THE HIPPO SIGNALING PATHWAY

IN ORGAN SIZE CONTROL AND REGENERATON

Jin Jiang, Ph.D. (Mentor)

_____

Michael Buszczak, Ph.D. (Chairman)

_____

James Brugarolas, Ph.D.

_____

Zhijian Chen, Ph.D.

_____

_____

DEDICATION

For my parents and friends.
For everyone that assists and encourages me with my research.

THE HIPPO SIGNALING PATHWAY

IN ORGAN SIZE CONTROL AND REGENERATON

by

FANGFANG REN

DISSERTATION

Presented to the Faculty of the Graduate School of Biomedical Sciences

The University of Texas Southwestern Medical Center at Dallas

In Partial Fulfillment of the Requirements

For the Degree of

DOCTOR OF PHILOSOPHY

The University of Texas Southwestern Medical Center at Dallas

Dallas, Texas

February, 2012

THE HIPPO SIGNALING PATHWAY

IN ORGAN SIZE CONTROL AND REGENERATON

Fangfang Ren

The University of Texas Southwestern Medical Center at Dallas, 2012
Supervising Professor: Dr. Jin Jiang, Ph.D.

The Hippo (Hpo) signaling pathway controls cell growth, proliferation and apoptosis in both *Drosophila* and vertebrates. Our lab has previously demonstrated that Hpo signaling regulates gene expression by inhibiting a transcription complex consisting of the transcriptional coactivator Yorkie (Yki) and the TEAD/TEF family of transcription factor Scalloped (Sd) in *Drosophila*. The inhibition of Yki activity is through modulating its phosphorylation status and subcellular localization by upstream kinase complex. I obtained both genetic and cellular evidence that 14-3-3 proteins are involved in this process. I also identified three Serine residues (S111, S168 and S250 of Yki as essential for restricting Yki activity. I found that 14-3-3 regulates Yki subcellular localization mainly through S168 but not the other two sites.

The recent identification of intestinal stem cells (ISCs) has made the *Drosophila* adult midgut an excellent model to study adult stem cell biology. Multiple signaling pathways have been implicated in the regulation of ISC proliferation, self-renewal and

differentiation. I obtained evidence that Hpo signaling plays an essential role in regulating ISC proliferation through both cell-autonomous and non-cell-autonomous mechanisms. Cytokines of the Upd family and multiple EGFR ligands were found to be ectopically induced when Hpo signaling is inactivated in differentiated cells, which in turn activate Jak-Stat and EGFR signaling pathways in ISCs to stimulate their proliferation. I also showed that tissue damaging reagent DSS-induced ISC proliferation is dependent on Yki activity in precursor cells.

Although several signaling pathways including Jak-Stat, EGFR and Hpo pathways have been implicated in damage-induced ISC proliferation, the cell intrinsic mechanisms have remained elusive. I found that the *Drosophila* homolog of *Myc* oncogene (*dMyc*), which encodes a transcription regulator that affects cellular growth and cell cycle progression, functions downstream of Hpo, Jak-Stat and EGFR pathways to mediate their effects on ISC proliferation. dMyc is also essential for adult midgut homeostasis as well as regeneration after exposure to damage reagents. I also demonstrated that the regulation of dMyc levels by Hipo, Jak-Stat and EGFR pathways is at the level of transcription.

TABLE OF CONTENTS

LIST OF FIGURES

CHAPTER ONE

Background Introduction

1. Hippo signaling pathway

Growth is a fundamental process for animal development, which requires the coordinated actions of different cellular activities. The mechanisms by which multicellular organisms control growth of individual organs to reach a particular size during both normal development and regeneration has long been a puzzle for developmental biologists. For example, how do cells sense the signals to exit cell cycle when the correct organ size is achieved? How do cells in damaged organs proliferate to restore organs to original size without causing any overgrowth? The answers to these questions are still far from being fully understood but studies of model organisms such as *Drosophila* have suggested that organ-intrinsic mechanisms such as intra- and inter-cellular signaling could play essential roles in determining these cellular behaviors (Bryant and Simpson, 1984; Conlon and Raff, 1999).

The recent discovery of the Hpo signaling pathway as a crucial growth regulator has shed light on these long-standing questions because it provides new information regarding the mechanisms of tissue growth and organ size control. Hpo pathway was initially discovered by genetic screens carried out in *Drosophila* and later was found to be conserved in mammals with respect to both key pathway components and basic signal transduction steps (Pan, 2010; Zhao et al., 2010). Hpo pathway also interacts with other important signaling pathways such as Wnt, Shh and BMP pathways in the

regulation of diverse cellular actions (Baena-Lopez et al., 2008).

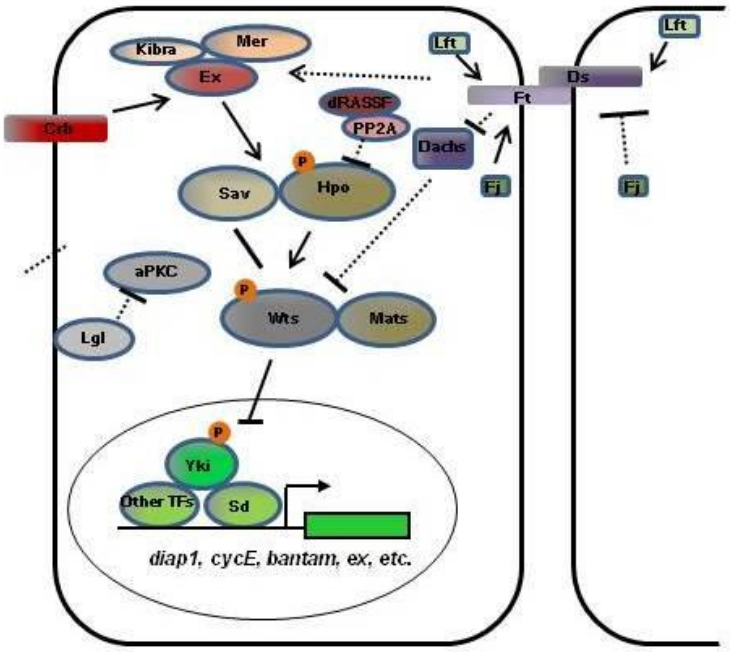

Figure 1-1. Schematic model of the Hpo signaling network (Modified from (Yue et al., 2012))

Figure 1-1 is a sketch diagram of Hpo signaling pathway in *Drosophila*, which mainly consists of three groups of proteins: core kinase cascade, upstream regulatory input and downstream transcriptional complex. I will briefly introduce components of Hpo pathway and how signaling is transduced.

1.1 The Hpo kinase cascade

The core kinase cascade is composed of four proteins: Hippo (Hpo), Salvador (Sav), Warts (Wts) and Mob-as-tumor-suppressor (Mats), loss of function of which lead to massive tissue overgrowth. Hpo, the *Drosophila* homolog of mammalian Ste20 family kinases MST1 and MST2, associates with the WW-domain containing scaffolding protein Sav to phosphorylate and activate the downstream kinase Wts, which belongs to the Nuclear Dbf-2-related (NDR) kinase family (Harvey et al., 2003; Jia et al., 2003; Justice et al., 1995; Pantalacci et al., 2003; Tapon et al., 2002; Udan et al., 2003; Wu et al., 2003; Xu et al., 1995). The activated Wts interacts with a cofactor Mats, which is also phosphorylated by Hpo (Lai et al., 2005; Wei et al., 2007), to phosphorylate and inactivate downstream transcriptional coactivator Yorkie (Yki), the *Drosophila* homolog of Yap (Huang et al., 2005), resulting in inhibition of cell proliferation and cell growth as well as promotion of cell death.

1.2 Upstream regulators

The network of upstream inputs of Hpo pathway is complex and composed of ever increasing number of proteins to relay signal to core kinase cascade. Although our

knowledge of the multiple upstream inputs is still incomplete, we can cluster those upstream regulatory components into several different complexes according to their physical and genetic interactions.

1.2.1 Fat signaling

Fat (Ft) is a large atypical cadherin that functions as a transmembrane receptor for Hpo pathway to control organ growth (Reddy and Irvine, 2008). It can also affect planar cell polarity (PCP) and proximal-distal patterning independent of Hpo signaling (Yang et al., 2002). Several other proteins have been identified that belong to the Fat branch of Hpo signaling, including its ligand Dachsous (Ds), which is also an atypical cadherin and mediates Ft function (Cho and Irvine, 2004; Matakatsu and Blair, 2004; Simon et al., 2010); the Golgi kinase Four-jointed (Fj), which phosphorylates extracellular cadherin domains of Ft and Ds to modulate their binding in the way that promotes Ft-Ds affinity by phosphorylating Ft while reduces Ft-Ds interaction by phosphorylating Ds (Brittle et al., 2010; Ishikawa et al., 2008); the casein kinase I Discs overgrown (Dco), which phosphorylates intracellular domain of Ft in a Ds dependent manner to activate Ft (Cho et al., 2006; Feng and Irvine, 2009; Sopko et al., 2009); the conserved cytoplasmic protein Lowfat (Lft), which influences the stability of Ft and Ds through binding to the intracellular domains of both proteins. The sub-apical localization of Ft and Lft are interconnected (Mao et al., 2009); the unconventional myosin Dachs, which functions downstream of Ft and whose activity and localization is related to Ft activation (Cho et al., 2006; Cho and Irvine, 2004;

Mao et al., 2006); the palmitoyltransferase Approximated (App), which regulates membrane localization of Dachs (Matakatsu and Blair, 2008).

Several mechanisms have been described for the regulation of Hpo signaling by Ft branch. Ft can influence Wts protein levels in a posttranslational manner *via* Dachs, which has been suggested as the primary mechanism of Ft actions on Hpo signaling (Cho et al., 2006). Ft can also modulate subapical membrane localization and levels of Expanded (Ex) protein (Bennett and Harvey, 2006; Feng and Irvine, 2007; Silva et al., 2006; Willecke et al., 2006). Moreover, the gradients of Ds and Fj expression modulates Hpo signaling in the way that the different levels of Ds and Fj between neighboring cells promote Hpo pathway target gene expression and cell proliferation (Cho et al., 2006; Cho and Irvine, 2004; Rogulja et al., 2008; Willecke et al., 2008; Zecca and Struhl, 2010). Ft branch is selectively required for Hpo signaling in different tissues, such as it is important in imaginal discs and neuroepithelia but not in ovary (Meignin et al., 2007; Polesello and Tapon, 2007; Reddy et al., 2010).

1.2.2 Merlin/Expanded/Kibra complex

Merlin (Mer) and Expanded (Ex) are two FERM (Four-point one, Ezrin, Radixin, Moesin) domain-containing proteins that function partially redundantly to modulate Hpo signalin (Hamaratoglu et al., 2006). Mutation of either *mer* or *ex* alone results in Yki activation and tissue growth while inactivation of both genes leads to stronger phenotypes (Hamaratoglu et al., 2006; Maitra et al., 2006; McCartney et al., 2000).

Mer and Ex can physically associate with each other and colocalize in epithelia cells, while genetically they are differentially required for Hpo signaling in different tissues as well as during different developmental stages (McCartney et al., 2000). The WW domain containing protein Kibra was recently identified to colocalize and form complex with Ex and Mer and function as their partner. Mutation of *kibra* also causes overgrowth phenotype similar to that of *mer* mutation (Baumgartner et al., 2010; Genevet et al., 2010; Yu et al., 2010). Ex/Mer/Kibra complex can associate with Hpo, Wts and Sav and promote phosphorylation and activation of Hpo and Wts, although the exact mechanism is not clear. Multiple members of Ex/Mer/Kibra complex and Hpo/Wts kinase cascade can physically interact with each other, such as Ex with Hpo, Mer with Sav and Kibra with Sav and Wts. Moreover, Ex can directly associate with Yki through WW domain- PPXY interaction and retain Yki in the cytoplasm independent of Yki phosphorylation status (Badouel et al., 2009; Oh and Irvine, 2009).

As FERM domain-containing proteins, Ex and Mer function as linkage of the F-actin cytoskeleton with cell membrane proteins since inactivation of Ex causes accumulation of apical F-actin (Sansores-Garcia et al., 2011). The membrane localization of Ex, Mer and Kibra is essential for their normal function. Although Ex, Mer and Kibra can interact with each other, their membrane association is independent of each other. Phosphatidylinositol 4-kinase (PI4KIIIα) is involved in apical membrane accumulation of Mer but not Ex or Kibra (Baumgartner et al., 2010;

Genevet et al., 2010; Yan et al., 2011; Yu et al., 2010).

1.2.3 Apical-basal polarity proteins

Several apical-basal polarity proteins have been associated with Hpo signaling, including transmembrane protein Crumbs (Crb), neoplastic tumor suppressor Lethal giant larvae (LgI) and the apical polarity regulator atypical protein kinase C (aPKC). Crb localizes in the sub-apical membrane of epithelial cells. It directly binds with Ex *via* the juxtamembrane FERM-binding motif (FBM) and therefore recruits Ex to the plasma membrane, through which to regulate Hpo signaling (Chen et al., 2010; Ling et al., 2010; Robinson et al., 2010). The sub-apical localization of Ex is reduced in *crb* mutant discs and Hpo signaling is attenuated. On the other hand, overexpression of Crb can also activate Yki and promote cell proliferation through mis-localizing Ex and reducing Ex levels. Therefore, either increased or decreased level of Crb causes inactivation of Ex and overgrowth phenotype.

Lgl localizes to the basolateral membrane of epithelial cells and forms complex with Discs large (Dlg) and Scribble (Scrib) (Bilder et al., 2000; Humbert et al., 2008). Inactivation of Lgl could result in Yki activation and increased cell proliferation as well as suppressed cell apoptosis, which can be blocked by overexpression of dominant-negative form of aPKC (Grzeschik et al., 2010; Menendez et al., 2010; Sun and Irvine, 2011). Overexpression of aPKC also leads to upregulation of Hpo pathway target genes. The mechanism by which Lgl and aPKC influence Hpo signaling is

likely through regulating the subcellular localization of Hpo and the *Drosophila* Ras-associated domain family protein (dRASSF). Loss of function of Lgl or activation of aPKC can trigger lateral mislocalization of Hpo and dRASSF (Grzeschik et al., 2010). Moreover, Jun kinase (Jnk) signaling is suggested to be involved in Yki activation and cell proliferation upon depletion of Lgl or activation of aPKC (Sun and Irvine, 2011). Therefore, Lgl and aPKC might regulate Hpo signaling through multiple mechanisms, which awaits further investigation.

1.2.4 Inhibitors of Hpo signaling

Since the central cascade of Hpo signaling is a kinase complex and phosphorylation events play important roles in Hpo signaling transduction, it is anticipated that phosphatases should be also involved in Hpo signaling pathway. Consistent with this notion, a PP2A phosphatase complex, STRIPAK (Striatin-interacting phosphatase and kinase) was identified as a negative regulator of Hpo signaling by both proteomic and genomic RNAi screening approaches. STRIPAK complex might function through dephosphorylating Hpo and inhibiting Hpo activity (Ribeiro et al., 2010).

dRASSF is another antagonist of Hpo signaling, which binds Hpo through a carboxyl-terminal Sav/Rassf/Hpo (SARAH) domain and inhibits phosphorylation of Hpo at Thr195 site (Polesello et al., 2006). dRASSF can also recruit STRIPAK complex to Hpo and contribute to STRIPAK activity.

Ajuba LIM protein (Jub) and Zyx102 (Zyx) protein are another group of negative regulator of Hpo signaling which target Wts (Das Thakur et al., 2010; Rauskolb et al., 2011). Jub belongs to the Ajuba protein family and Zyx is the member of the Zyxin family. Both proteins contain three C-terminal LIM domains. Jub can physically interact with both Sav and Wts and genetically function upstream of Wts but downstream of Hpo (Das Thakur et al., 2010). Zyx also binds with Wts and is involved specifically in the Fat branch of Hpo signaling through influencing the regulation of Wts protein levels by Fat (Rauskolb et al., 2011).

1.2.5 Other Yki regulators

Yki is the downstream transcriptional coactivator of Hpo signaling and contains two WW domains, which can interact with PPXY motif (X is any amino acid). Two PPXY-motif containing proteins, Wpb2 and Myopic (Mop) have been identified as regulators of Hpo signaling by associating with Yki (Gilbert et al., 2011; Zhang et al., 2011). Mop is the *Drosophila* homolog of His-domain protein tyrosine phosphatase (HD-PTP) and can retain Yki in the cytoplasm upon binding to Yki and affect the expression of a subset of Yki target genes but not the others. It is also involved in regulating Yki endosomal association (Gilbert et al., 2011). Wbp2 functions in opposition with Mop and promotes transcriptional activity of Yki (Zhang et al., 2011).

1.3 Downstream transcriptional complex and target genes

The downstream target of Hpo kinase cascade is the transcriptional coactivator Yki,

which contains no DNA binding domains and functions together with DNA-binding transcription factors to mediate outputs of Hpo signaling. The activity of Yki is dependent on its phosphorylation status and subcellular localization. Yki can be phosphorylated at Ser 111, Ser 168 and Ser 250 by Wts kinase and this causes Yki to associate with 14-3-3 proteins, which results in cytoplasmic retention of Yki and inhibition of its transcriptional activity (Oh and Irvine, 2008, 2009; Ren et al., 2010b). The first binding partner of Yki identified is the TEAD/TEF family transcription factor Scalloped (Sd), which binds with Yki at C-terminal and activates expression of downstream target gene *diap1* (Goulev et al., 2008; Wu et al., 2008; Zhang et al., 2008). From then on, more DNA-binding partners of Yki have been identified, including Homothorax (Hth) and Teashirt (Tsh), which act in eye imaginal discs to upregulate the growth promoting microRNA gene *bantam* (*ban*) (Peng et al., 2009). Besides, Mothers against Dpp (Mad) can also associate with Yki and promote expression of *bantam* (Oh and Irvine, 2011; Peng et al., 2009). The involvement of Mad in Hpo signaling suggests the existence of crosstalk between Hpo signaling and Dpp signaling. Those multiple DNA-binding partners of Yki play roles in a tissue-specific manner to target different downstream genes, such as Sd functions mainly in the wing disc and regulates *Diap1* while Hth acts primarily in the anterior eye disc and regulates *bantam*.

A broad spectrum of genes are regulated by Hpo signaling, which include cell growth promoter *ban* and *Myc* (Neto-Silva et al., 2010; Nolo et al., 2006; Thompson and

Cohen, 2006; Ziosi et al., 2010), cell cycle regulator *E2F1* and *cyclin E* (*cycE*) (Goulev et al., 2008; Silva et al., 2006; Tapon et al., 2002; Tyler and Baker, 2007), cell death inhibitor *Diap1* (Wu et al., 2003). Moreover, several upstream regulators of Hpo signaling like Kibra, Ex, Crb and Fj are subject to the regulation to form a negative feedback loop (Cho et al., 2006; Genevet et al., 2009; Genevet et al., 2010; Hamaratoglu et al., 2006). Hpo signaling can also target components of other signaling pathways such as ligands for Notch, Wnt, EGFR and Jak-Stat pathways (Cho et al., 2006; Karpowicz et al., 2010; Ren et al., 2010a; Shaw et al., 2010; Staley and Irvine, 2010; Zhang et al., 2009b) as well as Wg and BMP (Baena-Lopez et al., 2008), which suggests extensive crosstalk of Hpo signaling with other signaling pathways.

1.4 Crosstalk of Hpo signaling with other signaling pathways

As mentioned above, components of several other signaling pathways are downstream targets of Hpo signaling, implying the interaction of Hpo signaling with other signaling pathways. Hpo signaling can control the expression of the heparin sulphate proteoglycans Dally and Dally-like, which affect the extracellular levels of ligands for Hedgehog, bone morphogenetic protein (BMP), and Wnt pathways, and in turn regulate those morphogen signaling pathways (Baena-Lopez et al., 2008). On the other hand, some upstream components of Hpo signaling are subject to the regulation by morphogens, such as Wg and Dpp (Rogulja et al., 2008). Hpo signaling can also mediate the expression of the Notch ligands, Serrate and Delta as well as the

expression and subcellular localization of Notch, therefore influencing Notch signaling activity (Baena-Lopez et al., 2008). The expression of ligands for Jak-Stat and EGFR pathways can be ectopically induced by inactivation of Hpo signaling in *Drosophila* adult midgut, suggesting crosstalk of Hpo signaling with Jak-Stat and EGFR pathways (Ren et al., 2010a; Shaw et al., 2010; Staley and Irvine, 2010). Besides, in wing discs, Jun kinase signaling participates in modulating the activity of Hpo signaling (Sun and Irvine, 2011). Taken together, Hpo signaling is able to function as a mediator of multiple other signaling pathways and integrate physiological cues and extracellular signals to coordinately regulate cell growth, cell proliferation and cell apoptosis.

1.5 Hpo signaling in stem cell proliferation and tissue regeneration

Hpo signaling not only controls tissue growth during normal development but also is involved in stem cell proliferation and tissue regeneration. The role of Hpo signaling plays in regenerative growth was illustrated in two *Drosophila* model systems: imaginal discs and adult midgut. When *Drosophila* wing discs are exposed to tissue damage, no matter caused by surgery, genetic manipulation or irradiation, the damaged or missing parts can be replaced through increased proliferation of cells in intact parts, which is called compensatory cell proliferation (Fan and Bergmann, 2008). And Yki was found to be hyperactivated during this process and the high activity of Yki is essential for tissue to accomplish regeneration process (Grusche et al., 2011). Studies have shown that the hyperactivation of Yki during tissue

regeneration is dependent on Jnk signaling, which has already been demonstrated to participate in damage induced regeneration and compensatory cell proliferation in *Drosophila* (Sun and Irvine, 2011).

2. Intestine stem cells in adult *Drosophila* midgut

Adult stem cells are undifferentiated cells in adult animals and humans, which retain the ability of self-renewal to replenish dying cells in adult tissues. They possess multipotency and can differentiate to generate all types of cells in the adult tissue from which they originate (Presnell et al., 2002). Several signaling pathways have been reported to regulate self-renewal and differentiation of adult stem cells including Notch signaling, Wnt signaling and TGFβ signaling (Beachy et al., 2004; Dontu et al., 2004). There are many types of adult stem cells such as Hematopoietic stem cells, Mammary stem cells, Intestinal stem cells, Mesenchymal stem cells, Endothelial stem cells, Neural stem cells, Olfactory adult stem cells, Neural crest stem cells and Testicular cells. Adult stem cells have great therapeutic potential as they can differentiate into several types of progenies. Besides, adult stem cell have been implicated as a prime target for cancer (Presnell et al., 2002).

The adult *Drosophila* midgut is a newly developed and excellent model to study adult stem cell regulation during both homeostasis and regeneration because it bears a simple and well-defined cell linage and accessible to genetic manipulation. There are four different cell types in adult midgut epithelium. Intestinal stem cells (ISCs) are

located adjacent to the basement membrane of midgut epithelium, which are the only cell type that can undergo cell division (Micchelli and Perrimon, 2006; Ohlstein and Spradling, 2006). The asymmetric division of an ISC produces two daughter cells: one remains as an ISC, and the other one loses stem cell properties and converts to an enteroblast (EB) which can undergo differentiation to become either absorptive enterocytes (EC) or secretary enteroendocrine (ee) (Fig. 1-2) (Ohlstein and Spradling, 2006). The self-renewal, proliferation and differentiation of ISCs are regulated by multiple signaling pathways to maintain midgut homeostasis and are also responsive to injury stimulation to regenerate midgut epithelium (Jiang and Edgar, 2011).

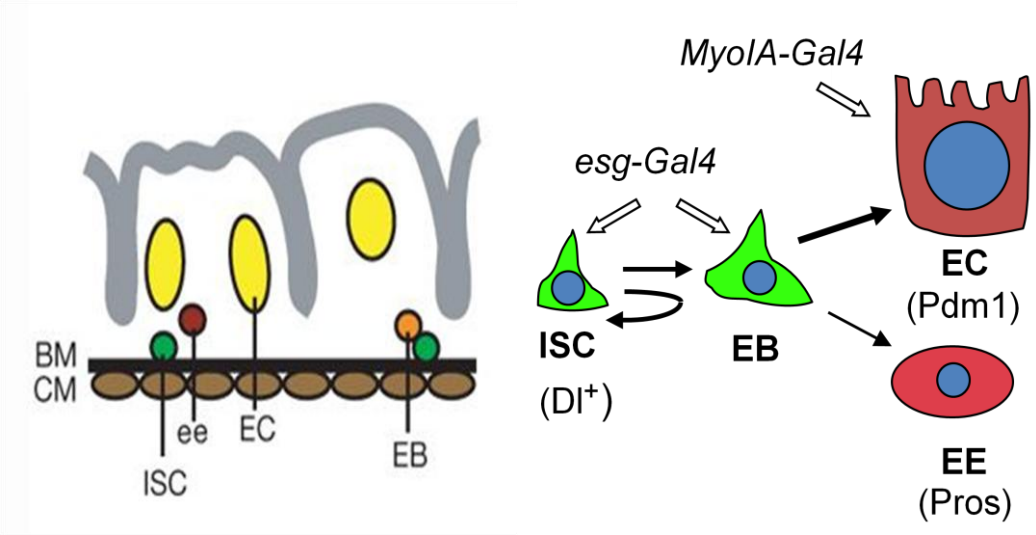

Figure 1-2. Structure and Cell linage in adult *Drosophila* midgut (Lin et al., 2008). BM: basement membrane; CM: circular muscle; ISC: intestine stem cell; EB: enteroblast; EC: enterocyte; ee: enteroendocrine(Ohlstein and Spradling, 2006)(Ohlstein and Spradling, 2006)(Ohlstein and Spradling, 2006)(Ohlstein and Spradling, 2006) (Ohlstein and Spradling, 2006).

