 390mM, glycine, 0.1% SDS, 5% methanol) and immunoblots were probed with primary antibodies: rabbit α -Ajuba (1:1000), mouse α -Ecadherin (1:1000), rabbit α - LIMD1 (1:1000) and Wtip: 1:1000. For quantification, blots were minimally exposed and then scanned for quantification using ImageJ software.

Figure 1: **Schematic representation of a polarized epithelial cell.** Adapted from Miyoshi J and Takai Y Jan 5; (57):815-55. Tight junction- TJ, Adherens junction- AJ. TJ proteins Occludin, Claudin and JAM are connected to the actin cytoskeleton via the ZO proteins. The E-cadherin-catenin system lies below the TJ proteins at the AJ.

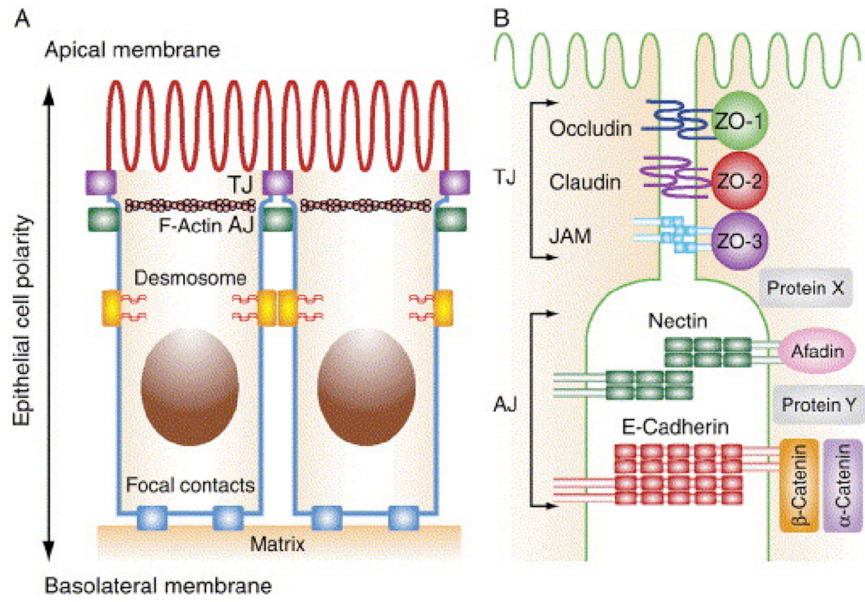
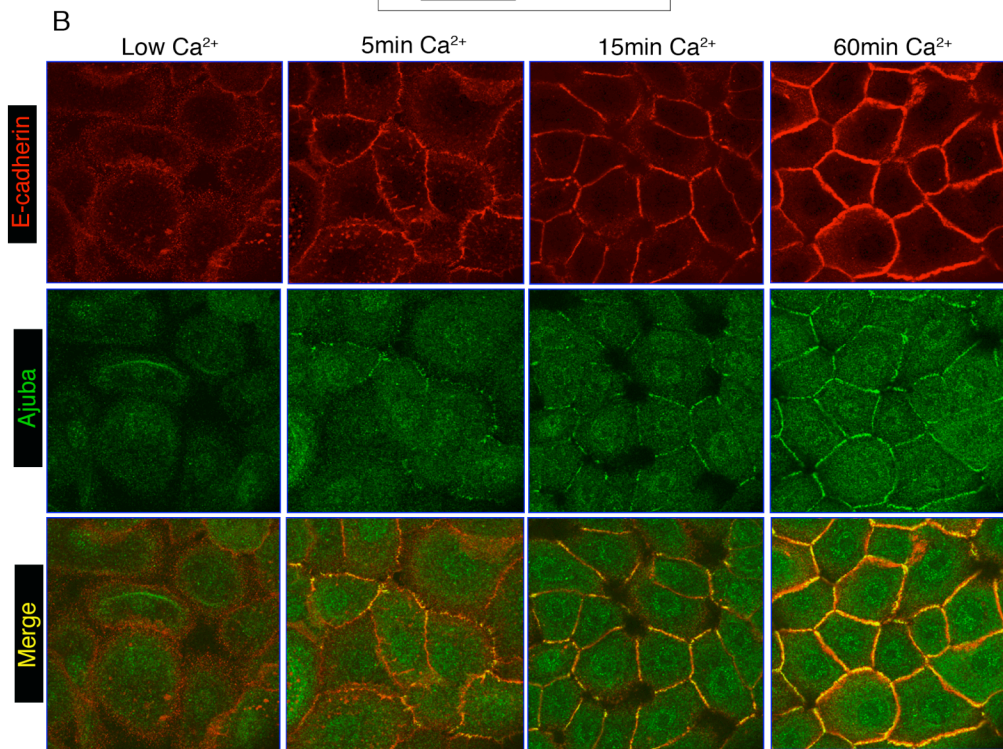
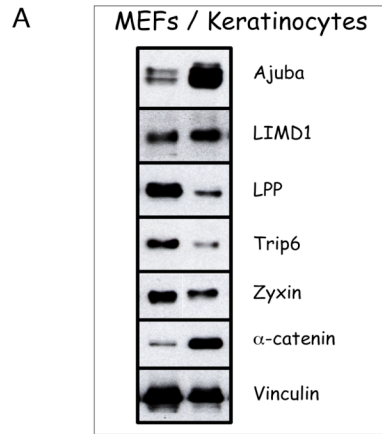


Figure 1

Figure 2: Ajuba is rapidly recruited to newly formed cell-cell contacts in keratinocytes. (A) Western blot analyses of Ajuba subfamily members (Ajuba and LIMD1) and Zyxin family members (LPP, TRIP6 and Zyxin) in primary mouse embryonic fibroblasts (MEF) (left column) and primary mouse keratinocytes (right column) with equal amounts of protein loaded in each lane. (B) Cells grown in low calcium medium loose cell-cell adhesion and E-cadherin (red) and Ajuba (green) proteins are internalized into the cell. Cells from Low calcium media (Column 1) were then transferred to standard medium for 5min (Column 2), 15min (Column 3) or 60 min (Column 4) to induce the formation of cell-cell contacts. Cells were stained for E-cadherin (*a, d, and g*) and Ajuba (*b, e, and h*). Merged images are shown in the bottom row. The four columns in the merge images show the distinct staining pattern of Ajuba (green) and E-cadherin (red) in the absence of junction formation. However with the addition of calcium and Ajuba and E-cadherin get recruited to the same compartments at the newly formed junctions.



Adapted from Marie et al. 2002

Figure 2

Figure 3: **Primary keratinocytes from Ajuba knock out mice display defects in junctional stability.** Adapted from Marie H et al. J. Biol. Chem. January 10, 2003; 278(2): 1220-1228. Primary keratinocytes isolated from Ajuba null and wild type newborn littermates. *A*, once confluent, cells were switched to low calcium medium, to initiate cell-cell junction formation, calcium was added back into the media. Cells were fixed and stained for E-cadherin. Significant gaps remain between cells even though E-cadherin is recruited to the cell surfaces normally.

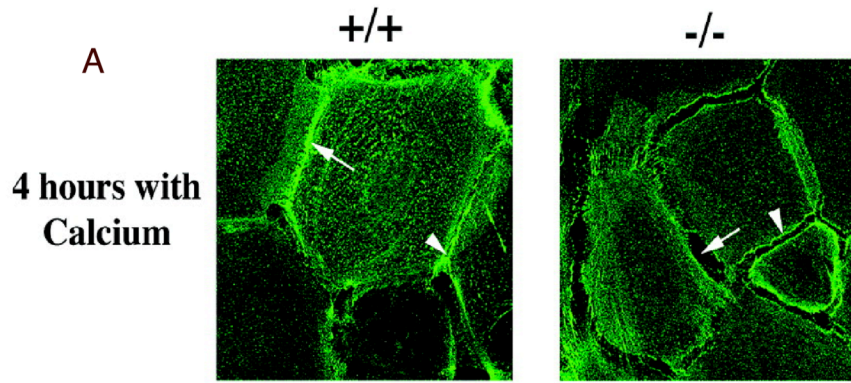


Figure 3

Figure 4: **Ajuba localizes to the Adherens junctions.** (A-A'') MDCK cells plated on collagen filters and allowed to polarize and then fixed and stained with E-cadherin (A), Ajuba (A') and merged (A''). (B) Z- stack view of the merged images from A'' showing the exact localization of Ajuba with respect to Adherens junction protein E-cadherin.

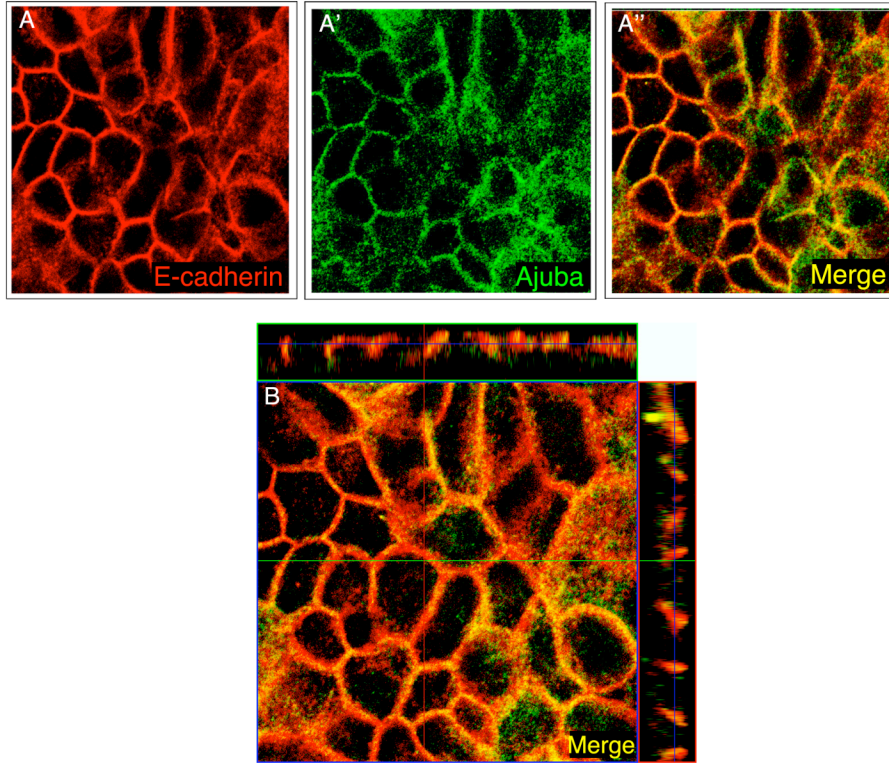


Figure 4

Figure 5: (A) **Map of the lentiviral vector pFLRu** containing 2 multiple cloning sites (MCS); MCS at 5' of Ubc for the shRNA expression cassette, and 3' of Ubc for the RNAi-resistant isoform of shRNA targeted gene containing an in-frame C-terminal GFP tag. The vector is also designed to contain a puromycin (puro) resistance cassette.

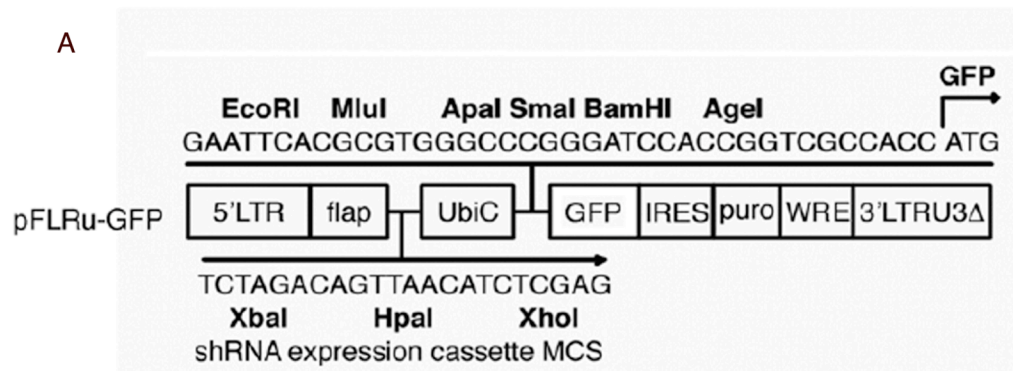


Figure 5

Figure 6. Ajuba knockdown in MDCK cells. Ajuba shRNAs were generated against canine Ajuba sequence and expressed in MDCK cells. Control samples expressed shRNAs against luciferase. (A) Immunofluorescence staining of cells expressing luciferase shRNA or Ajuba shRNA with GFP tags. (B) Western blot analysis of cell lysates from control, Ajuba kd and rescue cells, actin was used as the loading control. (C) MDCK cells knocked down for Ajuba and LIMD1 using transient Ambion dsRNA oligos using nucleofections and blotted for various junctional proteins as labeled.

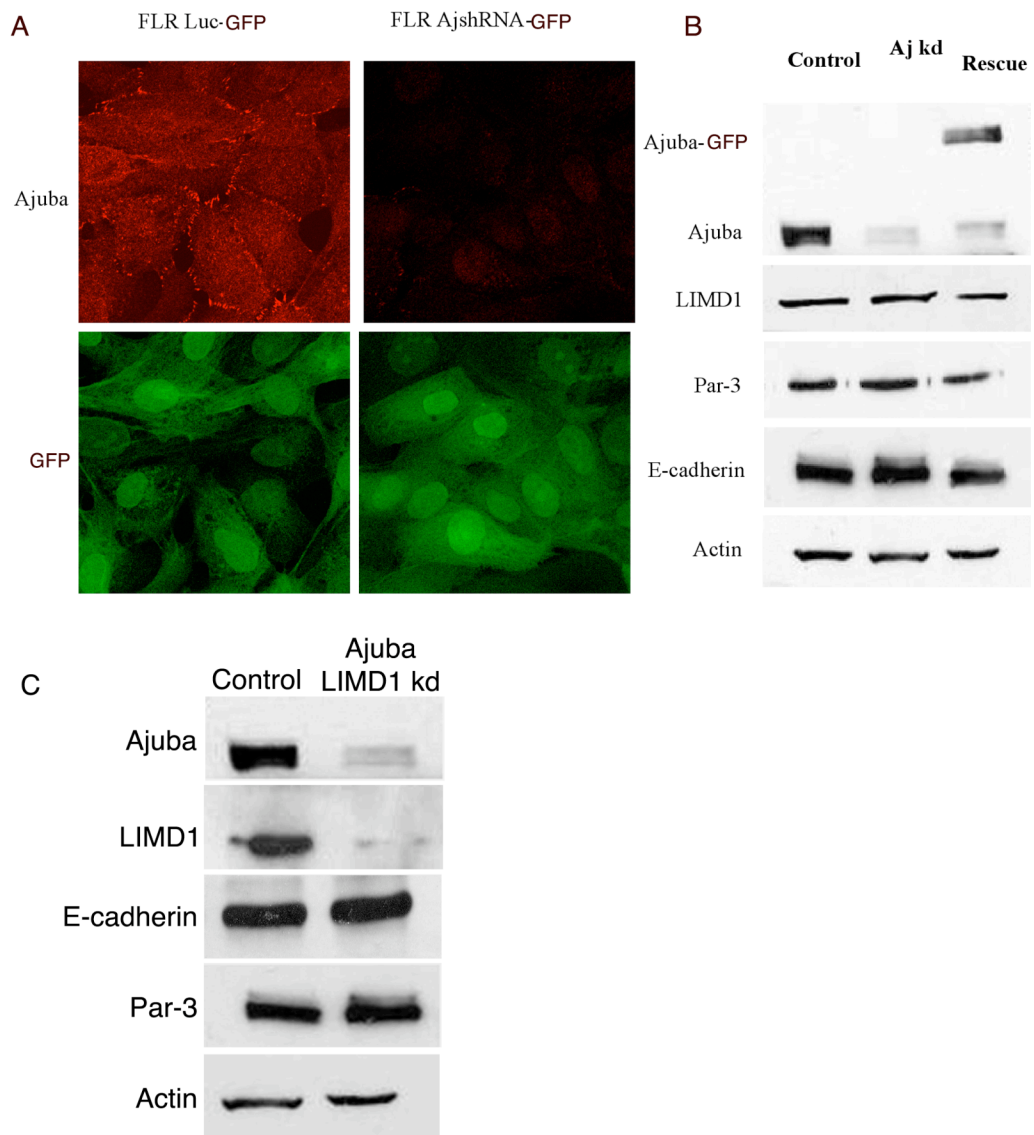


Figure 6

Figure 7: **Calcium Switch in MDCK cells.** (A) Cells are originally plated on collagen transwell filters so as to form a polarized monolayer of cells in normal calcium media. (B) When Calcium is withdrawn from the cell media the cells loose calcium dependant junctions and all junctional proteins become cellularized. (C) Finally the process can be reversed by the re-addition of calcium (a calcium switch), which allows for the rapid reformation of nascent epithelial junctions.

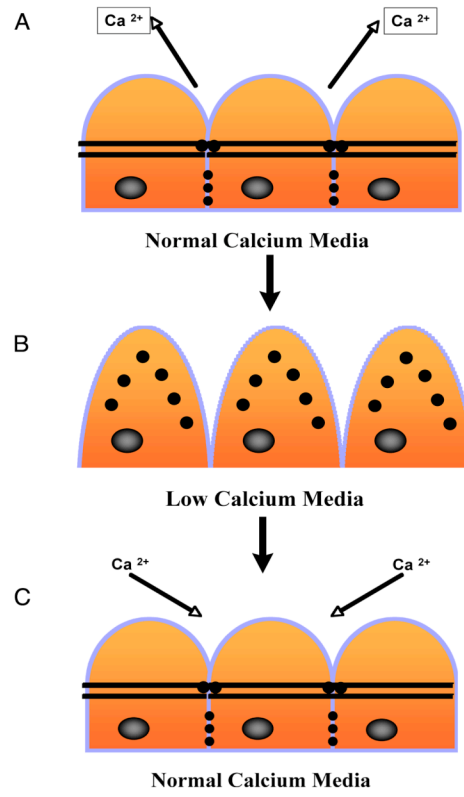


Figure 7

Figure 8. **Knockdown of the LIM proteins results in aberrant cell morphology.** (A-B) Phase contrast images of PDV control (A) and Ajuba knockdown lines showing altered cell morphology (B). (C-D) Confocal images of MDCK cells stained with E-cadherin in red and subject to control RNAI using Luc siRNA (C) and Ajuba and LIMD1 siRNA (D). (E-G) Z-stack analysis of confocal images of MDCK control (E), Ajuba knockdown (F), and rescue cell lines (G). YFP (green) shows infected cells, E-cadherin staining is shown in red.

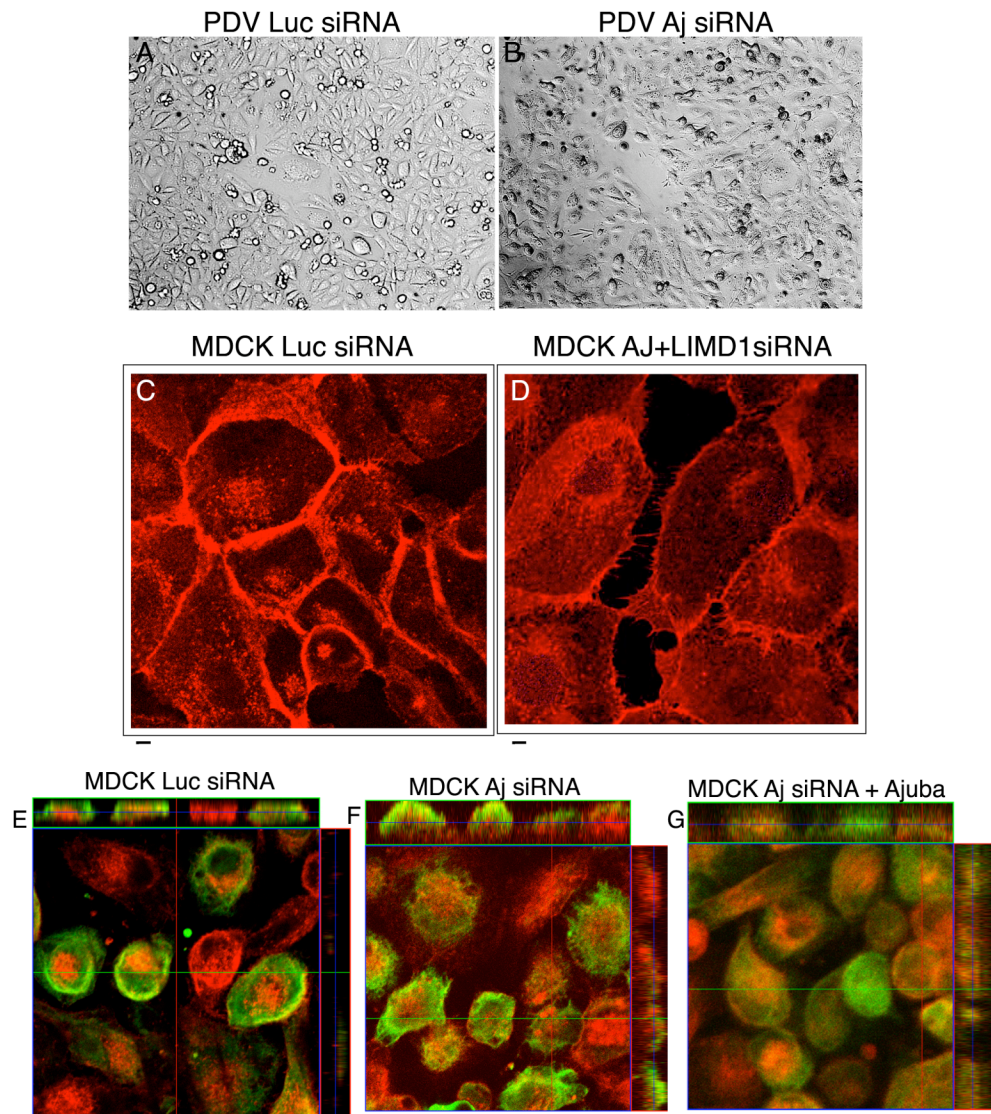


Figure 8

Figure 9: Ajuba deficiency delays recruitment of cadherin TJ protein Occludin and AJ protein E- to nascent junctions. (A-H) MDCK cells plated on collagen filters and grown in standard media until a confluent layer of cells was formed and then switched to LCM for 10-12 hrs, after which the media was switched back to HCM to induce a calcium switch. (A-D) Wild type cells (A-A''), Ajuba knock down cells (B-B''), LIMD1 knock down cells (C-C'') and Ajuba and LIMD1 double knock down cells (D-D'') stained for AJ protein E-cadherin, fixed before reintroducing calcium (0 min), 2 hours or 6 hours after addition of calcium. (E-H) Wild type cells (E-E''), Ajuba knock down cells (F-F''), LIMD1 knock down cells (G-G'') and Ajuba and LIMD1 double knock down cells (H-H'') stained for AJ protein E-cadherin, fixed before reintroducing calcium (0 min), 2 hours or 6 hours after addition of calcium.

