STUDIES OF THE HIPPO SIGNALING PATHWAY

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This dissertation is dedicated to my family.

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STUDIES OF THE HIPPO SIGNLAING PATHWAY

By TAO YUE

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STUDIES OF THE HIPPO SIGNLAING PATHWAY

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How multicellular organisms control their growth to reach proper organ size during development is a fascinating question. Recent studies, initially from *Drosophila*, have identified the Hpo tumor suppressor pathway as a crucial mechanism that controls tissue growth by inhibiting cell growth, proliferation and survival. Deregulation of the Hpo pathway has been implicated in various human cancers.

Central to the Hpo pathway is a kinase cassette consisting of four tumor suppressor proteins, the Ste20-like kinase Hpo, the WW domain-containing protein Salvador (Sav), the NDR family kinase Warts (Wts) and the Mob family protein Mats. The kinase activities of Hpo and Wts are facilitated by their regulatory proteins Sav and Mats, respectively. Activated Hpo/Sav complex phosphorylates and activates the Wts/Mats complex, which in turn phosphorylates and inactivates the transcriptional coactivator Yorkie (Yki). Phosphorylation of Yki restricts its nuclear localization through recruiting 14-3-3. When the activity of the Hpo/Wts kinase cassette is compromised, Yki forms complexes with transcription factors including Scalloped (Sd) and translocates to the nucleus to activate Hpo pathway target genes, including *cyclin E, diap1*, and the microRNA *bantam* that regulate cell growth, proliferation and survival.

To identify novel components of the Hpo signaling pathway, I carried out a genetic modifier screen in which flies carrying *GMR-Gal4* and *UAS-Yki* were crossed to a collection of transgenic RNAi lines from Vienna *Drosophila* RNAi center (VDRC) and Bloomington stock center, and looked for enhancers or suppressors of the overgrown eye phenotype caused by Yki overexpression. Through this screen, I have found that Echinoid (Ed), an immunoglobulin domain-containing cell adhesion molecule, acts as an upstream regulator of the Hpo pathway. Loss of Ed compromises Yki phosphorylation, resulting in elevated Yki activity that drives Hpo target gene expression and tissue overgrowth. Ed physically interacts with and stabilizes the Hpo-binding partner Sav at adherens junctions. Ed/Sav interaction is promoted by cell-cell contact and requires dimerization of Ed cytoplasmic domain. Overexpression of Sav or dimerized Ed cytoplasmic domain suppressed loss-of-Ed phenotypes. I propose that Ed may link cell-cell contact to Hpo signaling through binding and stabilizing Sav, thus modulating the Hpo kinase activity. Furthermore, the Cul4/WDR40A complex has also been identified as a genetic modifier for the Hippo signaling pathway. However, the exact mechanism by which this complex regulates the Hippo signaling pathway need to be further addressed.

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List of abbreviations

Ds - Dachsous

Ed - Echinoid

Ex - Expanded

Ft - Fat

Fj - Four- jointed

Hth - Homothorax

Hpo - Hippo

Lgl - Lethal giant Larvae

Mats - Mob as tumor suppressor

Mer - Merlin

Rassf - Ras associated family protein

Sav - Salvador

Sd - Scalloped

Vg - Vestigial

Wts - Warts

Yki - Yorkie

Chapter I

Introduction

"*Beauty depends on size as well as symmetry.*"—Aristotle

How multi-cellular organisms control their growth to reach proper organ size during development is a fascinating question in developmental biology. Understanding this process is not only to satisfy scientists' curiosity for nature, but more importantly to develop specific strategies to battles diseases resulted from deregulation of this normal process in growth control.

In the early 20th century, Sir D'Arcy Thompson, the pioneering biologist to study evolutionary constraints on size, raised an interesting hypothesis that physical forces such as gravity, diffusion constants, surface tension and others, are the determinants of animal size. However, the extent to which those physical factors shape an organism during development is yet to be determined even till today. In the past couple of decades, more and more studies have revealed that the size of an organism and its organ is regulated by systemic factors such as growth hormone and insulin-like proteins as well as intrinsic factors. These external and internal factors cooperatively control cell number, cell mass/volume and extracellular mass/volume in a tissue specific manner to determine the final organ size (Stanger, 2008).

Drosophila melanogaster, as one of the first organisms for genetic analysis, has been used to study growth control in developmental biology for almost a century. Imaginal discs, which are the embryonic precursors of eyes, wing, etc in flies, are single cell-layer structures that undergo most proliferation during the larval stage to produce mature tissue with characteristic size and shape, providing an extremely powerful genetic tool to study organ size control. Moreover, the fact that above 80% human genes have fly homologs and the high level of functional conservation from fly to the mammal has made the fly a useful entry point to identify novel mammalian genes and dissect signaling pathways(St John and Xu, 1997).

Extensive genetic screens have been performed to identify overgrowth mutation in mosaic flies. Through these screens, *warts/large tumor suppressor*, *Sav* and *hpo/dMST*, have been identified and fallen into an emerging tumor suppressor pathway, the so-called Hippo (Hpo) signaling pathway. This pathway serves as a crucial and evolutionarily conserved mechanism that controls tissue growth and organ size by simultaneously inhibiting cell growth/proliferation and promoting cell death (Halder and Johnson, 2011; Pan, 2010; Zhang et al., 2009). The Hpo signaling pathway has also been implicated in cell contact-dependent growth inhibition (Zhao et al., 2007), and deregulation of the Hpo pathway has been linked to a wide range of human cancers, such as hypermethylation of Mst1/2 in soft tissue sarcoma and Lats1/2 in astrocytoma and breast cancers, decreased expression of Mob1 in colorectal and lung cancers (Pan, 2010).

Kinase cassette of the Hpo pathway

Central to the Hpo pathway is a kinase cassette consisting of four tumor suppressor proteins, the Ste20-like kinase Hpo (Harvey et al., 2003; Jia et al., 2003; Pantalacci et al., 2003; Udan et al., 2003a; Wu et al., 2003), the WW domain-containing protein Salvador (Sav) (Kango-Singh et al., 2002; Tapon et al., 2002), the NDR family kinase Warts (Wts) (Justice et al., 1995; Xu et al., 1995) and the Mob family protein Mats (Lai et al., 2005a). The kinase activities of Hpo and Wts are facilitated by their regulatory proteins Sav and Mats, respectively.

All of these four components of the Hpo signaling pathway are initially identified through genetic screen in *Drosophila*. Loss of function of Hpo, Wts, Sav and Mats leads to dramatic overgrowth of imaginal discs and of corresponding adult structures by increasing cell proliferation and inhibiting apoptosis. Animals with *hpo* mutant eyes, produce strong overgrown eyes and heads that are accompanied by a characteristic distortion and folding of the normally smooth cuticular surface, resembling the hide of the hippopotamus. Therefore, the *hpo* gene was named due to this hpo-like phenotype (Fig 1.1). Further biochemical studies have discovered physical interaction among these four proteins. Sav acting as a scaffolding protein, binds to both Hpo and Wts, and facilitate the phosphorylation of Wts by Hpo. Hpo also phosphorylates Mats, and promotes the interaction between Mats and Wts, which is required for the activation of Wts. Activated Wts, phosphorylates and thereby inhibits the

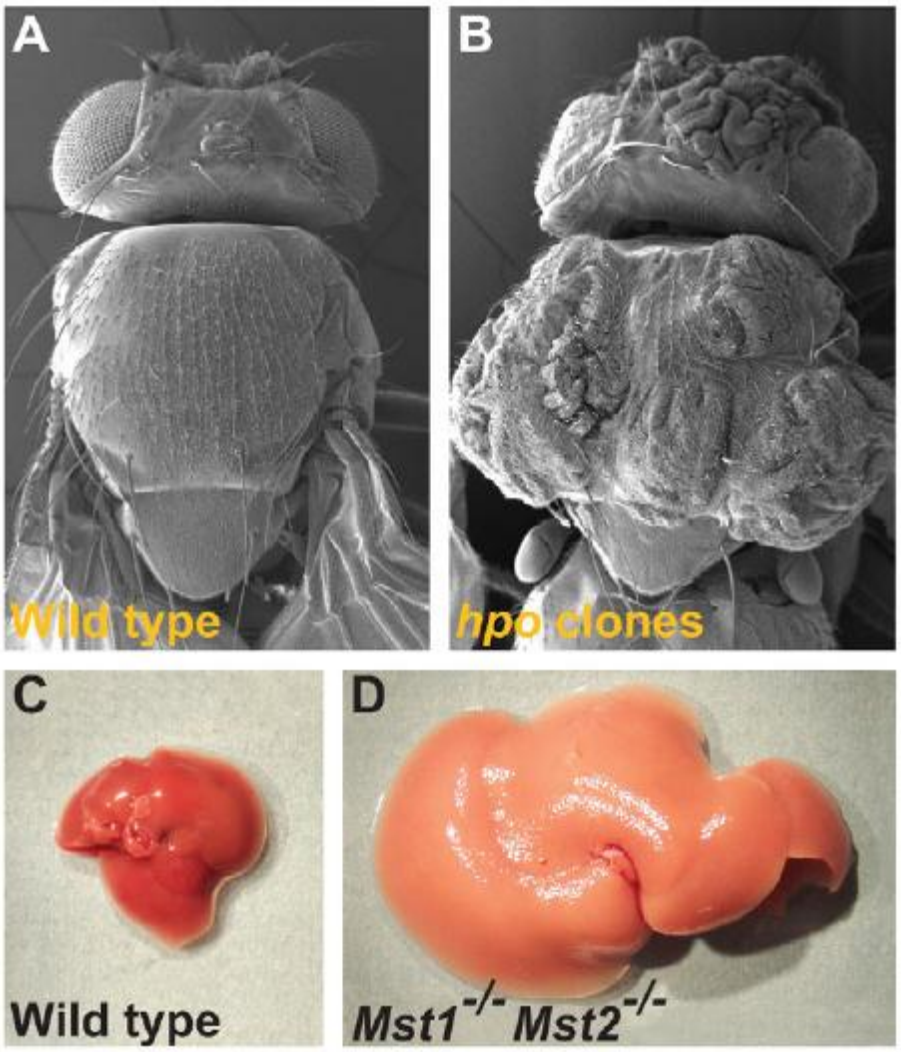

Fig 1.1 *Hpo* mutant phenotypes in *Drosophila* and mice (adapted from Georg Halder, Randy Johnson, 2010, Development). (**A, B**) scanning electron micrographs of a fly with *hippo* mutant clones exhibiting overgrowth of the adult cuticle. (**C, D)** mouse livers at 2 months of age from a wild-type mouse and a mutant mouse in which both Mst1 and Mst2, two mammalian *Hippo* homologs, have been conditionally inactivated in the developing liver (Lee et al., 2010; Lu et al., 2010; Song et al., 2010; Zhou et al., 2009).

transcriptional co-activator Yki, which is the critical effector of the Hpo signaling pathway.

Yki, the effector of the Hpo pathway

Unlike the components of the core kinase cassette, Yki, a non-DNA-binding transcriptional co-activator, was initially identified through a yeast two-hybrid screen for Wts-binding partner (Huang et al., 2005). It was mysterious how the core kinase cassette regulated *Cyclin E* (*CycE*) and d*iap1* expression, as the mutant clones of components of the Hpo signaling pathway show upregulated expression of the cell cycle regulator CycE and the cell death inhibitor Diap1 at both transcriptional and protein level. Identification of Yki filled this gap in our knowledge concerning the Hpo signaling pathway.

Genetic studies indicates that overexpression of Yki behaves similar to loss-of-function *wts* exhibiting increased Diap1 expression level and dramatic tissue overgrowth. In contrast, inactivation of *yki* causes reduced *diap1* transcription and is genetically epistatic to mutants of *hpo*, *sav* and *wts*. Biochemical characterization of Yki has found that phosphorylation of Ser168 is the most important event to introduce 14-3-3 binding site and thereby retaining Yki in the cytoplasm, although Wts directly phosphorylates Yki at three sites (Ser111, Ser168 and Ser250). When upstream kinase activity is compromised and Yki is not phosphorylated, Yki in conjunction with other transcription factors, translocates into the nucleus, and activates the expression of Hpo pathway target

genes(Wu et al., 2008; Zhang et al., 2008), including *cyclin E*(Tapon et al., 2002), *diap1*(Wu et al., 2003), *E2F1*(Goulev et al., 2008), the growth promoter *myc* and the growth and cell survival-promoting miRNA *bantam(*Nolo et al., 2006; Thompson and Cohen, 2006). Taken together, Yki promotes growth, and its activity is suppressed by tumor suppressors, Hpo, Sav, Wts and Mats.

Transcription factors of the Hpo pathway

To execute its function, Yki, a non-DNA binding transcriptional co-activator, has to interact with multiple transcription factors to activate downstream genes expression in a cell-type dependent manner. Scalloped (Sd), belonging to the TEAD/TEF family, is the first transcription factor identified as a Yki binding partner by a genome-wide yeast two-hybrid screen (Wu et al., 2008; Zhang et al., 2008). The complex of Sd and Yki directly resides to a minimal 26 bp Hpo response element that controls *diap1* expression in a Hpo-dependent fashion. Moreover, Sd promotes Yki nuclear localization and its inactivation suppresses tissue overgrowth caused by Yki overexpression or tumor suppressor mutations in the Hpo pathway. However, although Yki is required for normal growth and survival in all imaginal cells, Sd is only necessary for wing and neuronal development, indicating that other transcription factors regulate Hpo responsive genes expression in conjunction with Yki.

One recent study indicates that the transcription factor Homothorax (Hth), a TALE-homeodomain protein, forms a complex with Yki to promote cell survival

and proliferation anterior to the morphogenetic furrow in the eye imaginal disc by regulating the expression of the miRNA *bantam*, but not *diap1*. Chromatin immunoprecipitation analysis showed that Hth and Yki bound to the upstream of the *bantam* hairpin in eye imaginal disc cells, indicating that this is a direct regulation (Peng et al., 2009). Thus, both Sd and Hth mediate the activity of the Hpo pathway in a context-dependent manner. A comprehensive understanding of the transcriptional network in response to Hpo signaling will require identification of more Yki-interacting transcriptional factors.

Upstream regulators of the Hpo pathway

It has been a long puzzle that how organ growth signal is transduced into an individual cell to control the activity of Hpo signaling. Through candidate gene-based approached and forward genetic screens, researchers have been addressing this question by identifying multiple upstream regulators of the Hpo signaling pathway, including the WW and C2 domain-containing protein Kibra, the FERM domain proteins Merlin and Expanded, the protocadherins Fat and Dachsous, the cell polarity determinant Crumbs and Lethal giant Larvae and others.

(1) The Kibra, Expanded and Merlin complex

One critical upstream regulator modulating Hpo signaling is an apical protein complex containing Kibra, Expanded and Merlin (Baumgartner et al., 2010; Cho

et al., 2006; Genevet et al., 2010; Hamaratoglu et al., 2006; Pellock et al., 2007; Tyler and Baker, 2007; Willecke et al., 2006; Yu et al., 2010). Although individual mutations of these genes only produces relatively weak overgrowth phenotype in imaginal discs, double mutant combinations show stronger phenotype resembling *hpo* mutant phenotype, thus revealing a partial redundancy among them (Baumgartner et al., 2010; Genevet et al., 2010; Hamaratoglu et al., 2006; Maitra et al., 2006; Yu et al., 2010). In addition to being genetically upstream of of Hpo, they also form a physical complex which interacts with Hpo-Sav complex and promotes Wts phosphorylation in cultured cells probably by maintaining Hpo membrane association (Yu et al., 2010). Similar to many other signaling pathways harboring a negative feedback loop, the expression of these upstream regulators is transcriptionally regulated by Hpo signaling through Yki. Several lines of evidence also indicate that each of these three proteins regulates Hpo signaling to a different extent in a temporal and spatial manner, for example, Ex is required for Hpo signaling in the larval eye while Mer is required in the pupal eye (Milton et al., 2010).