2.1 ISC proliferation is stimulated by damage reagents

During normal adult midgut homeostasis, most of ISCs in epithelium are quiescent with low division rates. However, upon tissue damage, which could be caused by ingestion of chemical reagents such as Bleomycin or dextran sodium sulfate (DSS) , or bacterial infection such as *Pseudomonas entomophila*, or expression of cell death genes such as *reaper* in ECs, ISC proliferation is ectopically induced (Amcheslavsky et al., 2009; Apidianakis et al., 2009; Buchon et al., 2009a; Buchon et al., 2009b; Chatterjee and Ip, 2009; Jiang et al., 2009). This compensatory ISC proliferation results in the production of more newborn EBs, which can differentiate into ECs and ees to replenish epithelium cell pool and therefore midgut regenerates itself.

2.2 Notch signaling regulates ISC proliferation and differentiation

Notch pathway ligand Delta (Dl) is specifically expressed in ISCs but not EBs. During the process of ISC division, Dl is first expressed in both daughter cells. However, immediately after division, Dl is retained in the more basally located daughter cell but lost in the more apically located one, which in turn activates Notch target genes and reporters, such as Suppresser of Hairless (Su(H)). Notch signaling is involved in mediating the differentiation of EB and is essential for the production of EC fate (Ohlstein and Spradling, 2007). Dl expression levels in ISCs fluctuate therefore leading to different levels of Notch activation in neighboring EBs. When Dl is highly expressed in ISC, activation of Notch signaling in EB stimulates it to differentiate into the EC cell fate, while low levels of Dl in ISC promotes EB to ee

cell fate. Experiments also showed that ee can be produced even in the absence of Notch signaling. Besides, Notch signaling can influence ISCs proliferation, in the way that activation of Notch signaling in ISCs inhibits their proliferation and promotes the production of ECs while inactivation of Notch signaling in ISCs produces clusters of Dl-positive ISC-like cells without formation of ECs (Fre et al., 2005).

2.3 Wnt signaling mediates ISC proliferation and maintenance

ISC proliferation is subject to the regulation by Wnt signaling. Activation of Wnt signaling by overexpression of Wnt ligand, Wingless or a constitutively active form of β-Catein, Arm^{S10} or by mutation of Wnt signaling inhibitory components such as Shaggy (GSK3β), dAxin or Apc results in enhanced ISC proliferation (Lee et al., 2009; Lin et al., 2008). Studies also reported that Wnt signaling is essential for ISC maintenance, as prolonged inactivation of Wnt pathway components, such as Frizzled, Disheveled, or Armadillo gradually leads to ISC loss (Lee et al., 2009; Lin et al., 2008). It was proposed that Wnt signaling functions upstream of Notch signaling to maintain the balance of ISC self-renewal and differentiation (Lin et al., 2008).

2.4 Jak-Stat signaling regulates ISC proliferation and differentiation

Jak-Stat pathway is an important regulator of ISCs during midgut homeostasis and regeneration. Ectopic stimulation of Jak-Stat signaling activity in ISCs and EBs by overexpression of pathway ligands can significantly promote ISC proliferation and differentiation, eventually leading to midgut hyperplasia phenotype (Jiang et al., 2009;

Lin et al., 2009; Liu et al., 2010). On the other hand, inhibition of Jak-Stat signaling in ISCs and EBs by inactivation of pathway components such as *Drosophila* cytokine receptor Domeless (Dome), *Drosophila* Jak kinase Hopscotch (Hop) or Stat92E (Stat) results in the failure of midgut to regenerate (Jiang et al., 2009; Liu et al., 2010). As mentioned above, after exposure to different types of tissue injury, ISCs can be induced to enhanced levels of proliferation to regenerate midgut. It was reported that Jak-Stat signaling was activated during damage induced regenerative process. Studies have demonstrated that *Drosophila* cytokines, *Unpaireds* (*Upd*, *Upd2* and *Upd3*), which function as ligands of Jak-Stat signaling, are ectopically produced by damage stimulation, which activates Jak-Stat signaling in ISCs and EBs and therefore promotes ISC proliferation and EB differentiation to accomplish midgut regeneration (Jiang et al., 2009). *Upd* was reported to be expressed in midgut epithelial cells as well as in visceral muscle and *Upd3* was expressed in ECs (Buchon et al., 2009a; Jiang et al., 2009; Lin et al., 2009; Liu et al., 2010).

Jak-Stat signaling is also essential for EB differentiation under normal homeostatic development. ISC clones mutant for *dome, hop or stat* fail to produce mature differentiation cells ECs and ees, and are almost composed of ISCs and EBs (Beebe et al., 2009; Jiang et al., 2009; Liu et al., 2010). Studies also indicated that Jak-Stat signaling acted downstream of Notch signaling to regulate EB differentiation (Liu et al., 2010).

2.5 EGFR signaling mediates ISC proliferation

Like Jak-Stat signaling, EGFR signaling also plays crucial roles in midgut ISC proliferation in homeostatic conditions as well as in response to injury. Activation of EGFR signaling by overexpression of pathway ligands stimulates ISC division and produces hyperplasia phenotype while inactivation of EGFR signaling by mutation of many pathway components compromises ISC proliferation and maintenance (Biteau and Jasper, 2011; Buchon et al., 2010; Jiang et al., 2011; Xu et al., 2011). As with Jak-Stat signaling, after damage treatment of midgut, EGFR activity can be induced through ectopically enhanced expression of multiple EGFR pathway ligands such as Vein (Vn), Spitz and Keren, which are also essential for compensatory ISC proliferation and midgut regeneration (Buchon et al., 2010; Jiang et al., 2011). Vn is specifically expressed in visceral muscle and Spitz and Keren are produced by epithelial cells. These ligands act redundantly for EGFR activation (Biteau and Jasper, 2011; Buchon et al., 2010; Jiang et al., 2011; Xu et al., 2011). EGFR signaling and Jak-Stat signaling are dependent on each other to perform their functions. EGFR signaling is necessary for Jak-Stat signaling induced ISC proliferation and vice verse (Buchon et al., 2010; Jiang et al., 2011; Xu et al., 2011). EGFR signaling is also required for the proliferation of Notch mutation-induced ISC-like tumors (Xu et al., 2011).

2.6 Hpo signaling regulates ISC proliferation

Hpo signaling pathway, an evolutionarily conserved pathway implicated in organ size

control and tumorigenesis was also found to play an essential role in regulating ISC proliferation. Loss of Hpo signaling by either overexpression of downstream transcriptional coactivator Yki or inactivation of kinase Wts in ISCs and EBs or ECs stimulates ISC proliferation. Loss of Hpo signaling in ECs increases the production of cytokines of the Upd family and multiple EGFR ligands that activate Jak-Stat and EGFR signaling pathways in ISCs to stimulate their proliferation, thus revealing a novel non cell-autonomous role of Hpo signaling in blocking ISC proliferation (Cai et al., 2010; Karpowicz et al., 2010; Ren et al., 2010a; Staley and Irvine, 2010). The mechanism by which Hpo signaling mediates ISC proliferation in ISCs and EBs cells is still not clear. Moreover, Yki is required in ISCs and EBs for injury-induced ISC proliferation in response to tissue damaging reagents (Ren et al., 2010a; Shaw et al., 2010; Staley and Irvine, 2010).

2.7 JNK signaling regulates ISC proliferation in response to stress

In the adult midgut, *Drosophila* Jun N-terminal kinase (JNK) signaling pathway can be activated by certain types of damage such as oxidative stress or pathogenic infection, which results in ectopic expression of ligands of Jak-Stat and EGFR pathways and in turn activates these signaling pathways to stimulate compensatory ISC proliferation and midgut regeneration (Jiang et al., 2009). JNK signaling is activated in different cell types when midgut is exposed to various insults. For example, infection of midgut with *P. entomophila* or *aeruginosa*, which causes damage to ECs, leads to JNK signaling activation in ECs, while oxidative stress on

midgut stimulates JNK signaling in several types of epithelium cells including ISCs, EBs and ECs (Apidianakis et al., 2009; Biteau et al., 2008; Jiang et al., 2009). Activation of JNK signaling in ECs by either knocking down *puckered*(*puc*) or overexpressing an activated form of *hemioterous* (Hep^{Act}) can also lead to increased levels of ISC proliferation and midgut renewal (Jiang et al., 2009). Moreover, JNK signaling contributes to the failure of tissue homeostasis in aging midgut by promoting mis-differentiation of ISCs (Biteau et al., 2008).

2.8 ISC proliferation is responsive to cellular redox state

The levels of reactive oxygen species (ROS) are strictly regulated in stem cells to maintain stem cell properties and cellular redox state has been proposed to be important for stem cell behaviors (Kobayashi and Suda, 2011). As mentioned above, oxidative challenge on midgut can promote ISC proliferation, and study has found that a master regulator of the cellular redox state, Nrf2 is involved in controlling this process. Nrf2 is constitutively active in quiescent ISCs, which maintains ISCs in a low redox state and controls midgut homeostasis. When midgut is exposed to oxidative stress, Nrf2 is repressed by its negative regulator Keap1 leading to high ROS levels in ISCs to stimulate ISC proliferation. On the other hand, prolonged loss of Nrf2 in ISCs causes persistent high ROS levels, which can lead to uncontrolled ISC proliferation and disrupted homeostasis as well as age-related degeneration of midgut (Hochmuth et al., 2011).

3 *Myc* oncogene

3.1 Mammalian Myc

In mammals, the *myc* proto-oncogene family contains three primary members: *c-myc*, N-*myc* and L-*myc*, which exhibited differentiated patterns of expression. *c-myc* gene was observed to be expressed in a broad variety of tissues, while the expression of N- and L-*myc* was relatively limited . *Myc* encodes a transcription factor that influences a number of target genes to control a variety of cellular processes including cell proliferation, growth, apoptosis and metabolism. And Myc is frequently found to be overexpressed in many human and animal tumors (Eilers and Eisenman, 2008; Meyer and Penn, 2008)

Recently, the role of Myc in proliferation of stem/progenitor cells has been studied. During normal neurogenesis, N-Myc is essential for neuronal progenitor cell (NPC) proliferation as conditional deletion of N-*myc* in NPCs leads to reduced proliferation. (Knoepfler et al., 2002). Endogenous c-Myc is required for epidermal homeostasis since knocking down of c-myc in proliferative, basal keratinocytes results in premature differentiation (Watt et al., 2008; Zanet et al., 2005). Moreover, c-Myc-deficient HSCs fail to initiate differentaion because they are not able to be released from stem cell niche, therefore c-Myc maintains the balance of self-renewal and differentiation of hematopoietic stem cell (HSC) by mediating the interaction of HSCs with their niche (Dubois et al., 2008; He et al., 2008; Wilson et al., 2004). Loss of c-Myc in intestine stem cell-bearing cypts of adult mice leads to reduced cell sizes

and decreased cell numbers (Muncan et al., 2006). c-Myc is also crucial for self-renewal of murine ES cells in vitro (Cartwright et al., 2005; Kim et al., 2008) .

3.2 *Drosophila* Myc

A single homolog *Drosophila* Myc (dMyc) encoded by *diminutive* (*dm*) gene was identified in *Drosophila*, which makes it relatively easy to understand functions of Myc. Like its vertebrate counterparts, dMyc together with its partner dMax regulates expression of a large number of target genes (Hulf et al., 2005; Orian et al., 2003).

One of the most important roles of dMyc resides in its control of cellular growth and organism size. Flies carrying *dMyc* mutation exhibit reduced organ sizes with small cells while overexpression of dMyc promotes cellular growth. dMyc cell-autonomously regulates cell size by controlling expression of genes involved in ribosome biogenesis and therefore mediates ribosomal contents of cells (Grewal et al., 2005; Johnston et al., 1999). dMyc is also able to trigger cell competition and non-cell-autonomously induce cell apoptosis. dMyc induced cell competition occurs in female germ line stem cells in which stem cells with less dMyc are excluded from niche by neighboring wild type cells (Jin et al., 2008; Rhiner et al., 2009). Overexpression of dMyc accelerates passage through Gl phase but does not affect cell division rates as G2 phase is extended (Johnston et al., 1999). Moreover, dMyc is involved in imaginal disc regeneration after injury. In regenerating *Drosophila* wing imaginal discs, dMyc expression level is upregulated through a double repression

mechanism in which activated Wingless (Wg) signaling represses Notch, which otherwise would inhibit dMyc (Herranz et al., 2008; Smith-Bolton et al., 2009). The stability of dMyc is also subject to the regulation by Insulin signaling and TOR activity (Li et al., 2010). Besides, in wing imaginal discs, dMyc is transcriptionally upregulated by Yki/Sd, the Hpo pathway transcriptional complex and is necessary for Yki's activity to control target gene expression (Neto-Silva et al., 2010).

CHAPTER TWO

Hpo signaling regulates Yorkie nuclear localization and activity through 14-3-3 dependent and independent mechanisms

Introduction

Strict control of proper organ size is highly important during development, yet the underlying mechanisms of tightly controlling the growth of an organ in multicellular organism are still poorly understood. Although extrinsic stimulus, such as hormonal signals and nutrients supply, can influence tissue growth and organ size, the intrinsic mechanisms play essential roles in determining a proper organ size by coordinating cell growth, cell proliferation and cell death (Bryant and Simpson, 1984; Conlon and Raff, 1999). A number of tumor suppressors have been identified by genetic screens conducted in *Drosophila*, inactivation of which can cause overgrowth of imaginal disc derivatives (Hariharan and Bilder, 2006). Among which, several tumor suppressor genes, including *warts(wts)*(Justice et al., 1995; Xu et al., 1995), *salvador(sav)*(Tapon et al., 2002), *hpo*(also called *dMST*)(Harvey et al., 2003; Jia et al., 2003; Pantalacci et al., 2003; Udan et al., 2003; Wu et al., 2003), *fat*(Bennett and Harvey, 2006; Cho et al., 2006; Mahoney et al., 1991; Silva et al., 2006; Willecke et al., 2006), and *expanded* (*ex*)(Cho et al., 2006; Hamaratoglu et al., 2006; Maitra et al., 2006; Pellock et al., 2007), are defined to be included in a common tumor suppressor pathway, the so called Hpo pathway (Pan, 2007; Zhang et al., 2009a).

The core components of the Hpo pathway are four proteins, Hpo, Sav, Wts and Mats, which consist of the central kinase cascade. Hpo, the *Drosophila* homolog of mammalian Ste20 family kinases MST1 and MST2, associates with the WW-domain containing scaffolding protein Sav to phosphorylate and activate the downstream kinase Wts, which belongs to the Nuclear Dbf-2-related (NDR) kinase family (Harvey et al., 2003; Jia et al., 2003; Justice et al., 1995; Pantalacci et al., 2003; Tapon et al., 2002; Udan et al., 2003; Wu et al., 2003; Xu et al., 1995). The activated Wts interacts with a cofactor Mats, which is also phosphorylated by Hpo (Lai et al., 2005; Wei et al., 2007), to phosphorylate and inactivate a transcriptional coactivator Yki, the *Drosophila* homolog of Yap (Huang et al., 2005). The Hpo pathway regulates tissue growth and organ size by inhibiting cell growth and proliferation as well as by promoting apoptosis through modulating the expression of genes including *cyclin E*, *diap1* and *bantam* that are involved in these cellular processes (Harvey et al., 2003; Jia et al., 2003; Pantalacci et al., 2003; Tapon et al., 2002; Thompson and Cohen, 2006; Udan et al., 2003; Wu et al., 2003).

Recently, three groups have independently demonstrated that the TEAD/TEF family transcription factor Scalloped(Sd) is a transcription factor for the Hpo pathway. Sd both genetically and physically interacts with Yki and is essential for Yki's growth-promoting activity (Goulev et al., 2008; Wu et al., 2008; Zhang et al., 2008). Sd binds to the enhancer elements of *diap1* (Wu et al., 2008; Zhang et al., 2008). In addition, the association of Sd with Yki promotes Yki nuclear accumulation and

recruits Yki to the *diap1* promoter (Zhang et al., 2008). On the other hand, phosphorylation of Yki by upstream kinase cascade retains Yki in the cytoplasm (Dong et al., 2007; Oh and Irvine, 2008; Zhang et al., 2008). Serine168 of Yki is a critical site for both the activity and subcellular localization of Yki. When Ser168 is mutated to Ala, both Yki nuclear localization and ability to induce tissue overgrowth are increased. And Yki becomes more resistant to Hpo-mediated repression (Dong et al., 2007; Oh and Irvine, 2008; Zhang et al., 2008). This property of Yki is conserved in Yap and the corresponding Ser residue in Yap is Ser127 (Zhao et al., 2007). Phosphorylation of Yki/Yap at Ser168/Ser127 by Hpo signaling promotes their binding with 14-3-3 whereas YkiS168A/YapS127A fail to bind 14-3-3 (Dong et al., 2007; Oh and Irvine, 2008; Zhao et al., 2007). 14-3-3 often regulates subcellular localization including nuclear/cytoplasmic trafficking of its binding partern proteins, suggesting that phosphorylation of Yki/Yap at S168/S127 promotes 14-3-3 binding and therefore restricts their nuclear localization. However, there is still lack of direct genetic evidence for the involvement of 14-3-3 in the Hpo pathway and in regulating Yki /Yap activity.

14-3-3 proteins are highly conserved and ubiquitously expressed phosphor-S/T binding proteins, which can form homo- and hetero-dimers. 14-3-3 proteins participate in many signal transduction pathways through interacting with their binding partners and play important roles in many cellular processes (Mackintosh, 2004; Morrison, 2009). There are two isoforms of 14-3-3 in *Drosophila*, 14-3-3ε and

14-3-3ζ/ Leonardo that often function redundantly in many cellular processes (Acevedo et al., 2007; Benton and St Johnston, 2003). Here we show that both isoforms of 14-3-3 are involved in regulating Yki's activity through mediating its subcellular localization. And Yki can be regulated by both 14-3-3 dependent and independent mechanisms.

Results

Knockdown of 14-3-3 enhances Yki-induced overgrowth

To investigate whether 14-3-3 proteins affect Yki activity, *UAS-RNAi* trangenes of both isoforms of 14-3-3 were separately overexpressed in vivo to knockdown endogenous 14-3-3ε or 14-3-3ζ and examined whether reduction in 14-3-3 activity would modify Yki-induced overgrowth phenotype. *GMR-Gal4* driver was used to overexpress *UAS-Yki* posterior to the morphogenetic furrow (MF) (referred to as *GMR-Yki*) and enlarged eyes were exhibited (Fig. 2-1B, B') (Wu et al., 2008; Zhang et al., 2008). Coexpression of either *UAS-14-3-3ε-RNAi* or *UAS-14-3-3ζ-RNAi* transgene with *GMR-Yki* enhanced its eye overgrowth phenotype (Fig. 2-1C-D'), whereas knocking down either isoform of 14-3-3 alone with *GMR-Gal4* resulted in no visible eye phenotypes (data not shown).

To rule out the possibility that modification of the *GMR-Yki* phenotype by 14-3-3 RNAi was due to some off-target effects, we generated UAS transgenes that overexpress either 14-3-3ε or 14-3-3ζ. As shown in Fig.2-1, coexpression of *UAS-14-3-3ζ* but not *UAS-14-3-3ε* significantly blocked the enhancement of the

GMR-Yki phenotype caused by 14-3-3ε RNAi (Fig. 2-1E-F'). The suppression phenotype is similar for *14-3-3ζ* RNAi (Fig. 2-1G-H'). So the transgenic RNAi for each isoform did not significantly interfere with the expression of the other isoform and these two 14-3-3 isoforms regulate Yki activity in a redundant fashion.

Besides relying on RNAi methods, an alternative approach is to use loss-of-function mutations of 14-3-3 to reduce 14-3-3 activity. *14-3-3ε*^{j2B10} and *14-3-3ζ*^{A12BL} are strong loss-of-function alleles of *14-3-3ε* and *14-3-3ζ*, respectively (Flybase). Either *14-3-3ε*^{j2B10} or *14-3-3ζ*^{A12BL} heterozygosity could enhance the eye overgrowth phenotype of *GMR-Yki* (Fig. 2-1J-K'). The modification of the *GMR-Yki* phenotype by *14-3-3ε*^{j2B10} and *14-3-3ζ*^{A12BL} double heterozygosity was even more profound (Fig. 2-1L and L'). Taken together, all these observations above suggest that both 14-3-3 isoforms are involved in mediating Yki activity.

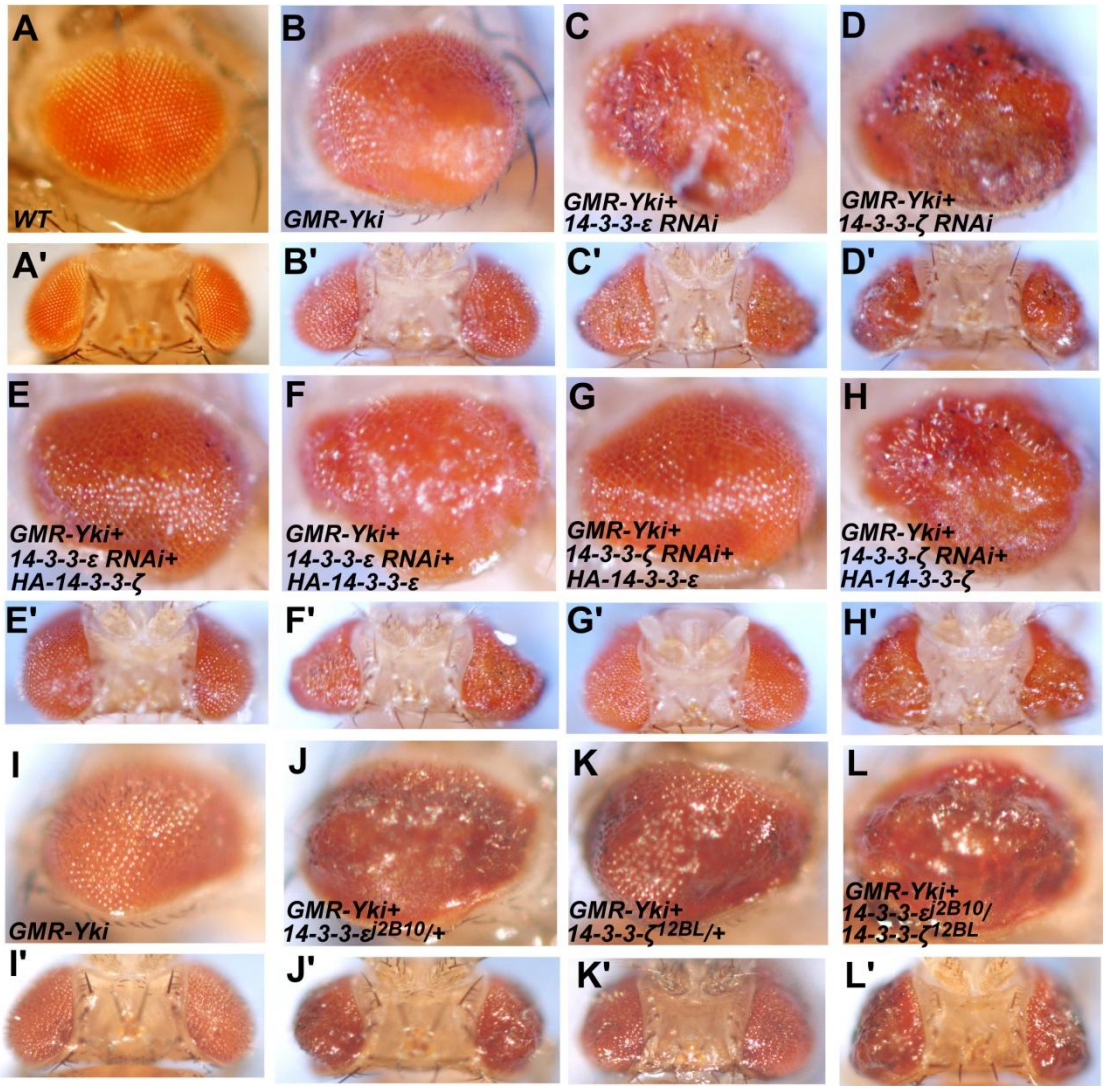

Figure 2-1. Loss of function of either 14-3-3 isoform can enhance Yki-induced eye overgrowth

(A-L') Side views (A-L) or dorsal views (A'-L') of adult eyes of *GMR-Gal4* (A and A'), *GMR-Gal4 UAS-Yki* (B and B'), *GMR-Gal4 UAS-Yki*; *UAS-14-3-3ε RNAi* (C and C'), *GMR-Gal4 UAS-Yki*; *UAS-14-3-3ζ RNAi* (D and D'), *GMR-Gal4 UAS-Yki*; *UAS-14-3-3ε RNAi*/ *UAS-HA-14-3-3ζ* (E and E'), *GMR-Gal4 UAS-Yki*; *UAS-14-3-3 ε RNAi*/*UAS-HA-14-3-3 ε* (F and F'), *GMR-Gal4 UAS-Yki*; *UAS-14-3-3 ζ RNAi*/*UAS-HA-14-3-3 ε* (G and G'), *GMR-Gal4 UAS-Yki*; *UAS-14-3-3 ζ RNAi*/*UAS-HA-14-3-3ζ* (H and H'), *GMR-Gal4 UAS-Yki* (I and I'), *GMR-Gal4*

UAS-Yki; *14-3-3ε* ^{j2B10}/+ (J and J'), *GMR-Gal4 UAS-Yki*; *14-3-3ζ* ^{12BL}/+ (K and K') and *GMR-Gal4 UAS-Yki*; *14-3-3ε* ^{j2B10}/+ *14-3-3ζ* ^{12BL}/+ (L and L'). Reduction of 14-3-3 either by RNAi knockdown or loss of function mutations enhanced the overgrown phenotype caused by Yki overexpression.