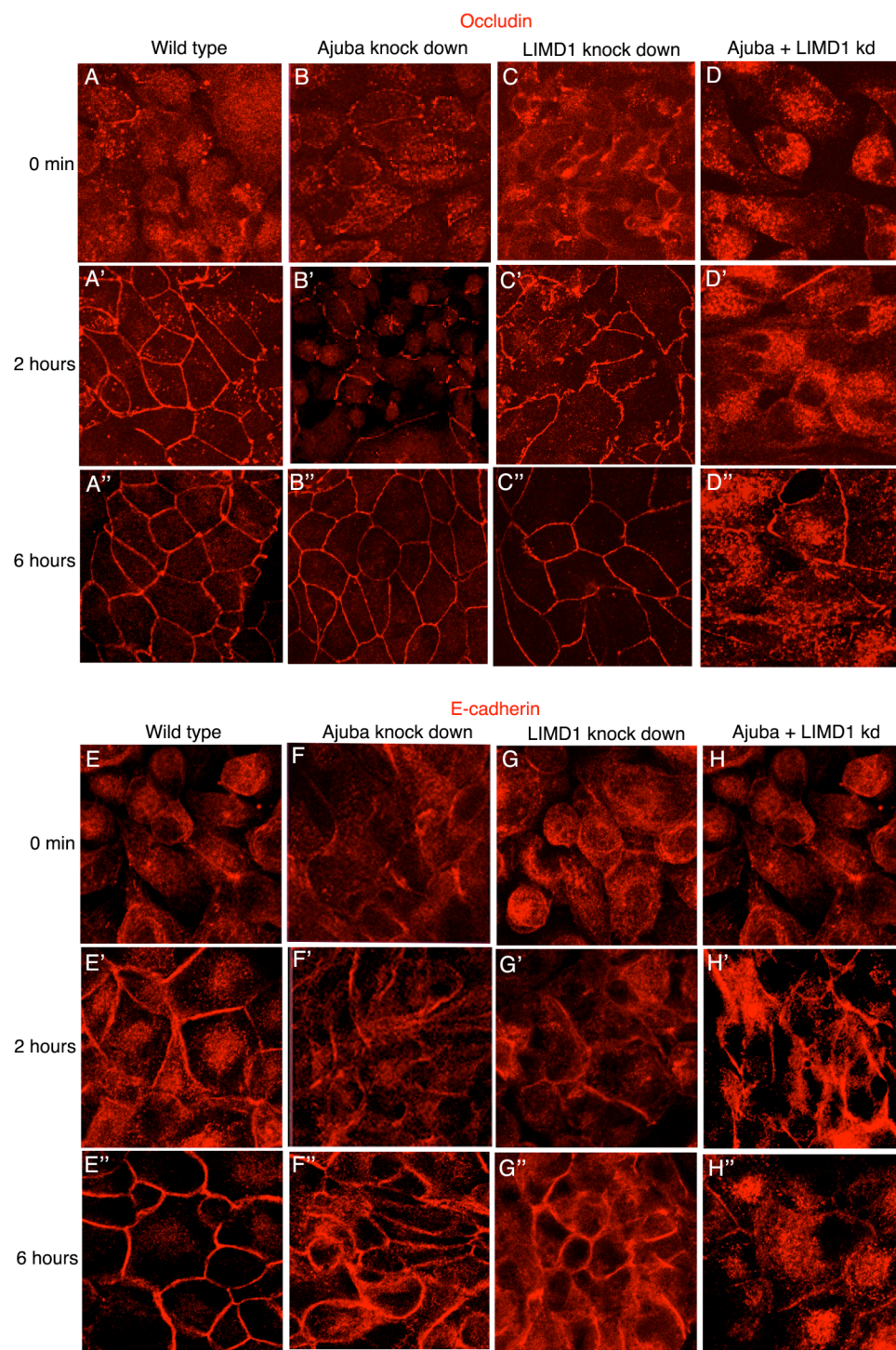


Figure 9

Figure 10: **Ajuba knock down cells have weakened cell-cell adhesion:** (A) Stable lines of MDCK epithelial cells, expressing either Luc shRNA or Aj shRNA were aliquoted into 50 ul drops and placed on the underside of the lid of a dish. The drops were allowed hang down for 4 hours. At every hour the number of aggregates (3-5 cell, 5-10 cells, 11-50 cells and >50 cells) formed were counted by phase microscopy before and after trituration (carried out by a 20ul pipette tip) for each cell line. (B-E) Control MDCK cells (B, C) and Aj RNAi cell (D, E) aggregates were counted for their size and number of cells within the aggregate before trituration (B and D respectively) and after trituration (C and E respectively). (F-I) The aggregate size and number were then graphically quantified for comparison.

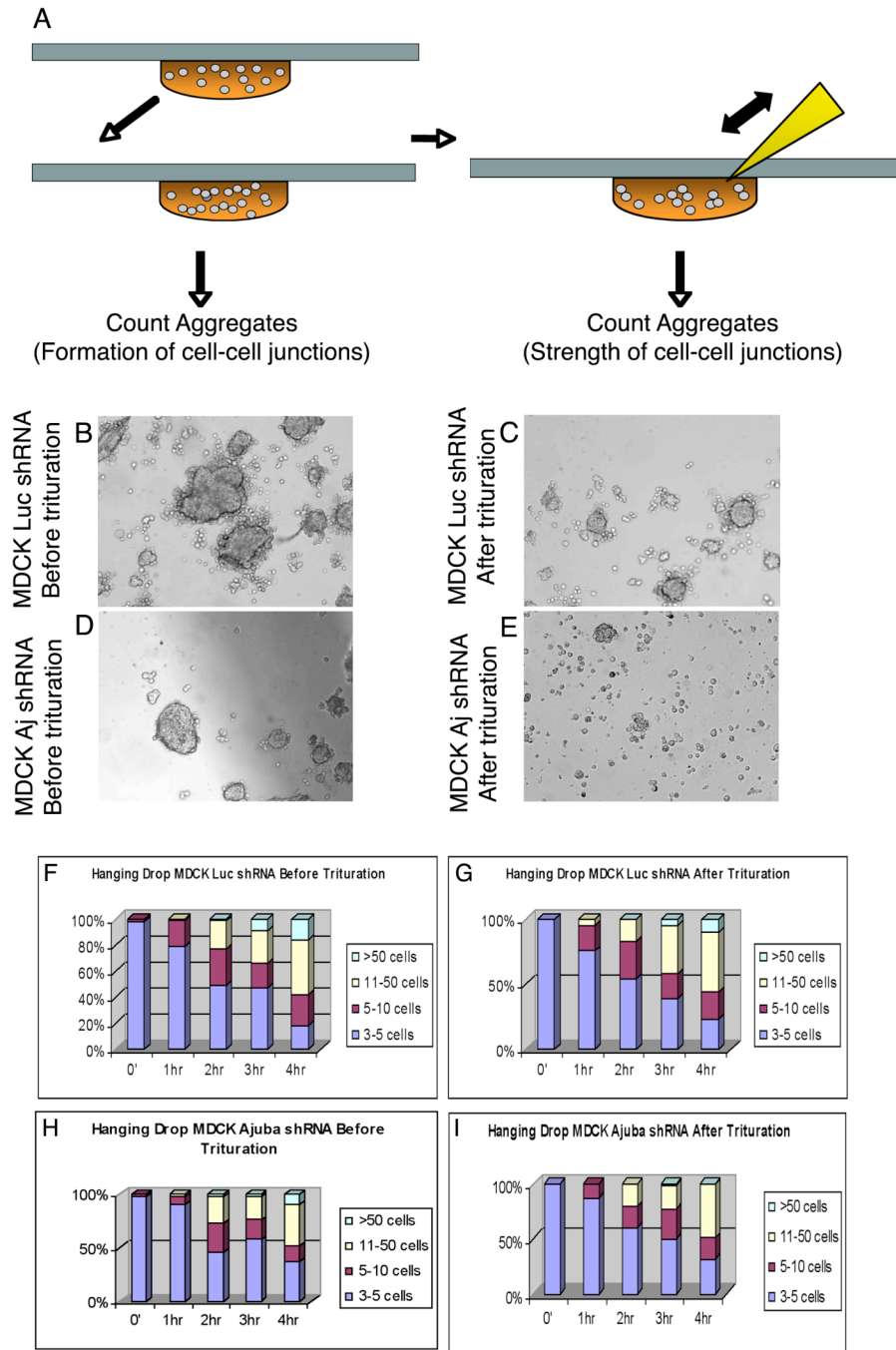


Figure 10

Figure 11: **Cells lacking Ajuba show delayed tight junctions formation.** (A) Stable lines of MDCK expressing either Luc shRNA, Aj shRNA or AjshRNA-mAj-YFP were plated on collagen filters. Once the cells formed a confluent layer, normal media was switched to low calcium media for 10-12 hrs, after which the media was switched back to high calcium media. The TER was then measured at every hour for 10 hours following the addition of calcium in the media. (B) A graphical representation of the TER measurements taken from control cells, Ajuba knock down cells and Ajuba knock down cells rescued using mouse wild type RNAi resistant Ajuba.

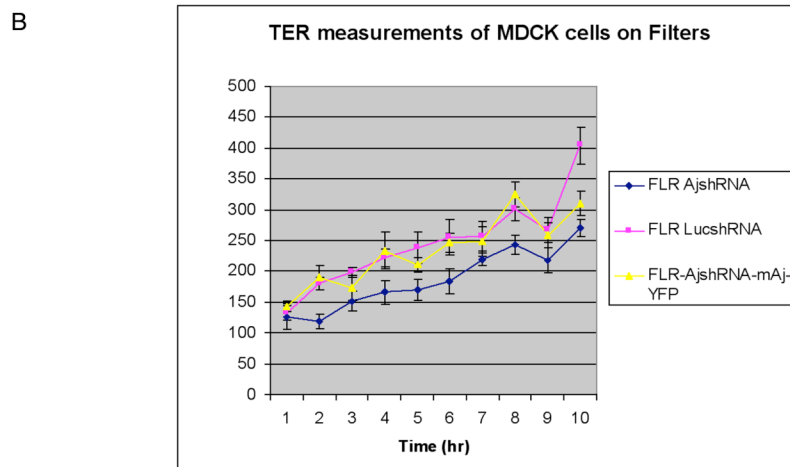
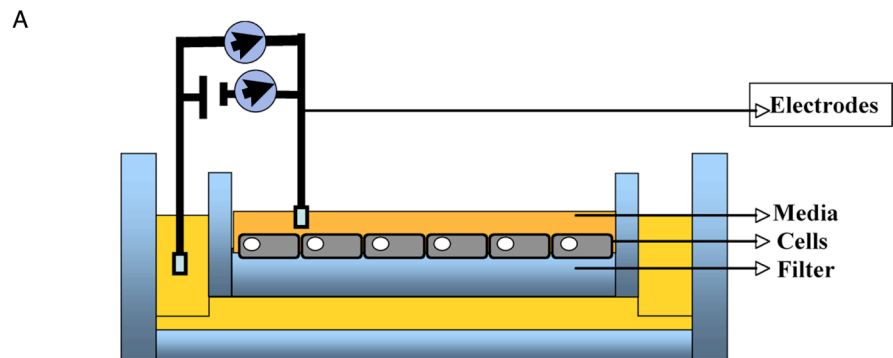


Figure 11

Figure 12: **WTIP knock down in MDCK cells via nucleofection.** (A-A'') MDCK cells stained for WTIP in green and E-cadherin in red in control Luc siRNA cells (A-A'') and MDCK cells knocked down for WTIP (B-B'').

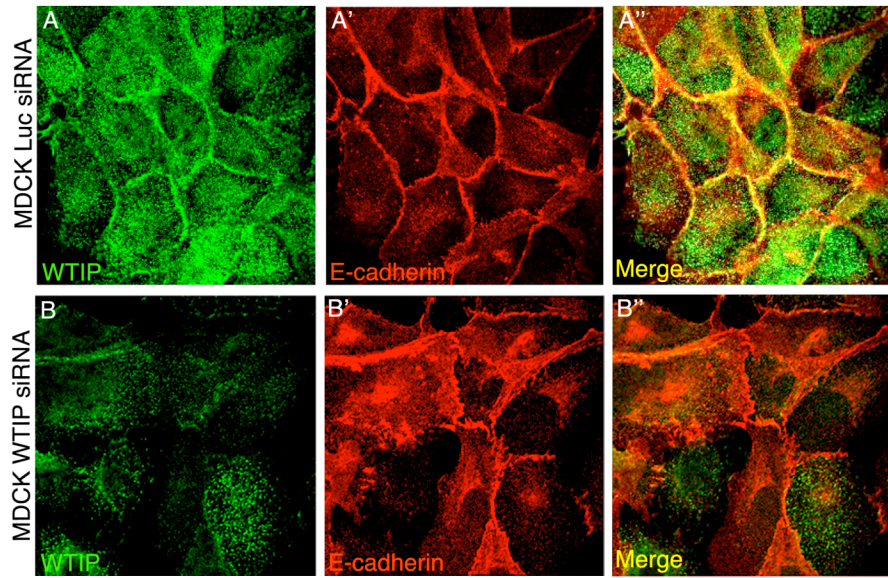


Figure 12

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Chapter 3

Ajuba LIM Proteins are Negative Regulators of the Hippo Signaling Pathway

Chapter 3 has been adapted from a submitted manuscript, Ajuba LIM Proteins are Negative Regulators of the Hippo Signaling Pathway. It also includes some unpublished data. All of this work was performed autonomously. The submitted manuscript has the following citation:

Meghna Das Thakur, Yunfeng Feng, Radhika Jagannathan, Midori J. Seppa, James B. Skeath and Gregory D. Longmore. Under review, 2010.

Abstract

The mammalian Ajuba LIM proteins (Ajuba, LIMD1, WTIP) are cytosolic adapter proteins recruited to nascent epithelial adherens junctions, where they are thought to contribute to junctional assembly and/or stability (Marie, Pratt et al. 2003). They also shuttle into the nucleus acting as corepressors of the Snail family of transcriptional repressors, thereby contributing to epithelial mesenchymal transition (Langer, Feng et al. 2008). As such they have the potential to communicate cell adhesive events with nuclear responses to remodel epithelia. Determining their role(s) *in vivo*, however, has been challenging due to shared interacting proteins, overlapping tissue expression and functional redundancy in cells. Thus, we turned to the *Drosophila* model system where a single gene, *CG11063* or *djub*, exists. The generation and analysis of *Drosophila* containing *djub* mutant loss-of-function alleles or depleted of dJub by RNAi identify *djub* as an essential gene required for normal development and a novel regulator of epithelial organ growth as a component of the conserved Hippo pathway, which has been implicated in both tissue size control and cancer development (Tapon, Harvey et al. 2002; Iida, Hirota et al. 2004; Lai, Wei et al. 2005). *djub*-deficient epithelial tissues were small due to decreased cell numbers resulting from increased apoptosis and decreased proliferation due to the downregulation of DIAP1 and cyclin E, phenocopying tissues deficient for Yorkie (Yki), the downstream target of the Hippo pathway. *djub* genetically interacts with the Hippo pathway, and genetic epistasis suggests that *djub* influences *wts* activity. In mammalian and *Drosophila* cells, Ajuba LIM proteins/dJub specifically interact with LATS/Wts and WW45/Sav to inhibit phosphorylation of YAP/Yki. This

work describes a novel role for the Ajuba LIM proteins as negative regulators of the Hpo signaling pathway.

Introduction

During development cell intrinsic as well as extrinsic factors coordinate to specify organ or tissue specific cell size and number. For all the diverse cell types, the final number of cells is determined by a balance of cell proliferation and cell death. Normal healthy tissue and the cells within cease to proliferate and grow once they have reached their final size. The question still remains of how these processes are regulated in normal healthy tissue and how cancer genes first initiate proliferation, which can then disrupt the intricate epithelial architecture.

Recent studies have established the role of the Hippo pathway in regulating size in *Drosophila* (Tapon, Harvey et al. 2002; Iida, Hirota et al. 2004; Lai, Wei et al. 2005) and has been implicated in cancer development in humans (Overholtzer, Zhang et al. 2006; Dong, Feldmann et al. 2007; Zhao, Wei et al. 2007; Steinhardt, Gayyed et al. 2008). The activation of the pathway enfold a kinase cascade where Hpo, a Ste-20-type kinase, forms a complex with Salvador (Sav), a WW-repeat adapter protein, and then activates Warts (Wts), a nuclear Dbf-2-related type kinase, by phosphorylation. Wts in turn binds to the Mats, Mob-as-tumor-suppressor protein, and phosphorylates the transcriptional coactivator Yorkie (Yki) at the Serine 168 site (Harvey, Pflieger et al. 2003; Pantalacci, Tapon et al. 2003; Udan, Kango-Singh et al. 2003). Phosphorylated Yki binds 14-3-3 proteins, which inhibit Yki from shuttling into the nucleus and induce the transcription of the Hpo pathway target genes. Yki has been ascertained to be an activator of proliferation and anti-apoptotic genes (Dong, Feldmann et al. 2007; Zhao, Wei et al. 2007). Also integral to the pathway is the ERM (ezrin/radixin/moesin) domain containing cytoskeleton proteins, Merlin (Mer) and Expanded (Ex) and the protocadherin Fat (Ft)

that are upstream of the pathway and function in the activation of the signaling cascade (Bennett and Harvey 2006; Badouel, Gardano et al. 2009).

Loss of function mutants of *hpo*, *sav*, *wts* and overexpression of *yki* all result in overgrowth of *Drosophila* epithelial tissue. The increase in proliferation and decrease in apoptosis is brought about by the misregulation of *yki* transcriptional targets such as cyclinE, *Drosophila* inhibitor of apoptosis protein DIAP1 and the microRNA bantam (Nolo, Morrison et al. 2006; Willecke, Hamaratoglu et al. 2006). Studies have also shown that the mammalian components of the Hpo/Sav/Wts/Yki pathway, namely Mst1/2, WW45, Lats1/2 and YAP and their size regulatory function are conserved in mammals. Furthermore, the *Drosophila* loss of function mutants for *yki*, *wts* and *hpo* can all be functionally rescued by their mammalian orthologs, YAP, Lats1 and Mst2 respectively (Tao, Zhang et al. 1999; Harvey, Pflieger et al. 2003; Dong, Feldmann et al. 2007).

Our studies have identified a novel negative regulator of the Hpo growth regulatory pathway, namely, the Ajuba LIM proteins. The family of Ajuba LIM proteins, namely Ajuba, LIMD1 and WTIP are closely related to the Zyxin (Zyxin, LPP, Trip6) family of LIM proteins. These proteins contain 3 homologous C-terminal protein interacting LIM domains and unique prelim region on their N-terminal region. Ajuba LIM proteins can be found in the cytosol, plasma membrane associated, or nuclear. In each subcellular compartment they appear to have distinct functions. They are present in most epithelia, in varying relative amounts. In epithelial cells Ajuba is actively recruited to newly forming cell-cell adhesions through an association with α -catenin bound to surface E-cadherin, where it can influence cell-cell adhesion formation or stabilization (Marie, Pratt et al. 2003). Ajuba LIM proteins also shuttle into the nucleus where they

have been shown to function as transcriptional co-repressors for Retinoblastoma protein (LIMD1) and Snail family proteins, where they can influence developmental epithelial mesenchymal transitions (EMT) (Goyal, Lin et al. 1999; Marie, Pratt et al. 2003). These results suggest that the Ajuba LIM proteins have the capacity to communicate cell surface events (adhesion) with nuclear responses (EMT, tumor transformation). These roles of the Ajuba family of LIM proteins make them excellent candidates for the coordination of cell surface roles resulting in a nuclear response. Since all three family members have largely overlapping expression in epithelia, albeit at different relative levels, a determination of their role in development, *in vivo*, has proven challenging. Both Ajuba^{-/-} and LIMD1^{-/-} mice are viable, and although LIMD1 mice are somewhat smaller, both develop normally. Likewise Ajuba/LIMD1 double null mice are also viable and develop normally.

To determine what role, if any, the Ajuba LIM proteins have in development, specifically in epithelial development, we decided to approach this problem in *Drosophila*. The main advantages of using the *Drosophila* as a model to ask our question was that flies have only a single Ajuba LIM protein family gene, *CG11063* on the X chromosome, thereby significantly reducing potential functional redundancy issues apparent in prior mouse experiments. Secondly, the fly system is well established as a model to study epithelium and the genetic tools available allow studying the effects of manipulating a gene in an *in vivo* system. To determine the role of Ajuba LIM proteins in epithelial development we generated drosophila lines expressing two different RNAs directed against *CG11063* and also a genetic null allele of *CG11063*, namely *djub*.