(2) the protocadherins Ft and Ds

Both Ft and Ds are large transmembrane protein containing 34 and 27 cadherin domains, respectively. Two different mechanisms have been found on how Ft controls Hpo signaling. In one mechanism, Ft acts on the Hpo pathway through an unconventional myosin Dachs, which associates with Wts and

promotes its proteolysis (Cho et al., 2006; Feng and Irvine, 2007). The preferential accumulation of the Dachs protein is regulated by Ft activity (Rogulja et al., 2008). In an alternative mechanism, the apical membrane localization and/or the stability of Ex is regulated by Ft, although no evidence shows that Ft interacts with Ex (Bennett and Harvey, 2006; Silva et al., 2006; Willecke et al., 2006).

Ft activity is regulated by Ds through a cadherin domain-mediated heterophilic interaction that is modulated by the kinase Four-jointed (Fj), which phosphorylates the extracellular domains of Ft and Ds (Bennett and Harvey, 2006; Brittle et al., 2010; Casal et al., 2006; Cho et al., 2006; Cho and Irvine, 2004; Ishikawa et al., 2008; Lawrence et al., 2007; Ma et al., 2003; Matakatsu and Blair, 2004; Silva et al., 2006; Simon et al., 2010; Strutt and Strutt, 2002; Willecke et al., 2006). Intriguingly, Ds and Fj are expressed in complementary gradients in developing imaginal discs, and their expression is controlled by morphogens including Decapentaglegic (Dpp), wingless, and Hedgehog (Casal et al., 2006; Rogulja et al., 2008; Yang et al., 2002), suggesting that the regulation of tissue size and tissue patterning is coordinated through Hpo signaling.

Interestingly, Ft activity is regulated by the steepness of the Ds gradient, but not by the amount of Ds. Only a shallow gradient of Ds activates Ft, but a steep gradient inhibits Ft (Rogulja et al., 2008; Willecke et al., 2006). Moreover, the requirement of Ds in both signal-sending cells and signal-responding cells indicates that Ds has both ligand and receptor functions in Hpo signaling (Casal

et al., 2006; Willecke et al., 2006). To understand how Ft and Ds control the growth, two non-mutually exclusive models, the polar coordinate model and the feed-forward model, have been raised to explain Ft/Ds roles in Hpo signaling (Rogulja et al., 2008; Willecke et al., 2006; Zecca and Struhl, 2010).

Based on the polar coordinate model, tissue growth is determined by the steepness of various morphogens in the tissue. A steep gradient induces growth, while a shallow gradient reduces growth. During development, neighboring cells, exposing to a steep gradient above a certain threshold, proliferate until the difference gets smaller below the threshold. Here, Ds and Fj serve as positional information due to their expression pattern. Ectopic expression of Ds and Fj in the neighboring cells of the imaginal discs induces proliferation at expression boundary whereas uniform expression of Ds and Fj leads to reduced growth.

The feed-forward model is also based on the expression pattern of Ds and Fj (Zecca and Struhl, 2010). High levels of Ds are expressed in the cells surrounding the wing pouch where Fj is highly expressed. The steep gradient of Ds and Fj inactivates Ft activity and induces the expression of Hpo responsive genes, including *vestigial* (*vg*), which is a transcriptional factor and specifying the wing cell fate. Induced Vg in newly specified wing pouch cells promotes Fj expression and thereby suppresses Ds expression, which pushes the Ds and Fj expression gradients outwards and leads to another cycle of recruitment of wing pouch cells. Although these two models can partially explain the control of growth in imaginal discs, ds and fj double mutant flies are still able to produce

wings, albeit small, suggesting that other unknown mechanisms may exist to regulate Hpo signaling.

(3) Cell polarity determinant Crumbs and Lgl

Recent several studies identified Crumbs as a new receptor for the Hpo signaling pathway (Chen et al., 2010; Ling et al., 2010; Robinson et al., 2010). Crumbs (Crb), an apical transmembrane protein, has been known for its role in establishing apical-basal cell polarity. Its extracellular domain contains 28 EGF-like repeats, and its intracellular region has a juxtamembrane FERM-binding motif (FBM) mediating the Crb-Ex interaction, and a C-terminal PDZ-binding motif (PBM). Both mutation and overexpression of *crb* resulted in overgrowth by upregulating Hpo target genes. Loss of Crb leads to mislocalizaton of Ex in the apical region. Interestingly, overexpression of Crb also depletes of apical Ex protein likely through a dominant-negative effect. Genetic analysis reveals that Crb functions upstream of Ex, but parallel with Mer and Kibra (Richardson and Pichaud, 2010; Robinson et al., 2010).

Another cell polarity determinant, Lethal giant larvae (Lgl), lately has been connected to the Hpo pathway (Grzeschik et al., 2010). Lgl locates the basolateral membrane of epithelial cells and organizes apical-basal cell polarity. Inactivation of Lgl leads to defects in cell polarity and dramatic overgrown imaginal discs with elevated cell proliferation, reduced apoptosis and Yki nuclear accumulation. Different from Crb, *lgl* mutant cells exhibit mislocaliztion of Hpo

and Ras associated family protein (Rassf), but not Ex. The exact mechanism underlying this regulation still remains unclear.

Although the mechanisms by which multiple inputs feed into the Hpo pathway are complicated, however, mutations in each of these genes only cause a relative weak overgrowth phenotype compared with mutations of components of the core kinase cassette, suggesting that these upstream regulators parallelly act in a coordinated manner at various levels within the signaling cascade (Fig 1.2). Another notable feature is that the relative contribution of the upstream Hpo pathway regulators varies depending on tissue types. For example, loss of Ft in wing imaginal discs results in strong activation of Hpo responsive genes and marked tissue overgrowth, but dose not perturb Hpo signaling in *Drosophila* ovarian follicle cells (Meignin et al., 2007; Polesello and Tapon, 2007). To the contrary, Kibra is a critical regulator of Hpo signaling in *Drosophila* ovarian follicle cells but its loss of function only weakly activates Hpo pathway genes and causes minor overgrowth of imaginal tissues (Genevet et al., 2010; Yu et al., 2010). Thus, the wiring of the Hpo signaling network may depend on the developmental context.

Conservation and divergence of the Hpo pathway between Drosophila and the mammal

The Hpo signaling pathway, initially identified in *Drosophila*, is highly

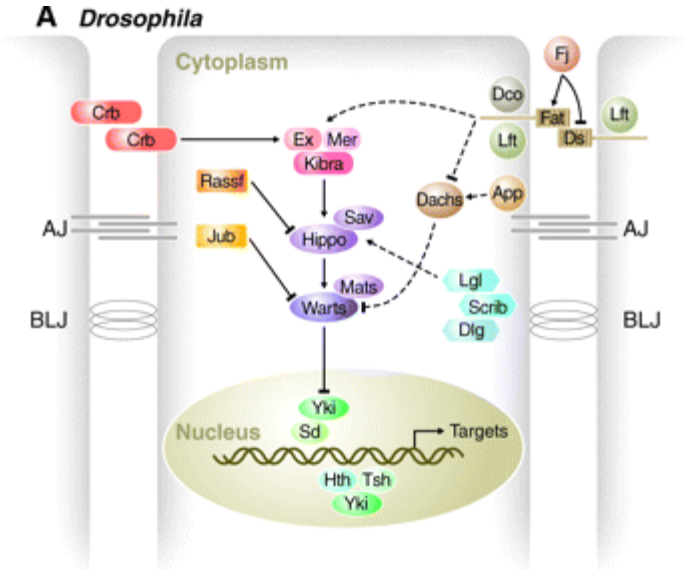

Fig. 1.2 Schematic representation of the Hippo pathway in flies (adapted from Georg Halder, Randy Johnson, 2010, Development). Cells (outlined in grey, nuclei in green) are shown with adherens junctions (AJ) and basolateral junctions (BLJ). (**A**,**B**) Hippo pathway components in *Drosophila* are shown in various colors, with pointed and blunt arrowheads indicating activating and inhibitory interactions, respectively. Continuous lines indicate direct interactions, whereas dashed lines indicate unknown mechanisms. See text for further details. Abbreviations: App, Approximated; Crb, Crumbs; Dco, Discs overgrown; Dlg, Discs large; Ds, Dachsous; Ex, Expanded; Fj, Four-jointed; Hth, Homothorax; Jub, *Drosophila* Ajuba; Lft, Lowfat; Lgl, Lethal giant larvae; Mer, Merlin; Mats, Mob as a tumor suppressor; Rassf, Ras-associated factor; Sav, Salvador; Sd, Scalloped; Tsh, Teashirt; Yki, Yorkie.

conserved in mammals, which has been experimentally confirmed by recent molecular and genetic studies. But at the same time, these studies also reported a higher level of complexity and divergence across the species.

In the mammal, the Hpo pathway consists of two Hpo homologs (Mst1 and Mst2), one Sav homolog (WW45), two Wts homologs (Lats1 and Lats2), two Mats homologs (MOBKL1A and MOBKL1B) and two Yki homologs (YAP and TAZ) (Table 1.1). These proteins assemble a conserved kinase cassette similar to their *Drosophila* counterparts (Callus et al., 2006; Chan et al., 2005; Praskova et al., 2008) and a few components of the *Drosophila* Hpo pathway can be functionally replaced by their mammalian homologs (Dong et al., 2007; Huang et al., 2005; Lai et al., 2005b; Tao et al., 1999; Wu et al., 2003; Wu et al., 2008).

Increasing evidence has indicated that the mammalian Hpo pathway regulates cell contact inhibition, organ size, and cancer development. Mice lacking Lats1 develop soft-tissue sarcomas, ovarian tumors. The human homolog of Sav is mutated in several cancer cell lines. Transgenic overexpression of Yap or liver-specific knockout of MST1/2 or Sav1 leads to increased liver size and ultimately induced hepatocellular carcinoma(HCC), indicating a conserved role for the Hpo pathway in regulating organ size in mammals (Fig 1.1).

Except Dachs, the mammal has homologs for all the reported upstream regulators of the Hpo pathway, containing two kibra homologs (KIBRA and WWC2), two Ex homologs (Ex1 and Ex2), one Mer homolog (NF2/Mer), one Ft homolog(Fat4/Fat-J), two Ds homologs (Dchs1 and Dchs2), one Fj homolog

Table 1. Components of the Hippo pathway in flies and mice

Drosophila gene	Mouse gene	Protein function
Core components		
Hippo (Hpo)	Mst1, Mst2*	Ste20 family Ser/Thr kinase
Salvador (Sav)	Sav1/WW45	WW-domain adaptor protein
Warts (Wts)	Lats1, Lats2	NDR family Ser/Thr kinase
Mob as tumor suppressor (Mats)	Mob1A, Mob1B	Wts co-factor
Yorkie (Yki)	Yap, Taz†	WW-domain transcriptional co-activator
Upstream modulators		
Fat	Fat4	Transmembrane cadherin
Dachsous (Ds)	Dchs1, Dchs2	Transmembrane cadherin
Four-jointed (Fj)	Fjx1	Golgi resident Ser/Thr kinase
N/A‡	CD44	Transmembrane receptor
Discs overgrown (Dco)	CKIδ, CKIε	Casein kinase Ser/Thr kinase
Lowfat (Lft)	Lix1, Lix1-L	Adaptor, unknown function
Dachs (D)	N/A§	Unconventional myosin
Approximated (App)	ZDHHC9, ZDHHC14, ZDHHC18	DHHC palmitoyltransferase
Crumbs (Crb)	Crb1-3	Transmembrane receptor
Expanded (Ex)	Ex1/FRMD6, Ex2¶	FERM-domain adaptor protein
Merlin (Mer)	Merlin/Nf2	FERM-domain adaptor protein
Kibra	Kibra	WW-domain adaptor protein
Rassf	Rassf1-6	RA-domain adaptor
Jub	Ajuba, LIMD1, WTIP	LIM-domain adaptor protein
Lethal giant larvae (Lgl)	Lgl1, Lgl2	WD40 scaffold protein
Downstream mediators		
Scalloped (Sd)	TEAD1-4	TEA-domain transcription factor
Teashirt (Tsh)	Tshz1-3	Zn-finger transcription factor
Homothorax (Hth)	Meis1-3, Prep1-2	Homeodomain transcription factor

*The mammalian Mst1 and Mst2 kinases are activated by caspase cleavage that removes an inhibitory C-terminal domain (Graves et al., 1998). *Drosophila* Hpo lacks consensus caspase recognition sequences and does not undergo a similar cleavage (Harvey et al., 2003; Jia et al., 2003; Pantalacci et al., 2003; Udan et al., 2003; Wu et al., 2003).

†Vertebrate Yap and Taz contain a C-terminal PDZ binding motif that is essential for Yap nuclear localization and pro-apoptotic signaling that is not present in *Drosophila* Yki (Kanai et al., 2000; Oka and Sudol, 2009). Phosphorylation by Lats primes Yap and Taz for secondary phosphorylation by a distinct kinase, resulting in b-TRCP mediated ubiquitination and degradation (Liu et al., 2010; Zhao et al., 2010b). The corresponding phosphorylation sites are not conserved in Yki (Zhao et al., 2010a).

‡Flies do not have CD44.

§No direct homolog of Dachs is known in vertebrates (Mao et al., 2006).

¶The mammalian Ex homologs are quite divergent in sequence from *Drosophila* Ex (Hamaratoglu et al., 2006).

Abbreviations: CKI, casein kinase I; Jub, *Drosophila* Ajuba; FERMD, 4.1-Ezrin-Radixin-Moesin domain protein; Lats, Large tumor suppressor; LIMD, Lim domain containing; Lix, Limb expression; Meis, Myeloid ecotropic viral integration site; Mob, Mps one binder; Mst, Mammalian sterile-20 like; NF2, Neurofibromatosis type 2; Prep, Pbx regulating protein; Rassf, Ras associated factor; Sav, Salvador; Taz, Transcriptional co-activator with PDZ-binding motif; TEAD, TEA-domain protein; Tshz, Teashirt-related zinc finger; WTIP, Wilms tumor protein 1-interacting protein; WW45, WW domain protein 45; Yap, Yes associated protein; ZDHHC, Zinc finger DHHC domain-containing palmitoyltransferase.

Table 1.1 Components of the Hippo pathway in flies and mice (adapted from Georg Halder, Randy Johnson, 2010, Development).

(Fjx1), and three Crb homologs (Crb1, Crb2 and Crb3). Among these mammalian homologs, NF2/Mer has been intensively studied over the time. Inactivation of NF2 results in hepatocellular carcinoma (HCC) and bile duct tumors, and this dual tumor phenotype is also revealed by mutations of Mst1/2 and Sav1, suggesting that deregulation of Hpo signaling could account for loss of NF2 phenotype.

However, mammalian homologs of Ft and Crb have not been linked to the Hpo signaling yet. Inactivation of Ft revealed the essential role for Fat4 in regulating planar cell polarity (PCP) in inner hair cell orientation and neural tube elongation. In human, *Crb1* mutation causes early-onset retinal degenerative diseases. Furthermore, the characteristic phenotype of inactivation of the mammalian Hpo signaling pathway has not been reported in Fat4 and Crb1 mutant cells. One possibility is that Fat4 and Crb1 regulate the Hpo signaling only in the certain context. Another simple explanation is that other Fats (1-3) or other Crb (2-3) function partially redundant to Fat 4 and Crb1 so that the loss-of-function phenotype is hardly revealed.

The aim of this study

Recent studies have greatly improved our understanding of the Hpo signaling pathway, many questions remain as described in the above. To address these questions, I have set up genetic modifier screens for suppressors and enhancers of a gain-of-Yki function phenotype generated by overexpression of Yki under GMR-gal4 control in

the developing eye (GMR-Yki). I expected to identify novel components for the Hpo signaling pathway. Here in my thesis, I described the strategy of my genetic screen and genetic modifiers identified through this screen. Echinoid (Ed), an immunoglobulin domain-containing cell adhesion molecule (Bai et al., 2001; Wei et al., 2005), has been found as a novel upstream regulator of the Hpo pathway. Through characterization of Ed function in Hpo signaling, I proposed a new mechanism by which the Hpo kinase cascade is regulated in *Drosophila*.

Chapter II

Identification and characterization of genetic modifiers of the Hpo signaling pathway

Genetic screen for novel components of the Hpo pathway

To identify additional components or regulators of the pathway, I have been carrying out a genetic modifier screen for enhancers and suppressors of the enlarged eye phenotype caused by overexpression of the Hpo pathway transcriptional effector, Yorki (Yki). Overexpression of Yki under the control of GMR-Gal4, which expresses in the posterior to the morphogenetic furrow of the eye imaginal disc, caused an overgrowth phenotype in the eye, accompanied by a characteristic distortion and folding of the normally smooth cuticular surface.