Overexpression of 14-3-3 suppresses Yki-induced overgrowth

Since reduction of 14-3-3 activity could enhance the *GMR-Yki* eye phenotype, the next question was whether increased 14-3-3 activity would have the opposite effects. Two copies of either *UAS-14-3-3ε*, *UAS-14-3-3ζ* or both were coexpressed with *UAS-Yki* under the control of the *GMR-Gal4* driver and eye phenotypes were observed. A dose dependent suppression of the *GMR-Yki* phenotype by 14-3-3 overexpression was clearly shown in Figure 2-2B-E'. Thus, gain of function study further demonstrated that both 14-3-3 isoforms can restrict Yki activity.

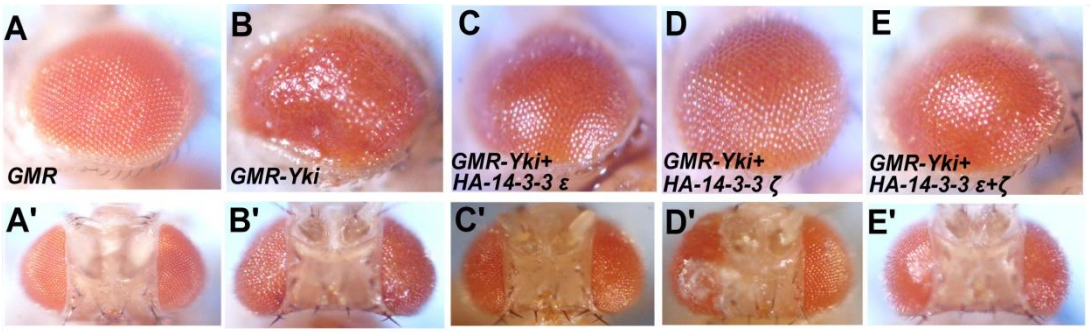

Figure 2-2. Overexpression of either 14-3-3 isoform can suppress Yki-induced eye overgrowth

(A-E) Side views (A-E) or dorsal views (A'-E') of adult eyes of *GMR-Gal4* (A and A'), *GMR-Gal4 UAS-Yki* (B and B'), *GMR-Gal4 UAS-Yki*; *UAS-HA-14-3-3ε* (C and C'), *GMR-Gal4 UAS-Yki*; *UAS-HA-14-3-3ζ* (D and D') and *GMR-Gal4 UAS-Yki*; *UAS-HA-14-3-3ε* + *UAS-HA-14-3-3ζ* (E and E').

Loss of 14-3-3 results in nuclear accumulation of Yki

To investigate whether 14-3-3 affects Yki activity by influencing Yki subcellular localization, we overexpressed both 14-3-3ε and 14-3-3ζ RNAi transgenes in wing discs under the control of the wing specific Gal4 driver *MS1096* and observed the subcellular localization of endogenous Yki. As 14-3-3 proteins are important for many cellular processes, we coexpressed a cell death inhibitor P35 (Hay et al., 1994) together with 14-3-3 RNAi transgenes to prevent possible cell lethality due to complete loss of 14-3-3 activity. Consistent with previous observations (Dong et al., 2007; Oh and Irvine, 2008), the endogenous Yki was predominantly localized in the cytoplasm in wild type wing discs (Fig. 2-3A and B). In wings discs of which both 14-3-3 isoforms were knocked down, endogenous Yki was translocated to the nucleus (Fig. 2-3C-D”), particularly in the dorsal compartment cells where *MS1096-gal4* expression is higher. These results demonstrated that 14-3-3 can prevent nuclear accumulation of Yki.

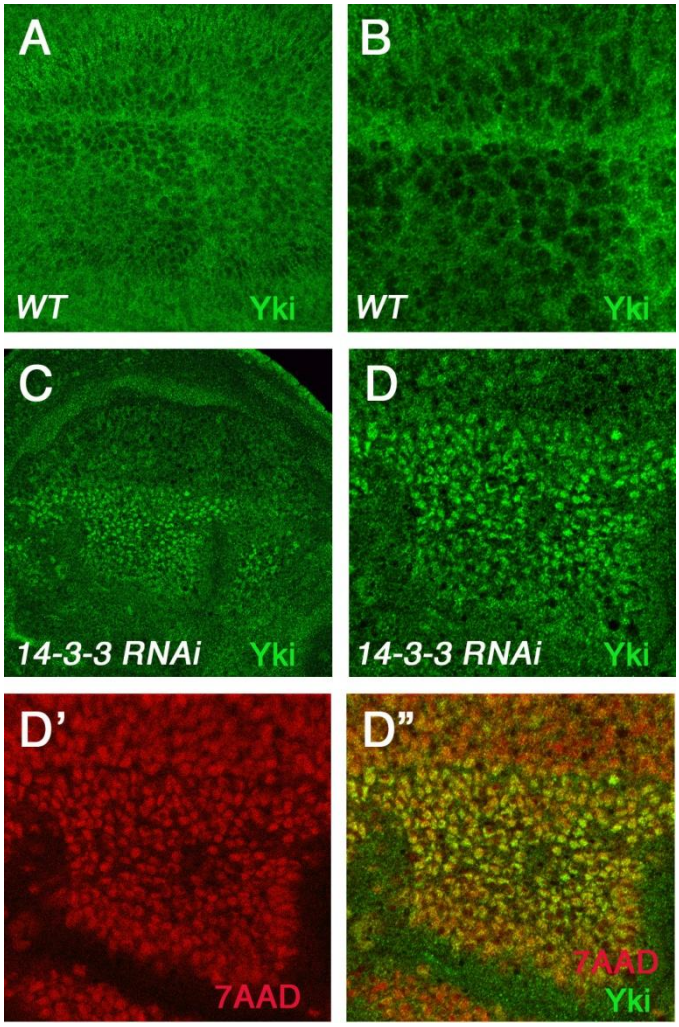

Figure 2-3. Loss of 14-3-3 results in nuclear accumulation of Yki

(A and B) Low (A) and high (B) magnification view of a wild type wing disc immunostained with an anti-Yki (green) antibody. Yki is primarily localized in the cytoplasm.

(C-D") Low (C) and high (D-D") magnification view of a wing disc expressing both *UAS-14-3-3ε RNAi* and *UAS-14-3-3ζ RNAi* together with *UAS-P35* using *MS1096* and immunostained with anti-Yki antibody (green in C, D and D") and 7-AAD (red in D' and D") to label the nuclei. The wing disc is oriented with anterior to the left and ventral up. Severe knockdown of both 14-3-3 isoforms in the dorsal region of the wing pouch resulted in nuclear accumulation of endogenous Yki.

Yki activity is regulated by multiple phosphorylation events

Studies on Yap have found that multiple phosphorylation sites that fall into the consensus sequence: HXRXXS contributed to Yap activity (Hao et al., 2008; Zhao et al., 2007). In Yki, three serine sites (Ser111, Ser168 and Ser250) fall into the same consensus (Fig. 2-6A). Previously, we demonstrated that Hpo signaling inhibits Yki activity partially through phosphorylating at Ser168 and restricts its nuclear localization (Dong et al., 2007; Oh and Irvine, 2008; Zhang et al., 2008). To determine whether phosphorylation at Ser111 and Ser250 also modulates Yki activity, Ser to Ala mutations were made in either wild type background ($Yki_{S111,250A}$) or in the background where Ser168 was also mutated into Ala (Yki_{3SA}). Their activities were then compared with those of wild type Yki and Yki_{S168A}. Because gene expression levels can be influenced by chromosomal position where it was inserted, the *phiC31* integration system was used to insert transformants for Myc-tagged wild type Yki (Myc-Yki) or Yki variants (Myc-Yki_{S168A}, Myc-$Yki_{S111, 250A}$ and Myc-Yki_{3SA}) on the same chromosome postion to ensure all the transgenes were expressed at the same level (Bischof et al., 2007). The *GMR-Gal4* driver was used to express all the transgenes posterior to the MF and adult eyes were observed. Consistent with our previous observation (Zhang et al., 2008), *GMR-Myc-Yki* induced only modest eye overgrowth (Fig. 2-4A, A'). In contrast, *GMR-Myc-*Yki_{S168A} showed dramatic eye overgrowth (Fig. 2-4B, B'). Interestingly, *GMR-Myc-*$Yki_{S111,250A}$ also exhibited enlarged eyes although the phenotype was weaker than that of *GMR-Myc-*Yki_{S168A} but stronger than that of *GMR-Myc-Yki* (Fig. 2-4C, C'). Finally, *GMR-Myc-*Yki_{3SA} resulted

in strongest eye overgrowth, which was more potent than that caused by *GMR-Myc-Yki$_{S168A}$* (Fig. 2-4D, D'). Since all the transgenes were expected to express at similar levels, the difference in the overgrowth phenotypes induced by different forms of Yki was due to the difference in their activities.

To further compare the relative contribution of multiple phosporylation sites to Yki activity, the *sd-luciferase* reporter assay was used to directly measure the activities of Yki variants (Zhang et al., 2008). *In vitro* luciferase results showed that Yki variants exhibited higher activity than the wild type Yki with the order of Yki_{3SA} > Yki_{S168A} > $Yki_{S111,250A}$ >Yki_{WT} (Fig. 2-4E), consistent with the *in vivo* activities. In addition, Yki variants were more resistant to Hpo-mediated inhibition (Fig. 2-4E). Taken together, these results demonstrated that, besides Ser168, Ser111 and Ser250 are also important phosphorylation sites for Yki activity, although playing a lesser extent role than Ser168.

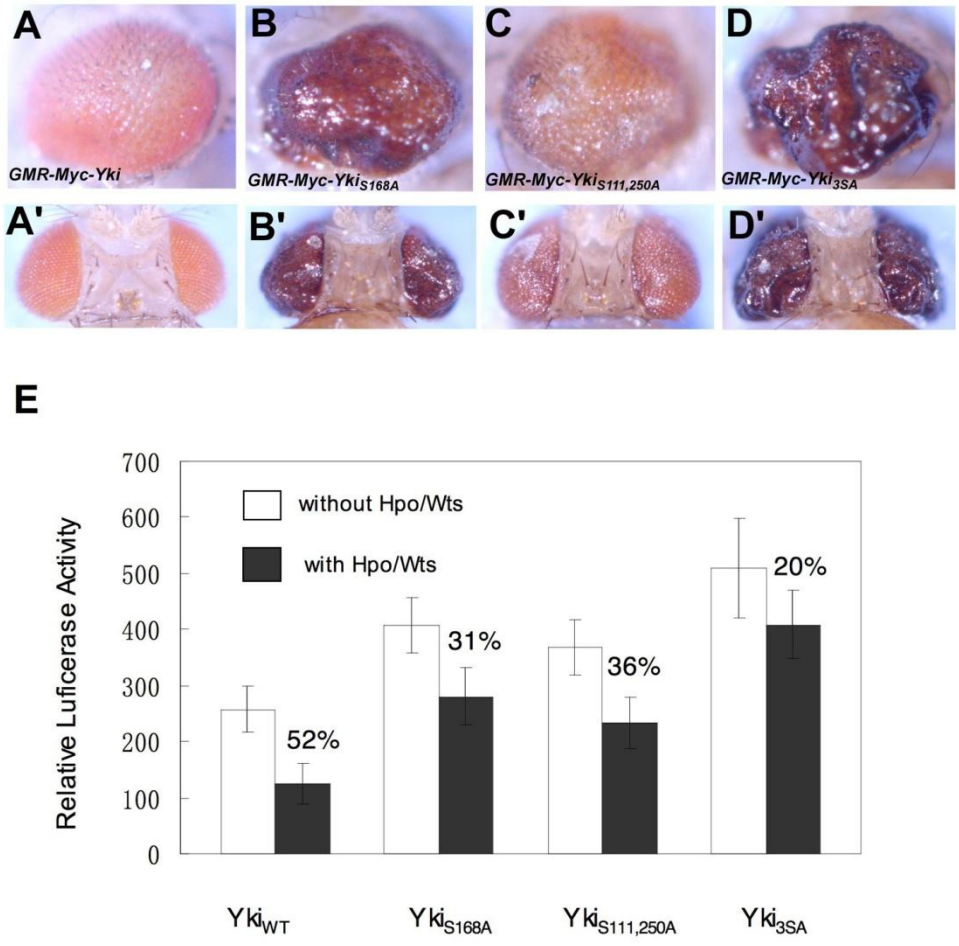

Figure 2-4. Multiple phosphorylation events contribute to Yki regulation

(A-D') Side views (A-D) or dorsal views (A'-D') of adult eyes expressing *UAS-Myc-Yki* (A and A'), *UAS-Myc-Yki$_{S168A}$* (B and B'), *UAS-Myc-Yki$_{S111,250A}$* (C and C') or *UAS-Myc-Yki$_{3SA}$* (D and D') with *GMR-Gal4*.

(E) S2 cells were transfected with the indicated Yki constructs together with Sd and an *Sd-luciferase* reporter gene, with or without Hpo and Wts expressing constructs. Cell lysates were subjected to dual luciferase assay. Error bars indicate standard deviation (triplicate wells). Numbers indicate degrees of suppression of Yki/Sd activity by Hpo/Wts.

Regulation of Yki subcellular localization by phosphorylation at S111 and S250

It has been shown that Hpo signaling inhibits Yki nuclear localization by phosphorylating Yki at S168. To determine whether phosphorylation at S111 and S250 mediates Yki activity by regulating its cytoplasm/nuclear localization, subcellular localization of Myc-Yki$_{S111,250A}$ were examined in S2 cells and wing disc cells. *HA-Sd* were coexpressed to facilitate Yki nuclear localization (Zhang et al., 2008). As shown in Figure 2-5, Myc-Yki$_{WT}$ exhibited significant nuclear signal when coexpressed with HA-Sd but was excluded from the nucleus in the presence of Flag-Hpo coexpression (Fig. 2-5A, A'). Myc-Yki$_{S168A}$ exhibited more nuclear signal than Myc-Yki$_{WT}$ when HA-Sd was coexpressed and a significant portion of Myc-Yki$_{S168A}$ remained in the nucleus when Flag-Hpo was coexpressed (Fig. 2-5B, B'). Myc-Yki$_{S111,250A}$ also exhibited higher levels of nuclear signal than Myc-Yki$_{WT}$ but lower than those of Myc-Yki$_{S168A}$ and nuclear Yki$_{S111,250A}$ signal was readily detectable when in the presence of Flag-Hpo (Fig. 2-5C, C'). Finally, Myc-Yki$_{3SA}$ was predominantly localized in the nucleus together with HA-Sd and the majority of Myc-Yki$_{3SA}$ remained in the nucleus even when Flag-Hpo was coexpressed (Fig. 2-5D, D').

A similar trend of nuclear localization of Yki variants in wing imaginal discs was also observed. In the presence of HA-Sd, Myc-Yki$_{S111,250A}$ exhibited higher level of nuclear signal compared with Myc-Yki$_{WT}$ (cf. Fig. 2-5G with 2-5E) whereas majority of Myc-Yki$_{S168A}$ and Myc-Yki$_{3SA}$ were localized in the nucleus (Fig. 2-5F, H). Therefore,

phosphorylating at S111 and S250 also restricted Yki nuclear localization, albeit the effect is less dramatic compared with S168.

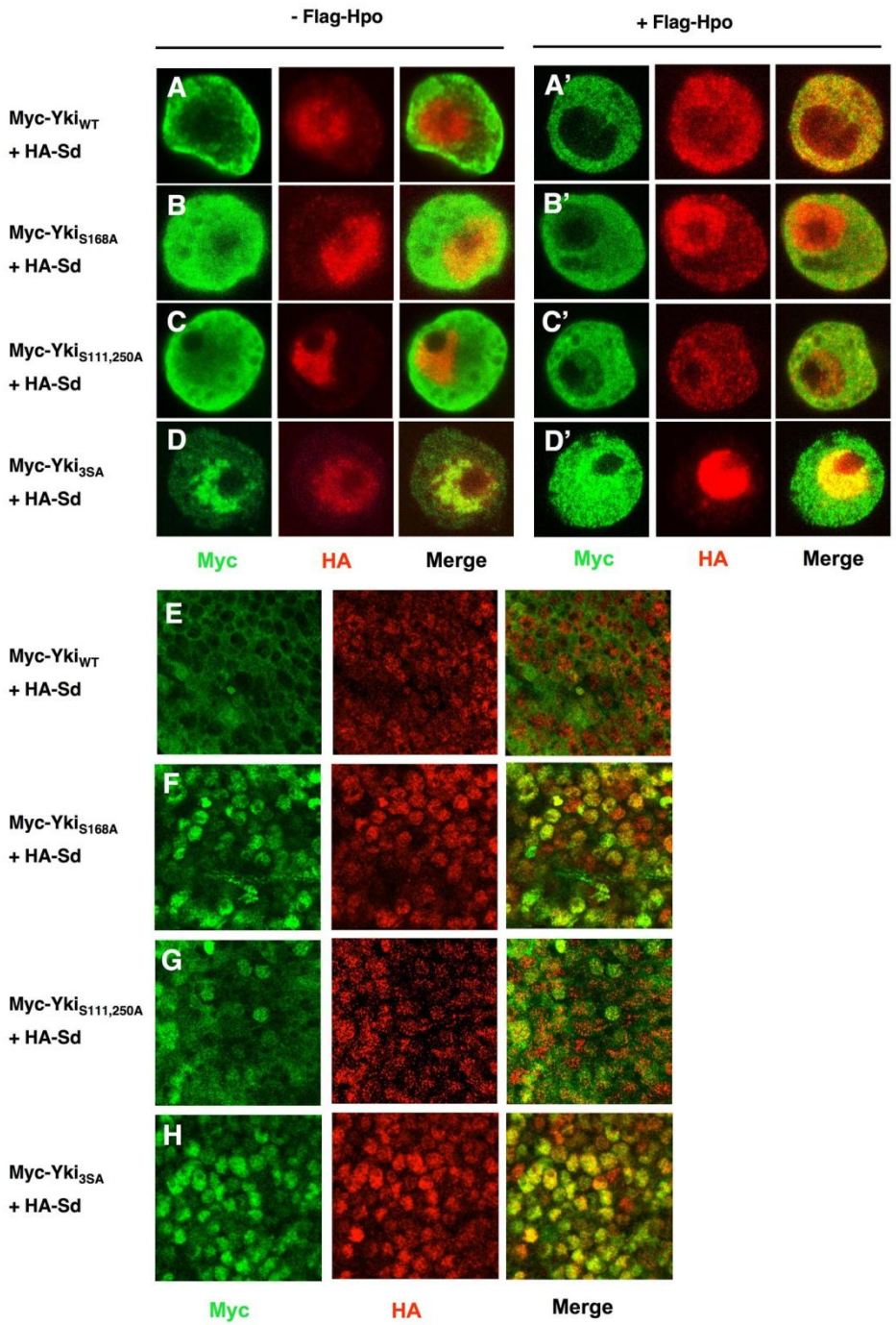

Figure 2-5. Phosphorylation at S111 and S250 restricts Yki nuclear localization

(A-D') S2 cells expressing HA-Sd together with Myc-Yki$_{WT}$ (A and A'), Myc-Yki$_{S168A}$ (B and B'), Myc-Yki$_{S111,250A}$ (C and C') or Myc-Yki$_{3SA}$ (D and D') with (A'-D') or without Fg-Hpo (A-D) were immunostained with anti-Myc (green) and anti-HA (red) antibodies.

(E-H) High-magnification views of wing discs expressing HA-Sd and Myc- Yki_{WT} (E), Myc-Yki_{S168A}(F), Myc-$Yki_{S111,250A}$(G) or Myc-Yki_{3SA} (H) using *MS1096* and immunostained with anti-Myc (green) and anti-HA (red) antibodies.

Regulation of Yki subcellular localization by 14-3-3 is mediated by phosphorylation at S168 but is independent of phosphorylation at Ser111 and S250

Phosphorylation at YkiS168/Yap127 generates a consensus 14-3-3 binding site that meets the requirement of $RXXS_pXP$ sequence (Dong et al., 2007; Oh and Irvine, 2009; Zhao et al., 2007). In contrast, phosphorylation at S111 or S250 fails to generate a 14-3-3 binding consensus site because of the lack of a critical Pro residue C-terminal to the phospho-Ser residue (Fig. 2-6A), suggesting that regulation of Yki by these two phosphorylation sites might be 14-3-3 independent. To confirm the hypothesis, co-immunoprecipitation experiments were performed to examine the interaction between 14-3-3 and various Yki mutants. As shown in Fig. 2-6B, HA-14-3-3 could interact with Myc-Yki_{WT} and coexpression of Hpo/Wts/Mats complex enhanced the interaction (Fig. 2-6B, lanes 1 and 5). Mutating S111 and S250 to Ala had little effect on either the basal or Hpo signaling-induced 14-3-3 binding of Yki (Fig. 2-6B, lanes 3 and 7). In contrast, when S168 was mutated to Ala, both the basal and Hpo signaling-induced 14-3-3 binding of Yki were completely blocked (Fig. 2-6B, lanes 2 and 6), implying that phosphorylation at S168 but not at S111 and S250 promotes 14-3-3 binding. Consistent with co-immunoprecipitation results, we observed that coexpression of 14-3-3 could further enhance Hpo-mediated inhibition of Myc-$Yki_{S111,250A}$ nuclear localization but not that of Myc-Yki_{S168A} in S2 cells (Fig. 2-6C). Thus, phosphorylation at S111 and S250 is not contributing to Yki/14-3-3 interaction and 14-3-3 role in inhibiting Yki nuclear localization.

We also observed similar phenotypes in adult eyes. Overexpreesion of 14-3-3 further enhanced the inhibition of the Hpo kinase domain (HpoN) on the overgrowth phenotypes caused by overexpression of Yki or $Yki_{S111,250A}$ but not that of Yki_{S168A} or Yki_{3SA} (Fig. 2-6D-G"). These observations confirm that 14-3-3 regulates Yki activity through phosphorylation at S168 whereas phosphorylation at S111 and S250 regulates Yki activity independent of 14-3-3.

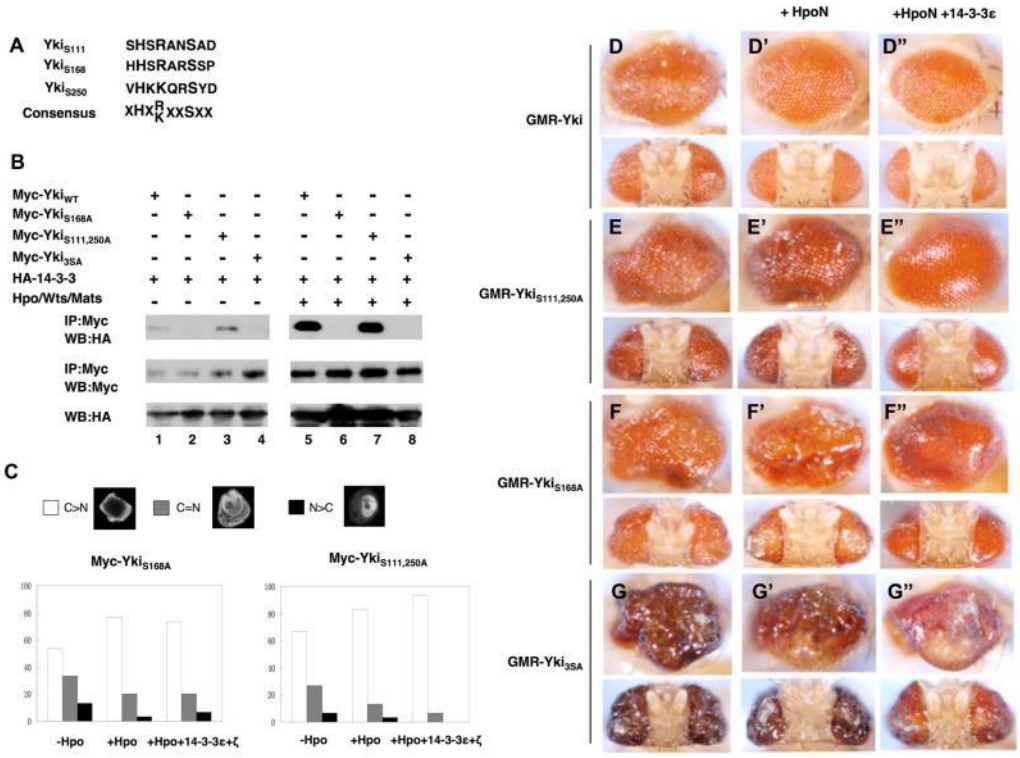

Figure 2-6. Phosphorylation at S111 and S250 regulates Yki independent of 14-3-3

(A) The alignment of Yki sequences surrounding S111, S168 or S250 with the consensus sequence for Wts phosphorylation site indicated underneath. X: any amino acid.