In the studies presented here we show that *djub* is an essential gene necessary for

normal *Drosophila* development. Moreover, our data suggest that *djub* regulates expression of CycE and DIAP1, key regulators of cell cycle progression and apoptosis. We provide evidence that *djub* acts through the Hippo pathway to regulate tissue size. Also *djub* acts genetically and biochemically upstream of Wts/LATS. Taken together the data suggest that *djub* is a negative regulator of the Hpo pathway.

Results

The *Drosophila* orthologue of mammalian Ajuba LIM proteins, dJub, regulates organ size

In *Drosophila* there is a single orthologue of the mammalian Ajuba subfamily of LIM proteins encoded by the *CG11063* locus in the X chromosome (Renfranz, Siegrist et al. 2003). *CG11063* exhibits greater sequence similarity to the three mammalian Ajuba subfamily proteins, than to *dZyx*, the *Drosophila* orthologue of the Ajuba-related Zyxin subfamily of LIM proteins (Zyxin, LPP, and Trip6) (Fig. S1A). We designate *CG11063* as *djub* (*Drosophila* Ajuba LIM proteins).

To determine the in vivo function(s) of *djub* in *Drosophila* we generated two different dJub RNAi lines: *djub-RNAi 22.5* and *djub-RNAi 18.1* (Fig. S1A). Ubiquitous expression of either, using *GAL4/UAS* and *actin-GAL4*, resulted in pharate lethality, suggesting that *djub* is an essential gene. Both RNAi constructs yielded similar phenotypes in all subsequent assays. Since *djub-RNAi 22.5* consistently induced stronger phenotypes we use RNAi 22.5 when referring to dJub RNAi.

Since Ajuba LIM proteins are abundant in mammalian epithelia (Goyal, Lin et al. 1999) and have been implicated in epithelia functions (Marie, Pratt et al. 2003), we selectively depleted *djub* function in larval wing and eye imaginal disc epithelium. *djub* RNAi expression in the wing, using *1096-gal4*, decreased wing size to 65% of wild type (Fig. 1B and E). Western blot analysis of tissues expressing dJub RNAi revealed an approximate 60% reduction of dJub protein level (Fig. 1H). The small wing phenotype was due to decreased cell number, not cell size, and wing patterning appeared unaffected (Fig. 1F). Similarly, *GMR-GAL4*-mediated expression of dJub RNAi in the pupal eye

epithelium resulted in a 25% reduction in interommatidial cells, without significant disruption to ommatidial patterning (Fig. 1J, K). These RNAi phenotypes were specific for dJub depletion, as overexpression of a wt *djub* transgene in dJub RNAi-expressing cells partially rescued both wing and eye phenotypes (Fig. S1D, H). Furthermore, overexpression of human LIMD1 (most closely related to dJub) also rescued the dJub RNAi wing phenotype (Fig. S1G, H), suggesting that this function of Ajuba LIM proteins is conserved between *Drosophila* and mammals. dJub and hLIMD1 overexpression in wt wings and eyes also resulted in a modest increase in size, due to increased cell number (Fig. 1C, E, F, R and data not shown). In pupal eye epithelium dJub localized to adherens junctions (AJs), predominantly in interommatidial cells, co-localizing with DE-cadherin in a punctate pattern (Fig. 1L-O). The HA-LIMD1 transgene also localized to AJs in wing larval disc epithelia (Fig. S1I). This cellular localization of dJub is similar to that for mammalian Ajuba LIM proteins in mammalian epithelia (Marie, Pratt et al. 2003).

We next generated *djub* mutant alleles using FLP-FRT based methods (Parks, Cook et al. 2004). Two distinct, yet overlapping, deficiencies of the *djub* locus were made (Fig. S2). The first allele, *djub*^I deletes *djub*, *CG11092* and the 5' region of *CG10997* (Fig. S2A). The second allele, *djub*^{II} removes *djub* and *CG32626* and the 3'most region of *CG11092* (Fig. S2A). In addition, both deficiencies yielded identical results for all phenotypic studies detailed below. Flies hemizygous for each deficiency died at late embryonic to first instar larval stage. Female flies (heterozygous for *djub*^I or *djub*^{II}) expressed 50% level of WAL-d protein, as determined by Western blot (Fig. S2C). Importantly, ubiquitous expression of wt *djub* transgene rescued lethality of both alleles, confirming that the loss of *djub*, and not the flanking genetic material, was responsible

for this phenotype and that *djub* as an essential gene.

When dJub was selectively deleted in the eye, using *eyeless*-FLP (EGUF/hid) to produce eyes composed of over 90% *djub*¹ mutant cells (Stowers and Schwarz 1999), adult eyes were severely reduced in size (Fig. 1Q). Genetic mosaic analysis of *djub*¹ mutant and wt twin-spot clones in eye and wing imaginal discs resulted in *djub*¹ mutant clones (Fig 1T, U yellow arrows) that were significantly smaller than wt twin-spot clones (Fig 1S, U red arrow). To verify that these growth defects were specific to loss of *djub* function, we induced *djub*¹ mutant clones throughout the wing imaginal disc while simultaneously expressing a wt *mCherry*-tagged *djub* transgene only in the posterior half of the wing disc using *engrailed-gal4*. In the anterior compartment *djub*¹ mutant clones were small and few in number (Fig. S2E). In contrast, the posterior compartment contained more and larger clones, similar to wt clones induced in a wt background (Fig. S2D-F).

***djub* mutant clones exhibit reduced proliferation and increased apoptosis**

The growth phenotype of *djub*¹ mutant clones could result from decreased cell proliferation and/or increased apoptosis. In wt larval eye imaginal discs undifferentiated cells lie anterior to the morphogenetic furrow and undergo asynchronous cell divisions (Fig. 2A, white arrow). Posterior to the furrow cells either differentiate or undergo one more cell division – the second mitotic wave (Fig. 2A, yellow arrow) – after which they differentiate or die (Ready, Hanson et al. 1976; Tomlinson and Ready 1987). Bromodeoxyuridine (BrdU) labeling of wt and *djub*¹ mutant eye discs, generated via the EGUF-Hid method, revealed that *djub*¹ eye discs displayed a strong reduction in the

number of cells undergoing asynchronous cell division anterior to the furrow (Fig. 2A', white arrow) and a near complete loss of the second mitotic wave (Fig. 2A', yellow arrow). During eye development apoptosis determines the final number of cells in the eye (Baker 2001). Staining eye discs for activated caspase-3 revealed that *djub*¹ mutant eye discs contained increased number of caspase-3-positive cells (Fig. 2B, B'), and when the caspase inhibitor P35 was coexpressed throughout a *djub*¹ mutant adult eye the small eye phenotype was partially rescued (Fig. S3B, C). Relative to wt, *djub*¹ mutant clones exhibited decreased levels of *Drosophila* inhibitor of apoptosis-1 (DIAP1) (Fig. 2C) and Cyclin E (Fig. 2E). dJub appeared to control transcription of DIAP1 as *djub*¹ mutant clones expressed less lacZ, under control of the *diap1* gene promoter (Fig. 2D). These data indicate that dJub regulates organ size by inhibiting apoptosis and promoting cell proliferation through influencing DIAP1 and Cyclin E expression, respectively. Although loss of dJub reduced eye size, photoreceptors cells (ELAV-positive cells) still developed (Fig. S3A), suggesting that dJub did not influence cell specification steps and that the differentiated cells did not require dJub for their survival. Furthermore, dJub deletion did not affect adherens junction organization, as determined by DE-cadherin staining of *djub*¹ mutant clones (Fig. S3D).

DJub genetically interacts with the Hippo pathway

The *djub* loss of function phenotype resembles that of *yorkie* (*yki*), which encodes a transcriptional coactivator, the activity of which is antagonized by the Hippo signaling pathway. Active Yki promotes proliferation and inhibits apoptosis by facilitating transcription of Cyclin E and DIAP1 (Huang, Wu et al. 2005; Oh and Irvine 2008). Given

the similarity between the *djub* and *yki* loss of function phenotypes we hypothesized that dJub governs organ size by affecting Yki activity either directly, or indirectly by inhibiting Hippo pathway function. Hippo pathway mutants (*hpo*, *sav*, *wts*) produce overgrown adult eyes and pupal eyes with increased interommatidial cells (Fig. 3C, E, G, and J, J', L, L', N, N', P respectively) (Harvey, Pflieger et al. 2003; Udan, Kango-Singh et al. 2003). Removing a copy of *djub* reduced the magnitude of *hpo* and *sav* mutant phenotype (Fig. 3D, K, K', P and F, M, M', P), and modestly affected the *wts* phenotype (Fig. 3H, O, O', P). In a reciprocal manner, a 50% reduction in Wts suppressed the dJub RNAi small wing phenotype (Fig. S4A-E), while a 50% reduction of Yki enhanced this phenotype (Fig. S4F-J). Taken together these two analyses suggest the possibility that *djub* and the Hippo pathway genetically interact. If so, then *djub* specifically interacted with the Hippo pathway of organ growth control as no genetic interactions were observed between dJub and Myc or components of the Insulin receptor signaling pathway, known to regulate organ size by affecting cell size (Fig. S4K). DJub localization to AJ was unaltered in *wts*, *hpo*, and *sav* mutant pupal eyes (Fig. S3E-G).

Epistatic analysis suggests that *djub* acts upstream of *wts* and *yki* but downstream of *hpo*

If *djub* genetically interacts with the Hippo pathway then there should be an epistatic relationship to components of the Hippo pathway. To determine where in the Hippo pathway dJub acts we performed genetic epistasis experiments between *djub* and *yki*, *wts* and *hpo*. MARCM pupal eye clones of *djub*¹ alone result in small clones (Fig. 4B, I), whereas MARCM clones overexpressing Yki or depleted of Wts or Hpo result in

increased clonal area as well as overproliferation of interommatidial cells (Pantalacci, Tapon et al. 2003; Wu, Huang et al. 2003; Huang, Wu et al. 2005; Edgar 2006) (Fig. 4C, E, G, I and Fig. 5C, E, G and I, respectively). *djub*¹ mutant MARCM clones overexpressing Yki displayed a phenotype identical to overexpression of Yki alone (Fig. 4D, I and Fig. 5D, I). *djub*¹ mutant MARCM clones depleted of Wts, resembled *wts* RNAi clones (Fig. 4F, I and Fig. 5F, I), however, removing *djub* in *hpo* RNAi MARCM clones resulted in a *djub*¹-like phenotype (Fig. 4H, I and Fig. 5H and I). This epistatic analysis suggested that *djub* acts downstream to *hpo* but upstream of *wts* and *yki*, but since the core Hippo pathway proteins (Hpo, Sav, Wts, and Mats) are thought to function as a complex, a precise epistatic relationship is difficult to conclude.

Ajuba LIM proteins/dJub associate with LATS/Wts and WW45/Sav in mammalian and *Drosophila* cells, respectively, and influence YAP activity in mammalian cells

The Hippo pathway is highly conserved between *Drosophila* and vertebrates (Tao, Zhang et al. 1999; Wu, Huang et al. 2003; Lai, Wei et al. 2005; Dong, Feldmann et al. 2007), and human LIMD1 rescues the cell growth defects of dJub depleted *Drosophila* wings (Fig. S1G, H). To determine whether Ajuba LIM proteins interact with Hippo pathway components in cells, and if so whether these interactions are functionally relevant, we tested whether the mammalian homologs of dJub (Ajuba, LIMD1 and WTIP) associated with mammalian orthologues of Hippo pathway members in human HEK293 epithelial cells, through co-immunoprecipitation experiments. All three Ajuba subfamily members associated strongly with LATS1/2, and Ajuba and WTIP associated

with WW45, but none associated with MST1/2 or YAP (Fig. 6A-C). The interaction between LATS and WW45 and Ajuba family proteins was specific as Zyxin, the most closely related LIM protein to Ajuba family members, failed to associate with either LATS or WW45 (Fig. 6D). In transfected *Drosophila* S2 cells dJub associated with Wts and Sav but not Hpo (Fig. S4L left panel). A weak association between dJub and Yki was noted but this was >10 fold less than that observed for Wts and Sav, and may well be nonspecific as transfected Yki was massively overexpressed in S2 cells (Fig. S4L, right panel).

To determine if these protein-protein interactions were functionally relevant we asked whether Ajuba LIM proteins affected YAP activity (i.e., YAP phosphorylation) (Dong, Feldmann et al. 2007; Oh and Irvine 2008; Reddy and Irvine 2008). Transfection of MST1, WW45, LATS1/2 alone into HEK293 cells resulted in variable increase in phospho-S127-YAP levels, however, when co-transfected with LIMD1 phospho-YAP levels were decreased in all instances (Fig. 6E). Overexpression of dJub in drosophila imaginal discs did not appreciable change the level or subcellular localization pattern of Yki or other Hippo pathway targets namely, Ex and Diap1. This may be due to the fact that only a small 10% increase in wing size occurs in wings overexpressing dJub (Fig. 1C, E, F). In another approach, Ajuba and LIMD1 were RNAi-depleted in MDCK cells, and phospho-YAP levels determined in cultures of cells at differing density. Analysis of MDCK cells depleted of all three Ajuba LIM proteins was not possible as cells died, like drosophila cells lacking dJub. Compared to control MDCK cells, in cells depleted of Ajuba and LIMD1 basal phospho-YAP levels were increased 2.5 fold in all three densities (Fig. 6F). Taken together, these results demonstrated that mammalian Ajuba

LIM proteins and dJub specifically associate with LATS/Wts and WW45/Sav in cells, and in mammalian cells these associations antagonize the phosphorylation of YAP.

Discussion

The recently described Hippo pathway has been established as one of the vital mechanisms that restrict organ size in *Drosophila*, and is well conserved in mammals as well (Tapon, Harvey et al. 2002; Iida, Hirota et al. 2004; Lai, Wei et al. 2005). Moreover mutations in components of the Hippo pathway have been implicated in the development of human cancers (Tapon, Harvey et al. 2002). The pathway accomplishes this by controlling the function/subcellular localization of the transcriptional coactivator Yki through a core protein kinase cascade (Hpo and Wts) that leads to selective phosphorylation of Yki, by Wts, resulting in the cytoplasmic accumulation of Yki, and thus, transcriptional inactivity (Huang, Wu et al. 2005; Oh and Irvine 2008). We now report that the single *Drosophila* orthologue of the Ajuba LIM protein family, *djub*, impinges on the function of Yki by inhibiting the Hpo pathway. Both biochemical and genetic evidence implicates dJub as a negative regulator of the Hpo/Wts pathway. In the first instance, mammalian Ajuba, LIMD1 and WTIP as well as *Drosophila* dJub LIM proteins specifically associate with Lats (Wts) and WW45 (Sav) in cells and their overexpression limits the phosphorylation of YAP (Yki) (Fig. 6 and Fig. S4). In *Drosophila* *djub* genetically interacts with Hpo pathway mutants, and based on epistasis analysis *djub* appears to act upstream of *yki* and *warts* yet downstream of *hpo*.

We provide several lines of genetic evidence that places the activity of *djub* downstream of Hpo and upstream of Wts. First, the growth defect of *djub* null mutants is phenotypically similar to *yki* loss of function and *hpo* and *wts* gain of function (Hamaratoglu, Willecke et al. 2006), where tissue size is severely reduced due to an increase in apoptosis and a decrease in cell proliferation with a corresponding

downregulation of the target genes DIAP1 and Cyclin E. (Fig. 1P, Q and Fig.2 C-E). Second, the phenotypes induced by removing *hpo*, *wts* or *sav* in the eye are reversed by removing one copy of *djub* (Fig. 3). Similarly, removing one genomic copy of *wts* suppresses the dJub RNAi phenotype while removing one genomic copy of *yki* enhances the dJub phenotype (Fig. S4). Third, the epistasis experiments show that overexpressing Yki and Wts RNAi in the absence of *djub* manifests as a Yki overexpression and a Wts RNAi phenotype respectively, and the Hpo RNAi phenotype persists in the absence of *djub*, indicating that the Wts RNAi and Yki overexpression phenotypes do not require the activity of *djub*, suggesting that *djub* functions upstream of *wts* and *yki*. The fact that the Hpo RNAi phenotype is masked in the absence of *djub* suggests that *hpo* functions upstream of *djub* (Fig. 4, 5, and 6). These genetic and biochemical data (Fig. 6) suggest the possibility that dJub may influence the Hpo pathway by affecting Wts activity. Precisely how Ajuba LIM proteins (dJub) influence LATS/Wts mediated inactivation of YAP/Yki remains to be determined, but possibilities include: inhibition of activation of LATS/Wts by upstream kinases (MST/Hpo), inhibition of the ability of LATS/Wts to phosphorylate YAP/Yki, or affecting the subcellular localization of LATS/Wts or WW45/Sav and thus their access to the Hippo pathway. Moreover, the regulatory relationship between Ajuba LIM proteins (dJub) and LATS/Wts may not be simply unidirectional as LATS has been shown to phosphorylate Ajuba (Abe, Ohsugi et al. 2006).

Although this is the first time the Ajuba family of LIM proteins have been implicated in the Hpo pathway, prior work has described an interaction between Ajuba and LATS at centrosomes that influences mitotic centrosome/spindle organization. That

study showed that Ajuba and Lats2 co-localize to centromeres and further, associate with each other during mitosis. This interaction requires the kinase activity of Lats2 and results in the phosphorylation of the LIM protein Ajuba (Abe, Ohsugi et al. 2006). With respect to the Hippo pathway there is some data that shows Mats and Wts colocalize at the centrosome (Shimizu, Ho et al. 2008). Therefore we cannot exclude the possibility of the centrosome being a functional site for the interactions, as mitotic damage can lead to apoptotic cell death, nor that dJub's effects upon the Hpo pathway are cell cycle dependent.

Ajuba LIM proteins are components of AJs in mammalian and *Drosophila* epithelia. Upstream members of the Hippo pathway include atypical cadherins (Fat, dachsous), and Expanded and Merlin – also localize to adherens junctions, leading to the hypothesis that AJs could be nodal points for initiation/regulation of Hippo signaling (Bennett and Harvey 2006; Cho, Feng et al. 2006; Silva, Tsatskis et al. 2006; Willecke, Hamaratoglu et al. 2006; Feng and Irvine 2007; Tyler and Baker 2007; Reddy and Irvine 2008), however how these upstream components actually activate MST/Hpo kinase is unknown. The Hippo pathway is thought to regulate cell contact growth inhibition (Zhao, Wei et al. 2007). Interestingly, in sub-confluent non-contacted cells, Ajuba LIM proteins are cytosolic while YAP is nuclear and cells proliferate (Zhao, Wei et al. 2007). When cells achieve confluence Ajuba proteins are recruited to AJs while YAP is phosphorylated and re-localized to the cytosol and cell proliferation ceases. Whether these events are related is not known, but given that Ajuba proteins associate with and inhibit LATS/Wts-mediated phosphorylation of YAP raises the possibility that the recruitment of Ajuba proteins/dJub to AJ in confluent cell cultures may “release”

LATS/Wts allowing for Hippo pathway mediated YAP/Yki phosphorylation, inactivation, and growth arrest.

Experimental Procedures and Materials

Drosophila genetics and strains

***djub* RNAi lines**

djub RNAi constructs were cloned as inverted repeats (Bao and Cagan 2006). Briefly, an approximately 500bp fragment of the coding region was amplified from a *Canton S* cDNA library and subcloned into pGem-WIZ (Bao and Cagan 2006). Subsequently a copy of the fragment was inserted in the opposite direction. The mini-white gene separated the inverted fragments. The entire piece “fragment – mini-white – inverted fragment” was then sub-cloned into pUAST, and this vector was used to generate transgenes via standard P element-mediated transformation (Rainbow Transgenics, Inc). *UAS-djub-RNAi 22.5* targets a 445bp fragment starting at 226bp before the start codon and *UAS-djub-RNAi 18.1* targets the 593bp fragment starting 500bp after the start codon (Fig. S1A).

Generation of *djub* deficiency lines

Djub deficiencies were generated as described in Parks, A.L et al (Parks, Cook et al. 2004). *djub*^I was generated by FRT-mediated recombination between PA (*P[XP]d05713*) and PB (*PBac[RB]CG11063^{e03614}*) in flies heterozygous for chromosomes bearing each P element (Fig. S1A). *djub*^{II} was generated by FRT-mediated recombination between PX (*PBac[RB]CG11092^{e03640}*) and PY (*P[XP]d02874*) (Fig. S1A). Genomic deletion was confirmed by PCR, and immunoblotting for *djub* gene product (Fig. S1B).

Construction of *djub-mCherry* and *HA-LIMD1* transgenes

cDNA was prepared from tissue obtained from 3rd instar larvae. The tissue was homogenized in Trizol and chloroform (1:5) and then subjected to centrifugation. The mRNA was precipitated with 100% isopropanol and the pellet was washed in RNase free 75% ethanol. The pellet was dissolved in DEPC-dH₂O and the DNA digested by DNase treatment. The mRNA was extracted with a pheno-chloroform isoamyl alcohol mixture and after centrifugation precipitated with 3 volumes of RNase free 100% ethanol. The pellet was washed with 75% ethanol and reverse-transcribed using standard techniques. The PCR products were confirmed to represent dJub cDNA by sequence analysis. dJub was amplified by PCR and cloned into pUAST+N-mCherry vector to construct *UAS-djub-mCherry* and transgenic flies were generated as detailed above. To construct the HA-LIMD1 construct, human LIMD1 cDNA was cloned into HA-pUAST vector.

Twin-spot analysis and gene expression studies.