My genetic screen scheme is described in Fig 2.1. First, I crossed GMR-Yki with deficiency lines from exelixis deficiency line collection to look for modifiers which are able to enhance or suppress GMR-Yki overgrowth phenotype. Each deficiency line has about ten genes deleted. Identified modifiers through the primary screen were taken to the second test. In this step, I first confirmed the effect on modification then crossed with GMR-Insulin receptor^{CA} which also caused enlarged eye but not through the Hippo pathway. Through the second test, I knew the specificity of the modification. At last, I analyzed genes deleted in bona fide modifers and ordered RNAi lines for those of interest to further narrow down. Since RNAi libraries became available about one year after,

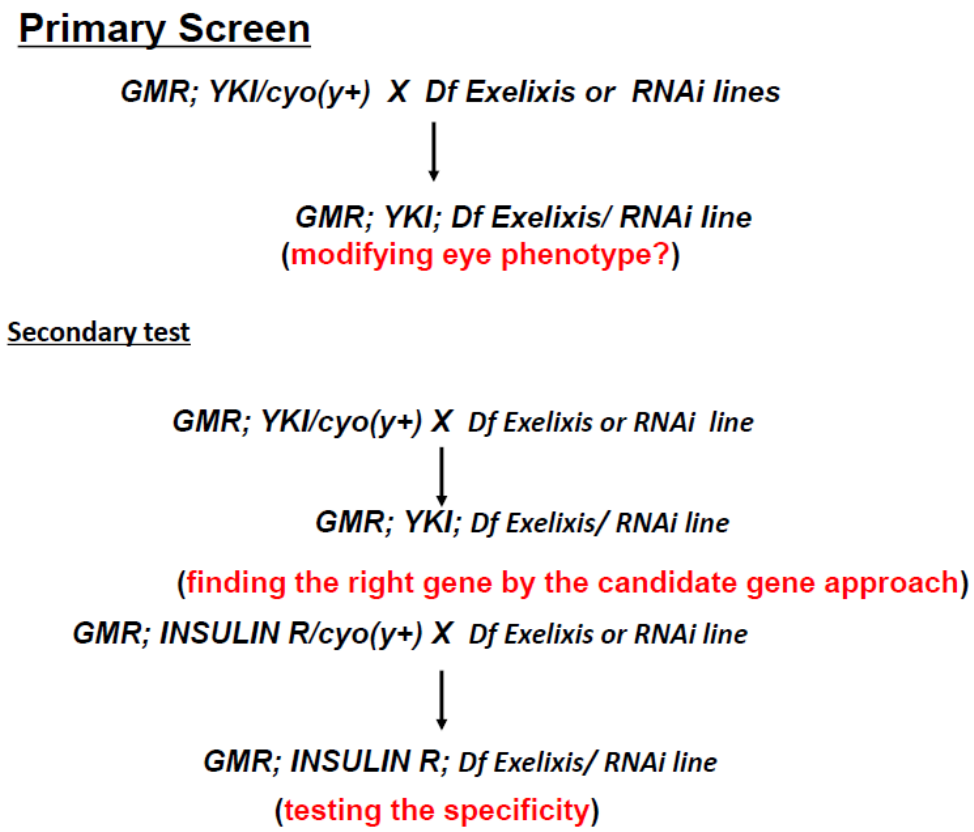

Fig. 2.1 The scheme of genetic modifier screen for enhancers or suppressors of GMR-Yki.

I started this screen, I was able to take candidate gene approach to directly screen RNAi library for those genes involved in many processes such as ubiqitination and cell adhesion.

So far I have screened 141 deficiency lines, covering one-third fly genome. Among these deficiency lines, six deficiency lines enhanced GMR-yki and one suppressed (Table 2.1). Meanwhile, I also screened over 500 RNAi lines.

Deficiency line (7704) enhances GMR-Yki phenotype, however, this line combined with GMR-Gal4 also produces overgrown eye, indicating that overexpression of some flanking gene surrounding the deleted region is sufficient to drive overgrowth. More careful analyses are required to identify the gene responsible for this overgrowth phenotype. Through a candidate gene approach, *CG4655 (Cad86C)* RNAi line has been identified as a suppressor for GMR-Yki. Although *Cg4655* encodes a cadherin domain containing protein, surprisingly, it appears to be required for growth and function opposite to Ft or Ds. It has been reported that its expression is induced by Hedgehog and Dpp signaling and ectopic expression of CG4655 in either eye disc or wing disc leads to apical construction and shortening of cells along the apical-basal axis (Schlichting and Dahmann, 2008). So far it is not clear whether CG4655 directly regulates Hpo signaling probably through the interaction with Fat or regulates other growth pathways.

Through these screens, three genes have been identified to have genetic interaction with the Hippo pathway. Two of them are WD40 repeat-containing protein, and Cullin

E3 ligase. My following study indicated these two proteins may form a complex regulating the Hippo pathway. A cell adhesion molecule, Ed, was also identified by the screen. My thesis project is focusing on these three modifiers.

The Cul4/WDR40A complex identified as a genetic modifier for the Hpo pathway

Through the screen, I found that RNAi line targeting *CG3313* genetically interacts with GMR-YKI and greatly enhanced its overgrowth phenotype. To rule out off-target effect, I made *UAS-CG3313* transgenic fly. Coexpression of *UAS-CG3313* indeed suppressed the modification of *CG3313* RNAi mediated enhancement on GMR-YKI, confirming the specificity of *CG3313* RNAi line. On the other hand, overexpression *CG3313* alone is sufficient to suppress GMR-Yki mediated overgrowth. Both gain- and loss-of-function studies indicated a genetic interaction between CG3313 and the Hpo signalin pathway (Fig 2.1).

Although little is known about CG3313 function, its mammalian homologue has been found to be a component of the DDB1-Cul4 ubiquitin ligase complex. The Cul4-DDB1 ubiquitin ligase complex has been shown to regulate cell proliferation, and survival, through targeted ubiquitination of key regulators by substrate receptors which dictates the specificity of this ubiquitnation machinary (Angers et al., 2006; He et al., 2006; Higa et al., 2006; Lee and Zhou, 2007). Recent work identified a family of WD40 proteins as substrate receptors (Angers et al., 2006). The mammalian homologue of CG3313 is a member of this family. One of structural signatures of this

Modifiers	**Cytology**	***GMR;YKi***	***GMR***	**Candidate gene**
7704	**1F2;2B1**	**enhancer**	**enhancer**	**unknown**
7731	**85E5 to 85E9**	**suppressor**	**No phenotype**	**unknown**
7639	**86E4;86E11**	**enhancer**	**No phenotype**	**unknown**
7966	**87A4;87A7**	**enhancer**	**No phenotype**	***CG3313(WD40)***
7649	**87F10;87F14**	**enhancer**	**No phenotype**	**unknown**
7676	**95D8;95E5**	**enhancer**	**No phenotype**	***tsc1***
7917	**100A4;100A5**	**enhancer**	**No phenotype**	***warts***

Table 2.1 Summary of genetic modifier screen for deficiency line collection.

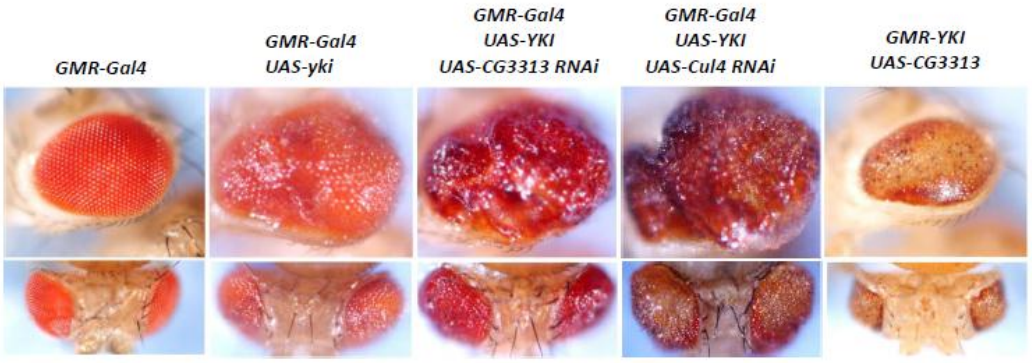

Figure 2.1 Genetic interaction between the the Cul4/WDR40A complex and the Hpo pathway. Both *CG3313* RNAi and *Cul4* RNAi enhance GMR-Yki phenotype, similarly. Conversely, Overexpression of CG3313 suppresses GMR-Yki mediated eye overgrowth.

WD40 family is that they all have two DXR motifs mediating the binding to DDB1. CG3313 also has these two motifs. Based on this, a simple hypothesis is that Cg3313 forms a complex with Cul4 to regulate the hippo pathway. This seems to be a case, because my genetic screen also identified *Cul4* RNAi as a genetic modifier for GMR-Yki. Expression of *UAS-Cul4* RNAi can greatly enhance GMR-yki phenotype as well. However, overexpression of *Cul4 RNAi* alone or *CG3313 RNAi* alone did not cause any significant overgrowth in the eyes (Fig2.1).

To see if this modification of adult phenotype is through the change of Hpo-responsive genes expression, I compared Hpo responsive genes expression such as *cyclin E, diap1*, between GMR-Yki and GMR-Yki; *UAS-Cul4 RNAi*. However, the expression of *cyclin E and diap1*did not have a significant upregulation if there is when *UAS-Cul4* RNAi was expressed, suggesting that other Hpo responsive genes may be involved. I am still in the process to identify the right or the sensitive Hpo target genes for this modification.

Ed identified as a genetic modifier for the Hpo pathway

To identify novel components of the Hpo signaling pathway, I carried out a genetic modifier screen in which flies carrying GMR-Gal4 and UAS-Yki (referred to as GMR>Yki) were crossed to a collection of transgenic RNAi lines from Vienna Drosophila RNAi center (VDRC) and Bloomington stock center, and looked for enhancers or suppressors of the overgrown eye phenotype caused by Yki overexpression. From this screen, I found that an RNAi line that targets Ed

(UAS-Ed-RNAi^{3087}) enhanced GMR>Yki mediated overgrowth although expression of UAS-Ed-RNAi^{3087} using GMR-Gal4 in otherwise wild type eyes did not cause any discernible overgrowth (Fig 2.2). However, expression of UAS-Ed-RNAi^{3087} using the eyeless-Gal4 (ey-Gal4) driver, which drives the expression of UAS transgene in eye discs starting at a much earlier stage than GMR-Gal4 (Halder et al., 1998), resulted in eye overgrowth (Fig. 2.2).

To further probe the role of Ed in growth control, I expressed several UAS-Ed-RNAi lines from VDRC in the posterior compartment of wing imaginal discs using hh-Gal4 (referred to as hh>Ed-RNAi). As shown in Fig. 2.2, inactivation of Ed by hh>Ed-RNAi produced adult wings with enlarged posterior compartments. RNAi-mediated knockdown of ed expression was confirmed by immunostaining with Ed antibody (Fig. 2.4). The overgrowth phenotype induced by hh>Ed-RNAi is unlikely due to an off-target effect because expression of three different transgenic RNAi lines (VDRC# 937, 3087, and 104279) targeting two non-overlapping regions of the ed coding sequence produced a similar overgrowth phenotype in adult wings (Fig. 2.3). I also generated a UAS-Ed-RNAi line that targets a C-terminal region of Ed (UAS-Ed-RNAiC ; Fig. 2.3) and found that expression of this line by hh-Gal4 also produced an enlarged posterior compartment of adult wings (Fig. 2.3). Furthermore, overexpression of a UAS-Ed transgene suppressed wing overgrowth caused by hh>Ed-RNAi (Fig. 2.2).

I then determined whether ed mutation could render tissue overgrowth. I generated mosaic eyes that contain wild type control clones or ed mutant clones using

the ey-FLP system (Newsome et al., 2000). As shown in Fig. 2.2, mosaic eyes containing ed mutant clones were overgrown compared with the control mosaic eyes. The overgrowth phenotype caused by the ed mutation is less severe compared with those caused by hpo mutations (Jia et al., 2003) but is comparable to those caused by kibra mutations (Baumgartner et al., 2010; Genevet et al., 2010; Yu et al., 2010).

Loss of Ed upregulates Hpo pathway target genes

The enlarged wing phenotype induced by *hh>Ed-RNAi* resembles those caused by loss of Hpo pathway components including Sav and Wts in posterior compartments (Fig. 2.2) (Genevet et al., 2010). I therefore determined whether loss of Ed led to deregulation of Hpo pathway target genes. As shown in Fig. 2.4, expression of *UAS-Ed-RNAi* by *hh-Gal4* led to the upregulation of *diap1-GFP* and *ex-lacZ* that faithfully report the expression of *diap1*and *ex*, respectively (Fig. 2.4 B-C') (Hamaratoglu et al., 2006; Zhang et al., 2008). Consistent with this, in *ed* mutant clones DIAP1 protein expression levels is upregulated (Fig2.4 F-F"). In addition, overexpression of a *UAS-Ed* transgene suppressed elevated *ex-lacZ* expression caused by *hh>Ed-RNAi* (Fig. 2.4 C''). Wg expression in the periphery of wing pouches is under the control of the Hpo signaling activity (Cho et al., 2006). I found that wing discs expressing *hh>Ed-RNAi* exhibited elevated Wg expression in these regions of the posterior compartment (indicated by the arrows in Fig. 2.4 D'), consistent with the notion that loss of Ed deregulates the Hpo signaling activity. In contrast, Wg expression along the dorsoventral compartment boundary, which is under the control of Notch (N) signaling activity, was not affected by loss of Ed (indicated by the

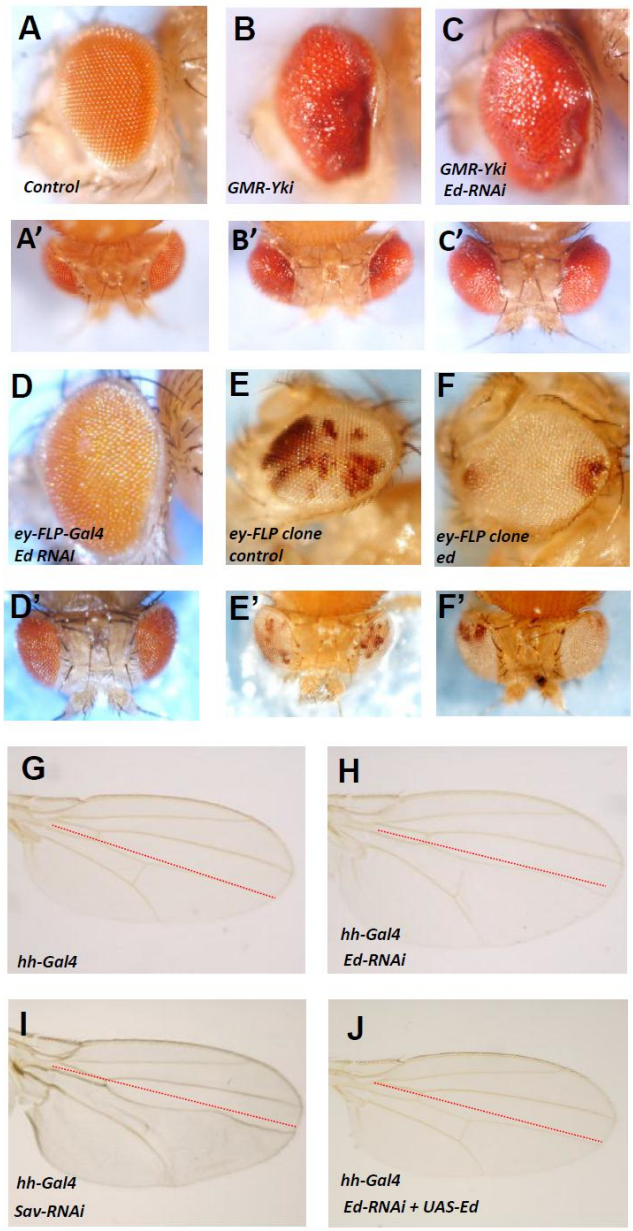

Figure 2.2 Inaction of Ed induces tissue overgrowth and enhances Yki gain-of-function phenotype.