(B) Mutating S111 and S250 does not affect 14-3-3 binding but mutating S168 abolishes 14-3-3 binding. S2 cells were transfected with indicated Myc-tagged wild type and mutant Yki constructs and HA-14-3-3 constructs with or without Hpo/Wts/Mats coexpression, followed by immunoprecipitation and western blot analyses with indicated antibodies.

(C) 14-3-3 regulates nuclear localization of $Yki_{S111, 250A}$ but not Yki_{S168A}. S2 cells were transfected with HA-SD, Myc-$Yki_{S111, 250A}$ or Myc-Yki_{S168A}, without or with coexpression of Hpo or Hpo plus 14-3-3. Cells were immunostained with Myc and

HA antibodies, and the subcellular localization of Myc-Yki$_{S111, 250A}$ or Myc-Yki$_{S168A}$ was monitored using confocal microscopy. Cells with different nucleocytoplasmic distributions of Myc tagged Yki were counted. A total of 100 cells were counted for each Yki construct. C>N: cells contain higher levels of Yki in the cytoplasm than in the nucleus. C=N: cells contain Yki equally distributed in cytoplasm and nucleus. N>C: cells contain higher levels of Yki in the nucleus than in the cytoplasm. The *y* axis indicates the percentage of cells in each category.

(D-G") Side (top) or (bottom) dorsal views of adult eyes expressing *GMR-Gal4 UAS-Yki* (D), *GMR-Gal4 UAS-Yki*$_{S111, 250A}$ (E), *GMR-Gal4 UAS-Yki*$_{S168A}$ (F) or *GMR-Gal4 UAS-Yki*$_{3SA}$ (G). HpoN (D'-G') or HpoN and 14-3-3 (D"-G") were coexpressed with the wild type or mutant Yki as indicated.

Discussion

The Hpo pathway is an evolutionarily conserved and functionally important pathway that regulates cell growth, cell proliferation and cell apoptosis. The transcriptional coactivator Yki/Yap is the critical downstream transcriptional regulator of the Hpo pathway, whose regulation determines the activity of this pathway (Pan, 2007; Zhang et al., 2009b). It has been demonstrated Yki activity largely depends on its phosporylation at Ser168 and subcellular localization, and the observation that the Yki can interact with 14-3-3 upon phosporylation at Ser168 suggests that 14-3-3 would be involved in regulating the nuclear localization and activity of Yki. Here we provide genetic evidence that 14-3-3 is an important tether protein for the cytoplasmic retention of Yki and restricts Yki activity.

First, both loss of function and gain of function studies suggest that both 14-3-3 isoforms participate in restricting Yki activity. Although either reducing or increasing 14-3-3 activity alone in wild type background resulted in no obvious eye phenotypes, knockdown of either 14-3-3ε or 14-3-3ζ enhanced the overgrowth phenotype induced by *GMR-Yki* and overexpressing either 14-3-3ε or 14-3-3ζ suppressed *GMR-Yki* phenotype. Second, the comparison of subcellular localization of endogenous Yki between wild type and 14-3-3 knocked-down wing imaginal discs confirms that the cytoplasmic retention of Yki is largely dependent on 14-3-3, suggesting that 14-3-3 inhibits Yki activity by restricting its nuclear localization.

Studies have proven that Hpo signaling restricts Yki nuclear localization and activity by phosphorylating Yki at S168, which is equivalent to S127 of Yap. Here we found that Yki has two additional phosphorylation sites Ser111 and Ser250 which also participate in regulating Yki subcellular localization and activity, although these two sites appear to be less potent than Ser168.

Consistent with the observation that Yki Ser168 but not Ser111 or Ser250 conforms to consensus 14-3-3 binding sites, we found that physical association of 14-3-3 with Yki depends on phosphorylation at Ser168 but not Ser111 or Ser250. Genetically, RNAi knockdown or overexpression of 14-3-3 did not significantly modulate the overgrowth phenotype caused by *GMR-Yki*$_{S168A}$ but could modify *GMR-Yki*$_{S111,250A}$ induced eye overgrowth phenotype. Moreover, the subcellular localization of Yki$_{S168A}$ was not influenced by overexpression of 14-3-3 but nuclear localization of Yki$_{S111,250A}$ was sensitive to 14-3-3 activity. Both the biochemical and genetic data suggest that regulation of Yki nuclear localization and activity by 14-3-3 is dependent on phosphorylation at Ser168 but independent of Ser111 or Ser250. It is possible that phosphorylation at Ser111 and Ser250 modulates Yki activity through other mechanisms or there might be other anchor proteins besides 14-3-3 that can restrict Yki nuclear localization after phosphorylation at these two serine sites.

CHAPTER THREE

Hpo signaling regulates Drosophila intestine stem cell proliferation through multiple pathways

Introduction

Most adult tissues depend on the proliferation of stem cells to maintain tissue homeostasis and repair tissue damage throughout adult life. The replenishment of lost differentiated cells by newly produced progenies of stem cells is under tight control to achieve normal tissue development and disruption of the regulatory mechanisms could lead to excessive proliferation of stem cells, which eventually results in tumor formation.

Recently, the *Drosophila* adult midgut has become an ideal model to understand how stem cell proliferation and differentiation are regulated not only because the cell lineage in this tissue is simple and well characterized but also because it bears structural and functional similarities to the mammalian intestine. Previous studies have identified intestinal stem cells (ISCs) in *Drosophila* adult midgut which are located adjacent to the basement membrane of midgut epithelium (Micchelli and Perrimon, 2006; Ohlstein and Spradling, 2006). The asymmetric division of an ISC produces two daughter cells: one remains as an ISC, and the other one loses stem cell properties and converts to an enteroblast (EB) which can undergo differentiation to become either absorptive enterocytes (EC) or secretary enteroendocrine (ee)(Ohlstein

and Spradling, 2006). Several important signaling pathways including Notch, Wingless, Jak-Stat and EGFR pathways are reported to regulate the proliferation and differentiation of ISCs (Lee et al., 2009; Lin et al., 2008; Ohlstein and Spradling, 2006). Moreover, it has been reported that tissue damage can induce ISC proliferation and differentiation to replenish dead cells, and the Jak-Stat and Insulin pathways are involved in this process (Amcheslavsky et al., 2009). However, the mechanisms underlying the regulation of ISC proliferation are still not fully understood, and it is highly possible that additional pathways participate in the regulation of ISC proliferation and differentiation.

The Hpo (Hpo) signaling pathway is a recently identified conserved tumor suppressor pathway that controls cell growth, proliferation and apoptosis, and deregulation of pathway activity can lead to several types of cancer. The core components of the Hpo pathway are four proteins, Hpo, Sav, Wts and Mats, which consists of the central kinase cascade. Upon pathway activation, the upstream kinase Hpo phosphorylates and activates downstream kinase Warts (Wts) to restrict the activity of the transcriptional coactivator Yorkie (Yki). When Hpo signaling is inactivated, Yki is released into the nucleus and forms a complex with the TEAD family of transcription factor Scalloped (Sd) to promote transcription of target genes involved in cell proliferation, cell growth and cell apoptosis (Pan, 2007, 2010).

Previous studies on the Hpo pathway were mainly focused on its function in

Drosophila appendage development, while its involvement in stem cell proliferation and tissue regeneration was not extensively investigated. In this study, we provided evidence that the Hpo pathway participates in regulating ISC proliferation in the adult midgut. We found out that the Hpo pathway negatively regulated ISC proliferation by both cell-autonomous and non-cell-autonomous mechanisms. Hpo signaling could act in ECs to restrict the production of cytokines and mitogens and thus reduce the activities of Jak-Stat and EGFR pathways, leading to suppressed ISC proliferation. In addition, Yki is required in the precursor cells for dextran sulfate sodium (DSS)-induced ISC proliferation.

Results

Loss of Hpo signaling in precursor cells stimulates ISC proliferation

Initially, to investigate the involvement of Hpo signaling in ISC proliferation in *Drosophila* adult midgut, a Wts transgenic RNAi line (*UAS-Wts-RNAi*) together with *UAS-GFP* was overexpressed in ISCs and EBs (collectively referred to as precursor cells; Fig. 3-1J) using a specific *esg-Gal4* driver to inactivate Hpo signaling in precursor cells. Compared with *UAS-GFP* expression alone, knocking down Wts led to more esg-GFP^{+} cells (compare Fig. 3-1B with 3-1A), implying an increase in the number of the precursor cell population. When *UAS-Yki-RNAi* or *UAS-Sd-RNAi* transgene was coexpressed with *UAS-Wts-RNAi*, the ectopically increased number of esg-GFP^{+} cells was blocked (Fig.3-1C-D), suggesting that Wts limits the precursor cell population through inhibiting Yki/Sd transcriptional complex. The expansion of

precursor cell population could be due to either an increase in ISC proliferation or a reduction in EB differentiation. To distinguish the two possibilities, a specific marker for mitotic cells, Phospho-Histone3 (PH3) was used to label dividing ISCs. Immunostaining with an antibody against PH3 indicates that *esg-Wts-RNAi* midguts contained more $PH3^+$ cells, which was suppressed by overexpressing *Yki-RNAi* or *Sd-RNAi* (Fig. 3-1B'-D', H). Overexpressing *UAS-Yki* transgene in precursor cells also increased the number of esg-GFP^+ and $PH3^+$ cells (Fig. 3-1E, E', H), and such increase was inhibited by knocking down of Sd (Fig.3-1H). Studies in adult midgut all suggest that ISC is the only cell population that can divide (Micchelli and Perrimon, 2006; Ohlstein and Spradling, 2006). To confirm that the ectopically induced $PH3^+$ cells in Wts RNAi or Yki overexpression midguts are ISCs, co-immunostaining with Dl antibody, which is a specific marker for ISCs (Ohlstein and Spradling, 2007), indicated that $PH3^+$ cells were all Dl^+ (Fig. 3-1F-G). Taken together, these results demonstrate that Wts functions in precursor cells to inhibit ISC proliferation by restricting the activity of Yki/Sd.

Since *esg-Gal4* is also expressed in adult midgut progenitor cells (AMPs) during larva stage, the ectopically induced ISC proliferation by reduced Hpo signaling observed above would be due to its effect on both larva and adult development. To confirm that perturbation of Hpo signaling at the adult stage is sufficient to affect ISC proliferation, temperature sensitive *tublin-Gal80* was used together with *esg-Gal4* to temporarily control target gene expression (referred to as esg^{ts}) (McGuire et al., 2004). 14 days

after transferring adult flies from 18^0C to 29^0C, *esg^{ts}-Wts-RNAi* and *esg^{ts}-Yki* adult midguts exhibited a dramatic increase in the number of esg-GFP$^+$ and PH3$^+$ cells (Fig. 3-1I, data not shown). Furthermore, Wts RNAi induced increase was suppressed by knockding down of Yki (Fig. 3-1I). All the results above demonstrate that Hpo signaling is involved in the adult midgut precursor cells to inhibit ISC proliferation.

Hpo pathway mutant clones exhibit elevated proliferation and produce differentiated cells

To further address the role of other components of Hpo signaling pathway in ISCs, we generated positively marked homozygous mutant clones in the ISC cell lineage using the MARCM system for several Hpo pathway components including *hpo* or *wts* (Lee and Luo, 2001). We then compared the clone size (measured by the number of GFP$^+$ cells per clone) between the control clones and mutant clones. After clone induction at 37^0C for 1 hour, the adult flies were grown at 18^0C for another 5 days. Then the flies were dissected and clone size was examined. The control clones normally contained 2-3 cells per clone, however, *hpo* or *wts* mutant clones contained 5-7 cells per clone (Fig. 3-1K-K", data not shown). Co-expressing *Yki-RNAi* in mutant clones reduced clone size to 2-3 cells per clone (Fig. 3-1M-M", data not shown). The increased clone size associated with Hpo pathway mutant clones suggests that Hpo signaling is required to restrict ISC growth and proliferation during adult midgut homeostasis.

Then we stained *hpo* or *wts* mutant clones with markers of different cell types.

Immunostaining with Dl revealed that there was often one Dl^{+} cell per isolated *hpo* or *wts* mutant clone (Fig. 3-1K-K", data not shown), indicating that Hpo pathway mutant ISCs can self-renew. Anti-Pdm1 and anti-Prospero (Pro) antibodies were used to label EC and ee respectively (Fig. 3-1J) (Lee et al., 2009; Micchelli and Perrimon, 2006; Ohlstein and Spradling, 2006). Pdm1 and Pro staining indicated that *hpo* or *wts* mutant clones contained Pdm1 and Pro positive cells (Fig. 3-1L-L", N-N", data not shown), implying that Hpo pathway mutant clones can still produce differentiated cells. Taken together, loss of Hpo signaling promotes ISCs proliferation, but it does not block ISC lineage differentiation.

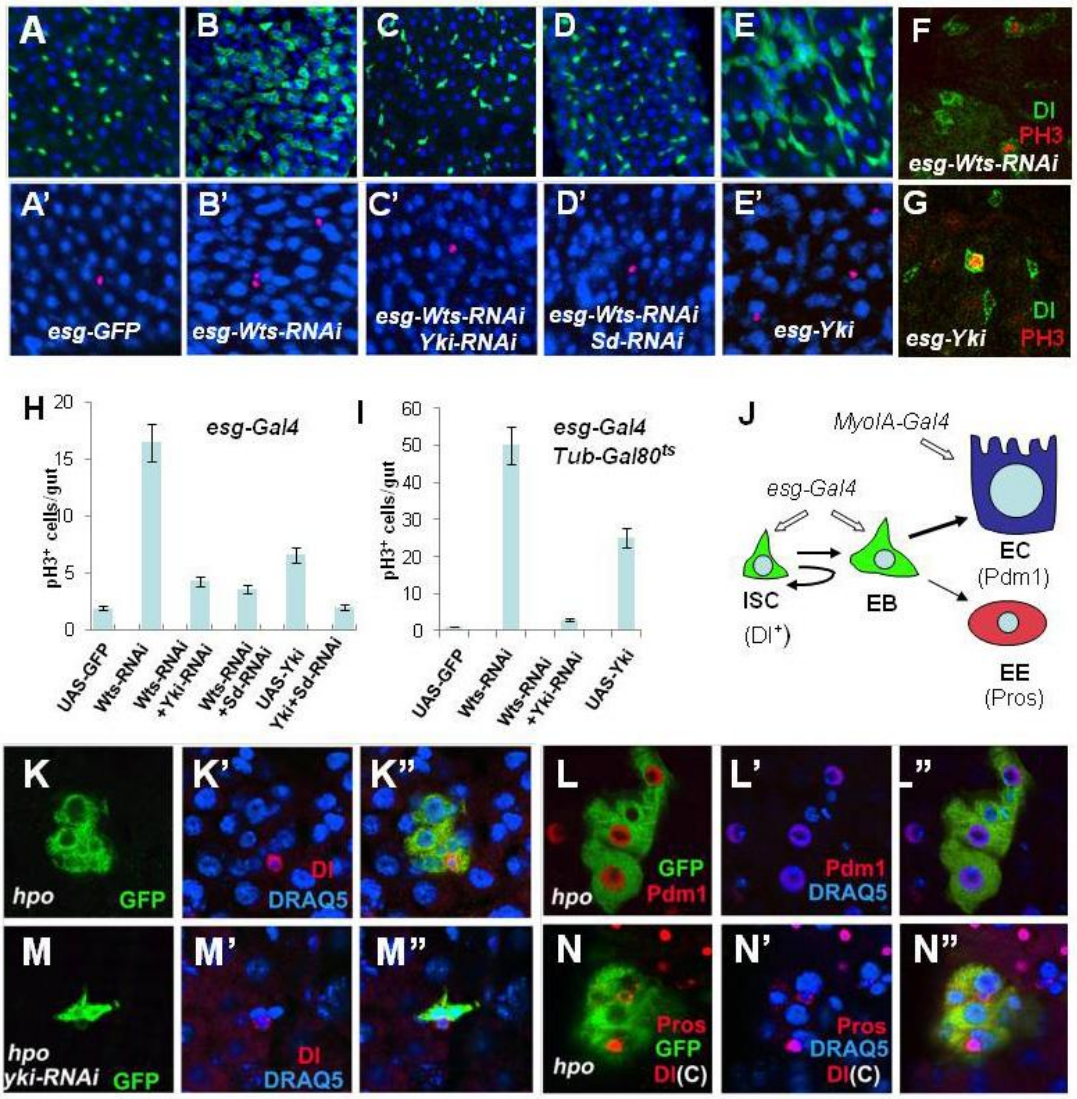

Figure 3-1. Hpo signaling restricts ISC proliferation by inhibiting Yki/Sd

(A-E') Three to five days old adult midguts expressing *esg-Gal4/UAS-GFP* without (A-A') or with *UAS-Wts-RNAi* (B-B'), *UAS-Wts-RNAi* + *UAS-Yki-RNAi* (C-C'), *UAS-Wts-RNAi* + *UAS-Sd-RNAi* (D-D') or *UAS-Yki* (E-E') were immunostained with GFP (green) and PH3 (red) antibodies, and a nuclear dye DRAQ5 (blue).

(F-G) Adult midguts expressing *esg-Wts-RNAi* (F) or *esg -Yki* (G) were immunostained with Dl (green) and PH3 (red) antibodies.

(H-I) Quantification of $PH3^+$ cells in midguts from adults of the indicated genotypes (mean ± s.d., n=15 for each genotype).

(J) The ISC lineage in *Drosophila* adult midguts. ISC: intestine stem cell; EB: enteroblast; EC: enterocyte; EE: enteroendocrine cell.

(K-N") Adult midguts containing GFP-labeled *hpo* clones (K-L", N-N") or *hpo* clones expressing *Yki-RNAi* (M-M") were immunostained to show the expression of GFP (green), Dl (cytoplasmic red in K-K", M-N"), Pdm1 (red in L-L"), Pros (nuclear red in N-N"), and DRAQ5 (blue). Guts were dissected out from adult flies grown at 18 ^{0}C for 5 days after clone induction.

hpo mutant clones can induce ISC proliferation non-autonomously

Staining with PH3 revealed that the overall number of $PH3^+$ cells increased in adult midguts carrying *hpo* mutant clones compared with those carrying control clones. The increase in the number of mitotic cells was not only distributed within the *hpo* mutant clones (Fig. 3-2A, B-B'), which is not surprised if Hpo signaling acts cell-autonomously to restrict ISC proliferation, but also located outside of the *hpo* mutant clones (arrows in Fig. 3-2B-B'). The phenomenon implies that *hpo* mutant cells may produce a secreted factor(s) that acts in a paracrine fashion to stimulate the proliferation of neighboring wild type ISCs.

The *hpo* mutant cells secreted factor(s) mentioned above could be produced by precursor cells, differentiated cells or both. To determine if Hpo signaling plays any roles in differentiated cells to stimulate ISC proliferation, an EC specific Gal4 driver, *Myo1A-Gal4* together with *tub-Gal80^{ts}* (*Myo1A^{ts}*) was used to overexpress *UAS-Wts-RNAi* or *UAS-Yki* in ECs. 2 to 3 days after shifted to 29^0C, a dramatic increase in ISC proliferation was observed in *Myo1A^{ts}-Wts-RNAi or Myo1A^{ts}-Yki* midguts, as indicated by the increased number of $PH3^+$ cells that are also Dl^+ positive (Fig. 3-2C-D", E). Coexpression of *Yki-RNAi* with *Wts-RNAi* or *Sd-RNAi* with *UAS-Yki* inhibited the ectopically induced ISC proliferation (Fig. 3-2E). These results suggest that the Hpo pathway could function in the ECs to restrict ISCs proliferation in a non-cell-autonomous manner.

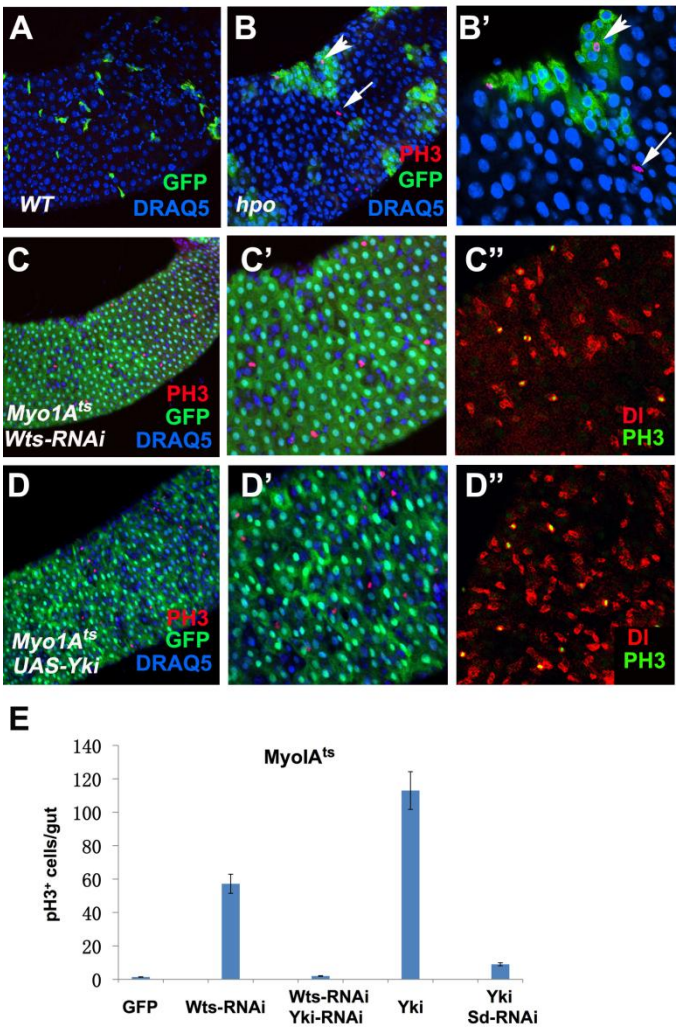

Figure 3-2 Loss of Hpo signaling can induce ISC proliferation nonautonomously

(A-B') Adult guts containing wild-type (*WT*) control clones (A) or hpo^{BF33} clones (B-B', B' is an enlarged view of B) were immunostained to show the expression of GFP (green), PH3 (red) and DRAQ5 (blue). The control and mutant clones were generated using the MARCM system and marked by GFP expression. *hpo* mutant clones stimulated cell division of neighboring wild type ISCs (arrows in B-B'). Arrowheads indicate dividing cells within *hpo* mutant clones.

(C-E) Adult midguts that expressed *UAS-Wts-RNAi* (C-C") or *UAS-Yki* (D-D") together with *UAS-GFP* using $MyoIA^{ts}$ were immunostained to show the expression of

PH3 (red in C-C' and D-D'; green in C", D"), Dl (red in C", D"), GFP (green in C-C', D-D') and DRAQ5 (blue). GFP marked ECs. Guts were dissected from adult flies grown at 29 ^{0}C for 5 days. (E) Quantification of $PH3^{+}$ cells in midguts of the indicated genotypes (mean ± s.d., n=15).

Inactivation of Hpo signaling increases the production of Upds and EGFR ligands

The non-cell-autonomous role of Hpo pathway in mediating ISCs proliferation suggests that there might be some secreted factors produced when Hpo pathway activity is reduced. To identify possible factors, we expressed *Wts-RNAi* or *UAS-Yki* in either ECs or precursor cells and examined the expression of ligands for several important pathways known to be involved in ISC proliferation, including the Wg/Wnt, Jak-Stat and EGFR pathways (Apidianakis et al., 2009; Buchon et al., 2009a; Jiang et al., 2009; Lin et al., 2008). The mRNA levels of Wg, the Jak-Stat pathway ligands: Unpaired (Upd), Upd2 and Upd3 (Arbouzova and Zeidler, 2006), and the EGFR ligands: Vein (Vn), Keren (Krn) and Spitz (Spi) (Shilo, 2005) were measured by reverse transcriptase quantitative PCR (RT-qPCR). We did not observe any increase in *wg* mRNA levels when Wts RNAi or Yki was overexpressed in ECs or ISC/EBs (Fig. 3-3A). On the other hand, Wts RNAi or Yki overexpression in ECs significantly up-regulated the mRNA levels of the three *Upd* genes as well as the three EGFR ligands, with *Upd3* up-regulated by ~40 folds and *Vn* by ~8 fold with Yki overexpression (Fig. 3-3A). However, overexpression of Wts RNAi or Yki in precursor cells only slightly increased the production of Upds and EGFR ligands (Fig. 3-3A). We also used a reporter gene *upd-lacZ* to monitor upd expression (Jiang et al. 2009). In *hpo* mutant clones, the expression *upd-lacZ* was ectopically induced, most notably in mutant ECs (arrows in Fig. 3-3B-B”). These results reveal that inactivation of Hpo signaling, mainly in ECs, leads to ectopic production of multiple ligands for

the Jak-Stat and EGFR pathways.