All mutant clones were induced using the FLP/FRT system (Xu and Rubin 1993). Clones in the eye were generated in flies/larvae of the following genotype: *djub,FRT19a /Ubi-GFP, FRT19a; eyeless-FLP* and *NeoFRT19a /Ubi-GFP, FRT19a; eyeless-FLP*. *Ubi-GFP, FRT19a; eyeless-FLP* (from N. Dyson). For rescue experiments in the wing imaginal discs, clones were generated in flies of the following genotype: *djub,FRT19a/ Ubx-FLP tub-GFP FRT19a;en-gal4/ UAS-djub-mcherry* and *NeoFRT19a/ Ubx-FLP tub-GFP FRT19a* as a wild type control. *Ubx-FLP tub-GFP FRT19a* (J.A Knoblich, IMBA, Austria).

Generation of *djub* EGUF/HID clones

Eyes composed almost entirely of mutant tissue were generated by the EGUF/Hid method (Stowers and Schwarz 1999). *djub* mutant eyes were created in flies of the following genotype: *FRT19A djub/GMR-hid, FRT19A, l(1)Cell Lethal¹; eyeless-GAL4-ey,UAS-FLP/+* and the control flies were of the genotype: *NeoFRT19A/GMR-hid, FRT19A, l(1)Cell Lethal¹; eyeless-GAL4,UAS-FLP/+*. *djub* mutant eyes in which cell death was blocked by eye-specific expression of P35 were generated in flies of the genotype: *FRT19A djub/GMR-hid, FRT19A, l(1)Cell Lethal¹;eyeless-GAL4,UAS-FLP/UAS-P35*. For the genetic interaction studies, female fly eyes mutant for *sav*, *wts*, *hpo* and heterozygous for *djub* were of genotypes: *FRT19A djub/+; FRT42D Hpo^{KC202}/FRT42D, GMR-hid, l(2)CL-R¹; eyeless-GAL4,UAS-FLP/+, FRT19A djub/+;eyeless-GAL4,UAS-FLP/+;FRT82B sav^{shrp3}/FRT82B GMR-hid, l(3)CL-R¹ and FRT19A djub/+;eyeless-GAL4,UAS-FLP/+;FRT82B wts^{p1}/FRT82B GMR-hid, l(3)CL-R¹. *GMR-hid,FRT19A,l(1)CL¹/FM7a;eyeless-GAL4,UAS-FLP, FRT42D,GMR-hid,l(2)CL-R¹/Cyo; eyeless-GAL4,UAS-FLP, eyeless-GAL4,UAS-FLP/Cyo;/FRT82B GMR-hid, l(3)CL-R¹/TM2 and UAS-P35* (Bloomington *Drosophila* Stock Center).*

Generation of MARCM clones

MARCM clones(Lee and Luo 1999) for the epistasis experiments were generated by heat shocking third instar larvae for 1 h at 37°C and dissecting female pupal eyes of the following genotypes 40 h APF : (a) *hsFLP, tub-gal80, FRT19A/+; UAS-GFP, UAS-lacZ/+; UAS-yki /tub-gal4* (b) *djub, FRT19a/ hsFLP, tub-gal80, FRT19A; UAS-GFP, UAS-lacZ/+; UAS-yki/tub-gal4* (c) *hsFLP, tub-gal80, FRT19A/+; UAS-hpoRNAi/UAS-*

GFP, UAS-*lacZ*; *tub-gal4/dicer* (d) *djub*, *FRT19a/* hsFLP, *tub-gal80*, FRT19A; UAS-*hpoRNAi*/UAS-GFP, UAS-*lacZ*; *tub-gal4/dicer* (e) hsFLP, *tub-gal80*, FRT19A/+; UAS-GFP, UAS-*lacZ/dicer*; UAS-*wtsRNAi/tub-gal4* and (f) *djub*, *FRT19a/* hsFLP, *tub-gal80*, FRT19A; UAS-GFP, UAS-*lacZ/dicer*; UAS-*wtsRNAi tub-gal4*. UAS-*yki* (K. Irvine, Rutgers University), UAS-*hpoRNAi* (N. Tapon, Cancer Research UK) and UAS-*wtsRNAi* (Vienna *Drosophila* RNAi Center).

GAL4/UAS analysis

Gene over-expression and RNAi assays were carried out using the GAL4/UAS system (Brand and Perrimon 1993). GAL4 driver lines used: *1096-gal4*, *GMR-gal4*, and *engrailed-GAL4*. UAS lines used: UAS-*djub-mCherry*, UAS-*djubRNAi* (22.5 and 18.1) and UAS-*P35*.

Fly lines and staging of pupae

All crosses took place at 25°C. Pupae were staged at 0hrs after puparium formation (APF) as white pre-pupae and maintained at 25°C. , Wandering third instar larvae were used for third instar imaginal disc dissections.

Mounting of Adult wings, cell counting and statistics

Adult flies were stored in 80% ethanol until ready for dissections. Only female flies were used for analyses. Wings were removed in 75% glycerol (in PBS) for mounting. Coverslips were sealed with nail polish. Wing cells were counted in the area bounded by the L4 and L5 veins and the second intervein, and the average and standard deviation

were plotted using Image J and Microsoft Excel. The Mann-Whitney U non-parametric test was used to calculate statistical significance of the number of cells, and the area of the wing region between various genotypes.

Other fly strains

DIAP1-LacZ and *UAS-Ex* flies were from G. Halder (University of Texas M. D. Anderson Cancer Center), *Ft⁴²²* flies were from K. Harvey (Peter MacCallum Cancer Centre, Australia), *INR^{Ex15}* flies were from L. Pick (University of Maryland), *Chico¹* flies were from D.L Stern (Princeton University), *Foxo²⁵* flies were from E. Hafen (Institute of Molecular Systems Biology, Switzerland) and *dmyc¹* flies were from P. Gallant (Zoologisches Institut, Switzerland).

Scanning Electron Microscopy

Scanning electron microscopy (SEM) of adult flies was carried out as previously described (Cordero, Larson et al. 2007). Adult fly samples were dehydrated and sputter-coated before being imaged using a Hitachi S-2600H scanning electron microscope (Department of Otolaryngology, Washington University in St Louis).

Immunohistochemistry

Pupal retina and eye and wing imaginal disc dissections were carried out in PBS following which the tissue was fixed in 4% paraformaldehyde dissolved in PBS. After fixation, tissues were washed in PBST (PBS + 0.5% Triton X-100) and incubated at 4°C overnight with primary antibody diluted in PAXDG buffer (0.1% BSA, 0.3% Triton X-

100, 0.3% deoxycholate, 5% normal goat serum in PBS). Tissues were then washed 3 times with PBST for 10 mins each, and incubated for 3 hrs at room temperature or overnight at 4°C with secondary antibody diluted in PAXDG (1:1000). Tissues were then rinsed with PBST and transferred onto slides for mounting in vectasheild mounting medium containing DAPI (Vector Labs). Images were captured at room temperature on a LSM 510 Zeiss confocal microscope using 63x oil objective. Image J and Photoshop (Adobe) were used to process images. The following antibodies were used: rat α -DE-cadherin (IC) (1:10, DSHB), rabbit α -dJub (1:400), mouse α -DIAP1 (1:200, B. Hay, California Institute of Technology), mouse α -BrdU (1:20, Becton-Dickinson), mouse α -CyclinE (1:40, H. Richardson, Peter MacCallum Cancer Center, Australia), rabbit α -Activated Caspase3 (1:100, Abcam) and mouse α -b-gal (1:2000, Promega). Secondary antibodies used were Alexa Fluor 488 and 568 (Invitrogen), rabbit α -HA (1:100, Abcam) and Cy5 (Jackson ImmunoResearch Laboratories).

Immunoprecipitation

HEK 293 T cells (4×10^5 plated in 12-well plates 24 hours prior to transfection) were transfected with the specified constructs. Drosophila S2 cells were cultured at room temperature in Express Five SFM media (Invitrogen) and 50 μ g/mL penicillin/streptomycin were transiently transfected by using FuGene6. 48 hours after transfection, cells were harvested by washing with cold PBS, and lysed with 200ml of IP buffer (20 mM HEPES [pH 7.5], 120 mM NaCl, 5 mM NaF, 1 mM sodium orthovanadate, 0.5 mM EDTA, 1 mM DTT, 5% glycerol, 0.1% NP-40, and protease inhibitor cocktail from Sigma). Extracts were sonicated briefly, and then subjected to

centrifugation at 15,000xg for 15 min for clearing. 10ml of lysate was saved (mixed with 20ml of SDS sample buffer) as 5% input. For each IP (HEK 293T cells), the remaining cell lysate was mixed with 5 ml of IP buffer washed M2AG beads (1:1 [vol/vol], Sigma), for 1 hour at 4°C with gentle rotation. For S2 cells, lysates were precleared with protein G beads alone for 1 hr, then incubated overnight with 1mg of mouse α -myc antibody (Upstate), following which Protein G beads were then added for 1 hr. The immunoprecipitates were then washed with 1ml of IP buffer 4 times and finally boiled in 25 μ l SDS loading buffer. 8ml of boiled samples were run on SDS-PAGE under reducing condition and then transferred to nitrocellulose membranes for Immunoblot analysis.

MDCK cell culture and transfection

MDCK canine kidney cells were cultured in 1X MEM (Gibco) containing 10% FBS (Invitrogen) and 50 μ g/mL penicillin/streptomycin. Nucleofections (Amaxa cell line Nucleofection Kit L) was used to transfect MDCK cells with siRNAi directed against Ajuba and LIMD1, according to the manufacturer's instructions. Equal numbers of cells were immediately plated on three different sized dishes so as to have cells plated at low, medium and high densities. Forty-eight hours after nucleofection the cells were harvested for immunoblot analysis.

Immunoblotting and Antisera used.

Fly tissue, S2 cells or HEK 293T cells were lysed in RIPA buffer [150 mM NaCl, 10 mM Tris-HCl (pH 7.5), 1mM EDTA, 1% Triton-X100, 0.35% DOC, 0.25% NP-40, 0.1%SDS], PMSF and protease inhibitor cocktail (Sigma). Lysates were then cleared by

centrifugation and boiled for 5 minutes in SDS sample buffer and resolved by 10% SDS-PAGE, under reducing conditions. Proteins were transferred to nitrocellulose in transfer buffer (48mM Tris, 390mM, glycine, 0.1% SDS, 5% methanol) and immunoblots were probed with primary antibodies: rabbit α -dJub (1:1000), mouse α -Flag (1:10000, Sigma), rabbit α -YAP (1:1000, Cell Signaling), rabbit α -P-YAP(S127) (1:1000, Cell Signaling), mouse α -myc (0.1 μ g/ml, A. Shaw, Washington University St. Louis), rabbit α -Warts (1:1000, K. Irvine, Rutgers University), rabbit α -YFP (1:1000, Invitrogen), rabbit α -Ajuba (1:1000) (RAKESH K. GOYAL and ANTHONY J. MUSLIN) and rabbit α -LIMD1 (1:1000) (Feng, Zhao et al. 2007). For quantification, blots were minimally exposed and then scanned for quantification using ImageJ software.

Djub Antibody Generation

Rabbits were immunized with a dJub N-terminal peptide (TTQRTQTQARNPGNSDSYETL) coupled to KLH. Polyclonal antiserum was affinity-purified using the immunizing peptide (Proteintech Group, Inc).

Figure 1. dJub regulates tissue size by controlling cell number. (A-C) Wings from females wt flies (A), female flies expressing dJub RNAi (B), or dJub-mCherry transgene (C). *1096-Gal4* was used to drive RNAi or transgene expression. (D) Outlines of the wings in panels A-C. (E, F) Quantification of relative wing areas (E) and cell numbers (F) of genotypes in A-C. Area and cell number measurements were taken from the wing region located between veins L4 and L5, and wt defined as 100% (N=20 for each). (G) Extracts of mammalian HEK293 cells transfected with myc-dJub immunoblotted with dJub antiserum (left) or Myc antiserum (right). (H) Immunoblot analysis of dJub protein levels in wt or dJub RNAi-expressing larval eye imaginal discs. Actin serves as loading control. Relative amount of dJub protein is indicated below each lane. Mid-pupal wt eyes (I) or dJub RNAi expressing eyes (J) stained for DE-cadherin. Secondary (arrows) and tertiary (arrowheads) interommatidial cells are highlighted. Loss of interommatidial cells in dJub RNAi expressing pupal eyes denoted by arrows (J). (K) Quantification of relative numbers of interommatidial cells in wt versus dJub RNAi pupal eye. Interommatidial cells were counted in 20 fields, each containing a cluster of at least 7 ommatidia. (L-O) Mid-pupal wt eyes stained for DE-cadherin (L), dJub (M), and merged image (N). Z-stack analysis of line in N is shown above panel N (N). (O) Immunostaining with dJub antiserum preabsorbed with immunizing peptide. (P-R) Scanning electron micrographs (SEMs) of female adult eyes. WT (Q), *djub*¹ generated via the EGUF-Hid method, which results in eyes composed almost entirely of mutant tissue (Q), and *GMR-gal4* driven overexpression of UAS-dJub-mCherry transgene (R). (S-U) Female third-instar larval eye imaginal discs containing wt (S) or *djub*¹ mutant (T, U) clones marked by the absence of GFP expression (black). (U) Enlarged view of *djub*¹ and wt twin spot clones. Yellow

arrow identifies *djub*¹ clones, red arrow identifies wt twin spot clone containing two copies of Ubi-GFP, and white arrow identifies tissue carrying one copy of Ubi-GFP. In all experiments wings and eyes were dissected from female flies. In graphs data are shown as mean percentages +/- standard deviation, with N= 20 for each genotype. (***) Represents p-value ≤ 0.001 and (*) represents p-value ≤ 0.05 . Anterior is to the left for all larval imaginal discs.

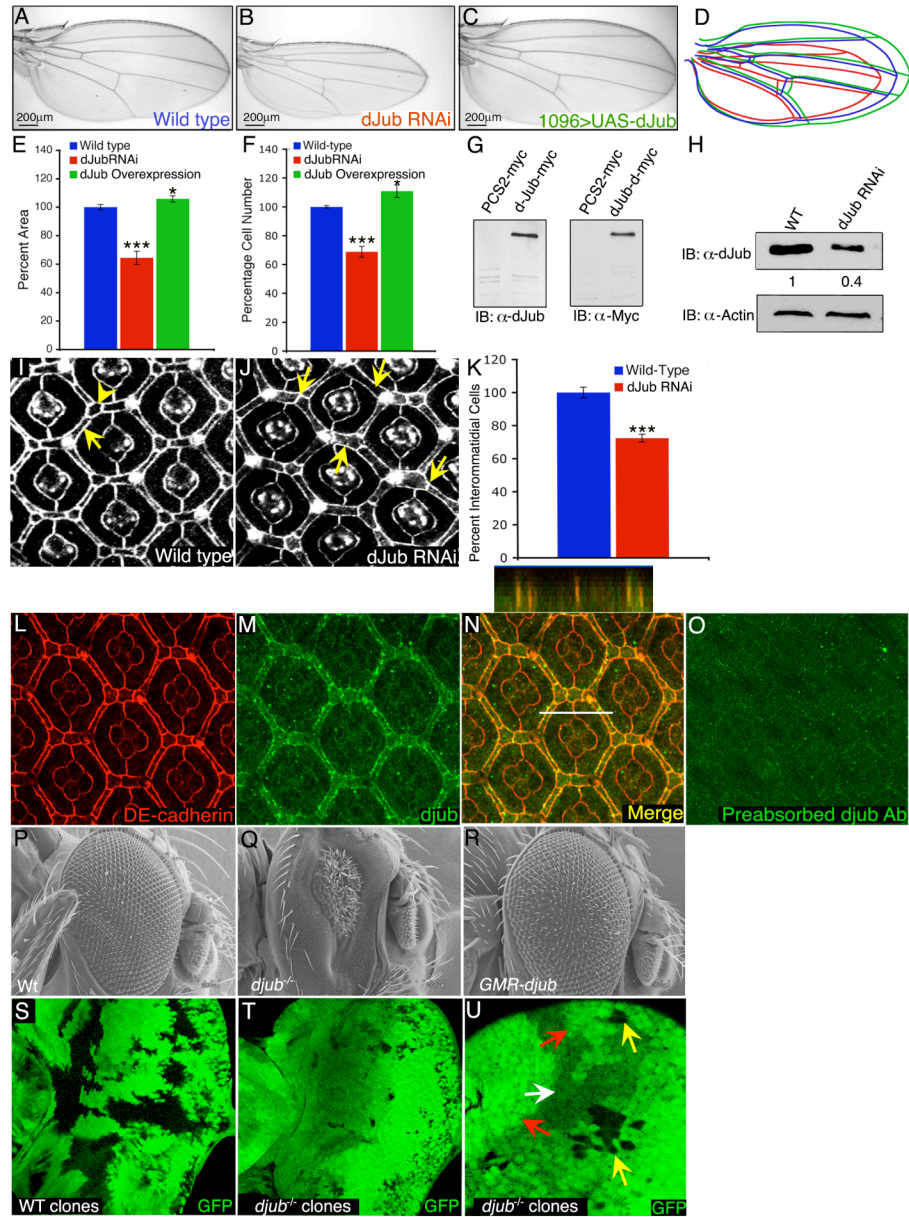


Figure 1

Figure 2. *djub* affects cell proliferation and apoptosis and affects expression of **DIAP1 and Cyclin E**. Wt (A, B) or *djub*^I (A', B') Female larval eye imaginal discs, composed almost entirely of cells homozygous for either a lethal-free FRT19A chromosome (A, B), or an FRT19A chromosome carrying *djub*^I deficiency (A', B'). (A, A') Proliferation detected by BrdU incorporation (green). Cells anterior to the morphogenetic furrow (white arrowhead). Cells posterior to the furrow (yellow arrow). (B, B') Apoptosis detected by an antibody specific for activated caspase 3. (C-E) Third-instar larval eye imaginal discs containing *djub*^I clones (GFP -ve, yellow arrows) stained for DIAP1 (C'), DIAP1-lacZ (D'), or Cyclin E (E') expression. Anterior is to the left for all larval eye imaginal discs.

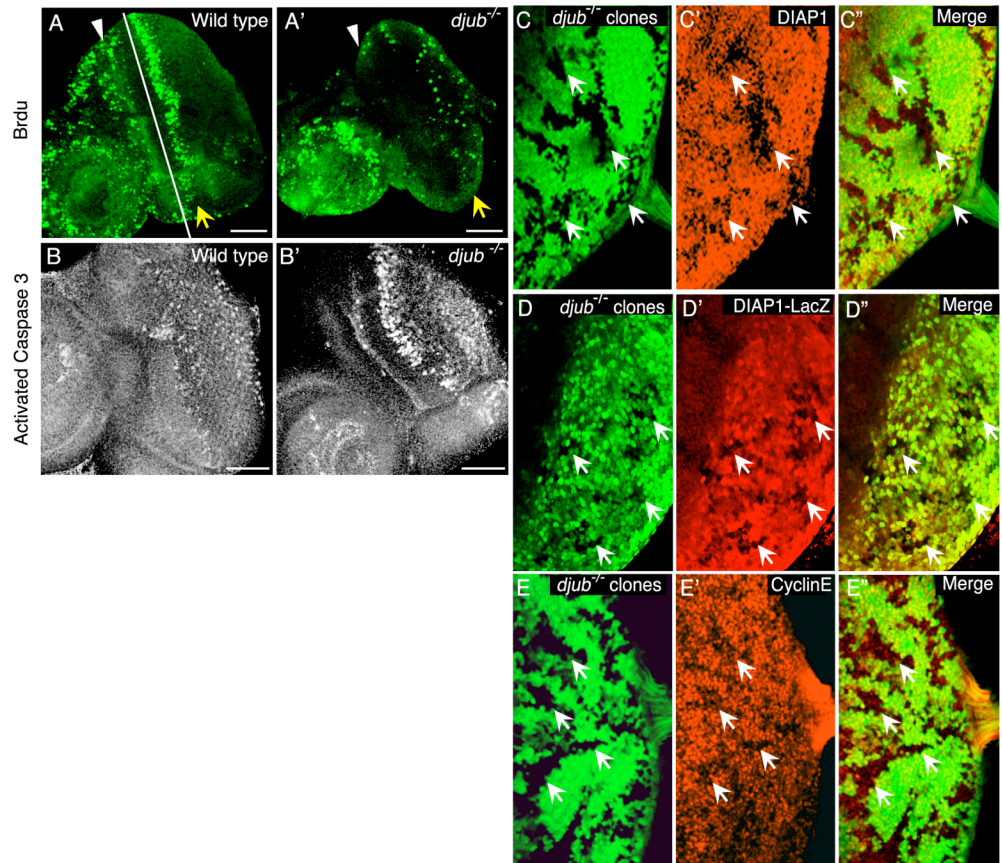


Figure 2

Figure 3. ***djub* genetically interacts with the Hippo pathway.** (A-H) Genetic interaction analyses. SEMs of adult female *Drosophila* eyes of wt and Hippo pathway mutants (A,C,E,G) and *djub*¹ or Hippo pathway mutants containing a deletion of a single copy of *djub* (B,D,F,H), as indicated. Mid-pupal eye dissections of wt and Hippo pathway mutants at low and high magnifications (I, J, L, N and I', J', L', N' respectively) or Hippo pathway mutants containing a deletion of a single copy of *djub* (K, M, O and K', M' and O'), as indicated, and stained for DE-cadherin to identify interommatidial cells. Scale bars in (A-H) equal 100mm and (I-O) equal 10mm. (P) Quantification of interommatidial cell numbers in 10 random fields containing 10 ommatidia each of genotypes in A-H. Data are shown as mean percentages +/- standard deviation and (***) represents p-value ≤ 0.001 .