(A-D') Side (A-D) and dorsal (A'-D') views of a control adult fly eye (A-A') or eyes expressing *UAS-Yki* (B-B') or *UAS-Yki* + *UAS-Ed-RNAi*^{3087} (C-C') with *GMR-Gal4*, or expressing *UAS-Ed-RNAi*^{3087} with *ey-Gal4* (D-D').

(E-F') Side (E-F) and dorsal (E'-F') views mosaic eyes that contain control clones (E-E') or *ed* clones (F-F') induced by *ey-FLP*.

(G-J) A control adult fly wing (G) and wings expressing *UAS-Ed-RNAi*^{937} (H), *UAS-Sav-RNAi* (I), or *UAS-Ed-RNAi*^{937} + *UAS-Ed* (J) with *hh-Gal4*. Red dashed lines indicate the A/P compartment boundary.

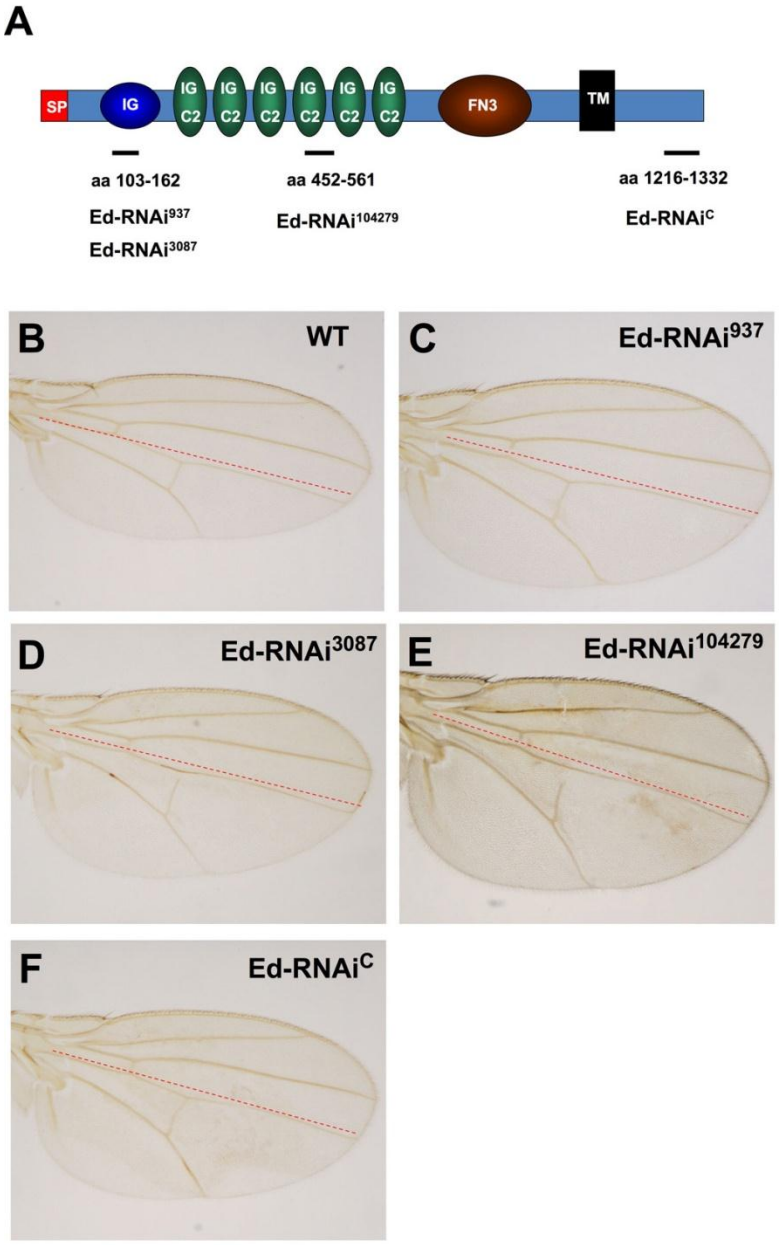

Fig. 2.3 Wing overgrowth induced by expressing different Ed-RNAi lines.
(A) A schematic drawing of Ed showing different domains. Black lines underneath the drawing indicate regions targeted by individual *UAS-Ed-RNAi* lines. SP: signal peptide;
IG: immunoglobulin domain; IG C2: immunoglobulin C2-set domain; FN3: fibronectin domain 3; TM: transmembrane domain.
(B-F) A wild type adult wing (B) or wings expressing the indicated *UAS-Ed-RNAi* lines (C-F) with *hh-Gal4*. Red dashed lines demarcate the A/P compartment boundary. Loss of Ed in posterior compartment wing imaginal disc cells resulted in enlarged posterior compartments of adult wings. Of note, Ed-RNAi^{3087} and Ed-RNAi^{104279} resulted in higher degree of lethality than Ed-RNAi^{937}, suggesting that they are stronger lines.

asterisk in Fig. 2.4. D'). Similarly, ed mutant clones exhibited elevated Wg expression at the peripheral of wing pouches (arrows in Fig. 2.4. G-G'). In contrast, Wg expression along the D/V compartment boundary remained unchanged in ed mutant clones (asterisks in Fig. 2.4. G-G').

I also examined the expression of a *bantam* sensor, *bantam-GFP*, which inversely reports the expression level of the *bantam* microRNA (Thompson and Cohen, 2006). As shown in Fig. 2.4.E', wing discs expressing *hh>Ed-RNAi* exhibited reduced levels of *bantam-GFP* expression in posterior compartment cells**,** suggesting that loss of Ed upregulates *bantam*. Taken together, these observations demonstrate that loss of Ed increases the expression of multiple Hpo pathway target genes and further suggest that tissue overgrowth caused by Ed inactivation is due to deregulation of Hpo signaling activity.

Ed regulates Yki phosphorylation and nuclear localization

The expression of Hpo pathway target genes is controlled by the transcriptional coactivator Yki, whose activity is primarily regulated by phosphorylation-mediated nuclear exclusion. Phosphorylation of Yki at S168 recruits 14-3-3 that restricts Yki nuclear localization, thereby limiting Yki activity in the nucleus (Dong et al., 2007; Oh and Irvine, 2008; Ren et al., 2010; Zhang et al., 2008). The increased expression of multiple Hpo pathway genes in *hh>Ed-RNAi* wing discs suggests that Yki activity is upregulated by loss of Ed. I therefore examined the Yki phosphorylation status using a phospho-specific antibody that recognizes phosphorylated S168 (pS168) (Dong et al., 2007). I found that expression of *UAS-Ed-RNAi* using the wing specific

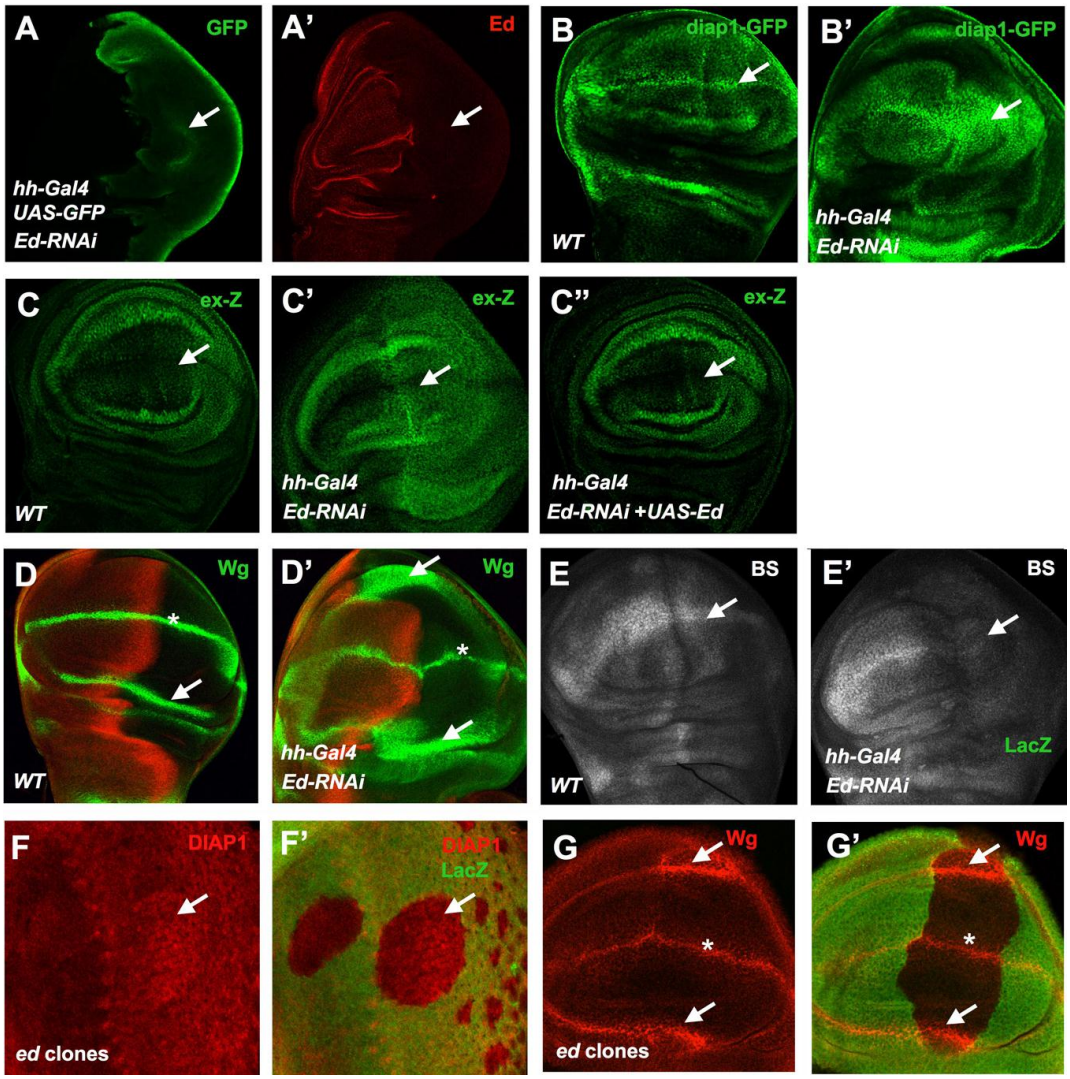

Figure 2.4 Inaction of Ed increases the expression of multiple Hpo pathway target genes.

(A-A') A wing disc expressing *UAS-GFP* + *UAS-Ed-RNAi*^{104279} with *hh-Gal4* and immunostained with anti-GFP and anti-Ed antibodies. GFP marked the posterior compartment (arrows) where *hh-Gal4* is expressed.

(B-E') Wild type wing discs (B, C, D, and E) or wing discs expressing *UAS-Ed-RNAi*^{104279} (B', C', D', and E') or *UAS-Ed-RNAi*^{104279} + *UAS-Ed* (C'') with *hh-Gal4* and immunostained to show the expression of *diap1-GFP* (B-B'), *ex-lacZ* (C-C''), Wg (D-D'), and *bantam* sensor (BS; E-E'). Arrows in B-C'' and E-E' indicate posterior compartments. Arrows and asterisks in D-D' indicate Wg expression at the periphery of the wing pouch region and along the D/V compartment boundary, respectively.

(F-F') A large magnification view of an eye disc containing *ed* mutant clones and immunostained to show the expression of LacZ (green) and DIAP1 (red). *ed* mutant clones are marked by the lack of LacZ expression and exhibit elevated DIAP1 expression (arrows).

(G-G') A wing disc carrying *ed* mutant clones was immunostained to show the expression of LacZ (green) and Wg (red). *ed* mutant cells are marked by the lack of LacZ expression. *ed* mutant cells exhibit elevated Wg expression at the periphery of the wing pouch region (arrows) but normal Wg expression along the D/V boundary (asterisks).

Gal4 driver *MS1096* led to a marked reduction in the level of S168 phosphorylation (Fig. 2.5. A). Consistent with diminished S168 phosphorylation, loss of Ed led to increased nuclear localization of Yki in *hh>Ed-RNAi* wing discs (Fig. 2.5. B-B”). These results suggest that Ed inactivation may compromise the Hpo/Wts kinase activity, leading to reduced Yki phosphorylation and increased Yki nuclear localization and activity.

If upregulation of Yki activity is responsible for the phenotypes induced by Ed inactivation, one would expect that reduction of Yki activity should attenuate the phenotypes caused by loss of Ed. Indeed, Yki RNAi suppressed the overgrowth phenotype induced by loss of Ed (Fig. 2.5. D).

Ed acts upstream of Sav and in parallel with Ds/Ft

I next investigated how loss of Ed compromised the activity of the Hpo/Wts kinase cassette. Ed is a multi-functional protein that has been implicated in restricting the activity of EGFR (Bai et al., 2001; Spencer and Cagan, 2003). However, EGFR RNAi did not block the upregulation of ex-lacZ induced by Ed RNAi, and overexpression of a constitutively active form of MAP kinase (MAPKCA) by hh-Gal4 failed to activate ex-lacZ expression in wing discs (Fig. 2.6. B-C’). Thus, Ed does not act through the EGFR pathway to regulate Hpo pathway responsive genes.

Ft regulates the Hpo/Wts kinase cassette through the non-conventional Myosin Dachs (Cho et al., 2006). Inactivation of Dachs by RNAi effectively blocked tissue overgrowth and elevated expression of ex-lacZ and Wg induced by Ft RNAi (Fig.2.6.

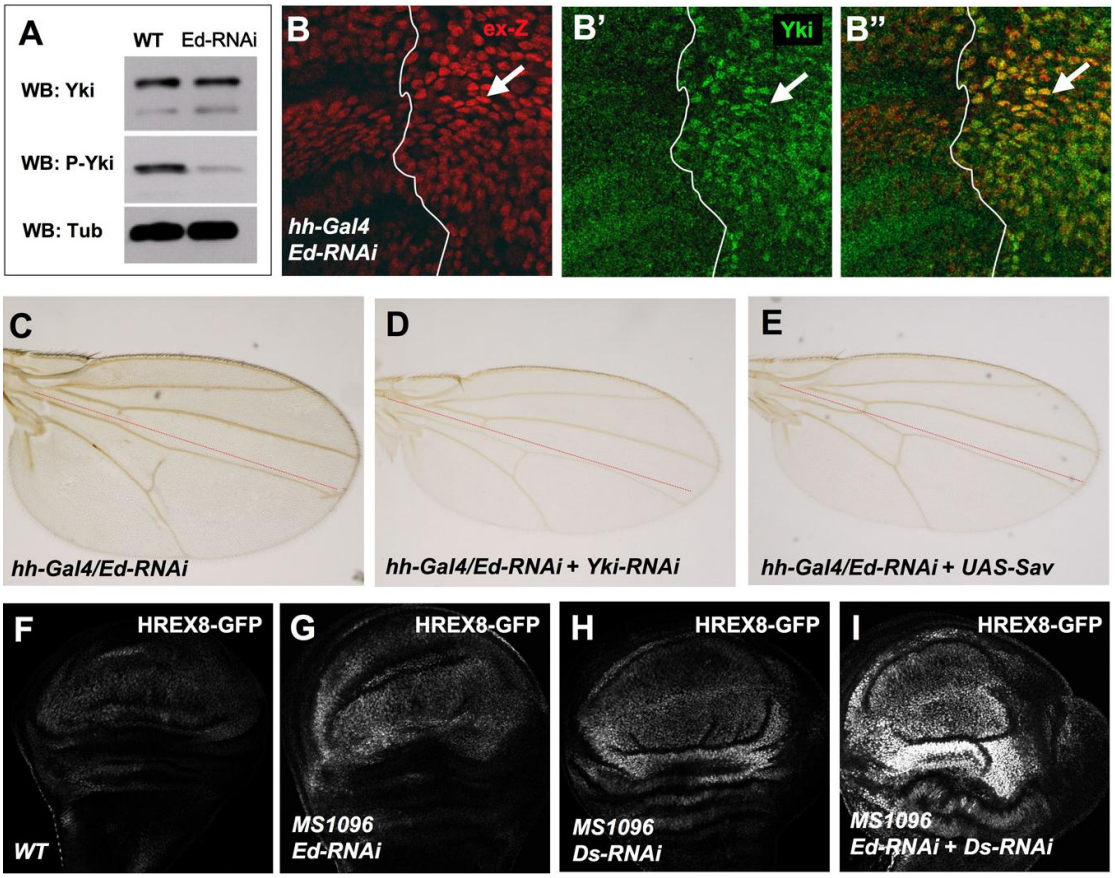

Figure 2.5 Ed acts upstream of Sav to regulate Yki phosphorylation and nuclear localization.