Excessive Yki activity in ECs activates Jak-Stat and EGFR signaling in ISCs

It is highly possible that the ectopic expression of Upds and EGFR ligands upon loss of Hpo signaling may result in activation of Jak-Stat and EGFR pathways. Indeed, RT-qPCR results revealed a ~10-fold increase in the expression of *Socs36E*, a Jak-Stat pathway target gene, when Wts RNAi or Yki was overexpressed in ECs, whereas Wts RNAi or Yki overexpression in ISCs/EBs resulted in a much lesser increase (< 3 fold) (Fig. 3-3A). To further monitor the Jak-Stat pathway activity, the expression of a pathway reporter gene, *10XSTAT-dGFP* was examined (Bach et al., 2007). Consistent with previous studies (Jiang et al., 2009), *10XSTAT-dGFP* was expressed in precursor cells, with ISCs exhibiting weaker levels of expression than EBs (arrows in Fig. 3-3C-C"). When Yki was overexpressed in ECs but not in ISCs/EBs, an obvious increase in the level of *10XSTAT-dGFP* expression was observed, not only in precursor cells but also in large differentiating cells (arrows Fig. 3-3D-D").

For EGFR pathway activity, we immunostained with a phospho-specific antibody to detect expression of phosphorylated *Drosophila* ERK (dpERK) as pathway activity readout (Gabay et al., 1997). In wild type midguts, dpERK signals were weakly detected in ISCs and EBs (arrows in Fig. 3-3E-E'). Acute activation of Yki in ECs (1 day after temperature shift of *MyoIA^{ts}-Yki* guts) resulted in a dramatically higher levels of dpERK signal in precursor cells (arrows Fig. 3-3F-F'). The results above

demonstrate that loss of Hpo signaling in ECs can ectopically induce the expression of Upds and EGFR ligands and in turn activate Jak-Stat and EGFR signal pathways in ISCs.

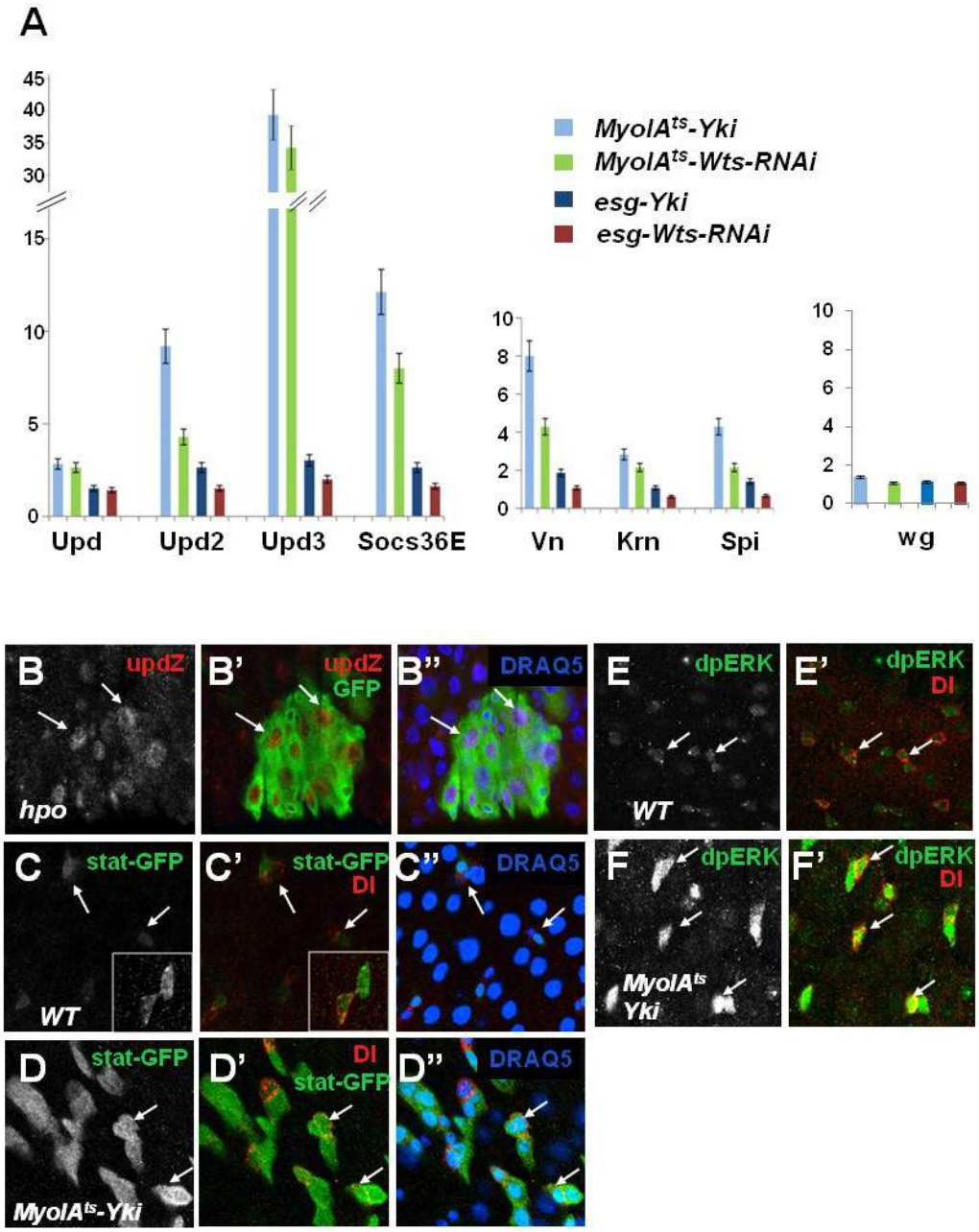

Figure 3-3. Hpo signaling regulates the Jak-Stat and EGFR pathways

(A) Relative mRNA levels of Upds, Socs36E, EGFR ligands and Wg in whole midguts of the indicated genotypes measured by RT-qPCR. Numbers indicate the fold of activation over the control guts. Wts RNAi or Yki overexpression in ECs (*MyoIA*^{ts}), and to a much lesser extent in precursor cells (*esg-Gal4*), induced elevated mRNA levels of the Jak-Stat and EGFR pathway ligands as well as the Jak-Stat pathway

target Socs36E.

(B- B") Expression of *upd-lacZ* in midguts carrying *hpo* mutant clones induced by the MARCM system. *upd-lacZ* was ectopically expressed in *hpo* mutant clones as well as some wild type cells adjacent to mutant clones. The expression of *upd-lacZ* was not detectable in control guts.

(C- D") Expression of the Jak-Stat reporter, *10XSTAT-dGFP* (STAT-GFP) in wild type (C-C") or *MyoIA*^{ts}*-Yki* guts. Insets in C-C' show images obtained by scanning at increased gain. *10XSTAT-dGFP* is expressed in the precursors with lower levels in ISCs. Yki overexpression in ECs induced increased expression of *10XSTAT-dGFP* in ISCs (arrows in D-D") as well as associated EBs and differentiating cells.

(E-F') Expression of phosphorylated ERK (dpERK) in wild type (E-E') and *MyoIA*^{ts}*-Yki* guts. *MyoIA*^{ts}*-Yki* flies were shifted to 29 ^{0}C for 1 day before dissection. Low levels of dpERK signal were detected in ISCs and associated EBs in control guts (E-E'). Yki overexpression in ECs increased the levels of the dpERK signal in ISCs (arrows) and associated EBs (F, F').

Jak-Stat and EGFR pathway activities are required for elevated ISC proliferation induced by loss of Hpo signaling

To determine whether the activities of Jak-Stat and EGFR pathway are important for the elevated ISC proliferation by deregulation of Hpo signaling, we carried out genetic epistasis experiments. We used MARCM system to produce *hpo* mutant clones in which Jak-Stat or EGFR signaling was inactivated by knocking down Jak-Stat pathway receptor, Dome (*dome-RNAi*) or EGFR pathway receptor, EGFR/TOP (*EGFR-RNAi*) (Shilo, 2005). *hpo* mutant clones expressing *Dome-RNAi* (referred to as *hpo*$^{-}$ *Dome*$^{-}$) or *EGFR-RNAi* (referred to as *hpo*$^{-}$ *EGFR*$^{-}$) exhibited reduced clone size compared with *hpo* mutant clones (Fig 3-4A-C'). Moreover, PH3 staining revealed that Dome RNAi or EGFR RNAi suppressed the elevated ISC proliferation inside but not outside of the *hpo* mutant clones (Fig. 3-4A-C', F). Thus, inactivating the Jak-Stat or EGFR pathway blocked ISC proliferation induced by loss of Hpo signaling. The sustained increased ISC proliferation outside *hpo*$^{-}$ *Dome*$^{-}$ or *hpo*$^{-}$ *EGFR*$^{-}$ clones suggests that these mutant clones may still produce excessive ligands for the Jak-Stat or EGFR pathways that can stimulate the proliferation of neighboring wild type ISCs in a paracrine fashion.

To further dissect the epistatic relationship between the Hpo and the Jak-Stat/EGFR pathways in the regulation of ISC proliferation, we ectopically activated Jak-Stat or EGFR pathway by overexpressing Upd or an activated EGFR ($EGFR^{A887T}$) (Lesokhin et al., 1999) and simultaneously inactivated Yki by coexpressing *Yki-RNAi* in

precursor cells using the *esg*^{ts} system. Both the numbers of esg-GFP^{+} and PH3^{+} cells indicated that inactivation of Yki had no effect on Upd or EGFR^{A887T} overexpression induced ISC proliferation (Fig. 3-4D-E, G), consistent with the conclusion that Jak-Stat and EGFR pathways acting downstream of the Hpo pathway to control ISCs proliferation.

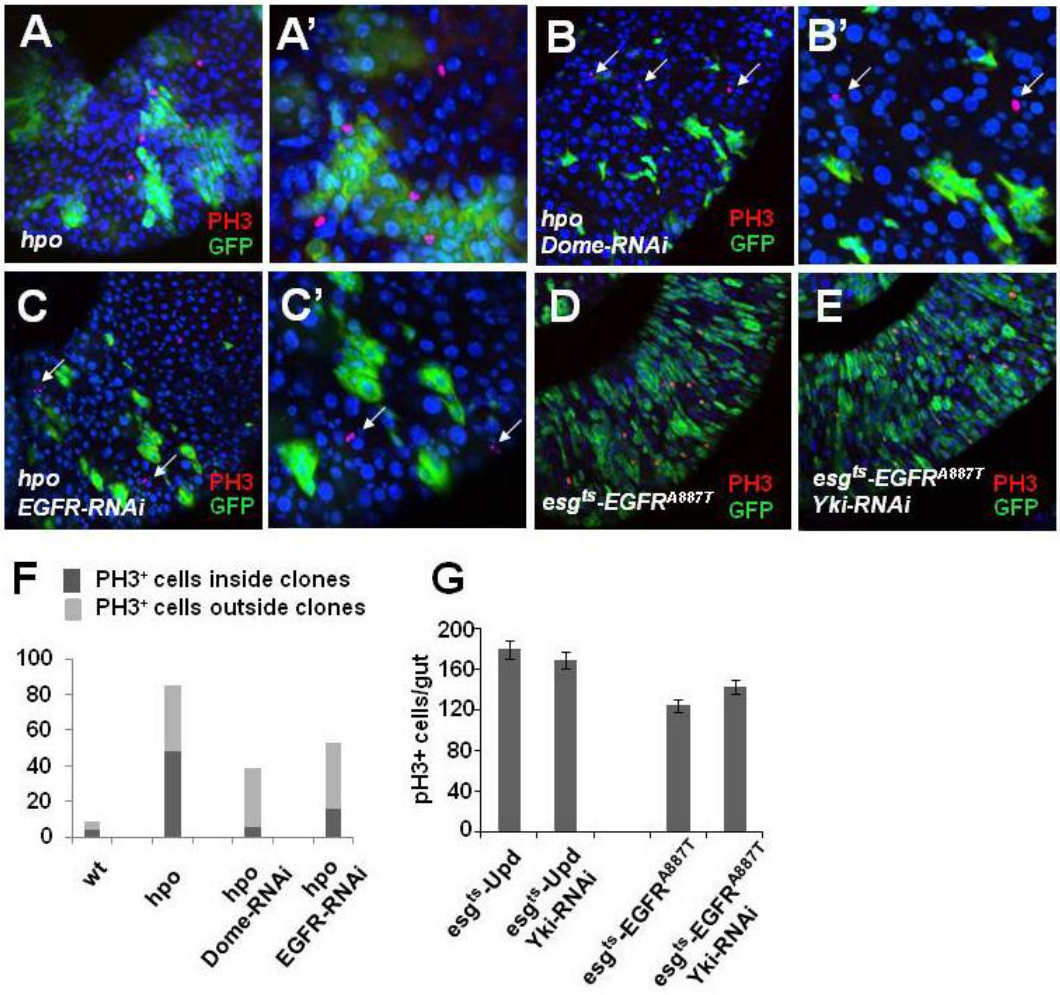

Figure 3-4. Inactivation of Jak-Stat or EGFR signaling in *hpo* mutant ISCs suppressed their proliferation

(A-C') Low (A, B, C) and high (A', B', C') magnification images of adult midguts carrying *hpo* mutant clones (A-A'), *hpo* mutant clones expressing *Dome-RNAi* (B-B'), or *hpo* mutant clones expressing *EGFR-RNAi* (C-C') and stained with anti-PH3 (red) and anti-GFP (green) antibodies. The mutant clones were generated by the MARCM system and the guts were dissected out from flies grown at 25 ^{0}C for 5 days after clone induction. *hpo* mutant clones expressing *Dome-RNAi* or *EGFR-RNAi* exhibited reduced clone size and contained less PH3^{+} cells within the clones; however, ectopic PH3 signals can be readily detected in wild type cells near the mutant clones (arrows

in B-C').

(D-E) esg-GFP (green) and PH3 (red) expression in adult midguts expressing an active form of EGFR, $EGFR^{A887T}$, in the absence (D) or presence (E) of *Yki-RNAi* transgene using the esg^{ts} system. Adult flies were shifted to 29^0C for 6 days before dissection.

(F) Quantification of PH3 positive cells inside or outside the control or mutant clones of the indicated genotypes. A total of 12 guts were counted for each genotype.

(G) Quantification of PH3 positive cells from guts of the indicated genotypes (mean ± s.d., n=5).

Yki is required in precursor cells for DSS-induced ISC proliferation

Previous studies have indicated that tissue damage caused by feeding flies with DSS or bleomycin, or by bacterial infection (such as *Pseudomonas entomophila* or *PE*) stimulates ISC proliferation (Amcheslavsky et al., 2009; Apidianakis et al., 2009; Buchon et al., 2009a; Jiang et al., 2009). To determine if the Hpo pathway participates in these processes, flies expressing *Yki-RNAi* in either precursor cells or ECs were treated with various tissue damaging reagents. Yki RNAi in ISCs/EBs but not in ECs suppressed DSS-induced ISC proliferation, as indicated by the reduction in the number of esg-GFP^{+} and PH3^{+} cells (Fig. 3-5A-C). These observations suggest that Yki is required in ISCs/EBs for DSS-stimulated ISC proliferation. In contrast, bleomycin or *PE*-induced ISC proliferation was not significantly affected by Yki inactivation in either ISCs/EBs or ECs (Fig. 3-5A-C).

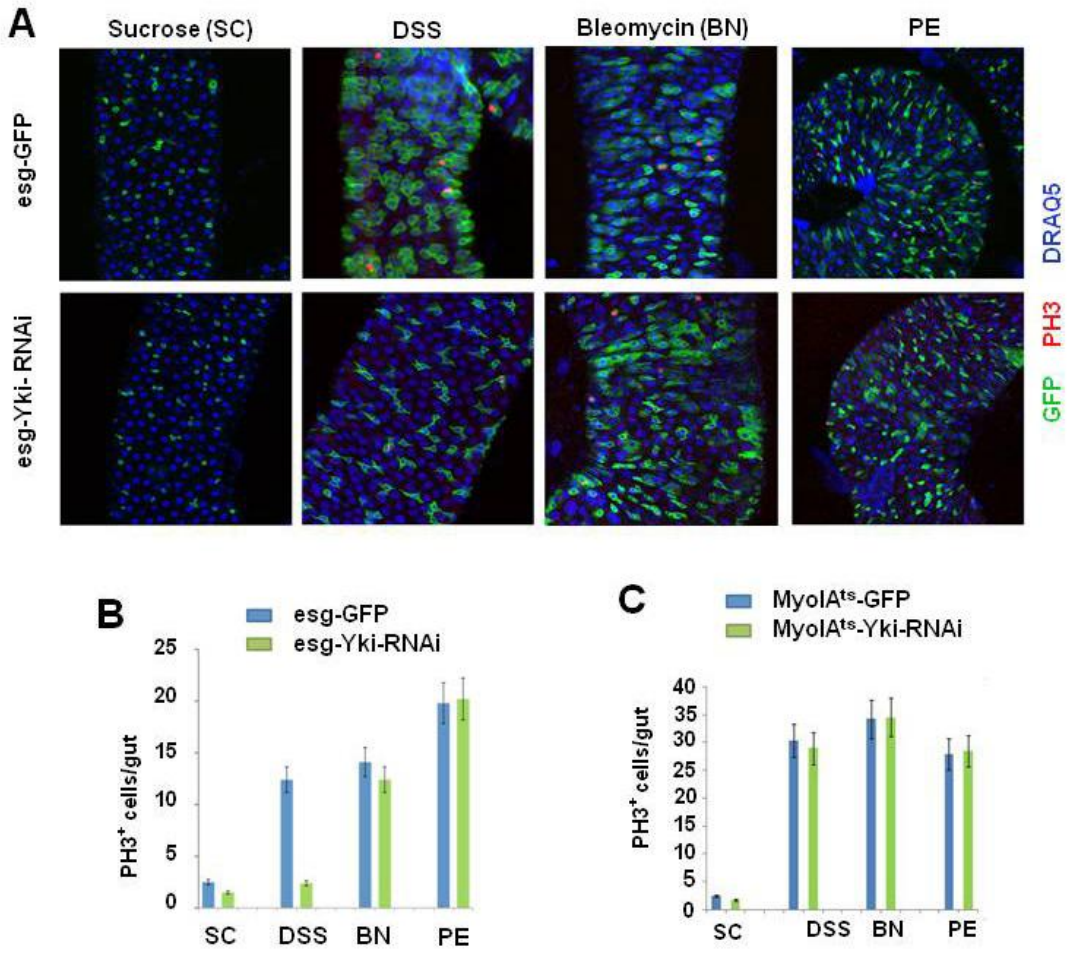

Figure 3-5. Yki is required in the precursor cells for DSS-stimulated ISC proliferation.

(A) Adult flies expressing *esg-Gal4/UAS-GFP* (top) or *esg-Gal4/UAS-GFP* + *UAS-Yki-RNAi* (bottom) were treated with sucrose (SC), DSS, bleomycin (BN), or *PE* for 3 days before midguts were dissected out and immunostained with GFP (green) and PH3 (red) antibodies and DRAQ5 (blue).

(B-C) Quantification of $PH3^{+}$ cells in control adult midguts or adult midguts expressing *esg-Yki-RNAi* (B), *MyoIA^{ts}-Yki-RNAi* (C) treated with sucrose, DSS, bleomycin, or *PE* (mean ± s.d., n=10 for each genotype).

Discussion

The Hpo tumor suppressor pathway controls organ size and mediates cell contact inhibition in both *Drosophila* and mammals (Pan, 2007; Zhang et al., 2009b); however, its role in stem cell regulation has not been extensively investigated. Using the *Drosophila* adult midgut as a model, we provided evidence that Hpo signaling plays an essential role in restricting adult stem cell proliferation during tissue homeostasis. In addition, we proposed a novel non-cell-autonomous manner by which Hpo signaling regulates stem cell proliferation.

The most common recognized mechanism through which Hpo pathway functions is that Hpo signaling acts cell-autonomously to control cell growth, proliferation and apoptosis by mediating genes involved in these processes (Pan, 2007; Zhang et al., 2009b). Consistent with this view, we found that Hpo signaling could function in precursor cells to inhibit ISC proliferation by restricting the activity of Yki/Sd. However, it was unexpectedly observed that *hpo* mutant clones can promote the proliferation of neighboring wild type ISCs, suggesting a possible non-cell-autonomous role of Hpo signaling. To probe that, we further found that loss of Hpo signaling or overexpression of Yki in enterocytes significantly stimulated ISC proliferation, proving the non-cell-autonomous mechanism of Hpo pathway actions. Moreover, we provided evidence that inactivation of Hpo signaling in enterocytes resulted in increased production of cytokines and mitogens that in turn activate the Jak-Stat and EGFR pathways in ISCs to promote their proliferation. Interestingly, a

recent study in mammals showed that activation of the mammalian orthologue of Yki, Yap, in cultured breast epithelial cells activates an EGFR ligand that can drive cell proliferation non-autonomously *in vitro*(Zhang et al., 2009b), suggesting that the non-autonomous mechanism proposed here seems to be conserved.

Although Wts RNAi or Yki overexpression in precursor cells could stimulate ISC proliferation, we did not observe an obvious increase of either the Jak-Stat or EGFR pathway activity in ISCs. Thus, the induced ISC proliferation by loss of Hpo signaling in precursor cells may reflect a cell-autonomous role of Hpo signaling in regulating ISC proliferation. Interestingly, we found that tissue damage reagent DSS stimulated ISC proliferation specifically required Yki activity in ISCs/EBs. A possible explanation is that DSS treatment may alleviate the inhibition of Yki activity by Hpo signaling and promote a cell-autonomous role of Yki to drive ISC proliferation. Previous studies suggest that DSS disrupts basement membrane organization whereas bleomycin and PE cause damage in enterocytes. As ISCs contact the basement membrane, our finding thus raises an interesting possibility that the Hpo pathway may sense ISC interaction with the basement membrane to restrict ISC proliferation and that disruption of basement membrane organization may alleviate Hpo pathway-mediated contact inhibition of ISC proliferation, which needs to be investigated in the future.

Our finding also has an important implication on tumorigenesis. Deregulation of Hpo

signaling has been linked with several types of human cancer. Indeed, liver specific overexpression of Yap or knockout of MST1/2 (the mammalian orthologue of Hpo) caused hepatocellular carcinomas (Dong et al., 2007; Lu et al., 2010; Zhao et al., 2010). Furthermore, MST1/2 mutant livers contain proliferative oval cells that are facultative stem cells contributing to both the hepatocyte and biliary lineages, implying that Hpo signaling may repress adult liver stem cell activation (Lu et al., 2010). Thus, it would be important to determine whether Hpo signaling is more widely involved in adult stem cell regulation and whether Hpo pathway activity is deregulated in response to tissue injury that is thought to contribute to tumorigenesis (Beachy et al., 2004). Our finding that Hpo signaling can regulate stem cell proliferation through non-cell-autonomous mechanisms implies that deregulation of Hpo signaling in tumor microenvironments may also contribute to tumorigenesis.

CHAPTER FOUR

Drosophila Myc integrates multiple signaling pathways to regulate intestine stem cell proliferation and gut regeneration in response to injury

Introduction

Most adult tissues depend on stem cells to maintain tissue homeostasis during development as well as to repair damaged tissue by increased levels of stem cell proliferation to replenish lost cells. The *Drosophila* adult midgut is an ideal model system to understand how stem cell proliferation is regulated because not only the cell lineage in this tissue is simple and well characterized but also it is functionally and structurally similar to mammalian intestine. Previous studies have identified intestinal stem cells (ISCs) in *Drosophila* adult midgut which are located adjacent to the basement membrane of midgut epithelium (Micchelli and Perrimon, 2006; Ohlstein and Spradling, 2006). The asymmetric division of ISC produces two daughter cells: one remains as an ISC, and the other one loses stem cell properties and converts to an enteroblast (EB) which can undergo differentiation to become either absorptive enterocytes (EC) or secretary enteroendocrine (ee) (Ohlstein and Spradling, 2006). Several important signaling pathways including the Notch, Wnt, Hpo, Jak-stat and EGFR pathways are reported to regulate the proliferation and differentiation of ISCs (Jiang et al., 2011; Jiang et al., 2009; Karpowicz et al., 2010; Lin et al., 2008; Micchelli and Perrimon, 2006; Ren et al., 2010a; Shaw et al., 2010; Staley and Irvine, 2010). Moreover, studies have shown that exposure to tissue damage can ectopically

induce ISC proliferation and differentiation to compensate for the damaged cells (Amcheslavsky et al., 2009). Although ISC proliferation is subject to strict control to ensure both normal homeostasis and proper regeneration after damage, the cell intrinsic mechanisms that tune the rate of ISC proliferation and differentiation in response to injury are still unknown.

Drosophila Myc (dMyc), encoded by the *diminutive (dm)* gene, is an evolutionally conserved transcription factor that controls the expression of a large number of target genes. It is functionally involved in multiple cellular processes, which include cellular growth, cell cycle progression, DNA replication, cell apoptosis and cell competition (de la Cova et al., 2004; Grandori et al., 2005; Vita and Henriksson, 2006). Besides, dMyc also functions in stem cell. For example, in *Drosophila* female germline, germline stem cells (GSCs) maintains higher levels of dMyc while their differentiating daughters cystoblasts contain lower levels of dMyc. Ectopical expression of dMyc in cystoblasts can convert them to a stem-cell like morphology, suggesting that low level of dMyc is essential for GSCs differentiation (Rhiner et al., 2009).

Although dMyc and mammalian Myc have been implicated in stem cell biology, their precise roles in stem cell proliferation and maintance under both normal development and regeneration have not been defined. Here, we provide evidence that dMyc is essential for ISC proliferation during normal homeostasis as well as after exposure to

damage treatment. We also demonstrated that damage treatment could upregulate dMyc through Hpo, Jak-Stat and EGFR pathways at the transcrpitioanl level.