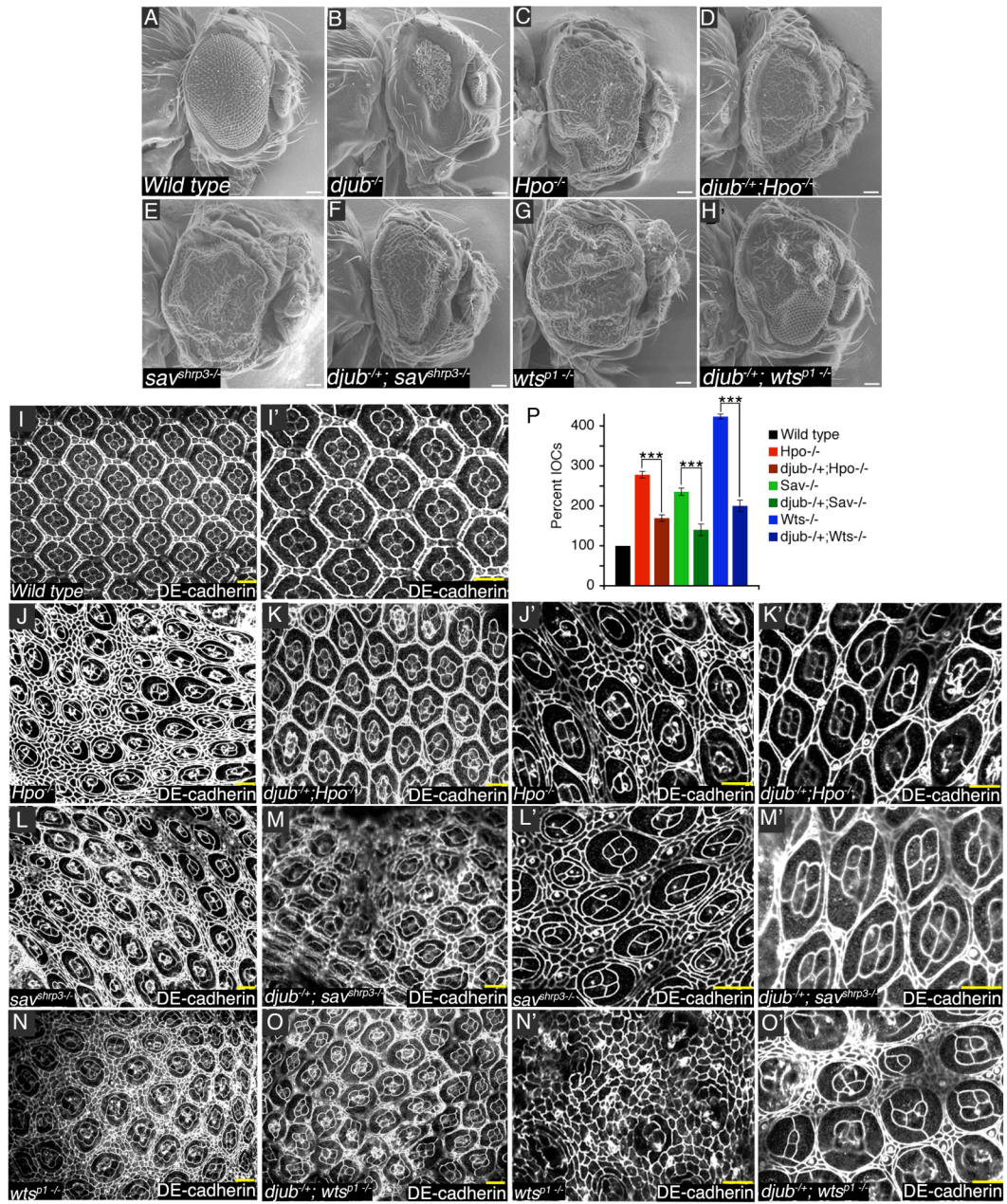


Figure 3

Figure 4. ***djub* is epistatic to *hpo* based on clonal area.** (A-H) Genetic epistasis analysis. Female mid-pupal eyes stained for DE-cadherin (red). Wt showing normal sized GFP positive wt MARCM clones (A). (B) *djub*¹ MARCM clones (GFP +ve) are smaller than Wt. (C) MARCM clones overexpressing Yki (GFP +ve). (D) MARCM clones mutant for *djub*¹ and overexpressing Yki (GFP +ve). (E) MARCM clones expressing wts RNAi (GFP +ve). (F) MARCM clones mutant for *djub*¹ and expressing wts RNAi (GFP +ve). (G) MARCM clones expressing hpo RNAi (GFP +ve). (H) MARCM clones mutant for *djub*¹ and expressing hpo RNAi (GFP +ve). (I) Graphical representation of the clonal area (GFP +ve) for each genotype as a percentage of the entire pupal eye area. In graphs data are shown as mean percentages +/- standard deviation, with N= 10 for each genotype. (***) Represents p-value ≤ 0.001 . Scale bars in (A-H) equal 20mm.

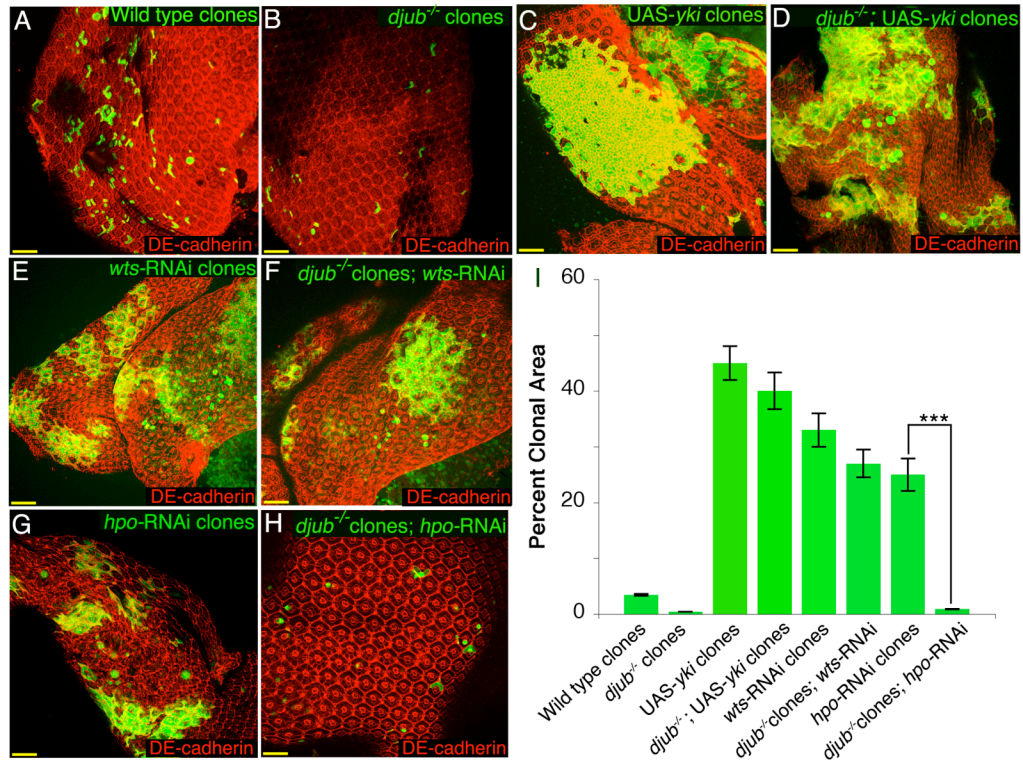


Figure 4

Figure 5. ***djub* is epistatic to *hpo* based on interommatidial cell number.** (A-H) Genetic epistasis analysis. Female mid-pupal eyes stained for DE-cadherin (red/white). Wt showing normal pattern of secondary and tertiary interommatidial cells and GFP positive wt MARCM clones (A, A'). (B, B') *djub*¹ MARCM clones (GFP +ve) showing a loss of interommatidial cells. (C, C') MARCM clones overexpressing Yki (GFP +ve). (D, D') MARCM clones mutant for *djub*¹ and overexpressing Yki (GFP +ve). (E, E') MARCM clones expressing wts RNAi (GFP +ve). (F, F') MARCM clones mutant for *djub*¹ and expressing wts RNAi (GFP +ve). (G, G') MARCM clones expressing *hpo* RNAi (GFP +ve). (H, H') MARCM clones mutant for *djub*¹ and expressing *hpo* RNAi (GFP +ve). Arrows identify changes in interommatidial cell numbers. (I) Graphical representation of the percent increase of interommatidial cells within the clonal area (GFP +ve) as compared to wild type (set at 100% IOCs) for each genotype. In graphs data are shown as mean percentages +/- standard deviation, with N= 10 for each genotype. (***) Represents p-value ≤ 0.001 . Scale bars in (A-H) equal 10mm.

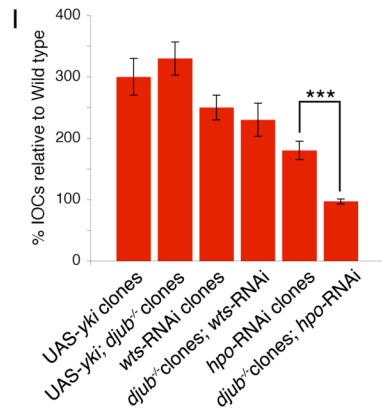
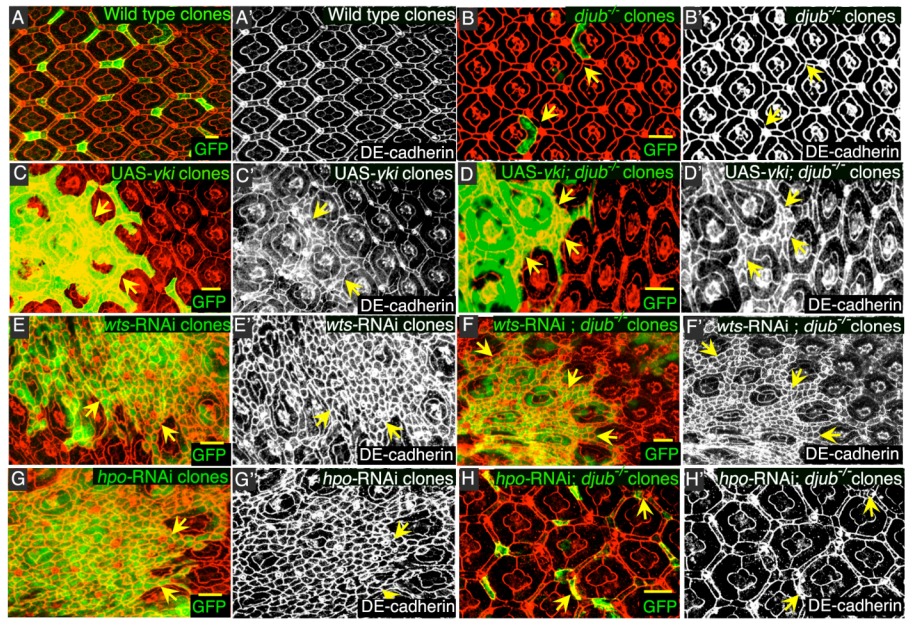


Figure 5

Figure 6. Ajuba LIM proteins associate with components of the Hippo pathway in mammalian cells and influence YAP phosphorylation.

(A-D) HEK293 cells were co-transfected with LIMD1-YFP (A), Ajuba-YFP (B), Myc-WTIP (C), or Myc-Zyxin (D) and Flag-tagged Mst1, Lats1/2, WW45 or YAP, as indicated. Cell lysates were immunoprecipitated for each Hippo pathway member (anti-Flag), and bound products immunoblotted (IB) for the presence of each LIM protein (anti-YFP or anti-Myc). Immunoblots of input controls (5%) are shown on the right side of each panel.

(E) HEK293 cells were transfected with the indicated member of the Hippo pathway in the absence or presence of LIMD1-YFP. Levels of phospho-S127-YAP (upper panel) or total YAP (lower panels) were then determined by immunoblot (IB) analysis. Relative amounts of phospho-S127-YAP with respect to total YAP protein is indicated below each lane. (F) MDCK cells were transfected with control Luc siRNA (lanes 1-3) or Ajuba and LIMD1 siRNAs (lanes 4-6) and then plated at low (LD), medium (MD) and high density (HD). Amount of S127-YAP phosphorylation relative to total YAP for each density within control and Ajuba/LIMD1 depleted cells was determined by immunoblotting. The relative level of YAP phosphorylation for each density between control and Ajuba/LIMD1 depleted cells was determined and is indicated above the lanes. (G) Working model, based upon results herein, for how Ajuba LIM proteins could influence Hippo pathway signaling.

Supplemental Figure 1. **dJub is the *Drosophila* ortholog of the Ajuba sub-family of mammalian LIM domain-containing proteins and dJub regulation of wing size is evolutionarily conserved.** (A) Schematic of the human and *drosophila* members of the Ajuba and Zyxin sub-families of LIM proteins. Orange circles denote LIM domains in each protein. LIM domain homology is listed to the right. Red lines above indicate the regions in dJub targeted by two RNAi hairpin constructs (22.5 and 18.1). Adult fly wings from wt (B, E), dJub RNAi 22.5 (C, F), dJub RNAi plus dJub-mCherry rescue transgene (D), and dJub RNAi plus human HA-LIMD1 rescue transgene (G) flies. In all cases *1096-GAL4* was used to drive transgenes specifically in the wing. (H) Quantification of wing area relative to wt wings. Data are shown as mean percentages +/- standard deviation, with N= 20 for each genotype. (***) Represents p-value ≤ 0.001 and (*) represents p-value ≤ 0.05 . (I) Immunolocalization of human HA-LIMD1 transgene in larval wing imaginal discs. DE-cadherin staining (I, red), HA-LIMD1 staining (I', green), and merged images (I'').

A

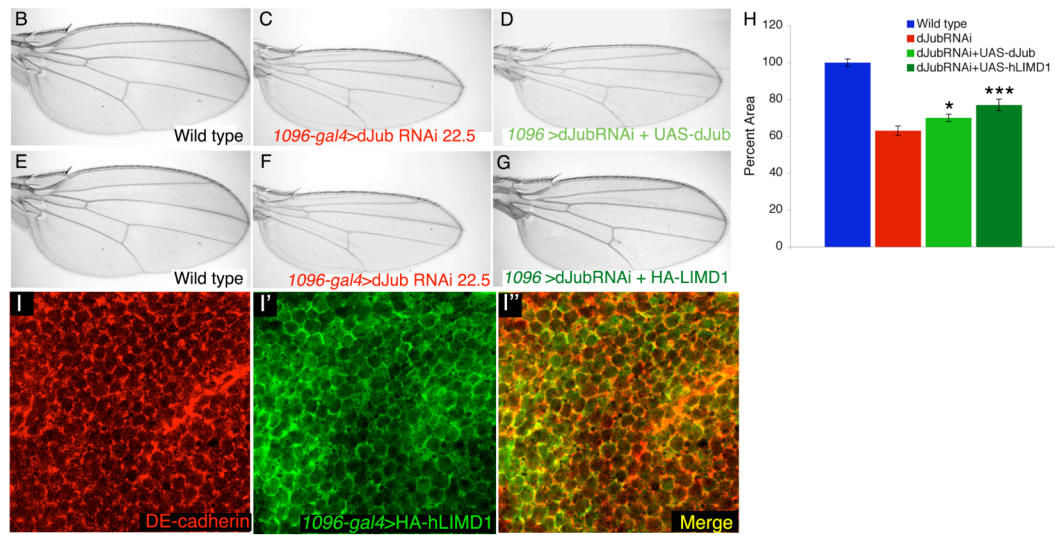
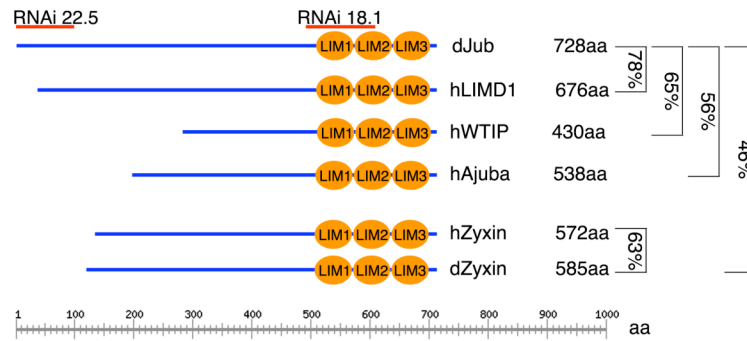


Figure S1

Supplemental Figure 2. **Generation of *djub* deficiency alleles and rescue of *djub* null phenotype.** (A) Schematic of the genomic region containing *djub*, including the location of the FRT-bearing P elements (black/grey triangles) used to generate the *djub* deficiencies. A1 through C2 refer to PCR primers used to verify the presence of each FRT-mediated deletion. (B) Genomic PCR confirmation of deficiencies. Due to the size of intervening genomic DNA, primer pairs A1-A2 and B1-B2 fail to amplify a PCR product unless the desired deletion has occurred. Primer Pairs C1-C2 amplify a product only if the desired deletion has not occurred. (C) Immunoblot blot (IB) analysis of dJub protein levels in cell lysates prepared from larval eye imaginal discs from wt or heterozygous *djub*¹ flies. Actin serves as loading control, and the relative amount of dJub protein is listed below each lane. (D-F) Third-instar larval wing imaginal discs containing wt clones (GFP -ve) (D), or *djub*¹ mutant clones (GFP -ve) (E and E') expressed throughout the wing discs using Ubx-flp. dJub-mCherry transgene (red) was expressed only in the posterior compartment of the wing using *en-gal4* (E or E'). *djub*¹ mutant + dJub-mCherry clones in the posterior compartment (red arrows E'), anterior compartment *djub*¹ mutant only clones (yellow arrows E'). (F) Quantification of the relative area occupied by wt clones (from D), posterior compartment *djub*¹ mutant clones co-expressing *djub-mCherry* (from E), and anterior compartment *djub*¹ mutant clones (from E) calculated as percent area occupied by GFP-negative clones relative to total tissue area (wt clonal area was calculated relative to entire wing). Anterior is to the left for all larval imaginal discs.

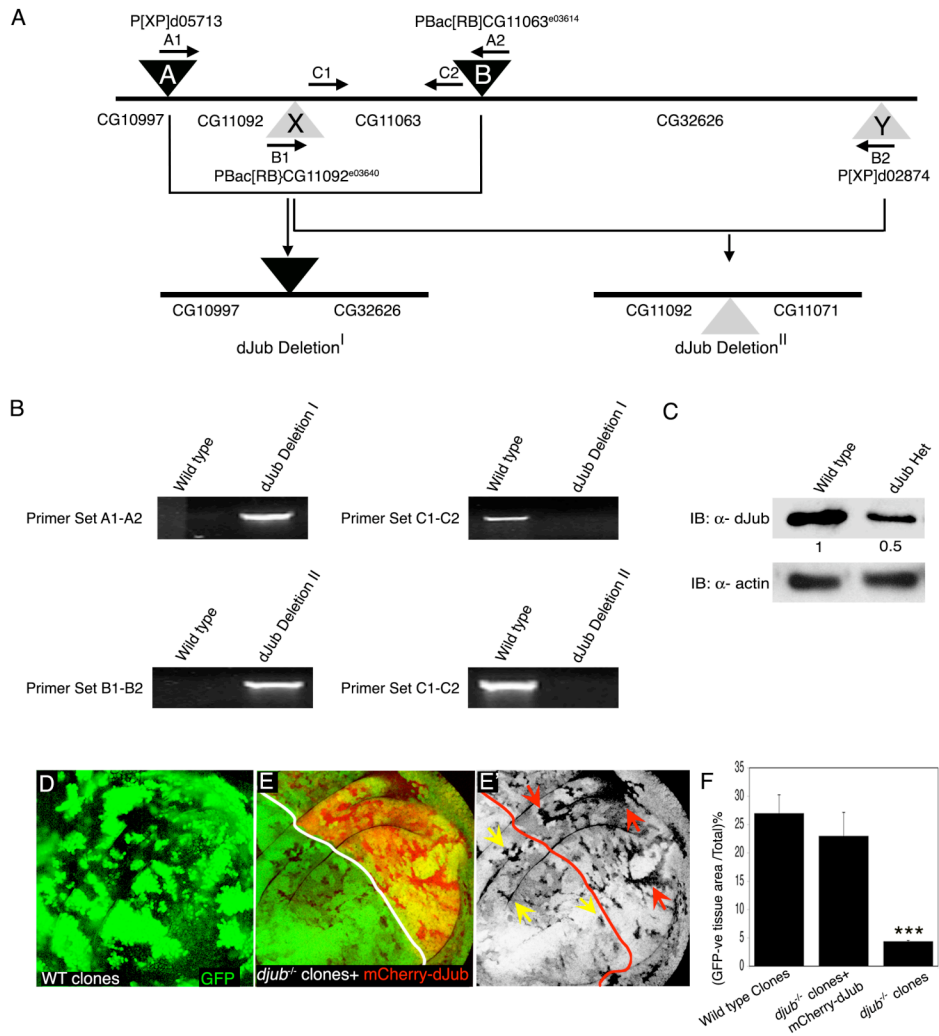


Figure S2

Supplemental Figure 3. **Loss of *dJub* function does not affect neuronal differentiation or adherens junctions organization. Loss of function Hippo pathway mutants do not affect dJub localization.** (A) Larval eye imaginal disc containing *djub*¹ mutant clones (GFP negative), generated by *Eyeless-flp*, and stained for the neuronal marker ELAV. White arrows indicate ELAV-positive photoreceptors. (B) SEM of *djub*¹ mutant adult eye (EGUF-Hid method). (C) SEM of *djub*¹ mutant eyes also expressing the anti-apoptotic factor P35 in all eye cells via *GMR-GAL4*. (D, D') Third-instar larval eye imaginal discs containing *djub*¹ mutant clones marked by the absence of GFP expression (black) stained for DE-cadherin (red in D, white in D'). (D'') Enlarged view of *djub*¹ mutant clones; white arrow identifies normal DE-cadherin staining in *djub*¹ mutant clones. (E-G) Mid-pupal eyes from *wts*^{x1} (E), *hpo* (F), and *sav*^{shrp3} (G) loss of function mutants stained for DE-cadherin (red) and dJub (green).