(A) Western blot analysis of Yki and phospho-Yki for wild type wing discs or wing discs expressing *UAS-Ed-RNAi*^{104279} with the *MS1096* Gal4 driver. The bottom panel is western blot for α-tubulin as a loading control.

(B-B") A wing disc expressing *ex-lacZ* and *UAS-Ed-RNAi*^{104279} with *hh-Gal4* was immunostained with Yki (green) and LacZ (red) antibodies. The A/P boundary is demarcated by a white line. Of note, Yki colocalized with LacZ in the nucleus of posterior compartment cells (arrows).

(C-E) Adult fly wings expressing *UAS-Ed-RNAi*^{973} (C), or *UAS-Ed-RNAi*^{973} + *UAS-Yki-RNAi* (D), or *UAS-Ed-RNAi*^{973} + *UAS-Sav* (E) with *hh-Gal4*. Red dash lines demarcate the A/P compartment boundary.

(F-I) A wild type wing disc (F) or wing discs expressing *UAS-Ed-RNAi*^{104279} (G), *UAS-Ds-RNAi* (H), *UAS-Ed-RNAi*^{104279} + *UAS-Ds-RNAi* (I) with *MS1096.* The discs also expressed *UAS- dicer2* to enhance the RNAi efficiency.

E-E'). In contrast, Dachs RNAi did not suppress the elevated expression of ex-lacZ and Wg induced by Ed RNAi (Fig. 2.6. F-F'), suggesting that Ed does not act through the Ft/Dachs branch of the Hpo pathway. Consistent with this notion, I found that inactivation of Ds synergized with loss of Ed to activate an Hpo pathway reporter, HREx8-GFP, which contains 8 copies of a 32 bp Hpo responsive element (Fig. 2.5. F-I)(Wu et al., 2008). These observations suggest that Ed may act in parallel with Ds/Ft to regulate Hpo signaling.

I next examined the epistatic relationship between Ed and the Hpo kinase cassette. I coexpressed UAS-Sav in hh>Ed-RNAi wing discs and found that Sav overexpression suppressed the tissue overgrowth induced by loss of Ed (Fig. 2.5. E). These observations suggest that Ed acts upstream of Sav to regulate Hpo signaling.

Ed physically interacts with Sav/Hpo, Ex/Mer/Kibra and Yki through its intracellular domain

To understand the molecular mechanism by which Ed regulates Hpo signaling, I examined physical interactions between Ed and membrane proximal Hpo pathway components by coimmunoprecipitation experiments. S2 cells were transfected with a C-terminally Myc-tagged full-length Ed (Ed^{FL}-Myc) or truncated Ed that lacks the intracellular domain ($Ed^{\Delta C}$-Myc) (Fig. 2.7 A) and HA-tagged Ex, Mer, or Sav, or with HA-tagged Ed^{FL} or $Ed^{\Delta C}$ (Ed^{FL}-HA or $Ed^{\Delta C}$-HA) and Myc-tagged Kibra. Cell extracts were immunoprecipitated with the corresponding antibodies, followed by western blot analysis. As shown in Fig. 2.7., Ed^{FL}-Myc but not $Ed^{\Delta C}$-Myc was

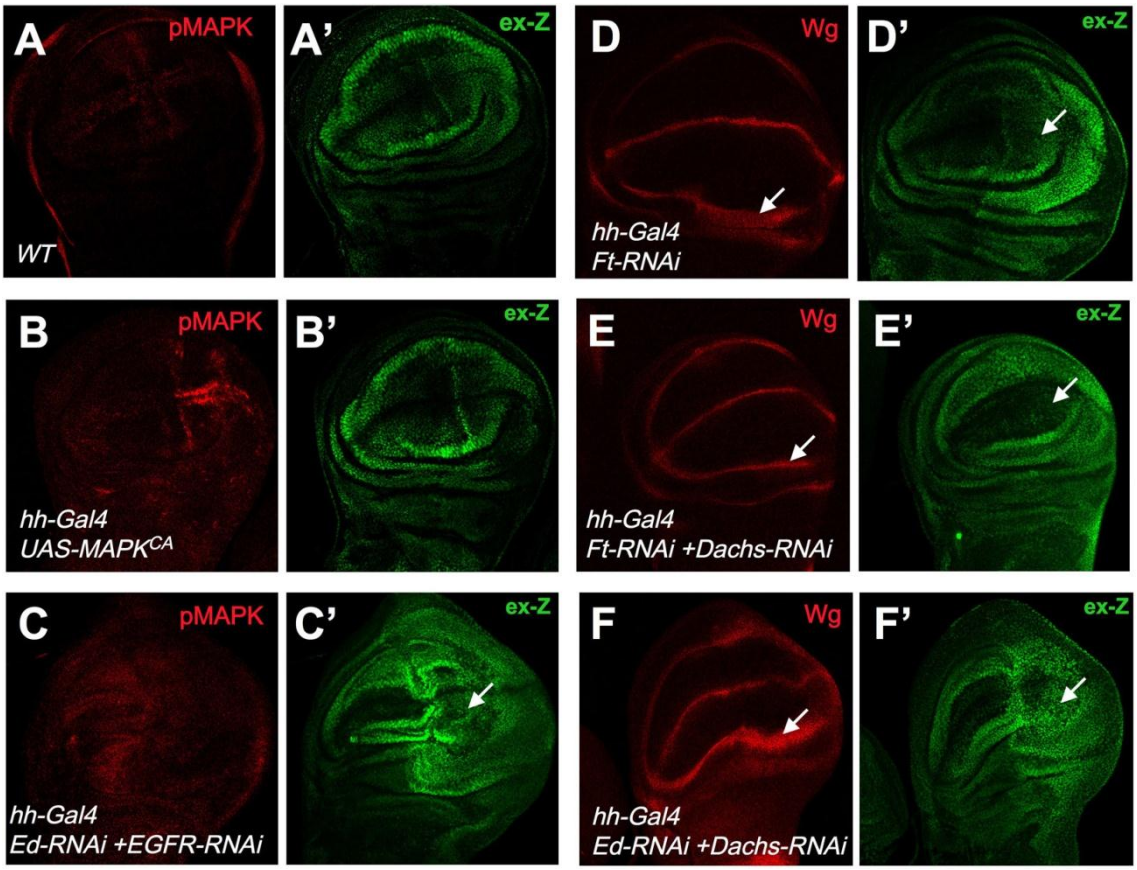

Figure 2.6 EGFR and Dachs are not required for the upregulation of Hpo pathway target genes induced by Ed RNAi.

(A-C') A wild type wing disc (A and A') or wing discs expressing *UAS-MAPK*^{CA} (B and B') or *UAS-Ed-RNAi*^{104279} + *UAS-EGFR-RNAi* (C and C') with *hh-Gal4* were immunostained to show the expression of phosphorylated MARK (pMARK) (red) and *ex-LacZ* (green). The arrow in C' indicates the region with elevated *ex-lacZ* expression. Overexpression of the active form of MAPK (MAPK^{CA}) did not upregulate the expression of *ex-lacZ* (B-B'). EFGR RNAi did not block the elevated *ex-lacZ* expression induced by Ed-RNAi (C-C').

(D-F') Wing discs expressing *UAS-Ft-RNAi* (D-D'), *UAS-Ft-RNAi* + *UAS-Dachs-RNAi* (E-E'), or *UAS-Ed-RNAi*^{104279} + *UAS-Dachs-RNAi* (F-F') with *hh-Gal4* were immunostained to show the expression of Wg (red) and *ex-LacZ* (green). Arrows indicate the regions with elevated expression of Wg or *ex-LacZ*. Inactivation of Dachs suppressed the elevated expression of Wg and *ex-LacZ* induced by loss of Ft (E-E') but not by loss of Ed (F-F').

coimmunoprecipitated with HA-Sav and HA-Ex (Fig. 2.7. B, D), and to a lesser extent with HA-Mer (Fig. 2.7. E). Myc-Kibra was coimmunoprecipitated with Ed^{FL}-HA but not $Ed^{\Delta C}$-HA (Fig. 2.7. F). These observations suggest that Ed interacts with Sav, Ex, Mer and Kibra through its intracellular domain. In addition, I found that Ed^{FL}-Myc was barely associated with Flag-Hpo and this interaction was markedly enhanced when HA-Sav was expressed (Fig. 2.7. C), suggesting that Sav may bring Hpo to Ed. Interestingly, I also found that Ed interacted with Yki through its intracellular domain but not associated with Sd (Fig. 2.7. G).

When expressed in S2 cells alone, HA-Sav and HA-Ex exhibited diffused patterns of cytoplasmic staining (Fig. 2.8. A, C). Coexpression of Ed^{FL}-Myc resulted in the recruitment of HA-Sav and HA-Ex to the plasma membrane where they colocalized with Ed^{FL}-Myc (Fig. 2.8. B, D). These observations further support the notion that Ed physically interacts with Sav and Ex and suggest that Ed can bring Sav and Ex to the cell membrane. I also confirmed that endogenous Ed and Sav form a complex in wing disc derived cl8 cells (Fig. 2.8. E).

I next generated an Ed variant lacking the N-terminal extracellular domain, which I named Ed^{TMC} (TMC stands for transmembrane plus cytoplasmic domain; Fig. 2.7. A). I found that C-terminally Myc-tagged Ed^{TMC} (Ed^{TMC}-Myc) interacted with HA-tagged Sav, Ex, Mer and Yki in coimmunoprecipitation assays (Fig. 2.7. H, Fig. 2.8. 4F-H). Deleting aa1217-1332 from Ed^{TMC} ($Ed^{TMC\Delta 1}$-Myc; Fig. 2.7.A) greatly reduced the binding to Sav, Ex and Mer whereas deleting aa1115-1332 ($Ed^{TMC\Delta 2}$-Myc; Fig. 2.7. A) abolished the binding to these proteins (Fig. 2.7.H, Fig. 2.8.4F-G),

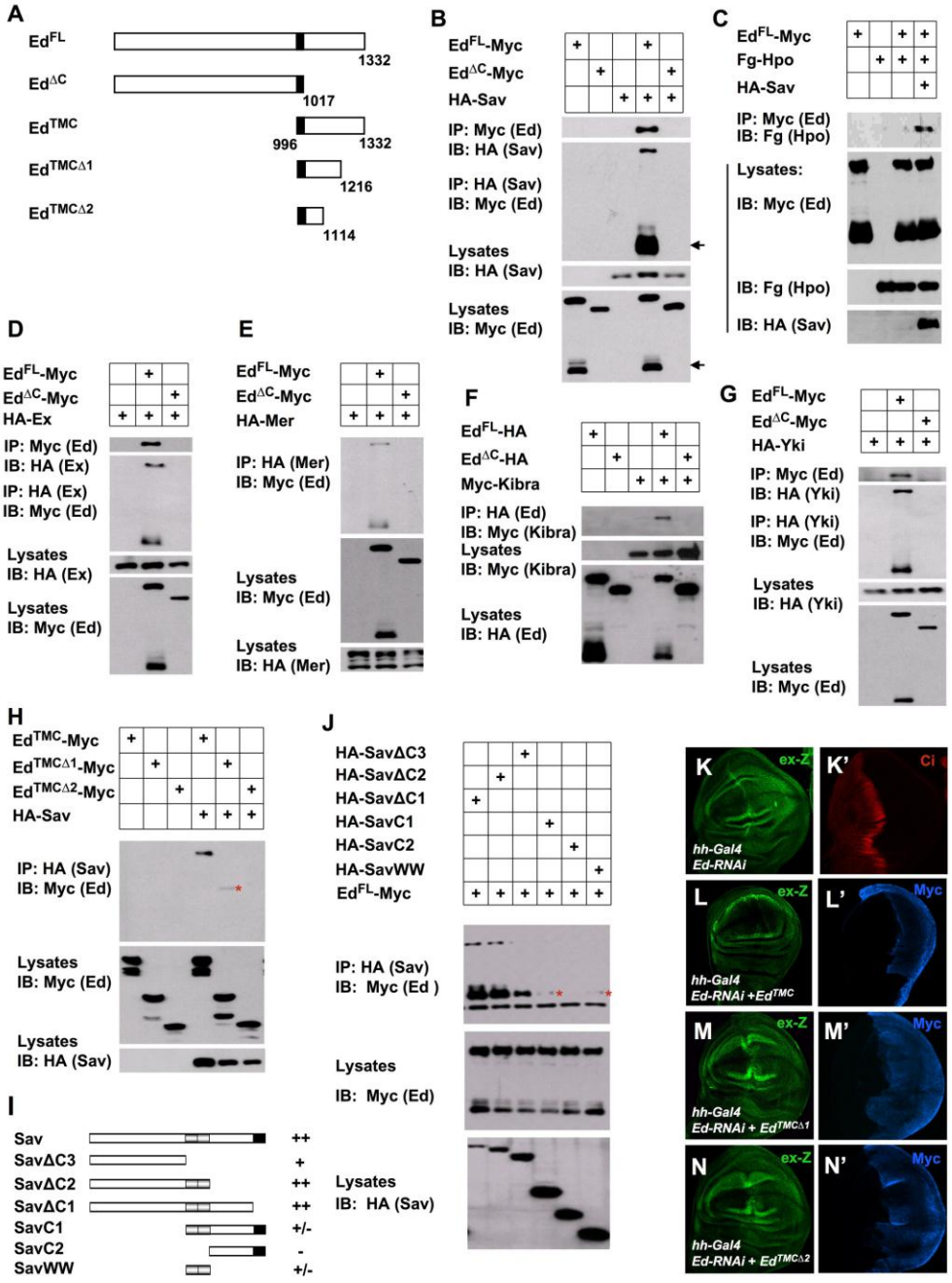

Figure 2.7 Ed interacts with multiple Hpo pathway components.

(A) A schematic drawing of full-length Ed (Ed^{FL}) and its deletion constructs. Black boxes denote the transmembrane domain of Ed.

(B-H) S2 cells were transfected with DNA constructs expressing epitope-tagged full-length or indicated truncated Ed and tagged Hpo pathway components including Sav (B-C, H), Hpo (C), Ex (D), Mer (E), Kibra (F), or Yki (G). Cell lysates were immunoprecipitated, followed by western blot analysis with the indicated antibodies (top panels), or directly subjected to western blot analysis with the indicated

antibodies (bottom panels). Asterisks in C and H indicate weak bands. Arrows in B indicate the cleaved Ed.

(I) A schematic drawing of full-length Sav and its deletion mutants. The WW and coiled-coil domains are indicated by shaded and black boxes, respectively. The ability of individual Sav constructs to interact with Ed is indicated by "+" or "-".

(J) S2 cells were transfected with DNA constructs expressing Myc-tagged Ed^{FL} and indicated HA-tagged Sav deletion constructs. Cell lysates were immunoprecipitated with anti-HA antibody, followed by western blot analysis with anti-Myc antibody (top panel), or directly subjected to western blot analysis with anti-Myc or anti-HA antibody (middle and bottom panels). Asterisks indicate weak bands.

(K-N') Wing discs expressing *UAS-Ed-RNAi*^{3087} (K and K'), or *UAS-Ed-RNAi*^{3087} + *UAS-Ed*^{TMC}*-Myc* (L and L'), or *UAS-Ed-RNAi*^{3087} + *UAS -Ed*$^{TMC\Delta 1}$*-Myc* (M and M'), or *UAS-Ed-RNAi*^{3087} + *UAS-Ed*$^{TMC\Delta 2}$*-Myc* (N and N') with *hh-Gal4* and immunostained to show the expression of Ci (red), *ex-lacZ* (green) and Myc (blue). Ed transgenes with comparable Myc expression levels were used for the rescue experiments.