Results

dMyc is required for ISC proliferation and midgut regeneration in response to tissue damage

To identify genes that play any role in midgut regeneration, we used the *esg^{ts}F/O* system (*esg-Gal4 tubGal80^{ts} UAS-GFP; UAS-flp Act>CD2>Gal4*) which can monitor epithelial turnover in the posterior midgut to perform genetic screens (Jiang et al., 2009). Tissue damage reagents such as DSS and Bleomycin were used to stimulate ISC proliferation and midgut regeneration (Amcheslavsky et al., 2009). Shifting *esg^{ts}F/O* adult flies to 29 ^{0}C resulted in excision of the Flp-out cassette to generate a ubiquitously expressed heritable Gal4 driver (Act>Gal4) so that GFP expression marked not only the precursor cells but also their progeny. 3 days after temperature shift, control *esg^{ts}F/O* flies and *esg^{ts}F/O* flies carrying library of UAS-RNAi lines were fed with either sucrose, DSS or Bleomycin for another three days. Compared with the mock treatment, *esg^{ts}F/O* flies treated with DSS or Bleomycin exhibited many more GFP positive cells in their midguts. In addition, DSS or Bleomycin treated *esg^{ts}F/O* midguts contained many differentiating or differentiated GFP positive cells with large nuclei whereas mock treated midguts contained GFP positive cells that are mainly precursor cells with small nuclei (Fig. 4-1A-C), suggesting that DSS and

Bleomycin accelerated midgut turnover. However, *esg^{ts}F/O* carrying a UAS-dMyc-RNAi line contained few GFP positive cells with small nuclei even after DSS or Bleomycin treatment (Fig. 4-1A'-C'). Therefore dMyc is identified as an important gene for midgut regeneration in response to damage induced by both chemicals.

Then we examined the requirement of dMyc in ISC proliferation. Adult flies expressing GFP alone or together with dMyc-RNAi under the control of *esg-Gal4* were fed with sucrose, DSS or Bleomycin for two days. Compared with sucrose treatment, midguts treated with DSS or Bleomycin exhibited increased number of GFP and PH3 positive cells (Fig. 4-1D-G), indicating elevated ISC proliferation. Knockdown of dMyc in precursor cells suppressed DSS or Bleomycin induced ISC proliferation (Fig. 4-1D'-F', 1G).

dMyc is upregulated in response to injury

We also found that both DSS and Bleomycin treatment upregulated dMyc levels in precursor cells (Fig. 4-1H-J''). In sucrose treated midgut, dMyc is expressed with high levels in ECs while with pretty low levels in precursor cells (Fig. 4-1H-H''). However, after DSS or Bleomycin treatment, dMyc levels are significantly upreagulated in precursor cells without much change in ECs (Fig. 4-1I-J'').

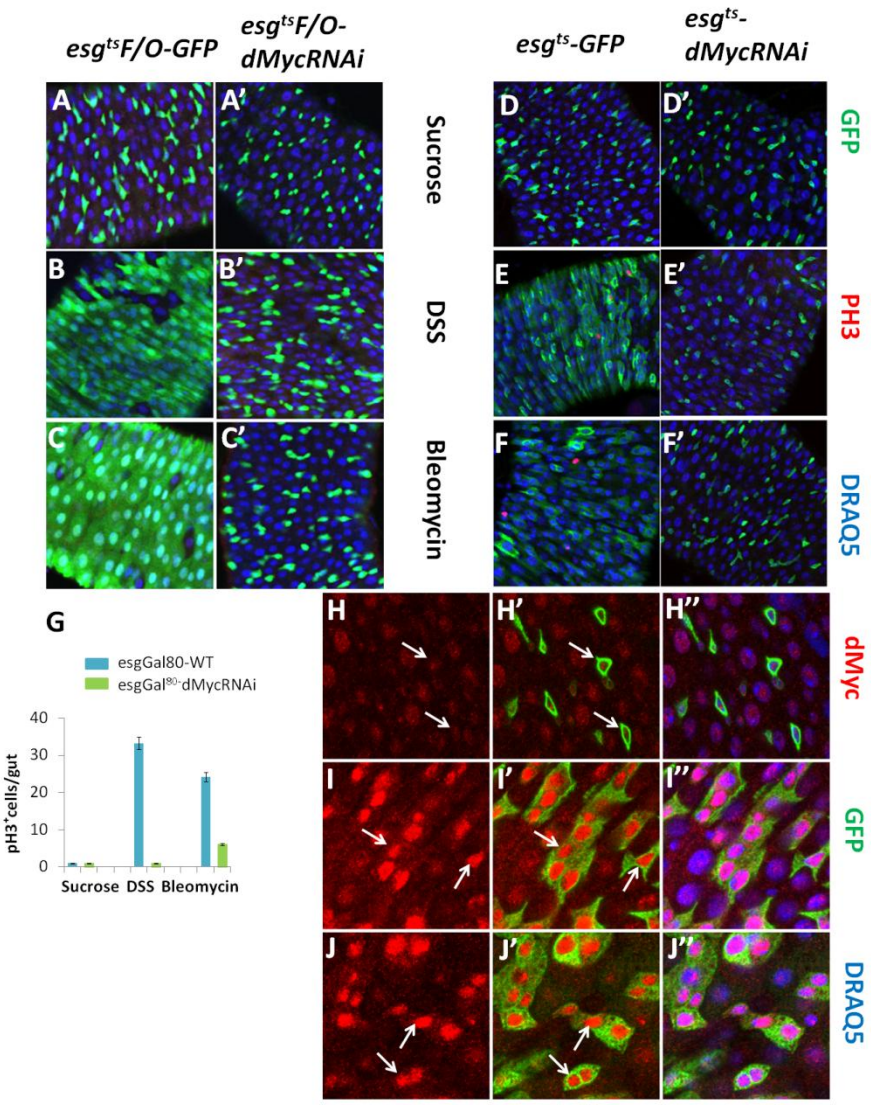

Figure 4-1. dMyc is required for ISC proliferation and midgut regeneration in response to tissue damage

(A-C') Adult midguts expressing *esg^{ts}F/O* without (A-C) or with *UAS-dMyc-RNAi* (A'-C') were treated with sucrose (A and A') or DSS (B and B') or Bleomycin (C and C') and immunostained with GFP (green) antibody and a nuclear dye DRAQ5 (blue). (D-F') Adult midguts expressing *UAS-GFP* without (D-F) or with *UAS-dMyc-RNAi* (D'-F') using the *esg-Gal4^{ts}* system were treated with sucrose (D-D') or DSS (E-E') or Bleomycin (F-F') and immunostained with GFP (green), PH3(red) antibodies and a nuclear dye DRAQ5 (blue). Adult flies were shifted to non-permissive temperature

(29 ^{0}C) for 3-5 days and then treated with different reagents for another 2 days before dissection.

(G) Quantification of $PH3^{+}$ cells in midguts of indicated genotypes (N=15 for each genotype).

(H-J'') Adult midguts expressing *esg-Gal4/UAS-GFP* were treated with sucrose (H-H'') or DSS (I-I'') or Bleomycin (J-J'') and immunostained with GFP (green) and dMyc (red) antibodies, and a nuclear dye DRAQ5 (blue).

dMyc is required for ISC proliferation, differentiation and maintenace during normal homeostasis

During normal development, the posterior midgut of wild type female adult usually turns over at a rate of about once per two weeks to maintain homeostasis (Jiang et al., 2009). Using the *esg^{ts}F/O* system to observe posterior midgut self-renewal, we found out that 15 days after temperature shift to 29 ^{0}C, most of the epithelial cells in the posterior region of *esg^{ts}F/O* midguts were GFP^{+}, indicating that the posterior midgut had renewed (Fig. 4-2A-A''). However, posterior midgut of esg^{ts}F/O-dMyc-RNAi flies contained very few GFP^{+} cells with small nuclei, suggesting they are precursor cells (Fig. 4-2B-B''). Loss of function of dMyc significantly slowed down turn over rate of normal midgut and dMyc is required for the self renewal and homeostasis of midgut.

To further determine if the basal dMyc activity is essential for ISC proliferation during normal midgut homeostasis, we generated GFP positively marked *dm4* mutant clones in the ISC cell lineage using the MARCM system (Lee and Luo, 2001). The adult flies were grown at 18^{0}C for 20 days after clone induction, the clone size for *dm4* mutant clones was greatly reduced compared to that of wild type control clones (Fig. 4-2C-D''), and *dm4* mutant clones do not contain ECs (Pdm+) or ees (Pros+) cells, suggesting that dMyc is required for ISC lineage differentiation (Fig. 4-2E-H''). Thus, the basal dMyc activity is critical for ISC proliferation and differentiation under normal gut homeostasis. We also observed the gradual loss of stem cell numbers in adult midgut when dMyc expression is reduced in precursor cells (Fig. 4-2I),

suggesting that dMyc is also required for stem cell maintenance in adult midgut.

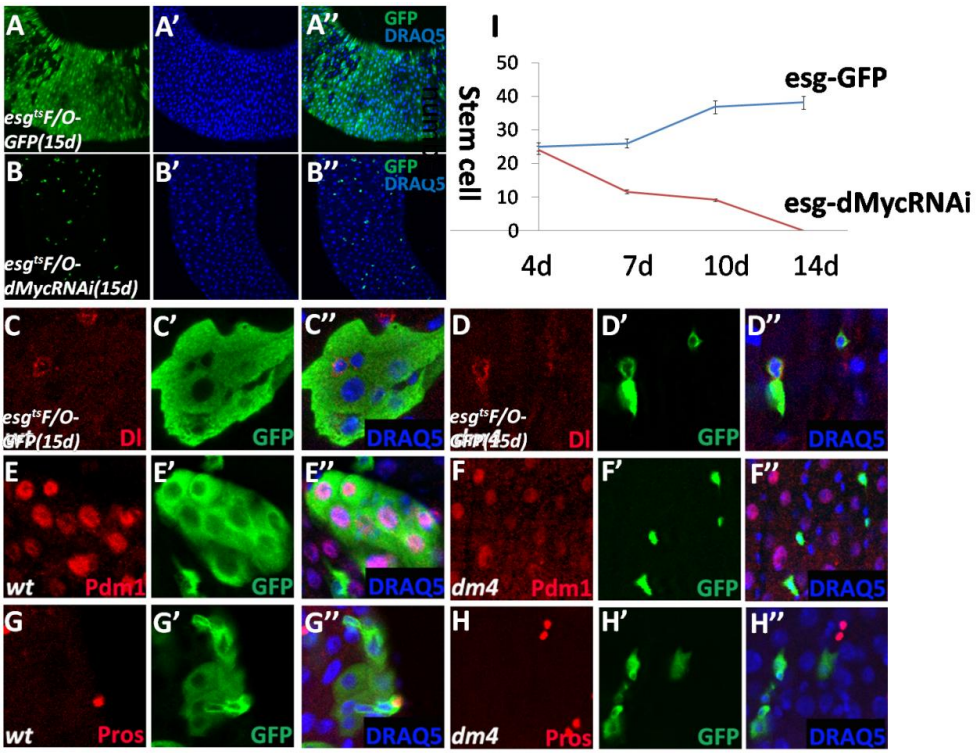

Figure 4-2. dMyc is important for midgut homeostasis

(A-B'') Adult midguts expressing *esg^{ts}F/O* without (A-A'') or with *UAS-dMyc-RNAi* (B-B'') were cultured at non-permissive temperature (29 ^{0}C) for 15 days and immunostained with GFP (green) antibody and a nuclear dye DRAQ5 (blue).

(C-H'') Adult midguts containing GFP positive wild-type (*WT*) clones (C-C", E-E'', G-G''), or *dm4* clones (D-D", F-F", H-H'') were immunostained to show the expression of GFP (green), Dl (red in C-D''), Pdm1 (red in E-F"), Pros (red in G-H"), and DRAQ5 (blue). *dm4* mutant clones grow slower and fail to differentiate into EC (Pdm1^{+}) or EE (Pros^{+}). The control and mutant clones were generated using the MARCM system. Guts were dissected out from adult flies grown at 18 ^{0}C for 20 days after clone induction.

(I) Quantification of stem cells number in adult midguts of indicated genotypes at different days old.

DSS induces dMyc upregulation through the Hpo pathway

Since DSS and Bleomycin treatment can upregulate dMyc levels in precursor cells and their induced ISC proliferation was dependent on dMyc activity, our previous study has shown that DSS can stimulate ISCs proliferation through a cell autonomous role of Hpo pathway transcriptional activator Yki in precursor cells, we wanted to ask whether tissue damage induced dMyc upregulation was through the activity of Yki.. Inactivation of Yki in precursor cells blocked DSS but not Bleomycin induced dMyc upregulation (Fig. 4-3A-E''), consistent with our previous findings that DSS promotes ISC proliferation through Yki activity in the precursor cells whereas Bleomycin stimulates ISC proliferation through Yki independent mechanisms (Ren et al., 2010a).

Then we wanted to explore the relationship of dMyc and Hpo signaling in midgut precursor cells. To interrupt Hpo pathway, we could either overexpress downstream transcription activator Yki or knock down upstream kinase Wts, both of which actions could inactivate Hpo pathway and promote cell proliferation. We found that overexpression of *yki* or knockdown of Wts in precursor cells can upregulate dMyc levels in these cells (Fig. 4-3F-H''). Furthermore, knockdown of dMyc in precursor cells suppressed the elevated ISC proliferation induced by excessive Yki or loss of Wts, indicated by the numbers of both GFP and PH3 positive cells (Fig. 4-3I-J', 3K). These results suggest that dMyc functions as a downstream mediator of Hpo pathway in the regulation of ISC proliferation in precursor cells.

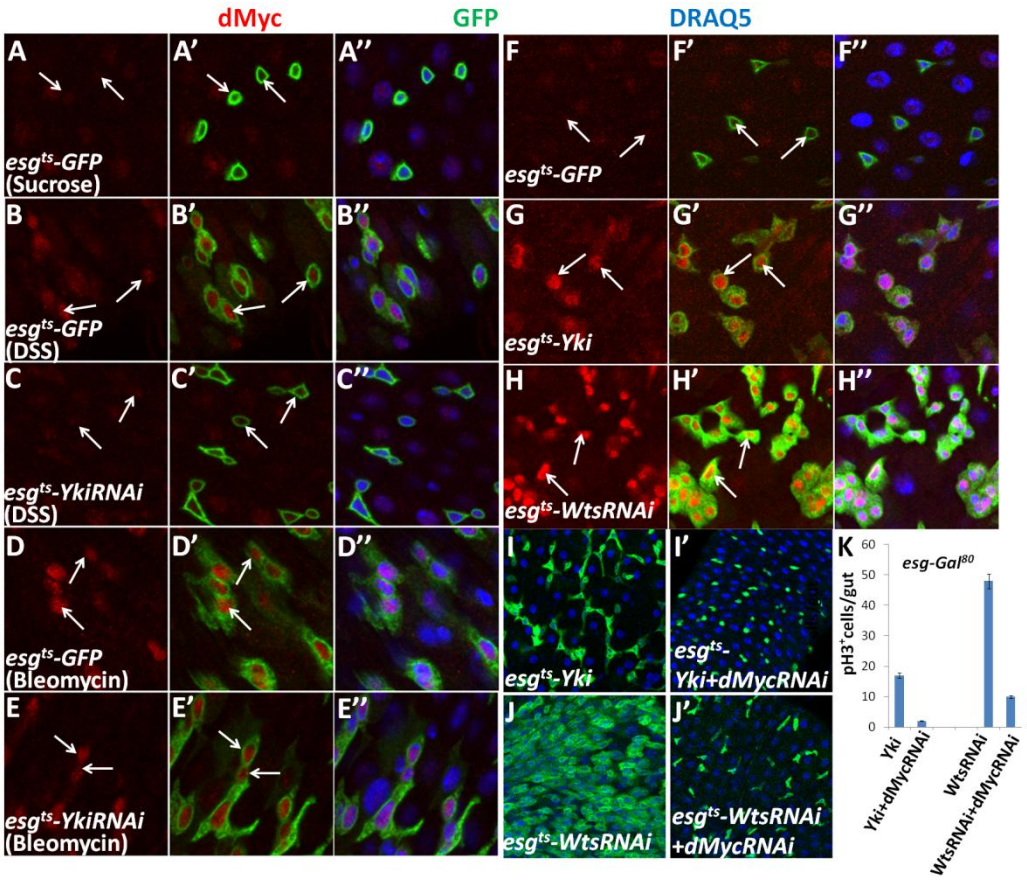

Figure 4-3. dMyc functions downstream of Hpo signaling pathway

(A-E'') Adult midguts expressing *UAS-GFP* without (A-A'', B-B'', D-D'') or with *UAS-Yki-RNAi* (C-C'', E-E'') using the *esg-Gal4*^{ts} system were treated with sucrose (A-A'') or DSS (B-B'', C-C'') or Bleomycin (D-D'', E-E'') and immunostained with GFP (green) and dMyc (red) antibodies, and a nuclear dye DRAQ5 (blue).

(F-H'') Adult midguts expressing *UAS-GFP* without (F-F'') or with *UAS-Yki* (G-G'') or with *UAS-Wts-RNAi* (H-H'') using the *esg-Gal4*^{ts} system were immunostained with GFP (green) and dMyc (red) antibodies, and a nuclear dye DRAQ5 (blue).

(I-J') Adult midguts expressing *UAS-GFP* with *UAS-Yki* (I) or *UAS-Yki+dMyc-RNAi* (I') or *UAS-Wts-RNAi* (J) or *UAS-Wts-RNAi+dMyc-RNAi* (J') using the *esg-Gal4*^{ts} system were immunostained with GFP (green) antibody and a nuclear dye DRAQ5

(blue).

(K) Quantification of $PH3^{+}$ cells in midguts of indicated genotypes (N=15 for each genotype).

Bleomycin induces dMyc through Jak-Stat and EGFR signaling pathways

We next investigated how dMyc is upregulated in precursor cells in response to Bleomycin treatment. Previous studies have demonstrated that tissue damage induced by Bleomycin feeding or bacterial infection promotes ISC proliferation through activating the Jak-Stat and EGFR pathways. Our finding that Bleomycin up-regulates dMyc independent of Yki has raised a possibility that Bleomycin regulates dMyc through Jak-Stat or/and EGFR pathways. Indeed, inactivation of Dome or EGFR in precursor cells partially and inactivation of both Dome and EGFR in precursor cells almost completely blocked Bleomycin induced dMyc upregulation in these cells (Fig. 4-4A-E'').

To determine whether Jak-Stat or EGFR pathway activation suffices to induce dMyc upregulation, we overexpressed a Jak-Stat pathway ligand Upd, or an activator form of EGFR ($EGFR^{A887T}$) in precursor cells using the *esg-Gal4/tub-Gal80* (esg^{ts}) system. We found that esg^{ts}>Upd and esg^{ts}> $EGFR^{A887T}$ flies exhibited elevated dMyc levels in ISCs and EBs after shifting to 29 ^{0}C (Fig. 4-4F-H''). To determine whether dMyc functions downstream of Jak-Stat or EGFR pathway in precursor cells to promote ISC proliferation, *UAS-dMyc-RNAi* was coexpressed with *UAS-Upd* or *UAS-EGFR*^{A887T} in precursor cells using esg^{ts} and ISC proliferation was examined. As shown in Fig. 4-4I-K, inactivation of dMyc markedly suppressed the ectopic ISC proliferation induced by overexpression of Upd or $EGFR^{A887T}$. Therefore dMyc is a common target of Hpo, Jak-Stat and EGFR pathways that mediates their effect on ISC proliferation.

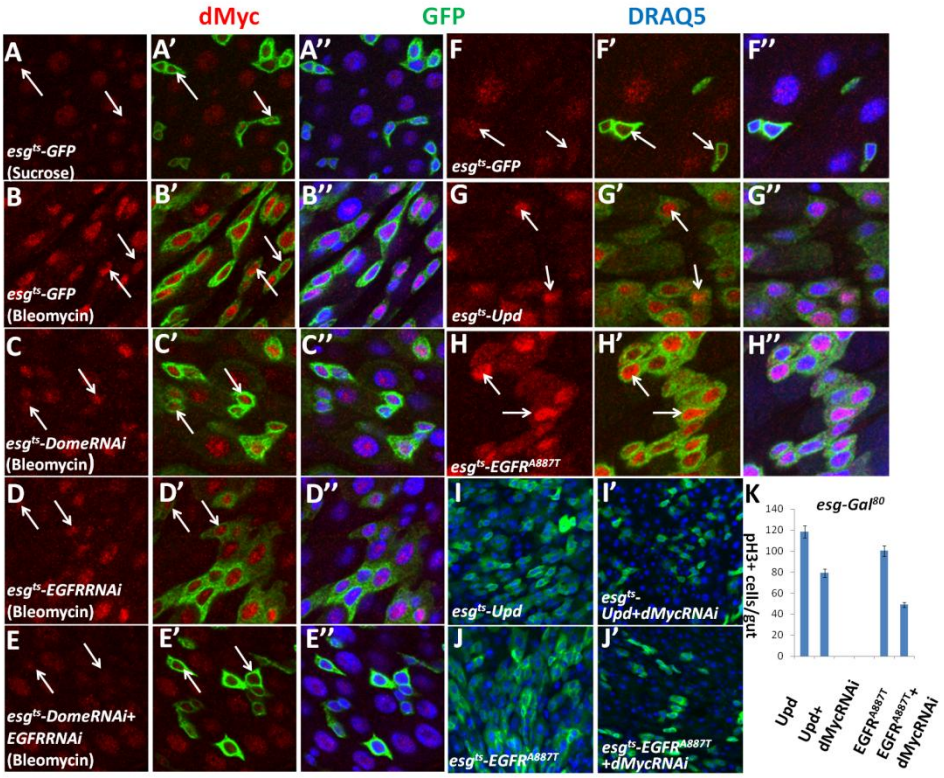

Figure 4-4. dMyc functions downstream of Jak-Stat and EGFR pathways

(A-E'') Adult midguts expressing *UAS-GFP* without (A-B'') or with *UAS-Dome-RNAi* (C-C'') or with *UAS-EGFR-RNAi* (D-D'') or with *UAS-Dome-RNAi+ UAS-EGFR-RNAi* (E-E'') using the *esg-Gal4*^{ts} system were treated with sucrose (A-A'') or Bleomycin (B-E'') and immunostained with GFP (green) and dMyc (red) antibodies, and a nuclear dye DRAQ5 (blue).

(F-H'') Adult midguts expressing *UAS-GFP* without (F-F'') or with *UAS-Upd* (G-G'') or with *UAS- EGFR*^{A887T} (H-H'') using the *esg-Gal4*^{ts} system were immunostained with GFP (green) and dMyc (red) antibodies, and a nuclear dye DRAQ5 (blue).

(I-J') Adult midguts expressing *UAS-GFP* with *UAS-Upd* (I) or *UAS-Upd +dMyc-RNAi* (I') or *UAS-EGFR*^{A887T} (J) or *UAS-EGFR*^{A887T}*+dMyc-RNAi* (J') using the *esg-Gal4*^{ts} system were immunostained with GFP (green) antibody and a nuclear

dye DRAQ5 (blue).