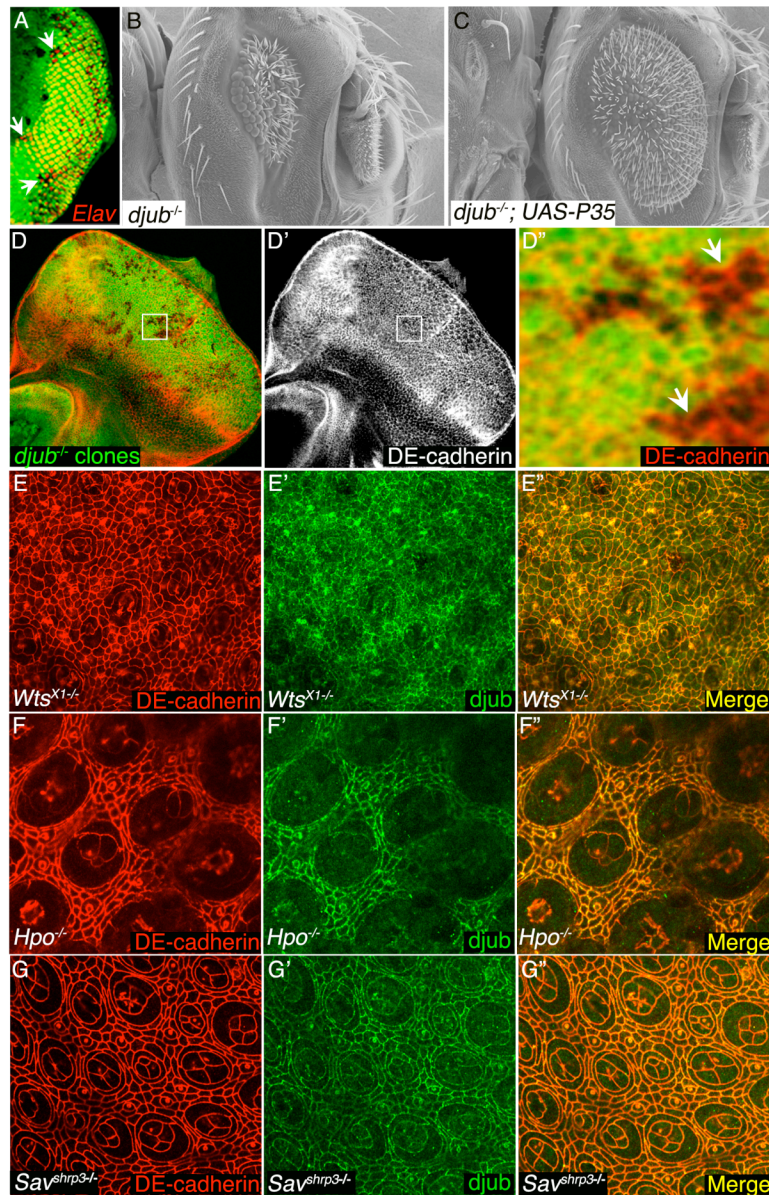


Figure S3

Supplemental Figure 4. **DJub genetically interacts with the Hippo pathway but not the Insulin Receptor Pathway or Myc** (A-J) Wings from adult wt flies (A, F), dJub RNAi-expressing flies (B, G), or expressing dJub RNAi in a background heterozygous for *wts^{X1}* (C) or *yki^{B5}* (H). (D and I) Schematics of the outline of wings shown in (A-C) and (F-H), respectively. (E, J) Quantification of the relative wing areas for the indicated genotypes. *1096-GAL4* was used for wing-specific expression of UAS-dJub RNAi. (K) Table summarizing whether enhancement or suppression of the *fat* eye and of the *djub* RNAi small wing phenotype occurred for the indicated genotypes (i.e., genetic interaction). Hippo pathway (Wts, Yki, Ex, Fat), Insulin Receptor pathway (INR, Chico, FOXO), and Myc. (L) S2 cells co-transfected with dJub-myc and Hpo-Flag, Sav-HA and Yki-HA and Wts, as indicated. Cell lysates were immunoprecipitated for dJub (anti-Myc), and bound products Immunoblotted (IB) for the presence of each Hippo pathway member. Immunoblots of input controls (5%) are shown on the left side of each panel. Data are shown as mean percentages +/- standard deviation, with n=20 for each genotype. (***) Represents p-value ≤ 0.001 and (*) represents p-value ≤ 0.05 .

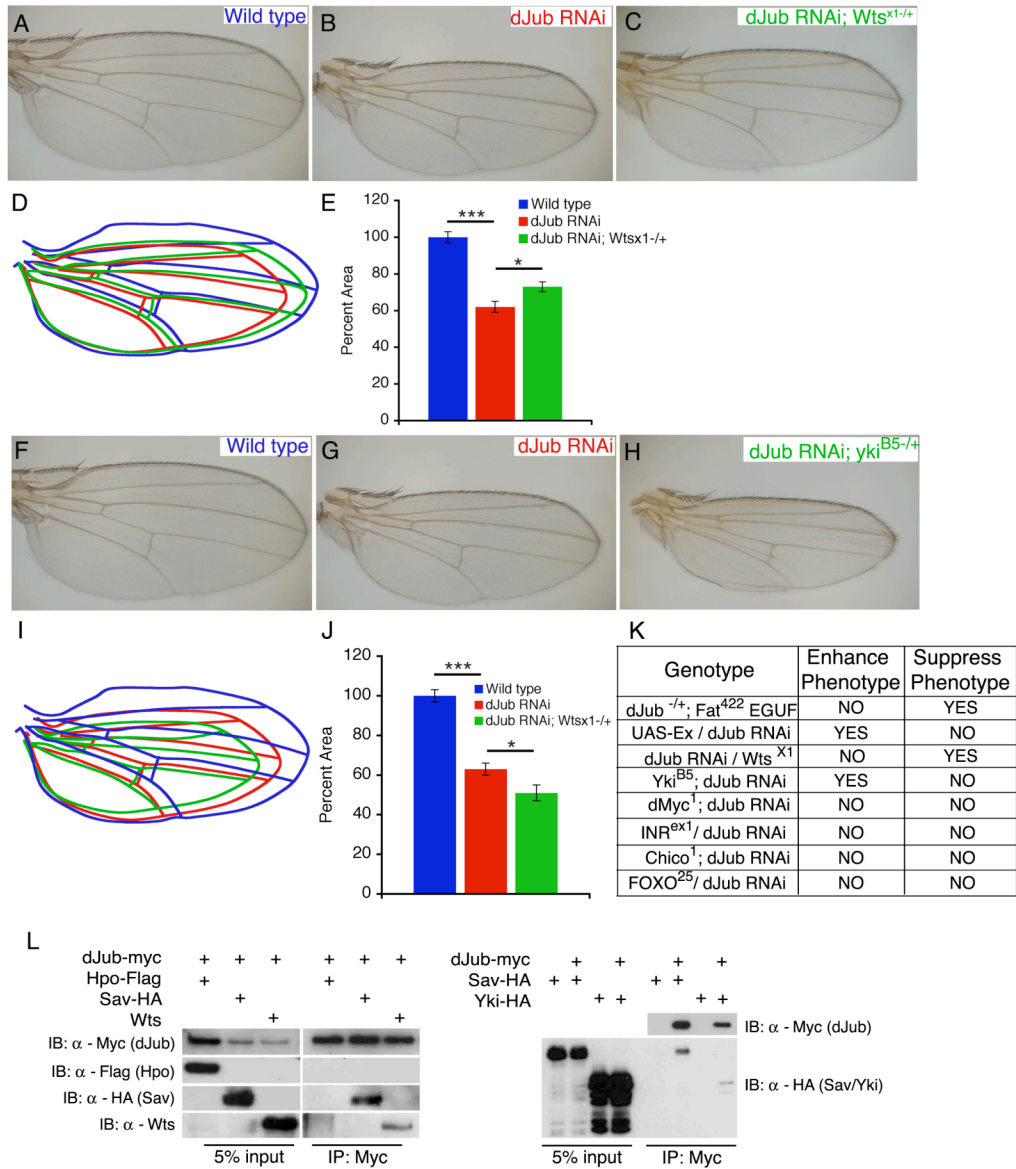


Figure S4

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Chapter 4

Apical junctions and growth control in mammalian and *Drosophila* cells

Introduction

A junctional role for the dJub and the Hippo pathway members in *Drosophila*

Studies carried out in this thesis as well as previous studies from the lab have shown that the Ajuba LIM proteins are highly expressed in epithelial cells, interact with α -catenin, and localize specifically to adherens junctions (Figure 1). This has been shown to be true in both mammalian and *Drosophila* epithelial cells. In chapter 2, I show that Ajuba LIM proteins appear to regulate the kinetics of nascent junction formation by delaying the establishment of functional Adherens and Tight junctions, and in chapter 3 we establish a novel tissue-growth regulatory role for the Ajuba LIM proteins. So, the question remains if there is a link between the junctional and growth control roles of the Ajuba LIM proteins.

Interestingly, in *Drosophila*, mutations in a number of genes (*scribbled*, *discs large*, *lethal giant larvae*) have been shown to disrupt cell polarity and also induce overproliferation (Bilder, Li et al. 2000; Bilder and Perrimon 2000; Bilder 2004). Zygotic mutations in several of the *Drosophila* junctional proteins lead to a “giant larva” phenotype, the result of excessive growth and proliferation. Importantly, the epithelia in these mutants are not composed of the typical flat columnar cells, but rather a mass of rounded, poorly adhesive cells that sit on top of each other. These results suggest that a mutation in a single gene can, in fact, simultaneously induce junctional and growth defects, and also that these two mechanisms are under similar genetic control.

Based on these results, we decided to investigate how Ajuba functions to both stabilize junctions as well as to promote growth. Of note, unlike the genes previously

described (*scrib*, *dlg*, *lgl*) that are at junctions and induce overproliferation when perturbed, Ajuba LIM proteins have the opposite phenotype. That is, loss of function mutations in Ajuba LIM proteins in *Drosophila* leads to tissue atrophy. One possible mechanism by which the Ajuba LIM proteins carry out this dual function, junctional stability and growth promotion, is that the loss of stable junctions when the LIM proteins are depleted may lead to growth defects by leading to the activation of the Hippo pathway and therefore increased in cell death and decreased cell proliferation and/or by inappropriately triggering the poorly understood mechanisms of contact inhibition. Another possibility is that the Ajuba LIM proteins have other undiscovered interacting partners and co-ordinate different polarity and growth control pathways.

We have shown in Chapter 3 that the Ajuba LIM proteins directly interact with the Hippo pathway to negatively regulate the pathway's growth regulatory role. The Hippo pathway plays a critical role in regulating organ size by transcriptionally regulating growth and apoptosis genes. Mutations in the upstream members of the pathway induce massive hyperplastic overgrowth phenotypes of epithelial tissue (*hpo*, *wts*, *sav*). The upstream regulators of the Hippo pathway namely Fat, Expanded and Merlin all localize to the Sub-Apical Region (Figure 1), and thus may be involved in relaying information about cell density and organ size to the downstream elements of the Hippo Pathway. Whether or not, and how, these upstream members and/or the Ajuba LIM proteins are capable of linking signals from cell junctions to the level growth regulation is yet to be determined.

Recent studies have shown that *wts* is capable of enhancing the *dlg* phenotype in *Drosophila* ovaries (Zhao, Szafranski et al. 2008). These data are exciting since it provides a possibility of cross-talk between the Scrib/Lgl/Dlg pathway at the Sub-Apical region in *Drosophila* and the Hippo growth control pathway. However, in this study the upstream members of the Hippo pathway namely, Ft, Ex and Mer did not enhance the *dlg* mutant phenotype, suggesting that multiple signals may converge at the level of Wts. Whether the Ajuba LIM proteins are the connection from junctions to Wts is not known. The investigations in Chapter 3 were conducted in larval eye and wing tissue where dJub did not seem to affect cell junctions in any obvious manner. In this chapter we further analyzed the epithelial junctional role of dJub in *Drosophila* at other developmental stages namely, the embryo and in the pupal eye. We also analyzed Hippo pathway mutants to see if they also give rise to defects in cell junctions in addition to giving rise to the overproliferation phenotype.

The Ajuba LIM proteins and the Hippo pathway in the context of cell density and contact inhibition

The phenomenon of “contact inhibition” is a hallmark of cells growing in culture, where normal cells stop proliferating upon achieving confluency. Cancer cells, typically, have lost contact inhibition, and thus continue to grow even after reaching their final tissue size. However, the phenomenon of contact inhibition is still poorly understood. Recent studies have shown that components of the Hippo pathway may regulate contact inhibition. For instance, as cell density increases and cells become confluent, Mer becomes dephosphorylated and activated, which has been shown to be necessary and

sufficient to mediate contact inhibition and the cessation of proliferation (Shaw, McClatchey et al. 1998; Morrison, Sherman et al. 2001). In further support of a role for the Hippo pathway in mediating contact inhibition Lats (Wts) and WW45 (Sav) null mouse embryonic fibroblasts display a loss of contact inhibition as well. And finally, YAP is phosphorylated and translocates to the cytoplasm upon but not prior to cells achieving confluence (Zhao, Wei et al. 2007). In fact, the ACHN cancer cell line, which has a WW45 mutation and therefore activated YAP, does not respond to contact inhibition, instead, these cell continue to proliferate even after confluency is reached. However, the introduction of dominant negative form of YAP restores the ability of these cells to undergo contact inhibition (Zhao, Wei et al. 2007). Based on the data, a possible model is that when cells in culture reach confluence, cell-cell interactions trigger signaling events that lead to the activation of the Hippo pathway. The Hippo pathway mediates the downregulation of the pro-growth and anti-apoptotic genes sends the “stop proliferation” signal leading to contact inhibition. Identification of the upstream signal, and the method by which the pathway senses confluency is still a mystery. The results in this Chapter describe a possible role of the Ajuba LIM proteins in a cell density dependent manner in regulating the Hippo Pathway.

Results

***djub* is essential for organization of epithelia in the *Drosophila* embryo**

My work on the function of Ajuba LIM proteins in mammalian cell culture showed that epithelial cells require Ajuba LIM proteins to form nascent, stable, and functional adherens and tight junctions in a timely manner. However, the existence of the three Ajuba subfamily proteins, and the likelihood of functional redundancy between them made it difficult to assay the phenotype of simultaneously deleting all three via siRNA. Having established the fly system (Chapter 3), as an appropriate mode to assess the effect of removing Ajuba family function, we decided to investigate the role of *djub* in the *Drosophila* embryonic epidermis. The *Drosophila* embryonic epidermis provides a good model system for both genetic analysis as well as the establishment of epithelial polarity in vivo. During the process of *Drosophila* embryonic development, a fully functional epithelium forms, and it contains separate apical and basolateral compartments. Adherens junctions are the first junctional complex to form post cellularization, and these junctions gradually mature to form zonula adherens belts around the apex of the cells (Figure 1). This is the region to which the cadherin/catenin complexes localize. Also, unlike MDCKs or other cells in vertebrates, these cells do not have Tight junctions, but rather Septate Junctions below the adherens junction (Figure 1). In addition, a Subapical Region develops which is important for the cells to polarize. The two main complexes that localize to this region are the Crumbs complex and the Bazooka complex, both are essential for cells to establish polarity.

To see if *djub* may play a role in the formation of cell-cell junctions, we first asked

if we can detect dJub in *Drosophila* embryos. We stained early to late stage embryos with dJub as well as DE-cadherin (Figure 2). We found that similar to the pupal eye in *Drosophila* and to MDCK cells, dJub colocalized with DE-cadherin in fly embryos (Figure 2A-B). Next, we asked whether loss of *djub* function gives rise to a phenotype in *Drosophila* embryos. The removal of zygotic *djub* function did not yield any obvious embryonic phenotypes. However, *djub* is supplied maternally, thus we made germline clones that lack *djub* function and found that *djub* maternal clones yielded embryos with severe epidermis defects (Figure 2C). The phenotype of *djub* null embryos are reminiscent of mutations in genes like *crumbs* and *bazooka*, which are required to establish the initial apical-basal polarity of cells (Figure 2D-E). These data suggest that dJub may play a role in establishing the polarity of epithelial cells in embryos, either by affecting the localization or activity of other polarity complexes, or by directly stabilizing the junctions.

***Djub* effects primary cells in *Drosophila* pupal eye**

The above results suggest that *djub* governs the formation of cell-cell junctions in mammalian as well as *Drosophila* epithelial cells. Thus, we decided to take a closer look at the function of *djub* in epithelial cells at later stages of development. We have established that cell junctions remain normal in the absence of *djub* (Chapter 3) both in larvae as well as in the pupal eye, as opposed to in the fly embryos where junctions do not seem to form properly. The difference between these tissues is that cell junctions are already formed in larval tissue and must still form in embryos. However, a closer look inspection of the pupal eye phenotype of *djub* mutant cells revealed that, in addition to

the cell death phenotype, we also observe a change in the primary cell size (Figure 3). It appears that primary cells lacking *djub* are often smaller than their pair. It is unclear what role dJub may have in maintaining the size and shape of these primary cells. This effect also seems to be specific to primary ommatidial cells. One possibility is that *djub* is necessary for the normal function of the cadherin-catenin complex at Adherens junctions, where *djub* localizes. Studies show that in *arm* mutations in follicle cells, the cadherin-catenin complex breaks down and disrupts the architecture of the apical domain causing the cells to lose their shape and size, but retain a monolayered epithelial arrangement (Tanentzapf, Smith et al. 2000). A similar effect is seen in the *djub* mutant pupal eyes, where only the primary cell size changes with no effect on the surrounding tissue architecture. Further experiments are necessary to understand the mechanisms behind maintenance of primary cells and exactly how dJub plays a part in this.

The Hippo pathway induces apical expansion

In addition to excessive proliferation and decreased apoptosis, *hpo* or *wts* mutant cells also show morphological defects consistent with perturbations to junctional complexes (Justice, Zilian et al. 1995; Xu, Wang et al. 1995; Wu, Huang et al. 2003). In fact previous studies have shown that clones that are homozygous null for *wts* produce abnormal bristles, and the mutant cells themselves show apical hypertrophy (Justice, Zilian et al. 1995), which in most ways is the opposite of the *djub* phenotype. During our analyses, we also made cull or overexpression clones for the Hippo pathway components. As expected, *wts*, *hpo* null clones and clones over expressing *yki* all had an overproliferation phenotype. However we noticed that in addition to the

overproliferation, the clones or cells marked with GFP, had an additional apical expansion phenotype (Figure 4A, B and C). In each image the blue or red arrows point at secondary interommatidial cells that show apical expansion as compared to the wild-type secondary cell marked by the yellow arrows. The apical expansion was seen in primary, secondary and tertiary interommatidial cells. This was different from the *djub* pupal eye phenotype, which only seemed to affect the primary interommatidial cells. Recent studies have shown that in the wing imaginal disc epithelium in clones deficient for Hippo pathway components, there is an increase in the expression levels of apical polarity proteins like aPKC, Crb and DE-cadherin which belong to the Bazooka and Crumbs polarity complexes (Genevet, Polesello et al. 2009; Hamaratoglu, Gajewski et al. 2009). Both in our results as well as these studies, basolateral marker Dlg is not affected. These studies also show that the accumulation of the apical proteins was not necessary for the Hippo overgrowth phenotype, suggesting that the polarity genes do not in fact contribute to the Hippo signaling based overproliferation phenotype. Thus the apical expansion as well as the hypertrophy may be a separate non-growth control function of the Hippo pathway. It may be that *djub* and the Hippo pathway have opposite phenotype in the context of apical expansion and may, in fact, functionally interact here as well, but further experiments are required to confirm this.

Ajuba LIM proteins and YAP localization correlate with Hippo activity in a cell density dependent manner

The data from Chapter 3 show that the Ajuba LIM proteins inhibit the Hippo pathway in the context of epithelial cells. We know from Chapter 2 that Ajuba LIM

proteins are important for the formation of nascent epithelial cell junctions. We next asked if there was a developmental context for this inhibition or if the Ajuba and Hippo pathway interaction along with Ajuba's role at cell junctions contributed to contact inhibition. The Hippo pathway transcriptional coactivator, YAP has been shown previously to localize in a cell density dependent manner. We confirmed this phenomenon in PDV mouse epithelial cells. In low cell density, YAP predominantly localized in the nuclei of the cells. In contrast, YAP translocated to the cytoplasm at high density (Figure 5A and B). As described in previous work. We know that under conditions of high density, the Hippo pathway turns on and this leads to the phosphorylation of YAP by Lats, which in turn directs YAP to the cytoplasm, where it is sequestered.

To examine for a possible role of djub in this process, we carried out the same experiment and assayed whether Ajub exhibits any cell density dependent function in its subcellular localization. At low cell densities, Ajuba localized mostly to the cytoplasm with almost no colocalization with E-cadherin (stains cell-cell junctions). However, at high densities, Ajuba localizes almost entirely to cell-cell junctions and completely colocalizes with E-cadherin (Figure 5C and D). These results were very intriguing as they again a tight functional link between the Hippo pathway and the Ajuba LIM proteins. For example, under conditions of low density, Ajuba may bind to Lats and in the cytoplasm and therefore inhibit Lats from phosphorylating YAP allowing cell proliferation to proceed. However at high densities, Ajuba LIM proteins may be recruited to the cell membrane by other means and this change in its subcellular localization may either free Lats to phosphorylate and inhibit YAP.