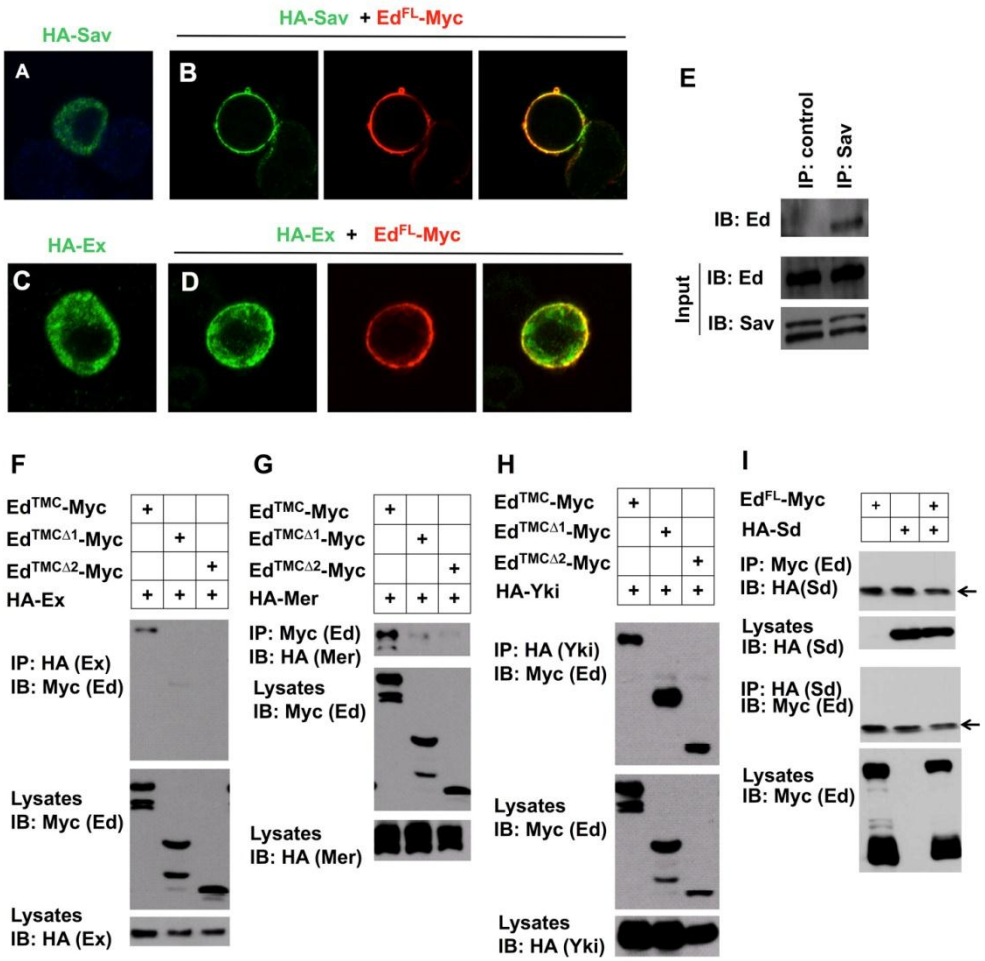

Figure 2.8 Characterization of physical interactions between Ed and Hpo pathway components

(A-D) Confocal images of S2 cells transfected with HA-Sav (A) or HA-Ex (C) alone or together with Ed^{FL}-Myc (B and D) and immunostained with antibodies against HA (green) and Myc (red). When expressed alone, both HA-Sav and HA-Ex exhibited diffused patterns of cytoplasmic staining (A and C); however, when Ed^{FL}-Myc was coexpressed, a large fraction of HA-Sav and HA-Ex were recruited to the plasma membrane and colocalized with Ed^{FL}-Myc (B and D). Of note, the tagged proteins were expressed using the *Gal4/UAS* system that drives gene expression at levels much higher than the tubulin promoter used in Fig. 7E-G".

(E). Detection of endogenous Ed/Sav complex in cl8 cells by immunoprecipitation using an anti-Sav antibody generated in this study.

(F-I) Immunoprecipitation and western blots analysis of cell extracts from S2 cells transfected with the indicated constructs. $Ed^{TMC\Delta1}$-Myc and $Ed^{TMC\Delta2}$-Myc exhibited diminished binding to HA-Ex and HA-Mer (F and G) but still formed a complex with HA-Yki (H). Ed^{FL}-Myc did not form a complex with HA-Sd (I). Arrows in (I) indicate IgG.

suggesting that the C-terminal 218 amino acids of Ed mediate its interaction with Sav and Ex/Mer. In contrast, both $Ed^{TMC\Delta1}$ and $Ed^{TMC\Delta2}$ interacted with Yki similarly to Ed^{TMC} (Fig. 2.7. 4H), suggesting that the membrane proximal region of Ed C-tail mediates Yki binding.

Deleting aa1217-1332 from Ed^{TMC} ($Ed^{TMC\Delta1}$-Myc; Fig. 2.7.A) greatly reduced the binding to Sav, Ex and Mer whereas deleting aa1115-1332 ($Ed^{TMC\Delta2}$-Myc; Fig. 2.7. A) abolished the binding to these proteins (Fig. 2.7.H, Fig. 2.8.4F-G), suggesting that the C-terminal 218 amino acids of Ed mediate its interaction with Sav and Ex/Mer. In contrast, both $Ed^{TMC\Delta1}$ and $Ed^{TMC\Delta2}$ interacted with Yki similarly to Ed^{TMC} (Fig. 2.7. 4H), suggesting that the membrane proximal region of Ed C-tail mediates Yki binding.

To map the domains in Sav that mediate interaction with Ed, I coexpressed full-length Ed with a number of truncated forms of Sav (Fig. 2.7.I). I found that the N- terminal region and WW domain of Sav are both required for its optimal binding to Ed, as deleting either domain from Sav (SavC1 or SavΔC3) greatly reduced its binding to Ed and combined deletion (SavC2) completely abolished the interaction (Fig. 2.7. J). In contrast, deleting the region C-terminal to the WW domain (SavΔC1 and SavΔC2) did not affect Sav/Ed interaction (Fig. 2.7. J).

Ed regulates Hpo signaling through its intracellular domain

Deleting the intracellular domain of Ed ($Ed^{\Delta C}$) blocked its ability to rescue the loss-of-Ed phenotypes both in wing discs and posterior follicle cells (Fig. S7C-C').

On the other hand, overexpression of Ed^{TMC} suppressed the loss-of-Ed phenotypes (Fig. 2.7. L-L'). These observations suggest that Ed regulates Hpo signaling through its intracellular domain, likely by interacting with multiple intracellular Hpo pathway components. Consistent with this notion, deleting the domain involved in binding to Sav/Hpo and Ex/Mer from Ed^{TMC} ($Ed^{TMC\Delta1}$ and $Ed^{TMC\Delta2}$) abolished its ability to suppress the elevated expression of *ex-lacZ* induced by loss of Ed in wing discs (Fig. 2.7. M-N').

Ed regulates Sav stability and subcellular localization

In the course of characterizing Ed/Sav interaction, I found that coexpression of Ed^{FL}-Myc stabilized transfected HA-Sav (Fig. 2.9. A). Stabilization of Sav is mediated by the intracellular domain of Ed as $Ed^{\Delta C}$ failed to stabilize Sav whereas Ed^{TMC} stabilized Sav albeit less effectively than Ed^{FL} (Fig. 2.9. A). I also measured the half-life of HA-Sav in the absence or presence of Ed^{FL}-Myc. HA-Sav was transfected into S2 cells with or without Ed^{FL}-Myc. The levels of HA-Sav were monitored at different time points after treatment with the protein synthesis inhibitor, cycloheximide (CHX). As shown in Fig. 2.9., the half-life of HA-Sav was less than two hours in the absence of Ed^{FL}-Myc; however, coexpression of Ed^{FL}-Myc increased the half-life of HA-Sav to 8 hours (Fig. 2.9. B-C). I also found that treating transfected cells with the proteasome inhibitor MG132 increased the steady state levels of HA-Sav (Fig. 2.9. D), suggesting that Sav was degraded by the ubiquitin/proteasome pathway.

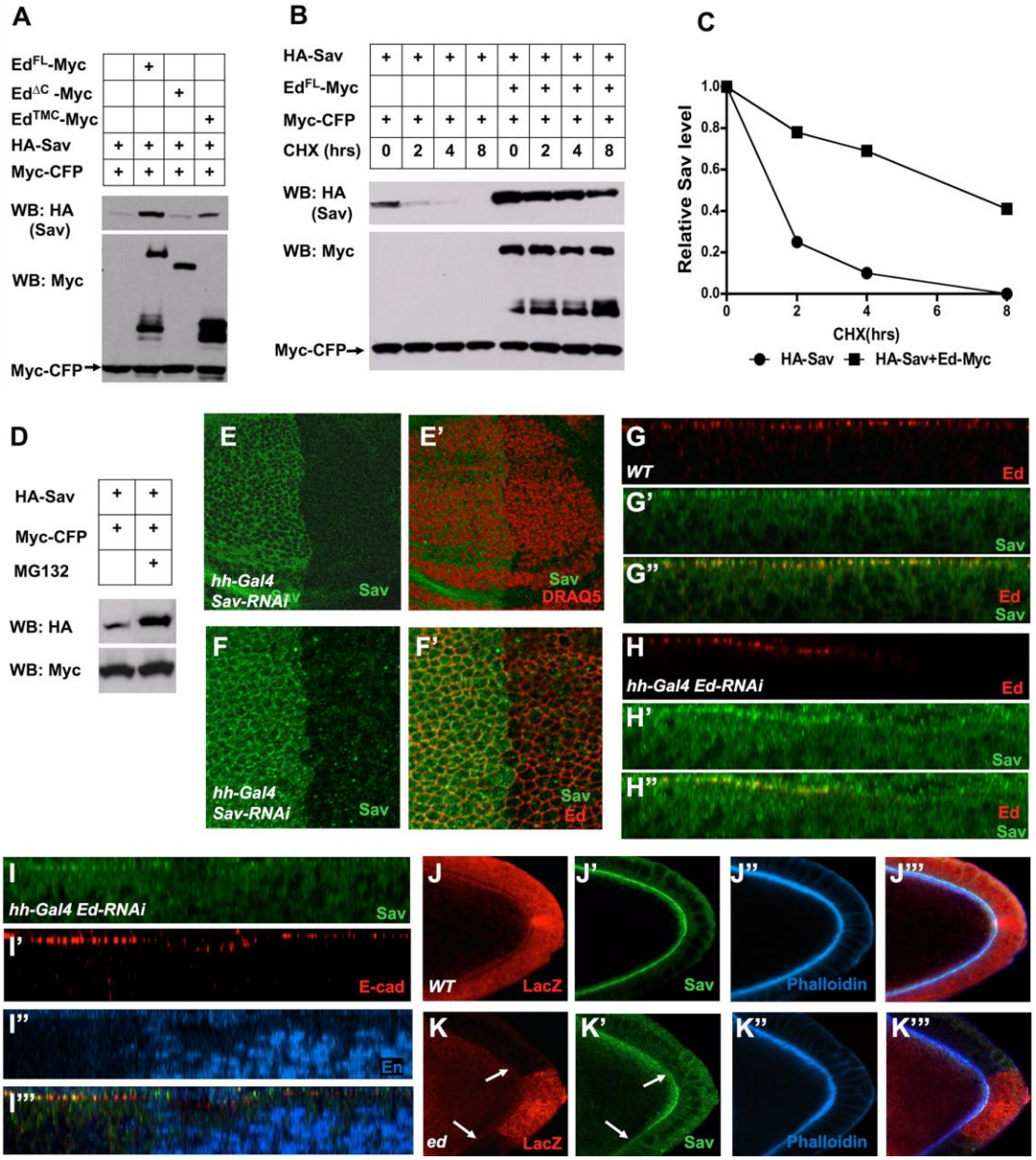

Figure 2.9 Ed regulates the stability and apical localization of Sav

(A) Western blot analysis of cell lysates from S2 cells transfected with the indicated constructs. Myc-CFP (arrow) was cotransfected as an internal control.

(B) S2 cells were transfected with HA-Sav with and without Ed^{FL}-Myc and treated with cycloheximide for the indicated time. Cell lysates were subjected to western blot analysis using anti-Myc antibody. Myc-CFP (arrow) was cotransfected as an internal control.

(C) Quantification of HA-Sav in the absence or presence of Ed^{FL}-Myc by western blot analysis performed in B.

(D) Western blot analysis of cell lysates from S2 cells transfected with HA-Sav and Myc-CFP and treated with MG132 for 4 hours before harvesting. Myc-CFP (lower panel) served as a transfection and loading control.

(E-F') Wing discs expressing *UAS-Sav-RNAi* with *hh-Gal4* were immunostained with an anti-Sav antibody (green) and the nuclear dye, DRAQ5 (red in E'), or an anti-Ed

antibody (red in F'). Sav exhibited cytoplasmic staining, which was abolished by Sav-RNAi.

(G-G") Transverse section view of wild type wing discs immunostained with Ed (red) and Sav antibodies (green). Sav was enriched at the apical plasma membrane where it colocalized with Ed at adherens junctions.

(H-I") Transverse section view of a wing disc expressing *UAS-Ed-RNAi*^{104279} with *hh-Gal4* and immunostained to show the expression of Sav (green), Ed (red in H, H") or E-cadherin (E-cad; red in I', I"") and En (blue in I", I"") that marks the P-compartment. Loss of Ed resulted in reduced Sav accumulation at the apical surface.

(J-K") Wild type (J-J") and mosaic egg chambers carrying *ed* mutant clones (K-K") were immunostained to show the expression of Sav (green), LacZ (red), and Phalloidin (blue). *ed* mutant follicle cells are marked by the lack of LacZ expression (arrows in K-K"). Posterior *ed* mutant follicle cells showed reduced Sav signals at the apical surface marked by Phalloidin (arrows in K-K").

To determine whether Ed regulates Sav stability and subcellular localization *in vivo*, I generated an anti-Sav antibody (see Experimental Procedures). The specificity of this antibody was confirmed by Sav RNAi (Fig. 2.9. E-F'). I found that Sav was distributed in cytoplasm with an enrichment at the apical surface where Sav colocalized with Ed at adherens junctions (Fig. 2.9. E-G''). Ed RNAi reduced the levels of Sav immunostaining at the apical region (Fig. 2.9. H-H''). Consistent with Ed regulating Sav at the posttranscriptional level, I found that Ed RNAi also reduced the levels of HA-Sav driven by a *UAS* transgene at adherens junctions (Fig. 2.11 A-B''). I also found that Sav was enriched at the apical surface of ovarian follicle cells and that the levels of apically localized Sav were reduced in posterior *ed* mutant follicle cells (Fig.2.9.J-J'').

Because I observed that Sav physically interacts with Ex/Mer, I also examined whether loss of Ed affects the level or subcellular localization of Ex/Mer. I found that levels of Ex and Mer were upregulated by Ed RNAi (Fig. 2.11 C-D''), likely due to the transcriptional upregulation of *ex* and *mer* expression in response to loss of Ed. However, loss of Ed did not affect the apical localization of Ex/Mer (Fig. 2.11. C-D'').

Regulation of Ed/Sav interaction by Ed dimerization and membrane localization

Although Ed^{TMC} interacts with Sav, the soluble Ed intracellular domain (Ed^{C}; Fig. 2.10.A) that lacks the transmembrane domain failed to bind Sav in our coimmunoprecipitation experiments (Fig. 2.10.B). One possibility is that Ed/Sav interaction may require association of Ed to the membrane. To test this possibility, a myristoylation signal was added to Ed^{C}

(Myr-Ed^{C}; Fig. 2.10.A). I found that Myr-Ed^{C}-Myc only weakly interacted HA-Sav, suggesting that tethering Ed C-tail to the membrane is not sufficient to confer efficient binding to Sav.