(K) Quantification of $PH3^{+}$ cells in midguts of indicated genotypes (N=15 for each genotype).

dMyc is transcriptionally regulated by Hpo, Jak-Stat and EGFR pathways

We next asked whether dMyc is regulated at transcriptional or post-transcriptional level by various pathways or in response to tissue damage. Two independent enhancer trap lines, referred to as dMyc-lacZ, which reported *lacZ* expression from *dm*/dMyc locus were used to monitor dMyc transcription and identical results were obtained (Neto-Silva et al., 2010). Under normal situation, *dMyc-lacZ* expression was barely detectable in precursor cells (Fig. 4-5A-A''). Inactivation of Wts or overexpression of Yki, Upd or $EGFR^{A887T}$ in precursor cells markedly increased dMyc-lacZ expression in these cells (Fig. 4-5B-E''), suggesting that dMyc was transcriptionally regulated by Hpo, Jak-Stat and EGFR pathways. Consistent with this, tissue damage reagents including DSS and bleomycin also stimulated *dMyc-lacZ* expression in precursor cells (data not shown). Thus, dMyc transcription is stimulated in response to tissue injury through multiple signaling pathways.

dMyc promoter/enhancer region contains consensus Sd, Stat and Pnt binding sites

The observation that dMyc is regulated by Hpo, Jak-Stat and EGFR pathways at the transcriptional level leads us to ask whether dMyc is a direct transcriptional target of these pathways. We searched *dm* gene region for binding sites of Sd, Stat and Pointed (Pnt), transcription factors of Hpo, Jak-Stat and EGFR pathways respectively. And several potential binding sites for each transcription factor were found to be primarily located in a region around 1kb length comprising the first extron and part of the first

intron (Fig. 4-5F). We then generated a *luciferase* reporter gene (*dMyc-En-luc*) containing this region and examined its response to the activity of Hpo, Jak-Stat or EGFR pathway in S2 cells. The basal activity of *dMyc-En-luc* was low but it could be significantly activated by coexpression of Yki+Sd or an activated form of Stat (StatΔNΔC)+Hop (Ekas et al.). When all the potential Stat sites were mutated, its response to StatΔNΔC and Hop was largely blocked (Fig. 4-5G). In contrast, *dMyc-en-luc* was not activated by an active form of Pointed (data not shown). It is possible that other regions of the *dMyc* locus are required for the EGFR pathway to active *dMyc*

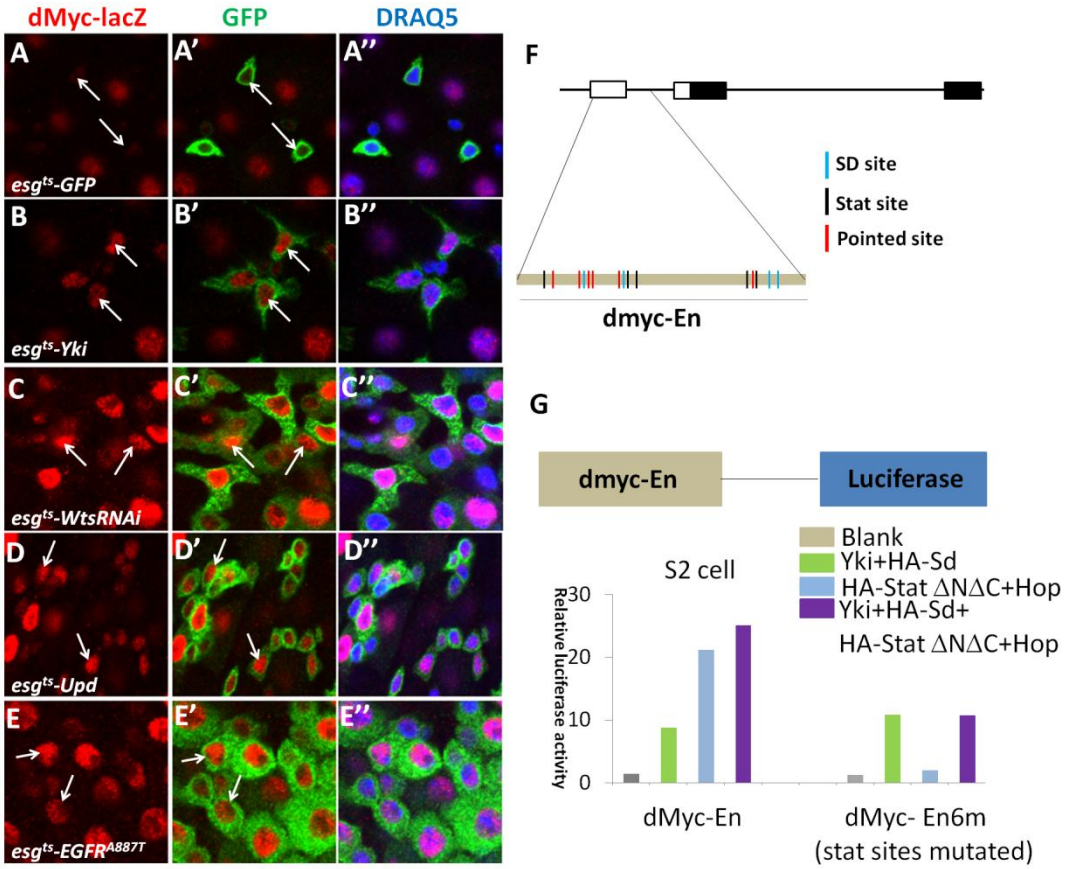

Figure 4-5. dMyc is regulated at transcriptional level

(A-E'') Adult midguts expressing *dMyc-laZ;UAS-GFP* without (A-A'') or with *UAS-Yki* (B-B'') or with *UAS-Wts-RNAi* (C-C'') or with *UAS-Upd* (D-D'') or with *UAS- EGFR*^{A887T} (E-E'') using the *esg-Gal4*^{ts} system were immunostained with GFP (green) and lacZ (red) antibodies, and a nuclear dye DRAQ5 (blue).

(F) Schematic representation of the *dm* locus. Black rectangles are coding regions, white rectangles are noncoding regions; lines denote introns. Blue bars indicate consensus Sd binding sites, Black bars indicate consensus Stat binding sites and red bars indicate consensus Pointed binding sites.

(G) Diagram of the dMyc-En reporter gene. dMyc enhancer region containing tandem binding sites for Sd, Stat and Pnt were placed upstream of the heat shock basal

promoter (hs) followed by the luciferase coding sequence. S2 cells were transfected by the indicated expression constructs plus the luciferase reporter gene, and the cell lysates were subjected to dual luciferase assay.

Discussion

The strict control of intestine stem cell proliferation is crucial to midgut homeostasis during adult *Drosophila* development. Evolutionarily conserved signaling pathways such as Hpo, Jak-Stat and EGFR pathways were reported to regulate the ISC proliferation. The data we present here indicated the growth-promoting transcription factor dMyc was subject to the regulation by Hpo, Jak-Stat and EGFR pathways and influenced their activities on the control of ISC proliferation in adult midgut. We also demonstrated that dMyc activity was not only essential for elevated ISC proliferation induced by tissue damage reagents but also crucial for normal ISC proliferation and maintenance in midgut homeostasis. Although our study here indicated that dMyc is necessary for ISC proliferation, overexpression of dMyc itself did not stimulate ISC proliferation (data not shown), suggesting that dMyc is not sufficient to promote ISC proliferation and injury may upregulate other genes that act in concert with *dMyc* to tune up ISC proliferation.

dMyc is a conserved transcription regulator that affects many fundamental processes of cell biology including cell growth, proliferation and apoptosis. Recently, the role of mammalian Myc in proliferation of stem/progenitor cells has been suggested. Studies on mouse small intestine have reported that although loss of c-Myc in intestine stem cell-bearing cypts of adult mice leads to reduced cell sizes, it does not affect cell proliferation or differentiation of crypt cells and the normal homeostasis of adult intestinal epithelium can be still maintained (Bettess et al., 2005). However, our

findings have shown that dMyc is necessary for normal ISC proliferation and maintenance in *Drosophila* adult midgut homeostasis. Since mammalian *myc* gene family contains three members: c-*myc*, N-*myc* and L-*myc*, they might function redundantly in mouse intestine. Inactivation of c-Myc alone may not be sufficient to cause an obvious homeostatic phenotype, which would possibly explain the difference observed between our study in *Drosophila* intestine and previous reports in mouse intestine. Besides, study in mammalian hematopoietic stem cells (HSCs) has suggested that proliferation and survival of relatively quiescent HSCs in the bone marrow is c-Myc independent while active progenitors require c-Myc for proliferation (Wilson et al., 2004). In contrast, our studies found that dMyc is essential for the maintenance of ISCs even when the division rate is low at homeostatic status. The mechanism by which dMyc maintains ISCs is unclear. It is possible that ISCs undergo precocious differentiation or gradually die without the activity of dMyc.

Inactivation of c-Myc in mice HSCs blocks HSCs to initiate differentaion because of their failture to be released from stem cell niche (Wilson et al., 2004). Similarly, the lack of differentiated cells in *dm* mutant clones implies that dMyc might be essential for precursor cells to undergo normal differentiation, although the exact mechanism is not clear. Our study also revealed the requirement of dMyc activity during *Drosophila* adult midgut regeneration after damage treatment and dMyc can be induced by different damage reagents. Whether Myc is also essential for adult tissue regeneration in mammals would be interesting to study.

Our report uncovered a role of dMyc in controlling intestine stem cell proliferation in midgut homeostasis and regeneration and established relationships of dMyc with multiple important signaling pathways. It will be interesting to investigate whether mammalian Myc can also integrate multiple signals to control stem cell proliferation during normal adult tissue homeostasis or in response to injury.

CHAPTER FIVE

Materials and Methods

Drosophila stocks and genetics

The following *Drosophila* stains were used for this study: *14-3-3-ζ -RNAi* transgene was obtained from VDRC (http://www.vdrc.at/). *14-3-3ε*^{j2B10} and *14-3-3ζ* ^{12BL} are strong loss-of-function alleles (http://flybase.org/). *hpo*^{BF33} (Jia et al., 2003); *wts*^{X1} (Xu et al., 1995); *yki*^{B5} (Huang et al., 2005); *ft*$^{G\text{-}rv}$ (Willecke et al., 2006); *esg-Gal4* (Micchelli and Perrimon, 2006); *esg*^{ts}*F/O* (*esg-Gal4 tubGal80*^{ts} *UAS-GFP; UAS-flp Act>CD2>Gal4*), *10XSTAT-dGFP, MyoIA-Gal4, UAS-Dome-RNAi*, *UAS-Upd* and *upd-lacZ* (Jiang et al., 2009); *UAS-Wts-RNAi* (VDRC #106174); *UAS-Yki-RNAi*, *UAS-Sd-RNAi* and *UAS-Yki* (Zhang et al., 2008); *UAS-EGFR-RNAi* (VDRC#43267); *UAS-EGFR*^{A887T} (BL#9534); *UAS-dMyc-RNAi* (VDRC #2948; BL #25784); *dm4* (Pierce et al., 2004); *dmyc-lacZ* (BL #11981 and #12247). To construct *UAS-14-3-3ε-RNAi* transgenes, genomic DNA fragment corresponding to *14-3-3ε* aa45-108 was amplified by PCR and subcloned between the *BglII* and *KpnI* sites of the *pUAST* vector. The corresponding cDNA fragment was inserted in a reverse orientation between *KpnI* and *XbaI* sites. Yki point mutations were generated by PCR-based site directed mutagenesis and verified by DNA sequence. The Yki coding sequence was amplified by PCR and subcloned in frame into the *pUAST-6Myc* vector (Zhang et al., 2008). To construct wild type and mutant forms *of attB-UAS-Myc-Yki* transgenes, a *pUAST* vector with *attB* sequence inserted upstream of the UAS-binding sites was used. The *vas-phi-zh2A-VK5* flies were used to generate Yki transformants

inserted at the 75B1 *attP* locus (Bischof et al., 2007). To construct *HA-14-3-3*, 14-3-3 coding sequences were amplified by PCR and subcloned in frame into the *pUAST-3HA* vector (Zhang et al., 2008). Mutant clones were generated using the MARCM system (Lee and Luo, 2001). Flies stocks were crossed and cultured at 18 ^{0}C. 5 days old F1 adults with the appropriate genotypes were subjected to heat shock at 37 ^{0}C for 1 hour. After clone induction, flies were raised at 18 ^{0}C or 25 ^{0}C for the indicated period of time before dissection. For experiments involving *tubGal80^{ts}*, crosses were set up and cultured at 18 ℃ to restrict Gal4 activity. F1 adult flies were then shifted to 29 ℃ to inactivate Gal80^{ts}. The genotypes for making mutant clones are described as below:

hpo clone
yw UAS-GFP hsflp; FRT42 tubulin Gal80/ FRT42 hpo^{BF33}; tubulin-Gal4/ +.

hpo clone expressing *Yki-RNAi* transgene
yw UAS-GFP hsflp; FRT42 tubulin Gal80/ FRT42 hpo^{BF33}; tubulin-Gal4/ UAS-Yki-RNAi.

hpo clone expressing *Dome-RNAi* transgene
yw UAS-GFP hsflp; FRT42 tubulin Gal80/ FRT42 hpo^{BF33}; tubulin-Gal4/UAS -Dome-RNAi.

hpo clone expressing *EGFR-RNAi* transgene
yw UAS-GFP hsflp; FRT42 tubulin Gal80/ FRT42 hpo^{BF33}; tubulin-Gal4/ UAS-EGFR-RNAi.

wts clone
yw UAS-GFP, hsflp; tubulin-Gal4/ +; FRT82 tubulin Gal80/ FRT82 wts^{x1}.

wts clone expressing *Yki-RNAi* transgene
yw UAS-GFP, hsflp; tubulin-Gal4/ UAS-Yki-RNAi; FRT82 tubulin Gal80/ FRT82 wts^{x1}.

ft clone
yw UAS-GFP, hsflp; FRT40 tubulin Gal80/ FRT40 ft^{G-rv}; tubulin-Gal4/ +

ft clone expressing *Yki-RNAi* transgene
yw UAS-GFP, hsflp; FRT40 tubulin Gal80/ FRT40 $ft^{G\text{-}rv}$; tubulin-Gal4/ UAS-Yki-RNAi.

yki clone
yw UAS-GFP, hsflp; FRT42 tubulin Gal80/ FRT42 yki^{B5}; tubulin-Gal4/ +.

Feeding experiments

5-10 day old female adult flies were used for feeding experiments. Flies were cultured in an empty vial containing a piece of 2.5 cm × 3.75 cm chromatography paper (Fisher) wet with 5% sucrose solution as feeding medium. Flies were fed with 3% of dextran sulfate sodium (MP Biomedicals) or 25 ug/ml bleomycin (Sigma) dissolved in 5% sucrose for 2-3 days at 29℃. For *PE* infection, flies were transferred to fly food laced with 0.5 ml of 10×concentrated overnight *P. entomophila* culture for 2 days at 29℃.

Cell counts

Mitotic indices were quantified by counting $PH3^+$ cells in >10 midguts of the proper genotype, time point or conditions and are presented as means ± standard deviation(SD).

Cell culture and transfection

S2 cells were cultured in *Drosophila* Schneider's Medium (Invitrogen) with 10% fetal bovine serum, 100U/ml of penicillin, and 100ug/ml of Streptomycin. Transfection was carried out using Calcium Phosphate Transfection Kit (Specialty Media)

according to manufacturer's instructions. A *ubiquitin-Gal4* construct was cotransfected with *pUAST* expression vectors for all the transfection experiments. 4ug DNA for ub-Gal4 and 2 ug DNA for each pUAST expression vector were used in a typical transfection experiment.

Immunoprecipitation and western blot analysis

S2 cells were prepared for analysis by washing twice in PBS buffer and were then lysed on ice for 30 min in lysis buffer. Insoluble material was sedimented by centrifugation at 10,000 × g for 15 min at 4^0C and the supernatant was used for immunoprecipitation. Lysates were precleared by incubation with protein A–Sepharose beads (Sigma) for 1 hour or overnight at 4^0C. After removal of the protein-A beads by centrifugation, the cleared lysates were incubated with purified antibodies for 2–4 hours at 4^0C. The immune complexes were collected by incubation with protein A–Sepharose beads for 1 hour at 4^0C, followed by centrifugation. The immunoprecipitates were then washed three times for 10 min each with lysis buffer and fractionated by SDS–PAGE. Antibodies used were mouse anti-HA (Santa Cruz), mouse anti-Myc (Santa Cruz).

Luciferase reporter assay

S2 cells were transfected with *3×Sd2-Luc* and *copia-renilla* luciferase reporter constructs (Zhang et al., 2008) in 12 well plate together with constructs expressing Sd and different mutant forms of Yki. Cells were incubated for 48 hr after transfection

and the luciferase reporter assay was performed using the Dual-Luciferase reporter assay system (Promega). Dual-Luciferase measurements were performed in triplicate using FLUOstar OPTIMA (BMG LABETCH).

Immunostaining

S2 cells were twice in PBS buffer and fixed in 1 X PBS plus 4% formaldehyde for 20 minutes. Cells then were washed in 1 X PBS, 0.5% BSA and 0.1% Triton X-100 for 3 times before incubated with primary and secondary antibodies in the same solution. For imaginal discs staining, larval or pupal heads were pulled or cut off and inverted in PBS buffer. Disc complexes were fixed in 1 X PBS plus 4% formaldehyde for 20 minutes. Samples were washed and incubated with primary and secondary antibodies in a solution containing 1 X PBS and 0.1% Triton X-100. Female flies were used for gut immunostaining in all experiments. The entire gastrointestinal tract was taken and fixed in 1 X PBS plus 8% EM grade Formaldehyde (Polysciences) for 2 hours. Samples were washed and incubated with primary and secondary antibodies in a solution containing 1 X PBS, 0.5% BSA, and 0.1% Triton X-100. The following primary antibodies were used: mouse anti-Delta (DSHB), 1:100; mouse anti-Prospero (DSHB), 1:50; rabbit anti-PH3 (UpSTATe Biotechnology), 1:2000; rabbit anti-GFP (Santa Cruz), 1:500; rabbit anti-Pdm1 (gift from Dr. Xiaohang Yang), 1:2000; mouse anti-dMyc, 1:2; rabbit anti-lacZ (Cell siganling), 1:1,000; rabbit anti-dpERK (Cell Signaling Technology), 1:500; DRAQ5 (Cell Signal); mouse anti-Myc (Santa Cruz), 1:50; rabbit anti-Flag(Santa Cruz), 1:200; rabbit anti-HA (Santa Cruz), 1:200; rabbit

anti-Yki (Oh and Irvine, 2008), 1:500 and 7-AAD (Molecular Probes).

RT-qPCR

RNA was extracted from 10 female midguts using RNAeasy Mini kit (QIAGEN), and cDNA was synthesized using the iScript cDNA synthesis kit (Bio-Rad). RT-qPCR was performed using iQ5 System (Bio-Rad). RT-qPCR was performed in duplicate on each of 3 independent biological replicates. RpL11 was used as a normalization control. Primer sequences are listed as below:

upd: Forward: 5' CCACGTAAGTTTGCATGTTG 3'
Reverse: 5' CTAAACAGTAGCCAGGACTC 3'

upd2: Forward: 5' ACTGTTGCATGTGGATGCTG 3'
Reverse: 5' CAGCCAAGGACGAGTTATCA 3'

upd3: Forward: 5' GAGCACCAAGACTCTGGACA 3'
Reverse: CCAGTGCAACTTGATGTTGC 3'

Socs36E: Forward: 5' CAGTCAGCAATATGTTGTCG 3'
Reverse: 5' ACTTGCAGCATCGTCGCTTC 3'

vn: Forward: 5' TCACACATTTAGTGGTGGAAG 3'
Reverse: 5' TTGTGATGCTTGAATTGGTAA 3'

Spi: Forward: 5' CGCCCAAGAATGAAAGAGAG 3'
Reverse: 5' AGGTATGCTGCTGGTGGAAC 3'

Krn: Forward: 5' CGTGTTTGGCAACAACAAGT 3'
Reverse: 5' TGTGGCAATGCAGTTTAAGG 3'

CHAPTER SIX

Conclusions and Future Studies

Conclusions

My thesis covers mainly three parts: The involvement of 14-3-3 protein in the regulation of Hpo signaling pathway transcriptional activator Yki; The regulation of intestine stem cell proliferation by Hpo signaling pathway and Identification of dMyc as downstream target genes of multiple signaling pathways to regulate intestine stem cell proliferation and gut regeneration.

For the first part, I provided genetic evidence that both isoforms of 14-3-3, 14-3-3ε and 14-3-3ζ, regulate Yki activity through modulating its subcellular localization. Inactivation of 14-3-3 by RNAi or genetic mutations enhanced whereas overexpression of 14-3-3 suppressed tissue overgrowth induced by Yki overexpression. Loss of 14-3-3 resulted in the accumulation of Yki in the nucleus. We found that regulation of Yki by 14-3-3 was mediated by phosphorylation of Yki at S168. In addition, we found that Hpo signaling also inhibited Yki nuclear localization and activity by phosphorylating Yki at S111 and S250, and this inhibition appears to be independent of 14-3-3.

For the second part, I demonstrated that the Hpo signaling pathway, an evolutionarily conserved pathway implicated in organ size control and tumorigenesis, plays an essential role in regulating ISC proliferation. Loss of Hpo signaling in either midgut

precursor cells or epithelial cells stimulates ISC proliferation. I provided evidence that loss of Hpo signaling in epithelial cells increases the production of cytokines of the Upd family and multiple EGFR ligands that activate Jak-Stat and EGFR signaling pathways in ISCs to stimulate their proliferation, thus revealing a novel non cell-autonomous role of Hpo signaling in blocking ISC proliferation. Finally, I showed that the Hpo pathway mediator Yki is also required in precursor cells for injury-induced ISC proliferation in response to tissue damaging reagent DSS.

For the third part, I demonstrated that the *Drosophila* homolog of oncoprotein Myc (dMyc) functions downstream of Jak-Stat, EGFR and Hpo signaling pathways to mediate their effect on ISC proliferation. While basal expression of dMyc is required for normal tissue homeostasis, dMyc transcription in ISCs is stimulated in response to tissue damage. I also demonstrated that tissue damage stimulates dMyc transcription through Jak-Stat, EGFR and Hpo pathways.

Future Studies

1 Examine whether dMyc is a direct transcriptional target of Hpo, Jak-Stat and EGFR pathways

My previous studies have indicated that dMyc can be regulated by Hpo, Jak-Stat and EGFR pathways at transcriptional level and a *dm* enhancer region containing potential binding sites for Sd, Stat and Pnt has been identified. This enhancer region is responsive to Hpo and Jak-Stat pathways *in vitro*, I will further investigate its activity

in vivo. I have made a construct putting this enhancer region upstream a GFP gene and injected into flies to make transformants. I will examine the expression pattern of this Enhancer-GFP transgene and whether it can respond to Hpo, Jak-Stat and EGFR pathways in *Drosophila* adult midgut. To distinguish whether dMyc is a direct or indirect target of Hpo, Jak-Stat and EGFR pathways, I will perform Chromatin Immunoprecipitation (Chip) experiment to examine whether Yki/Sd, Stat or Pnt can directly bind to *dm* gene locus in precursor cells. Since precursor cells constitute only a small portion of total cells in adult midgut, I can inactivate Notch signaling pathway to ectopically enrich stem cell pools and perform Chip experiment in this genetic background.

2 Investigate whether blocking ectopic upregulation of dMyc level could inhibit stimulated ISC proliferation

Previous results have shown that dMyc is essential for normal homeostasis as well as tissue regeneration after damage in *Drosophila* adult midgut, and dMyc is ectopically upregulated in precursor cells when midgut is exposed to damage reagents, I want to examine whether the enhanced ISC proliferation by damage reagents treatment is dependent on the ectopically induced dMyc. I will make *dm* mutant clones and express *UAS-dMyc* transgene under the control of *tublin-Gal4* driver inside clones and compare clone sizes with either *dm* mutant clones alone or wild type clones under normal homeostatic condition as well as after damage treatment. Moreover, I can coexpress *UAS-dMyc* transgene with *dMyc-RNAi* in precursor cells, and examine

dMyc level and ISC proliferation under homeostasis and damage treatment conditions.

3 Determine the mechanism of the loss of ISCs when dMyc is inactivated

When dMyc is inactivated in precursor cells by overexpressing dMyc-RNAi, I observed a gradual loss of stem cells in adult midgut. However, whether the ISCs loss is due to cell death or premature differentiation is not clear. To distinguish between these two possibilities, I can stain midguts overexpressing dMyc-RNAi in precursor cells with cell death marker. I can also coexpress a cell death inhibitor UAS-P35 transgene to see whether it can rescue ISCs loss. Besides, I can examine whether there is still any ISCs loss in the background of Notch inactivation in precursor cells, which can block differentiation.

BIBLIOGRAPHY

Acevedo, S.F., Tsigkari, K.K., Grammenoudi, S., and Skoulakis, E.M. (2007). In vivo functional specificity and homeostasis of Drosophila 14-3-3 proteins. Genetics *177*, 239-253.

Amcheslavsky, A., Jiang, J., and Ip, Y.T. (2009). Tissue damage-induced intestinal stem cell division in Drosophila. Cell Stem Cell *4*, 49-61.

Apidianakis, Y., Pitsouli, C., Perrimon, N., and Rahme, L. (2009). Synergy between bacterial infection and genetic predisposition in intestinal dysplasia. Proc Natl Acad Sci U S A *106*, 20883-20888.

Arbouzova, N.I., and Zeidler, M.P. (2006). JAK/STAT signalling in Drosophila: insights into conserved regulatory and cellular functions. Development *133*, 2605-2616.

Bach, E.A., Ekas, L.A., Ayala-Camargo, A., Flaherty, M.S., Lee, H., Perrimon, N., and Baeg, G.H. (2007). GFP reporters detect the activation of the Drosophila JAK/STAT pathway in vivo. Gene Expr Patterns *7*, 323-331.

Badouel, C., Gardano, L., Amin, N., Garg, A., Rosenfeld, R., Le Bihan, T., and McNeill, H. (2009). The FERM-domain protein Expanded regulates Hippo pathway activity via direct interactions with the transcriptional activator Yorkie. Dev Cell *16*, 411-420.

Baena-Lopez, L.A., Rodriguez, I., and Baonza, A. (2008). The tumor suppressor genes dachsous and fat modulate different signalling pathways by regulating dally and dally-like. Proc Natl Acad Sci U S A *105*, 9645-9650.

Baumgartner, R., Poernbacher, I., Buser, N., Hafen, E., and Stocker, H. (2010). The WW domain protein Kibra acts upstream of Hippo in Drosophila. Dev Cell *18*, 309-316.

Beachy, P.A., Karhadkar, S.S., and Berman, D.M. (2004). Tissue repair and stem cell renewal in carcinogenesis. Nature *432*, 324-331.

Beebe, K., Lee, W.C., and Micchelli, C.A. (2009). JAK/STAT signaling coordinates stem cell proliferation and multilineage differentiation in the Drosophila intestinal stem cell lineage. Dev Biol *338*, 28-37.

Bennett, F.C., and Harvey, K.F. (2006). Fat cadherin modulates organ size in Drosophila via the Salvador/Warts/Hippo signaling pathway. Curr Biol *16*, 2101-2110.