Ajuba LIM proteins and E-cadherin expression is related to cell density

Ajuba and LIMD1 proteins accumulate to higher levels in epithelial cells than in fibroblast cells. And, from Figure 5, we see that Ajuba localizes to distinct subcellular compartments in non-confluent vs confluent cells. Thus we hypothesize that Ajuba becomes stabilized once it localizes to Adherens junctions. Junctional proteins such as β -catenin have been shown to be expressed at higher levels and sequestered at junctions under conditions of high confluency in HaCaT human keratinocytes. Thus, we tested if Ajuba expression levels are altered as a result of cell density. We found that Ajuba is stabilized as a result of increasing cell density in multiple epithelial cell lines, namely PDV mouse cells as well as MDCK canine cells (Figure 6 A and C). Also, we found that LIMD1, like to Ajuba, also stabilized with increase in cell density in culture (Figure 6B). Interestingly we see a similar stabilization of E-cadherin with increased density of cell with equal actin loading (Figure 6 A-C). In addition to increased levels of both LIMD1 and Ajuba LIM proteins in these cell, we also noticed a mobility shift. It appeared that with increase in cell density the mobility of Ajuba on the gel increases, suggesting the presence of some form of modification (Figure 6A). However, we have not yet been able to establish the molecular nature of this modification, though phosphorylation is an obvious possibility. Ajuba may undergo a post-translational modification as a result of increased cellular junctions. Other studies have shown that Ajuba is capable of being phosphorylated (Hirota, Kunitoku et al. 2003; Haraguchi, Ohsugi et al. 2007). In many instances a phosphorylation modification of a protein, will strengthen or weaken interactions with other proteins. Further studies are necessary to establish if in fact the mobility shift is due to the phosphorylation of Ajuba, whether the phosphorylation is cell

density dependent, and the effect the absence or presence of the phosphorylation on Ajuba's signal transduction to regulate junctions or growth control.

Discussion:

Our experiments on cell density dependent localization, and possibly modification, link general cellular events to the modification and function of Ajuba proteins (Figure 5). For example the negative regulatory role of the LIM protein may, in fact, depend on the localization of the Ajuba LIM proteins to junctions. Ajuba's cytoplasmic as well as membrane localizations may both be necessary for its growth enhancing function. Based on these and previous experiments we think that Ajuba LIM proteins may play a critical role as scaffolding proteins that provide a regulated link between membrane proteins and the cytoskeleton and participate in signal transduction pathways. We are presently carrying out experiments where we will ask if mutant forms of the Ajuba LIM proteins that are either always tethered to the membrane via a CAAX motif or are unable to localize to junctions, effect the role of the Ajuba LIM proteins in tissue growth control.

We also observe that Ajuba and LIMD1 as well as E-cadherin seem to be stabilized at junctions. It appears that in low density cells or if the junctions are downregulated, Ajuba fails to be recruited or comes off the junctions. Other than the levels of the Ajuba LIM proteins we also observed that the Ajuba LIM proteins are modified in response to an increase in cell density. In denser cell cultures, Ajuba runs more slowly on the gel (Figure 6A) as compared to lower density cell cultures where Ajuba runs a little faster. Further investigations will clarify if the mobility shift is due to a phosphorylation modification or something else. Previous studies have shown that Ajuba can be phosphorylated, in one case GSK3 β phosphorylates Ajuba to stabilize it and in another Ajuba is phosphorylated by Lats during mitosis and is important for spindle

formation (Abe, Ohsugi et al. 2006; Haraguchi, Ohsugi et al. 2007). Further experiments are necessary to determine the other signals that appear to respond to cell density and modify Ajuba. Also how is Ajuba modified and what the effect of this modification is on Ajuba function, and interactions with other proteins.

The fact Ajuba can localize to regions of cell-cell contacts indicates a role in direct intercellular communication. The Ajuba/djub loss-of-function studies in this thesis indicate that Ajuba may play a role in contact inhibition and in the formation of stable adherens junctions. Ajuba/djub colocalize with and associate with adherens junction components in wild type cells. We know that Ajuba interacts with α -catenin and actin. These and/or other undiscovered interactions with Ajuba may control the assembly and stabilization of mature adherens junctions. The establishment of polarity, a crucial step for differentiation and morphogenesis may also depend on the function of djub/Ajuba proteins. In this way djub/Ajuba LIM proteins may also play a role in establishing or maintaining epithelial cell polarization. Thus by functioning to stabilize junctions in a density-dependent manner and establishing cell polarization the Ajuba LIM proteins/dJub might coordinate the control of cell-cell communication with cell proliferation in developing tissue.

Interestingly although the Hippo pathway components lead to tissue overgrowth in various somatic tissue, unlike *djub*, germline clones of *fat*, *expanded*, *hippo*, *sav* and *warts* do not display any overgrowth of the mutant cells (Sun, Zhao et al. 2008). However, we do see a role for the Hippo pathway components at the pupal stage. From the data presented here and from other studies, it is clear that the Hippo pathway has an

effect on junctions. Whether this happens through the junctional members of the pathway like Ex, Mer or dJub or if in fact the apical expansion phenotype is a result of one or several polarity genes that are transcriptional targets of Yki is yet to be determined. It is possible that dJub and the Hippo pathway interact to maintain junctions as well. This interaction may be similar or distinct from the growth control interaction.

The experiments in this chapter highlight the fact that the Ajuba LIM proteins as well as the Hippo pathway play a role in junctions and in return the junctions are important not only in the organization of epithelia but also in the regulation of tissue growth. The ability of the Ajuba LIM proteins to localize to junctions and also be involved in regulating the Hippo pathway suggests junctions might provide an ideal platform of communication between cells. Further the dual role of growth function and junction stabilization gives us a hint towards how junctional proteins integrate polarity and density signals from neighboring cells and consequently regulate the necessity for proliferation.

Methods:

Generation of MARCM clones

MARCM clones(Lee and Luo 1999) for the Hippo pathway apical expansion experiments were generated by heat shocking third instar larvae for 1 h at 37°C and dissecting pupal eyes of the different genotypes 40 h APF.

Immunohistochemistry for pupal eyes

Pupal retina dissections were carried out in PBS following which the tissue was fixed in 4% paraformaldehyde dissolved in PBS. After fixation, tissues were washed in PBST (PBS + 0.5% Triton X-100) and incubated at 4°C overnight with primary antibody diluted in PAXDG buffer (0.1% BSA, 0.3% Triton X-100, 0.3% deoxycholate, 5% normal goat serum in PBS). Tissues were then washed 3 times with PBST for 10 mins each, and incubated for 3 hrs at room temperature or overnight at 4°C with secondary antibody diluted in PAXDG (1:1000). Tissues were then rinsed with PBST and transferred onto slides for mounting in vectasheild mounting medium containing DAPI (Vector Labs). Images were captured at room temperature on a LSM 510 Zeiss confocal microscope using 63x oil objective. Image J and Photoshop (Adobe) were used to process images. The following antibodies were used: rat α -DE-cadherin (IC) (1:10, DSHB).

Generating X chromosome Germline Clones

The first step was to generate females that are heterozygous for the *djub* deletion and the *ovo*^{*D1*} (Bloomington Stock number 23880) DFS chromosome by crossing *ovo*^{*D1*} males in the first cross (Cross 1). The *djub* females were balanced over FM6 and contained a FRT site on 19A (deletion distal to the FRT site). Recombination was induced between FRT

sites on these chromosomes by heat shock to drive the *FLP* recombinase. For the cross (Cross 1) about 30 virgin females (*djub* FRT19a/FM6) were crossed to 10-15 males (OvoD1-hsp70hs*FLP* FRT 19A/>) for 2 days of egg laying before being transferred to a new vial. Flies were flipped everyday after the first 2 days period, and then heat shocked for two consecutive days for 1.5 hrs in 37C water bath. For the next cross, non-FM6 female virgin flies were collected that contained germlines with the homozygous *djub* mutation. These female flies were crossed to FM7-GFP males and the non-GFP embryos laid were *djub* null germline clones.

Collection of Germline clone embryos

Embryos were collected on a hard agar media made with grape juice, supplemented with some yeast paste after the agar has solidified. The females are more likely to lay their eggs on the yeast paste.

Immunohistochemistry for *Drosophila* embryos

Embryos were collected from grape caps placed underneath bottles containing the cross to generate the desired genotype. Collections were made overnight (0–16 h) and all day (0–8 h). Once embryos were collected, dead flies were removed with dissecting forceps. The embryos were then transferred into glass vials covered in mesh on one side and open on the other. Using a soft bristle brush and water, embryos were removed from the grape cap and transferred into the glass vials to dislodge the yeast paste. The yeast paste was completely removed using the brush and water running into the vial and flowing out the mesh where the embryos collected. The chorion layer of the embryos were removed by

placing the collection vial into a small beaker with a 50% bleach solution and incubated for 3–5 min. During the incubation, the embryos were gently swirled in the collection vials and rinsed with the bleach solution using a pasteur pipet. After this the embryos were washed extensively to remove the bleach. For the antibody staining, embryos were then fixed and stained using previously described methods (Patel 1994).

Figure 1: **Organization of *Drosophila* epithelium.** The proteins of the Baz and Cbs complexes localize to the Subapical region (green), apical to the Adherens junctions (blue). Scrib, Dlg and Lgl form a complex basally to the Adherens junctions (red). Some regulators of growth and proliferation signaling pathways, such as EGFR, Fat, Expanded, Merlin and Dachs, localize to the apical junction in the *Drosophila* epithelium.

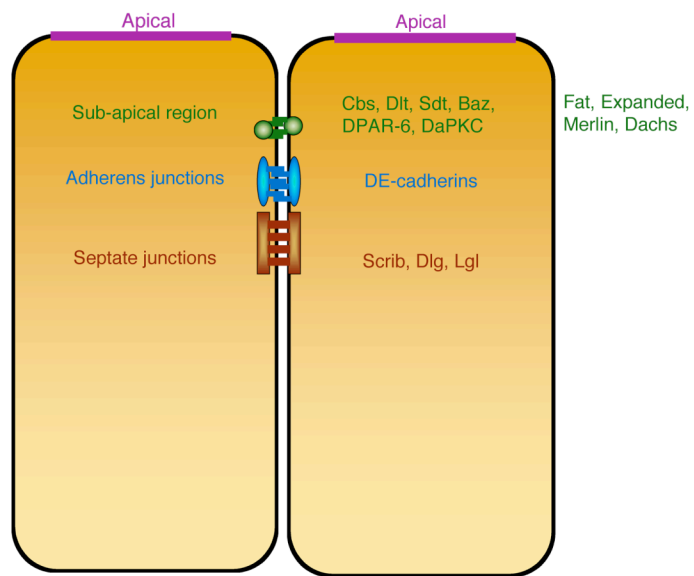


Figure 1

Figure 2: ***djub* germline clones have severe epithelial defects.** (A-B) Wild-type *Drosophila* embryos stained for dJub (Green) and DE-cadherin (Red) at early stages of embryonic development (A-A'') and towards the end of embryonic development (B-B''). *djub* germline clone without dJub staining, stained for DE-cadherin in red. (D-D') Crb11A22 mutant embryo TEM image (D) and stained for NrX (D'). BazXi zygotic mutant embryo stained for coracle (E).

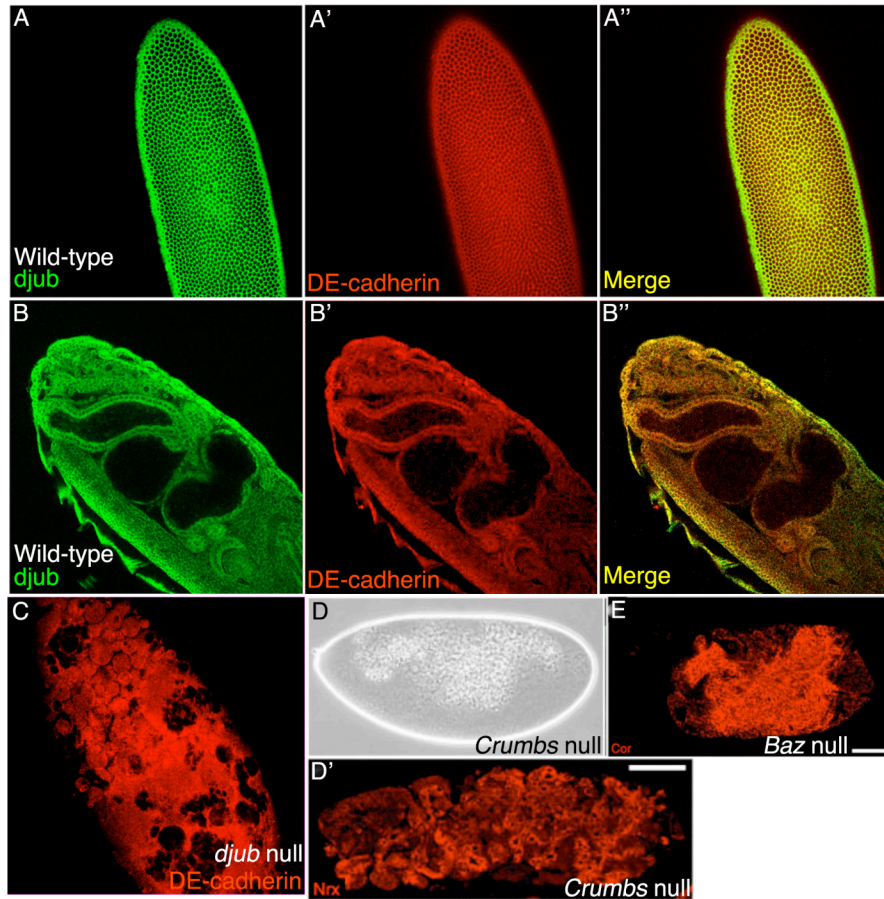


Figure 2

Figure 3: ***djub* mutant pupal eyes display primary cell defects.** Mid-pupal eye containing *djub* null mutant clones (GFP negative) expressed throughout the pupal eye using *Eyeless-FLP* and stained for DE-cadherin (red/white). Blue or red arrows point at primary cells that are mutant for *djub* or GFP negative. The white versus the red astrix (*), show a comparison of the pair of primary cells. The primary cells marked by red astrix are smaller than the primary cells marked with a white astrix.

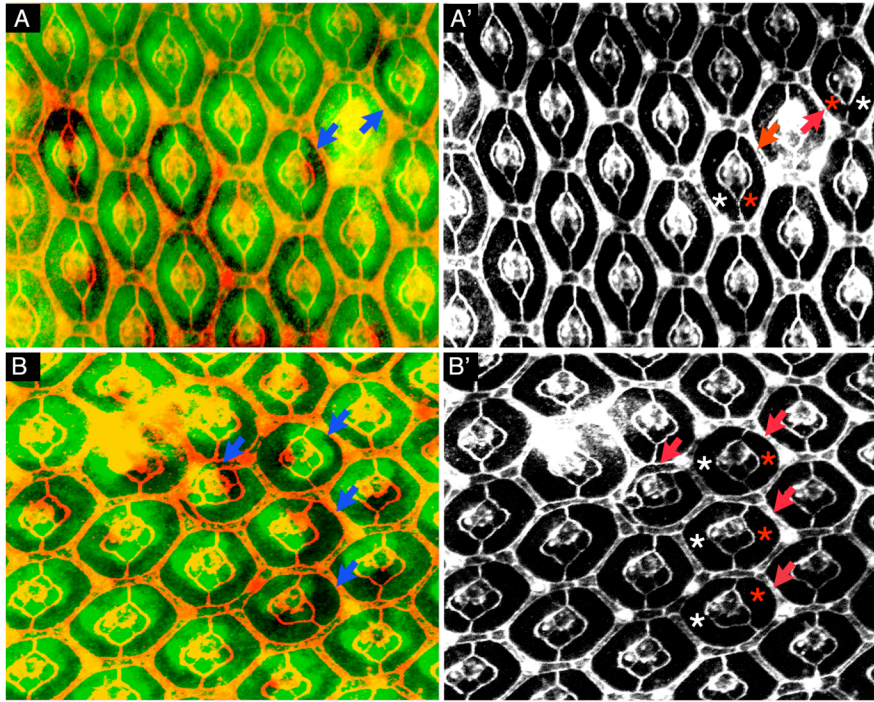


Figure 3

Figure 4: Apical expansion in Hippo pathway mutant cells. Mid-pupal eyes stained for DE-cadherin (red/white). GFP positive *hpo* null MARCM clones (A-A'); GFP positive *wts* null MARCM clones (B-B'); MARCM clones overexpressing Yki (GFP positive) (C-C'). Arrows identify apical expansion. Blue and red arrows in each genotype point at the same cell that mutant for *hpo* or *wts* or overexpressing Yki. The yellow arrows point at wild-type interommatidial cells.

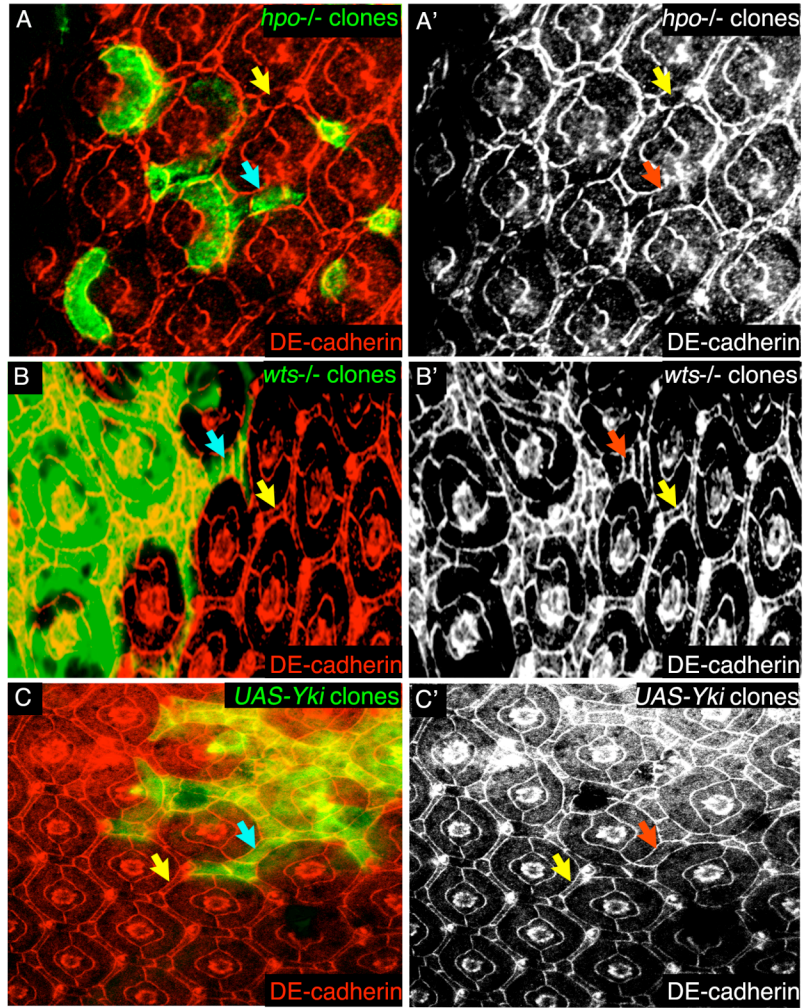


Figure 4

Figure 5: YAP and Ajuba localization change with respect to cell density. (Top two panels) YAP nuclear versus cytoplasmic localization is cell density dependent. PDV cells were cultured sparsely (Row 1) or to confluence (Row 2). YAP was stained with anti-YAP antibody (green). (Bottom two panels) Ajuba Cytoplasmic versus junctional localization is affected by cell density. PDV cells were cultured sparsely (Row 3) or to confluence (Row 4). Ajuba was stained with anti-Ajuba antibody (green). Cell outlines are visualized by E-cadherin staining (red).

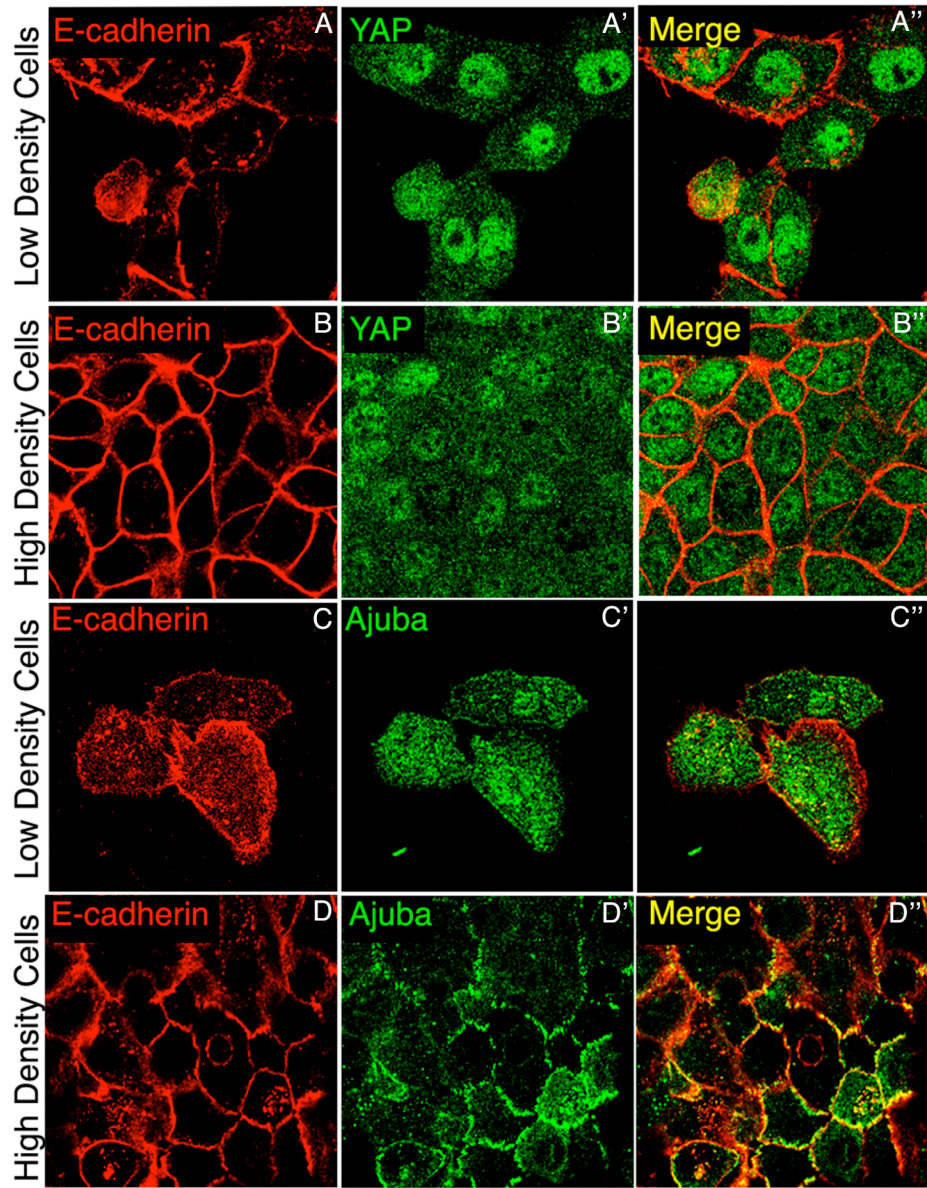


Figure 5

Figure 6: **Ajuba LIM proteins and E-cadherin expression levels are regulated by cell density.** (A-C) Cell lysates from PDV mouse epithelial cells immunoblotted for Ajuba. The black arrow points at the Ajuba band in low density cells and the red arrow shows the shift in Ajuba at higher densities as compared to the lower density (A). Cell lysates from PDV mouse epithelial cells immunoblotted for E-cadherin, LIMD1 and actin (B) and MDCK cells immunoblotted for E-cadherin Ajuba and Actin (C). PDV and MDCK epithelial cells were plated at increasing densities and then western blotted for the presence of E-cadherin, Ajuba and LIMD1. Actin served as a loading control.