A previous study reported that Ed forms a dimer or oligomer (Spencer and Cagan, 2003). Interestingly, I found that Ed^{TMC} but not Ed^{C} can self-associate when expressed in S2 cells (Fig. 2.10. C), suggesting that the TM domain of Ed can mediate dimerization/oligomerization. This observation further suggests that Ed/Sav association may require dimerization/oligomerization of Ed C-tail. To test this possibility, I added a coiled-coil dimerization motif (CC; see Experimental Procedures) to generate Myr-CC-Ed^{C} (Fig. 2.10. A) and found that Myr-CC-Ed^{C} could self-associate and interact effectively with Sav (Fig. 2.10. B-C). Adding the coiled-coil dimerization motif to soluble Ed^{C} (CC-Ed^{C}) did not result in strong association between CC-Ed^{C} and Sav (data not shown), suggesting that effective interaction between Ed and Sav requires both membrane localization and dimerization of the Ed intracellular domain. Consistent with this notion, I failed to detect an interaction between the Ed intracellular domain and Sav in a yeast two-hybrid assay (data not shown). Membrane association of EdTMC, Myr-EdC, and Myr-CC-EdC was confirmed by immunostaining of S2 cells transfected with these Ed variants (Fig. 2.12 G-K").

To determine the effect of C-tail dimerization on Ed activity *in vivo*, I generated transgenic flies expressing *UAS-Ed^{C}*, *UAS-Myr-Ed^{C}* and *UAS-Myr-CC-Ed^{C}*, and compared their activities by carrying out rescue experiments. I found that overexpression of Myr-CC-Ed^{C} but not Ed^{C} or Myr-Ed^{C} could suppress the elevated *ex-lacZ* expression induced by Ed RNAi (Fig. 2.12. A-F').

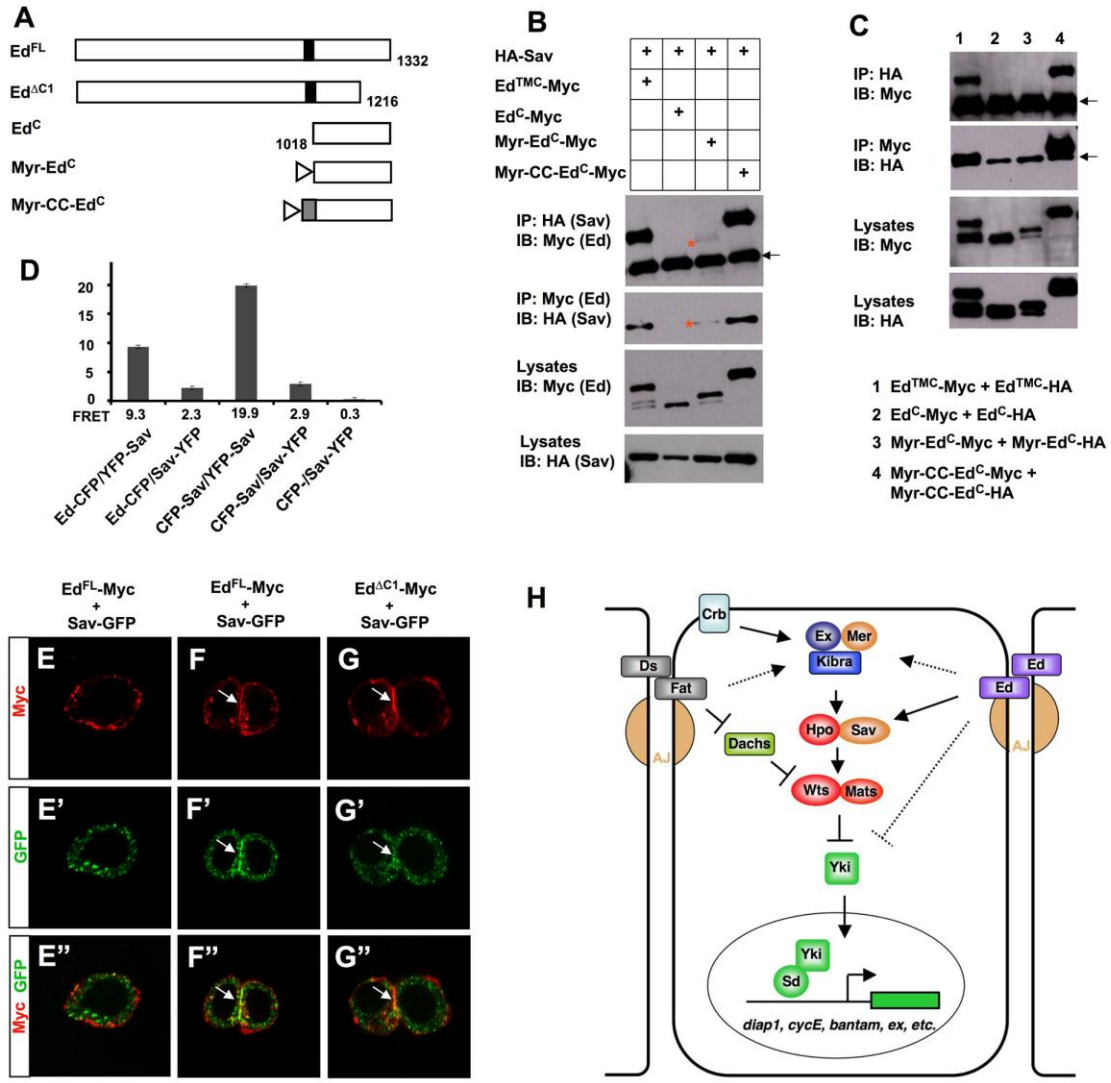

Figure 2.10 Regulation of Ed/Sav interaction by dimerization and cell-cell contact

(A) Schematic drawings of full-length Ed and Ed variants. Black bars indicate the Ed transmembrane domain. Triangle and grey box represent a myristoylation (Myr) signal and a coiled-coil (CC) dimerization domain, respectively.

(B-C) S2 cells were transfected with the indicated constructs. Cell lysates were immunoprecipitated with anti-HA or anti-Myc antibody, followed by western blot analysis with the indicated antibodies (top two panels) or directly subjected to western blot analysis with anti-Myc or anti-HA antibody (bottom two panels). Asterisks in B indicate weak bands. Arrows indicate IgG.

(D) FRET efficiency of indicated pairs of CFP/YFP constructs transfected into S2 cells (mean ± s.d., n≥10).

(E-G') S2 cells were transfected with tub-Sav-GFP and tub-Ed^{FL}-Myc or tub-Ed$^{TMC\Delta1}$-Myc. Immunostaining was carried out after cell aggregation was induced.

(H) A model for Ed in the Hpo signaling pathway. Dashed lines indicate less characterized interactions. See text for details.

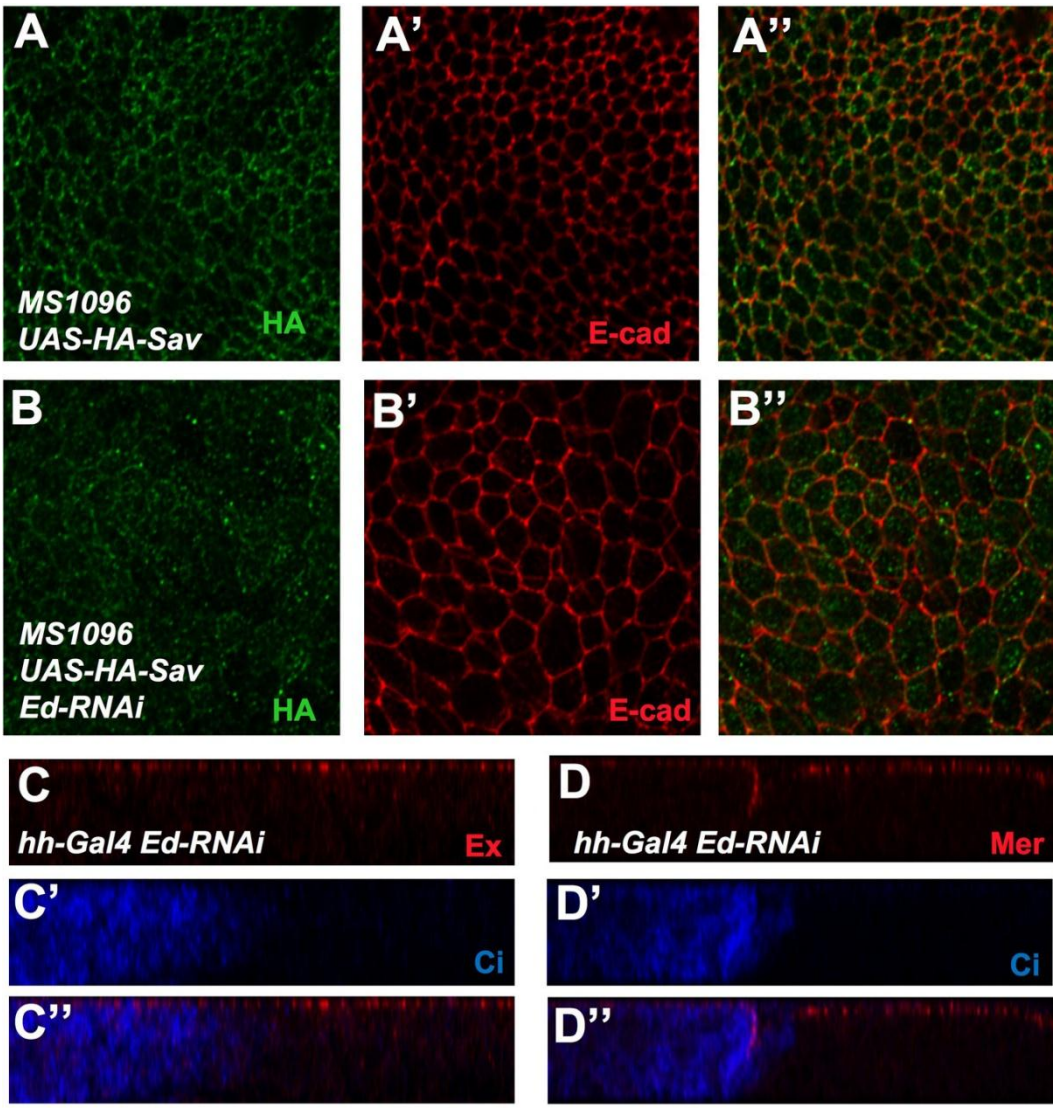

Figure 2.11 Loss of Ed reduced the levels of apical Sav but did not affect apical localization of Ex/Mer

(A-B'') Wing discs expressing *UAS-HA-Sav* (A-A'') or *UAS-HA-Sav* + *UAS-Ed-RNAi*^{104279} + *UAS-dicer2* (B-B'') with *MS1096* were immunostained to show the expression of HA-Sav (green) and E-cad (red). Loss of Ed caused diminished HA-Sav signal at adherens junctions marked by E-cad (B-B'').

(C-D'') Transverse section view of wing discs expressing *UAS-Ed-RNAi*^{104279} with *hh-Gal4* and immunostained with anti-Ex or anti-Mer and anti-Ci antibodies. Inactivation of Ed in posterior compartment cells (marked by the lack of Ci expression) did not affect the subcellular localization of either Ex or Mer.

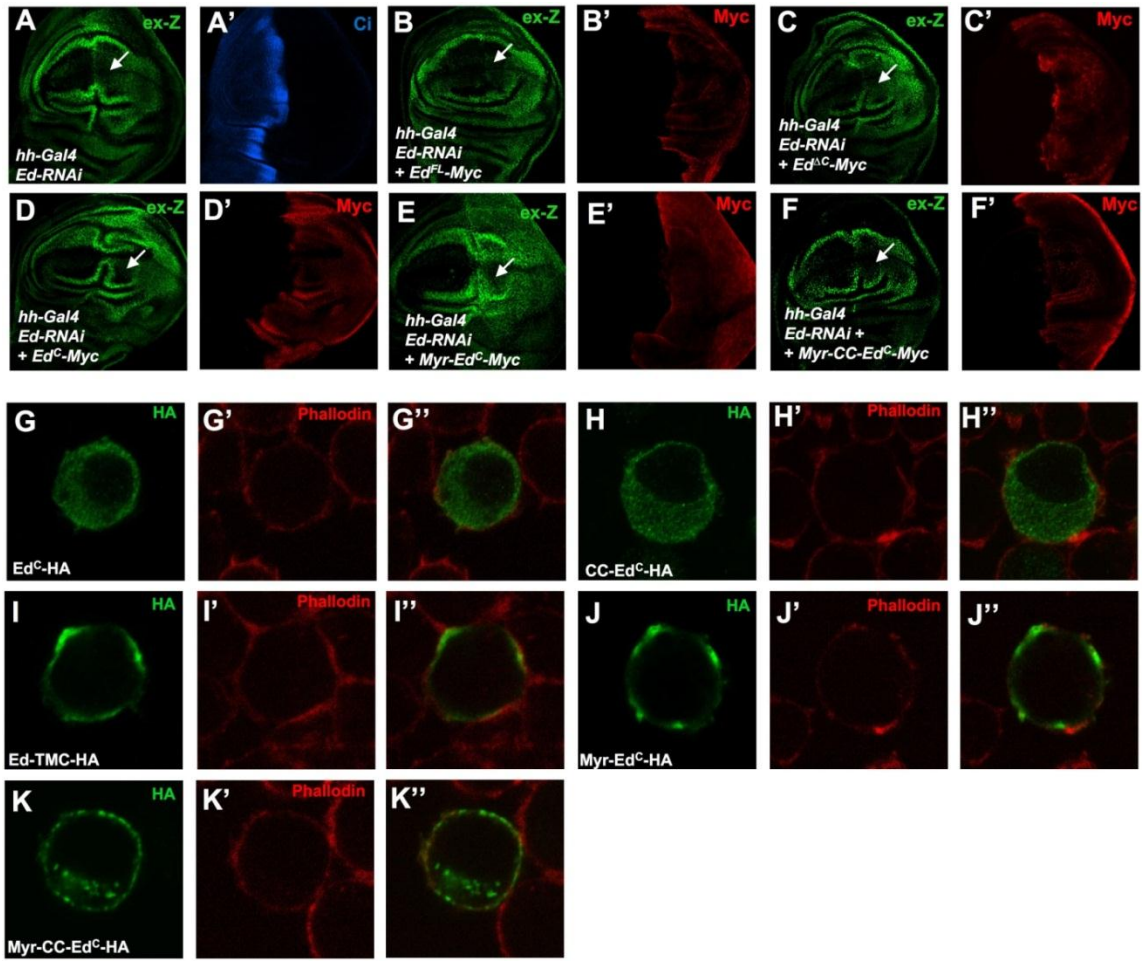

Figure 2.12 Regulation of Hpo signaling by Ed requires dimerization of its intracellular domain.

(A-F') A control *hh>Ed-RNAi*^{104279} disc (A-A') and *hh>Ed-RNAi*^{104279} discs expressing Ed^{FL}-Myc (B-B'), Ed$^{\Delta C}$-Myc (C-C'), Ed^{C}-Myc (D-D'), Myr-Ed^{C}-Myc (E-E'), or Myr-CC-Ed^{C}-Myc (F-F') were immunostained to show the expression of *ex-lacZ* (green), Myc (red), and Ci (blue). Ed^{FL}-Myc and Myr-CC-Ed^{C}-Myc but not Ed$^{\Delta C}$-Myc, Ed^{C}-Myc or Myr-Ed^{C}-Myc suppressed the elevated *ex-lacZ* expression (arrows). Of note, Myc staining indicates similar levels of transgenic expression for different Ed constructs. (G-K''') Subcellular localization of the indicated HA-tagged Ed constructs transfected into S2 cells. Phallodin marks the cell membrane.

Regulation of Ed/Sav interaction by cell-cell contact

A previous study showed that Ed transfected in S2 cells could mediate cell-cell adhesion though a homophilic interaction and that Ed was enriched at the cell contact site (Islam et al., 2003). I therefore determined whether cell-cell contact could modulate Ed/Sav interaction. To do this, I transiently expressed low levels of Ed^{FL}-Myc and Sav-GFP using the tubulin promoter (tub-Ed^{FL}-Myc and tub-Sav-GFP). In isolated cells, Ed^{FL}-Myc and Sav-GFP exhibited limited colocalization (Fig. 2.10.E-E''). Consistent with the previous finding (Bai et al., 2001), Ed^{FL}-Myc accumulated at the cell contact site upon cell-cell contact (Fig. 2.10. F). Strikingly, Sav-GFP also accumulated at the cell contact site where it colocalized with Ed^{FL}-Myc (Fig. 2.10. F'-F''). Deleting the C-terminal Sav-interacting domain in Ed ($Ed^{\Delta C1}$-Myc) prevented the recruitment of Sav-GFP to the cell contact region (Fig. 2.10. G-G''). Thus, cell-cell contact facilitates Ed/Sav association.