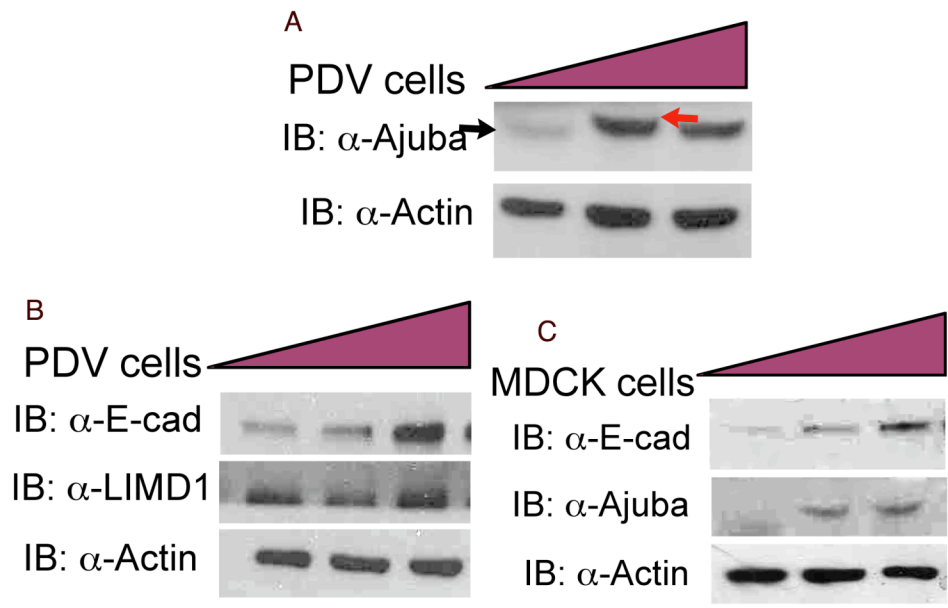


Figure 6

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Chapter 5

Conclusions and Future Directions

The Ajuba family of LIM domain containing proteins localize to cell-cell or cell matrix adhesion sites in epithelia and fibroblasts, respectively, and can influence the stability of cell adhesive complexes. Some LIM proteins can also shuttle to and from the nucleus giving them the potential to coordinate cell surface adhesive signals with nuclear responses (Goyal, Lin et al. 1999; Kanungo, Pratt et al. 2000; Langer, Feng et al. 2008). Compared to the Zyxin sub-family members, the Ajuba subfamily of LIM proteins are highly expressed in organs abundant in epithelia, like skin, kidney, liver etc. Previous studies have shown that Ajuba interacts with α -catenin at the cadherin adhesive complexes, and this interaction is required for efficient recruitment of Ajuba to cell-cell junctions. Ajuba also directly interacts with F-actin via its PreLIM domain (Marie, Pratt et al. 2003). However, to what extent Ajuba proteins act as a bridge to stabilize the cadherin adhesive complexes, and/or contribute to the stability, formation, and function of nascent junctions remains unclear. Furthermore, since three Ajuba subfamily members exist in mammals, Ajuba, LIMD1 and WTIP, functional redundancy greatly complicates the ability to dissect the functional roles of the proteins. Thus, creating a system where one can assay the Ajuba family null phenotype- as I have done during my thesis research- should accelerate the ability to shed light on novel roles of these proteins.

Ajuba LIM proteins in epithelia

Here, I have established that the Ajuba LIM proteins play a role in the establishment of epithelial cell-cell junctions. We show that Ajuba LIM proteins influence *de novo* assembly of tight junctions and adherens junctions, maintain stable cell-cell junctions, and are necessary for the establishment of epithelia polarity. More

importantly, we show that Ajuba and LIMD1 each display similar junctional phenotypes, which worsen when the function of both LIMD1 and Ajuba are depleted. To overcome the issue of functional redundancy, which has hampered precise and systematic investigation of Ajuba proteins in mammalian systems, I generated null mutations of the only member of the Ajuba family in flies, *djub*. I found that *djub* is an essential gene that when specifically depleted in the eye or wing epithelium inhibits growth by increasing apoptosis and decreasing proliferation simultaneously. Through the investigations carried out in this thesis, I observed genetic and biochemical evidence that dJub negatively regulates the Hippo pathway by activating Wts (Lats1/2) kinase. We also find that in both *Drosophila* S2 cells, as well as in mammalian cells, Ajuba, LIMD1, and WTIP associate specifically with Lats and WW45 to influence YAP phosphorylation in cells. Thus, as is observed with the function of the Hippo signaling pathway, these results suggest that the functional link between Ajuba proteins and the Hippo signaling pathway is also conserved from flies to humans.

Subcellular compartment-dependent regulation of the Hippo pathway by the Ajuba LIM proteins

My genetic and biochemical data (Chapter 3, Figure. 6) suggest that dJub influences the Hpo pathway by affecting Wts activity. Future experiments will focus on determining the molecular basis for Ajuba-mediated inhibition of Lats/Wts activity as well as the subcellular localization of this interaction. The possible modes by which Ajuba LIM proteins inhibit the activation of Lats/Wts is by inhibiting upstream kinases MST/Hpo from accessing and/or activating Lats/Wts, inhibiting the ability of Lats/Wts to

phosphorylate YAP/Yki, or affecting the subcellular localization of Lats/Wts or WW45/Sav and thus their access to the Hippo pathway. The other possibility is that the regulatory relationship between Ajuba LIM proteins (dJub) and Lats/Wts may not be simply unidirectional as Lats has been shown to phosphorylate Ajuba (Abe, Ohsugi et al. 2006). With respect to the subcellular localization of this interaction, accumulated studies of mammalian Ajuba LIM proteins have indicated that they function as adapter proteins (Langer, Feng et al. 2008) in multiple subcellular compartments, namely, adherens junctions, (Marie, Pratt et al. 2003), cytosol (Feng, Zhao et al. 2007), and the nucleus (Langer, Feng et al. 2008). Thus, the next question is, do the Ajuba LIM proteins need to be available in a specific compartment for their interaction and inhibition of the Hippo pathway. Future experiments will include using different constructs of LIMD1 (or Ajuba, dJub) where the proteins are either nuclear only, unable to translocate to the nucleus or tethered to the membrane using CAAX to check if the LIM proteins can still functionally regulate the Hippo pathway by measuring the phosphorylation state of Yap or Yki or if they can rescue the in vivo dJub phenotype.

Upstream regulation of the Hippo pathway

One important factor that still remains unknown is what cellular or molecular signal initiates the activation of the Hippo signaling pathway. While my thesis work has added additional players, and complexity to the Hippo signaling pathway, this fundamental question still remains unanswered. The Hippo pathway upstream members Expanded (Ex) and Merlin (Mer), both FERM domain-containing proteins localize to the

plasma membrane and act in parallel to regulate Hpo. Also, upstream member Ft acts through modulating levels of Wts, by affecting Wts stability (Cho, Feng et al. 2006). We have shown that dJub, like its mammalian orthologs, localizes to cell-cell junctions in *Drosophila* epithelia (Chapter 3, Figure 1L-O). Therefore, Ajuba LIM proteins may be an additional mode of regulation of the Hippo pathway besides the atypical cadherin Fat and Ex/Mer. There still remains the possibility that dJub influences the Hippo pathway higher up in the signaling cascade, at the level of Fat, Ex and Mer. Ajuba/dJub may act in conjunction with Mer and Ex or parallel to Hpo and lastly there is also the possibility of cross talk between these pathways all of which are as of yet undetermined. Ajuba/dJub may also influence the pathway indirectly by regulating the levels or localizations of these proteins. It is also worth mentioning that although dJub localizes to junctions, the staining appears to be punctate. We have yet to determine the significance of the non-uniform staining pattern. Thus, the regulation may not be linear, but occur via more complicated scenarios where the regulation of the Hippo pathway occurs downstream of Fat or in between Ex/Mer and Hippo.

Recent studies have described new findings that suggest an apical polarity complex regulation role for Hpo independent of the pathway's growth control function (Maitra, Kulikauskas et al. 2006; Genevet, Polesello et al. 2009; Hamaratoglu, Gajewski et al. 2009). This finding and also the data from Chapter 4 that describe the apical junctional phenotype for mutants of the Hippo pathway members further provides evidence for the possibility of dJub having a junctional role with respect to the Hpo pathway. Whether the Ajuba LIM proteins are capable of directly interacting with Fat, or

whether the Ajuba LIM proteins link Ex or Mer to Hpo or whether this role is played by some other component is yet to be determined.

Universality of the Hippo pathway

Another aspect of the Hippo pathway that needs further investigation is in what cell types does the Hippo pathway normally function to regulate apoptosis and cell proliferation. In case of *Drosophila* the pathway as well as dJub are expressed and function to restrict growth in imaginal discs, which are essentially columnar epithelia. So the question arises if the pathway modulates size of tissue or tumor formation only in a subset of cell types or human tissues that are similar to *Drosophila* imaginal discs in their properties of growth, proliferation and apoptosis. As far as the Ajuba LIM proteins go, they are most highly expressed in epithelial tissue in mammals (RAKESH K. GOYAL and ANTHONY J. MUSLIN). With respect to the Hippo pathway, interestingly there are multiple types of tumors that develop spontaneously in NF2 mice, which include bone tumors and malignant mesothelioma. However, in humans it is predominantly central nervous system tumors that develop as a result of NF2 deficiency (McClatchey and Giovannini 2005). Lats1 deficient mice will develop soft tissue sarcomas and ovarian tumors but there are reports on these mice developing nervous system tumors or even mesothelioma (St John, Tao et al. 1999). Thus, the Hippo pathway seems to be consistent in its regulation in other non-epithelial tissues as well. However, to what extent are the Ajuba LIM proteins expressed in these tissues and whether they continue to inhibit the Hippo pathway is not known.

Hippo signaling and the Ajuba LIM proteins role in cell adhesion and contact inhibition

We have established that Ajuba LIM proteins have a clear role in the overall morphology as well as stability and formation of epithelial cells. The depletion of Ajuba LIM proteins results in defects in stability or formation of epithelial sheets as well as cell-cell adhesion of MDCK and PDV cells in suspension (Chapter 2, Figure 10). We show in Chapter 4, components of the Hippo pathway namely, Hpo, Wts and Yki induce aberrations in epithelial cell morphology. These results are intriguing as they raise the possibility that the Hippo pathway may respond to adhesion or cell-cell contacts between cells. In the Hippo pathway, mutations in several components lead to a distinctly rounded morphology of the mutant clone, indicative of altered cell adhesion (Nolo, Morrison et al. 2006; Thompson and Cohen 2006). Furthermore, various mutations in the pathway appear to modify the adhesive properties of the cells in a way that the mutant cells preferentially bind to cells of the same genotype instead of a neighboring wild type cell. NF2-null MEFs have weakened cell-cell adhesion and do not undergo contact inhibition (Lallemand, Curto et al. 2003). In case of the Hippo pathway it is still not clear if the adhesion phenotype is a result of massive overproliferation or if there are downstream targets of the Hippo pathway that directly control cell-cell adhesion. However, the fact that several Hippo pathway activity regulators, namely, Ajuba LIM proteins, which are involved cell-cell adhesion strengths, and other upstream members such as Fat, Ex and Mer, which connect the cytoskeleton with the extracellular matrix receptors such as CD44 (Bretscher, Edwards et al. 2002), suggests that the Hippo pathway may in fact respond to adhesion or cell-cell contacts between cells.

Contact Inhibition and the Hippo pathway

Although we know that the Hippo pathway responds to contact inhibition, it is unclear how this process is carried out. In cell culture, high cell density activates the Hippo pathway, resulting in YAP shuttling into the cytoplasm, suggesting a role of YAP and the Hippo pathway on contact inhibition (Zhao, Wei et al. 2007; Lei, Zhang et al. 2008). In fact this is also seen in vivo, in the mouse embryo trophectoderm, YAP localizes to the nucleus in the outer peripheral cells, but toward the inner cell mass where the cell density is higher, YAP is phosphorylated by Hippo and localized to the cytoplasm (Nishioka, Inoue et al. 2009). These data suggest that the Hippo pathway and YAP may be able to receive signals from cell contacts and interpret these cues and accordingly activate different developmental pathways. Further investigation is necessary to define the molecular pathway that links the detection of cell density to Yki/YAP regulation. It is also not clear how Yki/YAP gets into the nucleus when the Hippo pathway is turned off. One mechanism for the nuclear translocation of Yki/YAP is through an interaction with target transcription factors such as *scalloped* as shown in *Drosophila* S2 cells with Yki (Goulev, Fauny et al. 2008; Wu, Liu et al. 2008), and with TEAD4 and YAP in mammalian cells. But in many cases, such as with *Sd*, the target transcription factor is only expressed in a small subset of tissue whereas Yki/YAP functions in a much more ubiquitous manner. Thus it will be interesting to see if different transcription factors play a role in regulating the nuclear localization of Yki/YAP and the mechanism by which this happens.

The data described in Chapter 4 regarding the subcellular localization of the Ajuba LIM proteins with respect to cell density is intriguing. The Ajuba LIM proteins are predominantly cytoplasmic at low cell densities, but at higher cell densities, Ajuba localization shifts to the junctions. At this point the implications of this change in localization with respect to cell density is not fully understood. Future experiments are necessary to measure the presence of a density dependent regulation of the Hippo pathway by the Ajuba LIM proteins. It will be interesting to see if the Ajuba LIM proteins interact with Lats in a cell density dependent manner. Like the rest of the Hippo pathway components, Fat mutant cells also have the rounded morphology and can impact Yki localization. Future experiments may also identify the extent to which Fat is involved in this process.

Mammalian roles of the Ajuba LIM proteins and the Hippo pathway

The novel growth-promoting role of the Ajuba LIM proteins, and their ability to inhibit the Hippo pathway has raised many new questions. Although these findings have begun to answer some of the queries regarding organ size regulation, as well as the role of the Ajuba family in epithelia, the studies were mainly carried out in a *Drosophila* model system. Thus, while the mammalian orthologs of Hippo and dJub proteins interact physically, it remains unclear whether they also control organ size in mammals. However, the Hippo pathway or components of the Hippo pathway have been implicated in tumorigenesis in mammals, for example, NF2 or mer is a known tumor suppressor gene and mutations in NF2 mutations can lead to neurofibromatosis. These studies alone, do not establish a role for the Hippo pathway members in size regulation, since many

other processes other than the Hippo pathway's size regulatory role can lead to tumorigenesis. Two complementary reasons for the absence of a clear demonstration of a size regulatory role of the Hippo pathway in mammals may be, the apparent genetic redundancy of specific Hippo components (eg. Lats1) resulting in knock-out mice that are either viable and lack any obvious overgrowth characteristics of the parallel *Drosophila* mutant (St John, Tao et al. 1999) or embryonic lethal making it difficult to assess the involvement in size regulation (eg. YAP) (McPherson, Tamblyn et al. 2004; Morin-Kensicki, Boone et al. 2006). Recent studies have begun characterizing the Hippo pathway in mammals, for instance transgenic models that overexpresses YAP specifically in the mouse liver gave rise to a Hippo regulated overgrowth phenotype (Dong, Feldmann et al. 2007).

Analysis of in vivo function of mammalian Ajuba family members, also suffers from the problem of genetic redundancy. For example, mice singly mutant for Ajuba or LIMD1 or doubly mutant for Ajuba/LIMD1 appear grossly normal and do not display any overt defects. No mouse model yet exists WTIP. Therefore, knock out or transgenic models, which allow us to manipulate the Ajuba LIM proteins and Hippo pathway components in a spatially and temporally restricted manner, are necessary. This would allow us to get around issues of lethality and more importantly investigate the role of these proteins in a developmental context. Further, such models would also allow the analysis of the Hippo pathway/Ajuba proteins function in other cellular processes, such as the possible role of the Hippo pathway in dendrite morphogenesis as has been recorded in *Drosophila* (Emoto, Parrish et al. 2006). Further investigation of the roles of the Hippo pathway in the mammalian system will also provide a way to ask whether the functions

of the Hippo pathway and Ajuba family proteins also intersect during the process of tissue organ regeneration, where tissues such as the liver regenerate back to, but not beyond, a critical size (Michalopoulos and DeFrances 1997). Clearly, future work on the nexus between Hippo pathway and Ajuba proteins in mammals holds promise to reveal new genetic and molecular insights into clinically relevant fields such as tumorigenesis and regeneration.

Hippo and Beyond

Mechanisms that coordinate both cell proliferation and cell death in flies and mammals are critical for the normal development and homeostasis of these organisms. The combination of the two processes is an important failsafe mechanism to inhibit inappropriate proliferation of somatic cells. In most cases, activation of an oncogene (eg. myc) or a mutation in a tumor suppressor, leads to unscheduled proliferation. This untimely proliferation in turn signals other pathways that counter the excessive proliferation by increased apoptosis. The Hippo pathway though is able to override this phenomenon since mutating the members of the pathway (Hpo, Wts, Sav, Mats) leads to increased cell proliferation accompanied by inhibition of cell death. The Hippo pathway's ability to both inhibit cell proliferation and promote apoptosis simultaneously makes it a great candidate for a robust mechanism of swiftly stopping organ growth during development. The pathway acts as an on/off switch for growth, making it susceptible to single component perturbations leading to detrimental uncontrolled growth.

It is important to note that the Hippo pathway is likely regulated at many nodes. In fact my thesis work suggests that Ajuba LIM proteins regulate Hippo signaling at the

level of Warts, whereas previous works have shown regulation of Hippo activity by Ex/Mer or by Fat. Fat itself will also regulate the levels of Wts via a parallel pathway through Dachs. We also don't know if there are players downstream of Ex/Mer that activate the pathway. Fat has been shown to regulate Ex, but the receptor upstream of Mer is yet to be identified. Finally, Chapter 4 suggests there may be still other players such as the junctional proteins Scrib/Dlg/Lgl, that cross-talk with the Hippo pathway.

Given the diversity of phenotypes that arise as a result of mutations in the Hippo pathway in *Drosophila* and mammals, the biological effect of Hippo signaling may be context dependent. For instance, in addition to its nuclear coactivating role and regulation of growth genes CyclinE and DIAP1, Yap can also promote cell death by binding to P73 (a p53 family member) in the nucleus (Strano, Munarriz et al. 2001; Strano, Monti et al. 2005). The fact that YAP can carry out these opposing roles suggests that YAP probably binds and activates different transcriptional factors to regulate progrowth versus proapoptotic genes. This may depend on the specific upstream signaling or a posttranslational modification that affects the stability and/or localization of YAP. In flies the Hippo pathway plays a role in several types of processes namely, retinal cell patterning, dendrite morphogenesis, regulation of oocyte polarity and even salivary gland degeneration. The role of Hpo in the salivary gland is Yki independent and PI3K dependent. This suggests that in addition to Yki there must be other downstream effectors of the pathway. In *Drosophila*, oocyte polarity by which the posterior follicle cell fate identity is determined during oogenesis is Fat independent, suggesting there are other upstream effectors that can regulate the Hippo pathway. Thus, the Hippo pathway is far

more complex than a direct linear regulatory mechanism of organ growth control. Future experiments will broaden our knowledge help us reconcile many of these findings.

In summary, the work described in this thesis shows that the Ajuba LIM proteins have a novel and crucial role in epithelial cells in regulating the overall size of an organ. The Ajuba LIM proteins mediate the effect by acting as direct negative regulators of the Hippo growth regulatory pathway. They play an influential role in both *Drosophila* as well as mammals to regulate the transcriptional output of the pathway by impacting the phosphorylated levels of Yki (or Yap in mammals) and thereby the transcriptional levels of growth-regulating and apoptotic genes. It will be important, in the future, to understand the precise mechanism by which djob or Ajuba/LIMD1/WTIP regulate the Hippo pathway, and where in epithelial cells this regulation occurs.

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