Discussion

The atypical cadherin Ft functions as a receptor for the Hpo pathway; however, Ft mainly acts through Dachs to control the stability of Wts (Cho et al., 2006). Our genetic study indicated that Ed does not act through Ft-Dachs to regulate Yki activity because inactivation of Dachs did not block Yki activation induced by loss of Ed. Furthermore, loss of Ed synergized with loss of Ds to induce the expression of Hpo responsive genes, supporting a model in which Ed acts in parallel with Ds/Ft in the

Hpo pathway (Fig. 2.10.H). Several lines of evidence suggest that Ed regulates Hpo signaling, at least in part, through Sav. 1) Using coimmunoprecipitation, colocalization and FRET assays, I demonstrate that Ed physically interacts with Sav. 2) Deleting the Sav-interacting domain in Ed compromises its *in vivo* activity. 3) Ed regulates the abundance and subcellular localization of Sav both *in vitro* and *in vivo*. 4) Overexpression of Sav suppresses tissue overgrowth induced by loss of Ed. Sav is a binding partner and activator of Hpo. Hence, Ed could influence the Hpo kinase activity by regulating the abundance and subcellular location of the Sav/Hpo complex. How Ed regulates Sav stability is currently unknown; however, I found that Sav is degraded in a proteasome-dependent manner. It is possible that binding of Ed to Sav leads to some modifications of Sav and prevents it from ubiquitin/proteasome-mediated degradation.

Sav binds Ed and Hpo through its N- and C-terminal regions, respectively (Fig. 5)(Jia et al., 2003; Udan et al., 2003b; Wu et al., 2003), and thus may function as a bridge to bring Hpo to Ed. Indeed, I observed enhanced Ed/Hpo association in the presence of cotransfected Sav. It has been suggested that apical membrane recruitment of Hpo promotes phosphorylation of Wts (Hergovich et al., 2005; Yu et al., 2010). Thus, it is conceivable that Ed may regulate the Hpo kinase by recruiting Sav/Hpo complex to the apical membrane. I found that Ed/Sav interaction requires membrane association and dimerization/oligomerization of Ed intracellular domain. As Sav also forms a dimer/oligomer (Fig. 7), dimerization/oligomerization of Ed intracellular domain may enhance binding to Sav through multimeric interactions. It is also

possible that membrane association and dimerization/oligomerization could lead to a modification of Ed intracellular domain, resulting in increased binding affinity toward Sav.

It has been shown that the Hpo pathway can mediate cell contact inhibition in mammalian cultured cells although the underlying mechanism has remained poorly defined. Interestingly, I found that cell-cell contact can facilitate the recruitment of Sav to Ed at the contact site. Cell-cell contact may facilitate homophilic interaction *in trans* and clustering of Ed intracellular domain, or induce post-translational modification of Ed C-tail at the contact site, leading to enhanced Sav association. I propose that regulation of Ed/Sav association may provide a mechanism for cell-cell contact to modulate Hpo signaling and tissue growth.

The mechanism by which Ed regulates Hpo signaling is likely to be more complex than simply regulating Sav/Hpo. For example, I also observed that Ed interacts with Ex/Mer/Kibra as well as Yki. It has been proposed that enrichment of Hpo pathway components to the apical membrane domain may facilitate the activation of the kinase cassette and increase the accessibility of Yki to its kinase (Genevet and Tapon, 2011). Our finding that Ed facilitates the apical localization of Sav lends further support to this notion. Through interacting with multiple components of the Hpo pathway, Ed could facilitate Hpo activation and Yki phosphorylation. I found that loss of Ed did not alter the apical membrane localization of Ex and Mer. The apical localization of Ex and Mer is likely to be mediated by other upstream components such as Ft and Crb in the absence of Ed. Indeed, Crb physically interacts with Ex and both loss and gain

of function of Crb caused mislocalization of a fraction of Ex to the basal region (Chen et al., 2010; Grzeschik et al., 2010; Robinson et al., 2010). It has been shown that Ex physically interacts with Yki, which may sequester Yki in the cytoplasm independent of Yki phosphorylation (Badouel et al., 2009). Our finding that Ed interacts with Yki through a domain distinct from those mediating binding to the upstream Hpo pathway components raises a possibility that Ed may also directly sequester Yki in the cytoplasm in addition to regulating its subcellular localization through phosphorylation.

Chapter III

Materials and methods

Mutants and transgenes

ed^{1X5} is a strong allele (Bai et al., 2001). To generate ed mutant or control clones in adult eyes, y w ey-FLP; l(2)cl-L3 P[w+] FRT40/Cyo (BL#5622) flies were crossed with ed1X5 FRT40 /Cyo or FRT40 flies. Marked ed clones in imaginal discs were generated using the Minute technology using the following genotype: hs-FLP; FRT40 M (2)24F arm-lacZ/ FRT40 ed1X5. FLP/FRT-mediated mitotic recombination was induced 2 days after egg laying by incubation the larvae at 37 for 1 hour.Transgenic RNAi lines used are: UAS-CG3313-RNAii (VDRC# 43758), UAS-Cul4-RNAi (VDRC# 44829), UAS-Ed-RNAi937 (VDRC# 937), UAS-Ed-RNAi3087 (VDRC# 3087), UAS-Ed-RNAi104279 (VDRC# 104279), UAS-Ft-RNAi (VDRC# 9396), UAS-Ds-RNAi (VDRC # 36219), UAS-Dachs-RNAi (VDRC# 12556), *UAS-EGFR-RNAi* (VDRC# 43267), *UAS-Yki-RNAi* (Zhang et al., 2008), *UAS-Sav-RNAi* (BL# 28006), and *UAS-Ed-RNAi*^{C} targeting the C-terminal region of Ed between aa1216-1332. VDRC# 104279 and 3087 appears to be stronger than other Ed RNAi lines and leads to more leathality when expressed by *hh-Gal4*. Other transgenes used in this study include *bantam sensor* (Brennecke et al., 2003), *ex-lacZ* (Hamaratoglu et al., 2006), *diap1-GFP* (Zhang et al., 2008), *UAS-Ed* (Fetting et al., 2009), and *UAS-HA-Sav* (Jia et al., 2003). To generate *UAS-Myr-Ed*^{C} and *UAS-Myr-CC-Ed*^{C} constructs, DNA fragments corresponding to a myristoylation signal, GNKCCSKRQ, and the leucine zipper coiled-coil dimerization domain of the

yeast GCN4 (O'Shea et al., 1991), RMKQLEDKVEELLSKNYHLEVEVARLKKLVGER, are synthesized and inserted into the N-terminus of Ed^{C}. Transgenic flies carrying *UAS-*Ed^{FL}*-Myc, UAS-*Ed^{TMC}*-Myc, UAS -*$Ed^{TMC\Delta 1}$*-Myc, UAS* $Ed^{TMC\Delta 2}$*-Myc, UAS-*Ed^{C}*, UAS-Myr-*Ed^{C} *and UAS-Myr-CC-*Ed^{C} were generated by P-element transformation. The genotypes for generating clones are as follows: *ed* clones: *y w hs-flp*; *FRT40* ed^{1X5}*/ arm-LacZ FRT40*; *ed* clones expressing various transgenes: *y w hs-flp*; *FRT40* ed^{1X5}*/FRT40 tub-Gal80*; *UAS-transgenes/tub-Gal4 UAS-GFP*.

Cell culture, transfection, immunoprecipitation, and western blot analysis

Drosophila S2 cells were cultured in *Drosophila* Schneider's medium (Invitrogen) with 10% fetal bovine serum, 100 U/ml of penicillin, and 100mg/ml of streptomycin. Transfection was carried out by Calcium Phosphate Transfection Kit (Specialty Media) according to manufacturer's instructions. The construct of *ubiquitin-Gal4* was cotransfected with *pUAST* expression vectors for all the transfection experiments except for cell-cell contact induced Ed/Sav interaction experiment in which tub-Ed^{FL}-Myc, tub-$Ed^{\Delta C1}$-Myc and tub-Sav-GFP were used. Immunoprecipitation and western blot analyses were carried out using standard protocols as previously described. MG132 (Calbiochem) was used at 50 μM. Transfected S2 cells were treated with Cycloheximide (Sigma) at a final concentration of 100 μM for the indicated time periods before harvesting. For endogenous protein detection, wing imaginal discs were collected from the later third instar larvae and lysed in NP40 cell

lysis buffer with protease inhibitor cocktail (Roche). Lysates were cleared by centrifugation and subjected to SDS-PAGE. Western blot was performed by using rabbit anti-Yki (1:1000, K. Irvine), rabbit anti-phospho-Yki (1:1000, D. Pan), mouse anti-α-Tubulin (1:100,000, Sigma), rat anti-Ed (1:1000, J. Hsu), and rabbit anti-Sav (1:1000).

Immunohistochemistry

To generate Sav antibodies, full-length Sav cDNA was cloned into pET30. His-Sav was expressed in BL21 E.coli (Invitrogen) by induction with 0.1 mM IPTG. Sav protein was purified on SDS-PAGE and used to immunize rabbits (Cocalico Biologicals). Other antibodies used were mouse anti-β-Gal (1:1000, Promega), rabbit anti-β-Gal (1:1000, Affinity Bioreagents) , mouse anti-Wg (1:100, DSHB), rat anti-Ci, 2A1 (Motzny and Holmgren, 1995), rabbit anti-Yki (1:1000, K. Irvine), Rabbit anti-GFP (Molecular Probes), guinea pig anti-Ex (1:5000) and guinea pig anti-Mer (1:5000, R. Fehon), rat anti-Ed (1:1000, J. Hsu), rat anti-E-cad (1:100, DHSB), rabbit anti-Sav (1:100), DRAQ5 (cell signaling), mouse anti-HA, mouse anti-Myc and mouse anti-Fg (Santa Cruz). Cy2-, Cy3- and Cy5- conjugated secondary antibodies were from Jackson ImmunoResearch Laboratories. Images were captured by Zeiss LSM510 confocal microscopy.

Chapter IV

Summary and future directions

The Hpo signaling pathway has emerged as a conserved regulatory pathway that controls tissue growth and organ size. Although the core pathway components, i.e., the Hpo/Sav/Wts/Mats kinase cassette and its effector Yki/Yap have been well defined, the upstream regulators, especially the membrane receptors that link cell-cell communication to Hpo signaling, remain poorly defined. Here I provide both genetic and biochemical evidence that the transmembrane cell adhesion molecule Ed functions as a novel upstream regulator of the Hpo pathway. I provide evidence that Ed physically interacts with Sav/Hpo and regulates the abundance of Sav at adherens junctions. Loss of Ed compromises the ability of Hpo/Wts kinase cassette to phosphorylate Yki, leading to elevated nuclear levels of Yki activity that drives tissue overgrowth. I find that Ed/Sav association is facilitated by cell-cell contact, raising an interesting possibility that Ed may serve as a mechanism that links Hpo signaling to cell contact inhibition. Thus, our study not only identified a novel upstream component for the Hpo pathway but also revealed a new mechanism by which the Hpo kinase cascade is regulated.

My thesis has mainly focused on the role of Ed in the Hpo signaling pathway. However, a few intriguing questions coming from my thesis study still remain to be answered. It is interesting to note that Ed is related to TSLC1, a tumor suppressor implicated in human non-small-cell lung cancer and other cancers including liver,

pancreatic, and prostate cancers (Kuramochi et al., 2001; Murakami, 2005). Like Ed, TSLC1 also mediates cell-cell adhesion through homophilic interactions (Masuda et al., 2002). TSLC1 interacts with MPP3, a human homolog of *Drosophila* tumor suppressor Discs large (Dlg) that has been implicated in the Hpo pathway (Fukuhara et al., 2003; Grzeschik et al., 2010), as well as DAL-1, a FERM-domain containing tumor suppressor related to Ex/Mer (Yageta et al., 2002). Therefore, it would be interesting to determine whether TSLC1 inhibits tumor formation through the Hpo pathway.

Through my genetic screen, several other deficiency lines (#7704, #7731, #7639, and #7649) also modified the overgrowth phenotype of GMR-Yki to different extents, but candidate genes selected from deleted regions were not responsive for this modification. It could be interesting to identify real responsible genes and characterize their functions. One simple strategy is to get RNAi lines targeting all genes deleted in the above deficiency lines, identify the right RNAi genes and then further analyze their functions.

Although I identified the Cul4/WD40 complex as a genetic modifier for Hpo signaling, we know little about how this complex modulates Hipo signaling. If the Cul4/WD40 complex directly regulates Hpo signaling, it is critical to show which Hpo target genes are upregulated of upon loss of Cul4 or CG3313. I am still in the process of identifying such physiologically relevant transcriptional targets. Immunoprecipitation analysis indicates that the Cul4/WD40 complex can interact with Yki, suggesting that Yki could be a potential target for this E3 complex, however,

in *Cul4* RNAi flip-out clones induced by heat shock, Yki protein level is not significantly upregulated, indicating that other potential targets may exist. Mammalian studies reveal that there is a physical interaction between human homolog of CG3313 and TGF-beta receptor. Consistent with this, I also find that CG3313 can be coprecipitated with baboon, *Drosophila* homolog of TGF-beta receptor. Given the increasing evidence to show that a crosstalk occurs between TGF signaling and Hpo signaling, it is also possible that the modification of GMR-Yki is due to elevated activity of TGF beta signaling. Then it is interesting to determine which TGF beta signaling pathway target genes have been upregulated in the absence of Cul4 or CG3313.

Ed has been shown to stabilize Sav in S2 cells probably by preventing proteasome-mediated degradation. However, the underlying mechanism remains poorly understood. A cell-culture based RNAi screen could be applied to identify an E3 ubiquitin ligase for Sav. Uncovering this degradation mechanism will add another layer of regulation for the Hpo signaling pathway, which has been complicated already. Moreover, Ed also physically interacts with multiple upstream regulators, such as Ex and Mer, suggesting that Ed may have more profound functions than just regulating Sav abundance at the AJ. While Loss of Ed is not able to change Ex and Mer protein localization in wing disc cells, it is possible that Ed functions as a scaffold protein to facilitate the assembly of an apical machinery containing Hpo, Sav, Ex, and Yki and thereby promotes Wts inhibitory phosphorylation on Yki (Genevet et al., 2010), although this model is difficult to prove in some sense.

Lastly, previous analysis indicates that Ds is genetically upstream of Dachs and functions as a ligand, however, other's and our studies have shown that Ds functions in both signaling sending cells and signal receiving cells in wing disc, and its intracellular domain is required for regulating Hpo target gene expression autonomously (Willecke et al., 2008). This observation can not be explained by Dr. Irvine's model in which Ds regulates Hpo signaling through polarizing Ft protein distribution. Moreover, Ds is also required for upregulated Hpo target gene expression in the cells surrounding Ds overexpression clones. Combining with its intracellular domain- mediated autonomous role, Ds clearly regulates Hpo signaling in more complicated fashion. One hypothesis is that Ds may directly regulate Dachs localization or activity. To test this possibility, co-immunoprecipitaion could be performed to examine the potential interaction between Ds and Dachs. As previous studies indicated, Ft regulates Wts proteolysis through Dachs. It is also possible that Ds regulates Hpo signaling by directly participating this proteolytic processing.

In the past several decades, we have gained tremendous understanding of how organ size is controlled. However, many important questions remain unanswered. For example, multiple signaling pathways have been identified to regulate tissue growth. But how these signaling are smoothly coordinated, translated into every single cell, and dictate organ growth to reach proper size? Other questions, such as how organ size is programed and how the organ senses the information to initiate or stops compensatory growth, are all interesting to be answered. Addressing these questions will not only provide great insight into the signaling

network underlying the control of organ size during normal development, but also help us understand how cancer cells hijiack the signaling network to favor their own survival and proliferation.

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