Benton, R., and St Johnston, D. (2003). Drosophila PAR-1 and 14-3-3 inhibit Bazooka/PAR-3 to establish complementary cortical domains in polarized cells. Cell *115*, 691-704.

Bettess, M.D., Dubois, N., Murphy, M.J., Dubey, C., Roger, C., Robine, S., and Trumpp, A. (2005). c-Myc is required for the formation of intestinal crypts but dispensable for homeostasis of the adult intestinal epithelium. Mol Cell Biol *25*, 7868-7878.

Bilder, D., Li, M., and Perrimon, N. (2000). Cooperative regulation of cell polarity and growth by Drosophila tumor suppressors. Science *289*, 113-116.

Bischof, J., Maeda, R.K., Hediger, M., Karch, F., and Basler, K. (2007). An optimized transgenesis system for Drosophila using germ-line-specific phiC31 integrases. Proc Natl Acad Sci U S A *104*, 3312-3317.

Biteau, B., Hochmuth, C.E., and Jasper, H. (2008). JNK activity in somatic stem cells causes loss of tissue homeostasis in the aging Drosophila gut. Cell Stem Cell *3*, 442-455.

Biteau, B., and Jasper, H. (2011). EGF signaling regulates the proliferation of intestinal stem cells in Drosophila. Development *138*, 1045-1055.

Brittle, A.L., Repiso, A., Casal, J., Lawrence, P.A., and Strutt, D. (2010). Four-jointed modulates growth and planar polarity by reducing the affinity of dachsous for fat. Curr Biol *20*, 803-810.

Bryant, P.J., and Simpson, P. (1984). Intrinsic and extrinsic control of growth in developing organs. Q Rev Biol *59*, 387-415.

Buchon, N., Broderick, N.A., Chakrabarti, S., and Lemaitre, B. (2009a). Invasive and indigenous microbiota impact intestinal stem cell activity through multiple pathways in Drosophila. Genes Dev *23*, 2333-2344.

Buchon, N., Broderick, N.A., Kuraishi, T., and Lemaitre, B. (2010). Drosophila EGFR pathway coordinates stem cell proliferation and gut remodeling following infection. BMC Biol *8*, 152.

Buchon, N., Broderick, N.A., Poidevin, M., Pradervand, S., and Lemaitre, B. (2009b). Drosophila intestinal response to bacterial infection: activation of host defense and stem cell proliferation. Cell Host Microbe *5*, 200-211.

Cai, J., Zhang, N., Zheng, Y., de Wilde, R.F., Maitra, A., and Pan, D. (2010). The Hippo signaling pathway restricts the oncogenic potential of an intestinal regeneration program. Genes Dev *24*, 2383-2388.

Cartwright, P., McLean, C., Sheppard, A., Rivett, D., Jones, K., and Dalton, S. (2005). LIF/STAT3 controls ES cell self-renewal and pluripotency by a Myc-dependent mechanism. Development *132*, 885-896.

Chatterjee, M., and Ip, Y.T. (2009). Pathogenic stimulation of intestinal stem cell response in Drosophila. J Cell Physiol *220*, 664-671.

Chen, C.L., Gajewski, K.M., Hamaratoglu, F., Bossuyt, W., Sansores-Garcia, L., Tao, C., and Halder, G. (2010). The apical-basal cell polarity determinant Crumbs regulates Hippo signaling in Drosophila. Proc Natl Acad Sci U S A *107*, 15810-15815.

Cho, E., Feng, Y., Rauskolb, C., Maitra, S., Fehon, R., and Irvine, K.D. (2006). Delineation of a Fat tumor suppressor pathway. Nat Genet *38*, 1142-1150.

Cho, E., and Irvine, K.D. (2004). Action of fat, four-jointed, dachsous and dachs in distal-to-proximal wing signaling. Development *131*, 4489-4500.

Conlon, I., and Raff, M. (1999). Size control in animal development. Cell *96*, 235-244.

Das Thakur, M., Feng, Y., Jagannathan, R., Seppa, M.J., Skeath, J.B., and Longmore, G.D. (2010). Ajuba LIM proteins are negative regulators of the Hippo signaling pathway. Curr Biol *20*, 657-662.

de la Cova, C., Abril, M., Bellosta, P., Gallant, P., and Johnston, L.A. (2004). Drosophila myc regulates organ size by inducing cell competition. Cell *117*, 107-116.

Dong, J., Feldmann, G., Huang, J., Wu, S., Zhang, N., Comerford, S.A., Gayyed, M.F., Anders, R.A., Maitra, A., and Pan, D. (2007). Elucidation of a universal size-control mechanism in Drosophila and mammals. Cell *130*, 1120-1133.

Dontu, G., Jackson, K.W., McNicholas, E., Kawamura, M.J., Abdallah, W.M., and Wicha, M.S. (2004). Role of Notch signaling in cell-fate determination of human mammary stem/progenitor cells. Breast Cancer Res *6*, R605-615.

Dubois, N.C., Adolphe, C., Ehninger, A., Wang, R.A., Robertson, E.J., and Trumpp, A. (2008). Placental rescue reveals a sole requirement for c-Myc in embryonic erythroblast survival and hematopoietic stem cell function. Development *135*, 2455-2465.

Eilers, M., and Eisenman, R.N. (2008). Myc's broad reach. Genes Dev *22*, 2755-2766.

Ekas, L.A., Cardozo, T.J., Flaherty, M.S., McMillan, E.A., Gonsalves, F.C., and Bach, E.A. Characterization of a dominant-active STAT that promotes tumorigenesis in Drosophila. Dev Biol *344*, 621-636.

Fan, Y., and Bergmann, A. (2008). Apoptosis-induced compensatory proliferation. The Cell is dead.

Long live the Cell! Trends Cell Biol *18*, 467-473.

Feng, Y., and Irvine, K.D. (2007). Fat and expanded act in parallel to regulate growth through warts. Proc Natl Acad Sci U S A *104*, 20362-20367.

Feng, Y., and Irvine, K.D. (2009). Processing and phosphorylation of the Fat receptor. Proc Natl Acad Sci U S A *106*, 11989-11994.

Fre, S., Huyghe, M., Mourikis, P., Robine, S., Louvard, D., and Artavanis-Tsakonas, S. (2005). Notch signals control the fate of immature progenitor cells in the intestine. Nature *435*, 964-968.

Gabay, L., Seger, R., and Shilo, B.Z. (1997). MAP kinase in situ activation atlas during Drosophila embryogenesis. Development *124*, 3535-3541.

Genevet, A., Polesello, C., Blight, K., Robertson, F., Collinson, L.M., Pichaud, F., and Tapon, N. (2009). The Hippo pathway regulates apical-domain size independently of its growth-control function. J Cell Sci *122*, 2360-2370.

Genevet, A., Wehr, M.C., Brain, R., Thompson, B.J., and Tapon, N. (2010). Kibra is a regulator of the Salvador/Warts/Hippo signaling network. Dev Cell *18*, 300-308.

Gilbert, M.M., Tipping, M., Veraksa, A., and Moberg, K.H. (2011). A screen for conditional growth suppressor genes identifies the Drosophila homolog of HD-PTP as a regulator of the oncoprotein Yorkie. Dev Cell *20*, 700-712.

Goulev, Y., Fauny, J.D., Gonzalez-Marti, B., Flagiello, D., Silber, J., and Zider, A. (2008). SCALLOPED interacts with YORKIE, the nuclear effector of the hippo tumor-suppressor pathway in Drosophila. Curr Biol *18*, 435-441.

Grandori, C., Gomez-Roman, N., Felton-Edkins, Z.A., Ngouenet, C., Galloway, D.A., Eisenman, R.N., and White, R.J. (2005). c-Myc binds to human ribosomal DNA and stimulates transcription of rRNA genes by RNA polymerase I. Nat Cell Biol *7*, 311-318.

Grewal, S.S., Li, L., Orian, A., Eisenman, R.N., and Edgar, B.A. (2005). Myc-dependent regulation of ribosomal RNA synthesis during Drosophila development. Nat Cell Biol *7*, 295-302.

Grusche, F.A., Degoutin, J.L., Richardson, H.E., and Harvey, K.F. (2011). The Salvador/Warts/Hippo pathway controls regenerative tissue growth in Drosophila melanogaster. Dev Biol *350*, 255-266.

Grzeschik, N.A., Parsons, L.M., Allott, M.L., Harvey, K.F., and Richardson, H.E. (2010). Lgl, aPKC, and Crumbs regulate the Salvador/Warts/Hippo pathway through two distinct mechanisms. Curr Biol *20*, 573-581.

Hamaratoglu, F., Willecke, M., Kango-Singh, M., Nolo, R., Hyun, E., Tao, C., Jafar-Nejad, H., and Halder, G. (2006). The tumour-suppressor genes NF2/Merlin and Expanded act through Hippo signalling to regulate cell proliferation and apoptosis. Nat Cell Biol *8*, 27-36.

Hao, Y., Chun, A., Cheung, K., Rashidi, B., and Yang, X. (2008). Tumor suppressor LATS1 is a negative regulator of oncogene YAP. J Biol Chem *283*, 5496-5509.

Hariharan, I.K., and Bilder, D. (2006). Regulation of imaginal disc growth by tumor-suppressor genes in Drosophila. Annu Rev Genet *40*, 335-361.

Harvey, K.F., Pfleger, C.M., and Hariharan, I.K. (2003). The Drosophila Mst ortholog, hippo, restricts growth and cell proliferation and promotes apoptosis. Cell *114*, 457-467.

Hay, B.A., Wolff, T., and Rubin, G.M. (1994). Expression of baculovirus P35 prevents cell death in Drosophila. Development *120*, 2121-2129.

He, C., Hu, H., Braren, R., Fong, S.Y., Trumpp, A., Carlson, T.R., and Wang, R.A. (2008). c-myc in the hematopoietic lineage is crucial for its angiogenic function in the mouse embryo. Development *135*, 2467-2477.

Herranz, H., Perez, L., Martin, F.A., and Milan, M. (2008). A Wingless and Notch double-repression mechanism regulates G1-S transition in the Drosophila wing. EMBO J *27*, 1633-1645.

Hochmuth, C.E., Biteau, B., Bohmann, D., and Jasper, H. (2011). Redox regulation by Keap1 and Nrf2 controls intestinal stem cell proliferation in Drosophila. Cell Stem Cell *8*, 188-199.

Huang, J., Wu, S., Barrera, J., Matthews, K., and Pan, D. (2005). The Hippo signaling pathway coordinately regulates cell proliferation and apoptosis by inactivating Yorkie, the Drosophila Homolog of YAP. Cell *122*, 421-434.

Hulf, T., Bellosta, P., Furrer, M., Steiger, D., Svensson, D., Barbour, A., and Gallant, P. (2005). Whole-genome analysis reveals a strong positional bias of conserved dMyc-dependent E-boxes. Mol Cell Biol *25*, 3401-3410.

Humbert, P.O., Grzeschik, N.A., Brumby, A.M., Galea, R., Elsum, I., and Richardson, H.E. (2008). Control of tumourigenesis by the Scribble/Dlg/Lgl polarity module. Oncogene *27*, 6888-6907.

Ishikawa, H.O., Takeuchi, H., Haltiwanger, R.S., and Irvine, K.D. (2008). Four-jointed is a Golgi kinase that phosphorylates a subset of cadherin domains. Science *321*, 401-404.

Jia, J., Zhang, W., Wang, B., Trinko, R., and Jiang, J. (2003). The Drosophila Ste20 family kinase dMST functions as a tumor suppressor by restricting cell proliferation and promoting apoptosis. Genes Dev *17*, 2514-2519.

Jiang, H., and Edgar, B.A. (2011). Intestinal stem cells in the adult Drosophila midgut. Exp Cell Res *317*, 2780-2788.

Jiang, H., Grenley, M.O., Bravo, M.J., Blumhagen, R.Z., and Edgar, B.A. (2011). EGFR/Ras/MAPK signaling mediates adult midgut epithelial homeostasis and regeneration in Drosophila. Cell Stem Cell *8*, 84-95.

Jiang, H., Patel, P.H., Kohlmaier, A., Grenley, M.O., McEwen, D.G., and Edgar, B.A. (2009). Cytokine/Jak/Stat signaling mediates regeneration and homeostasis in the Drosophila midgut. Cell *137*, 1343-1355.

Jin, Z., Kirilly, D., Weng, C., Kawase, E., Song, X., Smith, S., Schwartz, J., and Xie, T. (2008). Differentiation-defective stem cells outcompete normal stem cells for niche occupancy in the Drosophila ovary. Cell Stem Cell *2*, 39-49.

Johnston, L.A., Prober, D.A., Edgar, B.A., Eisenman, R.N., and Gallant, P. (1999). Drosophila myc regulates cellular growth during development. Cell *98*, 779-790.

Justice, R.W., Zilian, O., Woods, D.F., Noll, M., and Bryant, P.J. (1995). The Drosophila tumor suppressor gene warts encodes a homolog of human myotonic dystrophy kinase and is required for the control of cell shape and proliferation. Genes Dev *9*, 534-546.

Karpowicz, P., Perez, J., and Perrimon, N. (2010). The Hippo tumor suppressor pathway regulates intestinal stem cell regeneration. Development *137*, 4135-4145.

Kim, J., Chu, J., Shen, X., Wang, J., and Orkin, S.H. (2008). An extended transcriptional network for pluripotency of embryonic stem cells. Cell *132*, 1049-1061.

Knoepfler, P.S., Cheng, P.F., and Eisenman, R.N. (2002). N-myc is essential during neurogenesis for the rapid expansion of progenitor cell populations and the inhibition of neuronal differentiation. Genes Dev *16*, 2699-2712.

Kobayashi, C.I., and Suda, T. (2011). Regulation of reactive oxygen species in stem cells and cancer stem cells. J Cell Physiol *227*, 421-430.

Lai, Z.C., Wei, X., Shimizu, T., Ramos, E., Rohrbaugh, M., Nikolaidis, N., Ho, L.L., and Li, Y. (2005). Control of cell proliferation and apoptosis by mob as tumor suppressor, mats. Cell *120*, 675-685.

Lee, T., and Luo, L. (2001). Mosaic analysis with a repressible cell marker (MARCM) for Drosophila neural development. Trends Neurosci *24*, 251-254.

Lee, W.C., Beebe, K., Sudmeier, L., and Micchelli, C.A. (2009). Adenomatous polyposis coli regulates Drosophila intestinal stem cell proliferation. Development *136*, 2255-2264.

Lesokhin, A.M., Yu, S.Y., Katz, J., and Baker, N.E. (1999). Several levels of EGF receptor signaling during photoreceptor specification in wild-type, Ellipse, and null mutant Drosophila. Dev Biol *205*, 129-144.

Li, L., Edgar, B.A., and Grewal, S.S. (2010). Nutritional control of gene expression in Drosophila larvae via TOR, Myc and a novel cis-regulatory element. BMC Cell Biol *11*, 7.

Lin, G., Xu, N., and Xi, R. (2008). Paracrine Wingless signalling controls self-renewal of Drosophila intestinal stem cells. Nature *455*, 1119-1123.

Lin, G., Xu, N., and Xi, R. (2009). Paracrine unpaired signaling through the JAK/STAT pathway controls self-renewal and lineage differentiation of drosophila intestinal stem cells. J Mol Cell Biol *2*, 37-49.

Ling, C., Zheng, Y., Yin, F., Yu, J., Huang, J., Hong, Y., Wu, S., and Pan, D. (2010). The apical transmembrane protein Crumbs functions as a tumor suppressor that regulates Hippo signaling by binding to Expanded. Proc Natl Acad Sci U S A *107*, 10532-10537.

Liu, W., Singh, S.R., and Hou, S.X. (2010). JAK-STAT is restrained by Notch to control cell proliferation of the Drosophila intestinal stem cells. J Cell Biochem *109*, 992-999.

Lu, L., Li, Y., Kim, S.M., Bossuyt, W., Liu, P., Qiu, Q., Wang, Y., Halder, G., Finegold, M.J., Lee, J.S., *et al.* (2010). Hippo signaling is a potent in vivo growth and tumor suppressor pathway in the mammalian liver. Proc Natl Acad Sci U S A *107*, 1437-1442.

Mackintosh, C. (2004). Dynamic interactions between 14-3-3 proteins and phosphoproteins regulate diverse cellular processes. Biochem J *381*, 329-342.

Mahoney, P.A., Weber, U., Onofrechuk, P., Biessmann, H., Bryant, P.J., and Goodman, C.S. (1991). The fat tumor suppressor gene in Drosophila encodes a novel member of the cadherin gene superfamily. Cell *67*, 853-868.

Maitra, S., Kulikauskas, R.M., Gavilan, H., and Fehon, R.G. (2006). The tumor suppressors Merlin and Expanded function cooperatively to modulate receptor endocytosis and signaling. Curr Biol *16*, 702-709.

Mao, Y., Kucuk, B., and Irvine, K.D. (2009). Drosophila lowfat, a novel modulator of Fat signaling. Development *136*, 3223-3233.

Mao, Y., Rauskolb, C., Cho, E., Hu, W.L., Hayter, H., Minihan, G., Katz, F.N., and Irvine, K.D. (2006). Dachs: an unconventional myosin that functions downstream of Fat to regulate growth, affinity and gene expression in Drosophila. Development *133*, 2539-2551.

Matakatsu, H., and Blair, S.S. (2004). Interactions between Fat and Dachsous and the regulation of planar cell polarity in the Drosophila wing. Development *131*, 3785-3794.

Matakatsu, H., and Blair, S.S. (2008). The DHHC palmitoyltransferase approximated regulates Fat signaling and Dachs localization and activity. Curr Biol *18*, 1390-1395.

McCartney, B.M., Kulikauskas, R.M., LaJeunesse, D.R., and Fehon, R.G. (2000). The neurofibromatosis-2 homologue, Merlin, and the tumor suppressor expanded function together in Drosophila to regulate cell proliferation and differentiation. Development *127*, 1315-1324.

McGuire, S.E., Mao, Z., and Davis, R.L. (2004). Spatiotemporal gene expression targeting with the TARGET and gene-switch systems in Drosophila. Sci STKE *2004*, pl6.

Meignin, C., Alvarez-Garcia, I., Davis, I., and Palacios, I.M. (2007). The salvador-warts-hippo pathway is required for epithelial proliferation and axis specification in Drosophila. Curr Biol *17*, 1871-1878.

Menendez, J., Perez-Garijo, A., Calleja, M., and Morata, G. (2010). A tumor-suppressing mechanism in Drosophila involving cell competition and the Hippo pathway. Proc Natl Acad Sci U S A *107*, 14651-14656.

Meyer, N., and Penn, L.Z. (2008). Reflecting on 25 years with MYC. Nat Rev Cancer *8*, 976-990.

Micchelli, C.A., and Perrimon, N. (2006). Evidence that stem cells reside in the adult Drosophila midgut epithelium. Nature *439*, 475-479.

Morrison, D.K. (2009). The 14-3-3 proteins: integrators of diverse signaling cues that impact cell fate and cancer development. Trends Cell Biol *19*, 16-23.

Muncan, V., Sansom, O.J., Tertoolen, L., Phesse, T.J., Begthel, H., Sancho, E., Cole, A.M., Gregorieff, A., de Alboran, I.M., Clevers, H., *et al.* (2006). Rapid loss of intestinal crypts upon conditional deletion of the Wnt/Tcf-4 target gene c-Myc. Mol Cell Biol *26*, 8418-8426.

Neto-Silva, R.M., de Beco, S., and Johnston, L.A. (2010). Evidence for a growth-stabilizing regulatory feedback mechanism between Myc and Yorkie, the Drosophila homolog of Yap. Dev Cell *19*, 507-520.

Nolo, R., Morrison, C.M., Tao, C., Zhang, X., and Halder, G. (2006). The bantam microRNA is a target of the hippo tumor-suppressor pathway. Curr Biol *16*, 1895-1904.

Oh, H., and Irvine, K.D. (2008). In vivo regulation of Yorkie phosphorylation and localization. Development *135*, 1081-1088.

Oh, H., and Irvine, K.D. (2009). In vivo analysis of Yorkie phosphorylation sites. Oncogene *28*, 1916-1927.

Oh, H., and Irvine, K.D. (2011). Cooperative regulation of growth by Yorkie and Mad through bantam. Dev Cell *20*, 109-122.

Ohlstein, B., and Spradling, A. (2006). The adult Drosophila posterior midgut is maintained by pluripotent stem cells. Nature *439*, 470-474.

Ohlstein, B., and Spradling, A. (2007). Multipotent Drosophila intestinal stem cells specify daughter cell fates by differential notch signaling. Science *315*, 988-992.

Orian, A., van Steensel, B., Delrow, J., Bussemaker, H.J., Li, L., Sawado, T., Williams, E., Loo, L.W., Cowley, S.M., Yost, C., *et al.* (2003). Genomic binding by the Drosophila Myc, Max, Mad/Mnt transcription factor network. Genes Dev *17*, 1101-1114.

Pan, D. (2007). Hippo signaling in organ size control. Genes Dev *21*, 886-897.

Pan, D. (2010). The hippo signaling pathway in development and cancer. Dev Cell *19*, 491-505.

Pantalacci, S., Tapon, N., and Leopold, P. (2003). The Salvador partner Hippo promotes apoptosis and cell-cycle exit in Drosophila. Nat Cell Biol *5*, 921-927.

Pellock, B.J., Buff, E., White, K., and Hariharan, I.K. (2007). The Drosophila tumor suppressors Expanded and Merlin differentially regulate cell cycle exit, apoptosis, and Wingless signaling. Dev Biol *304*, 102-115.

Peng, H.W., Slattery, M., and Mann, R.S. (2009). Transcription factor choice in the Hippo signaling pathway: homothorax and yorkie regulation of the microRNA bantam in the progenitor domain of the Drosophila eye imaginal disc. Genes Dev *23*, 2307-2319.

Pierce, S.B., Yost, C., Britton, J.S., Loo, L.W., Flynn, E.M., Edgar, B.A., and Eisenman, R.N. (2004). dMyc is required for larval growth and endoreplication in Drosophila. Development *131*, 2317-2327.

Polesello, C., Huelsmann, S., Brown, N.H., and Tapon, N. (2006). The Drosophila RASSF homolog antagonizes the hippo pathway. Curr Biol *16*, 2459-2465.

Polesello, C., and Tapon, N. (2007). Salvador-warts-hippo signaling promotes Drosophila posterior follicle cell maturation downstream of notch. Curr Biol *17*, 1864-1870.

Presnell, S.C., Petersen, B., and Heidaran, M. (2002). Stem cells in adult tissues. Semin Cell Dev Biol *13*, 369-376.

Rauskolb, C., Pan, G., Reddy, B.V., Oh, H., and Irvine, K.D. (2011). Zyxin links fat signaling to the hippo pathway. PLoS Biol *9*, e1000624.

Reddy, B.V., and Irvine, K.D. (2008). The Fat and Warts signaling pathways: new insights into their regulation, mechanism and conservation. Development *135*, 2827-2838.

Reddy, B.V., Rauskolb, C., and Irvine, K.D. (2010). Influence of fat-hippo and notch signaling on the proliferation and differentiation of Drosophila optic neuroepithelia. Development *137*, 2397-2408.

Ren, F., Wang, B., Yue, T., Yun, E.Y., Ip, Y.T., and Jiang, J. (2010a). Hippo signaling regulates Drosophila intestine stem cell proliferation through multiple pathways. Proc Natl Acad Sci U S A *107*, 21064-21069.

Ren, F., Zhang, L., and Jiang, J. (2010b). Hippo signaling regulates Yorkie nuclear localization and activity through 14-3-3 dependent and independent mechanisms. Dev Biol *337*, 303-312.

Rhiner, C., Diaz, B., Portela, M., Poyatos, J.F., Fernandez-Ruiz, I., Lopez-Gay, J.M., Gerlitz, O., and Moreno, E. (2009). Persistent competition among stem cells and their daughters in the Drosophila ovary germline niche. Development *136*, 995-1006.

Ribeiro, P.S., Josue, F., Wepf, A., Wehr, M.C., Rinner, O., Kelly, G., Tapon, N., and Gstaiger, M. (2010). Combined functional genomic and proteomic approaches identify a PP2A complex as a negative regulator of Hippo signaling. Mol Cell *39*, 521-534.

Robinson, B.S., Huang, J., Hong, Y., and Moberg, K.H. (2010). Crumbs regulates Salvador/Warts/Hippo signaling in Drosophila via the FERM-domain protein Expanded. Curr Biol *20*, 582-590.

Rogulja, D., Rauskolb, C., and Irvine, K.D. (2008). Morphogen control of wing growth through the Fat signaling pathway. Dev Cell *15*, 309-321.

Sansores-Garcia, L., Bossuyt, W., Wada, K., Yonemura, S., Tao, C., Sasaki, H., and Halder, G. (2011). Modulating F-actin organization induces organ growth by affecting the Hippo pathway. EMBO J *30*, 2325-2335.

Shaw, R.L., Kohlmaier, A., Polesello, C., Veelken, C., Edgar, B.A., and Tapon, N. (2010). The Hippo pathway regulates intestinal stem cell proliferation during Drosophila adult midgut regeneration. Development *137*, 4147-4158.

Shilo, B.Z. (2005). Regulating the dynamics of EGF receptor signaling in space and time. Development *132*, 4017-4027.

Silva, E., Tsatskis, Y., Gardano, L., Tapon, N., and McNeill, H. (2006). The tumor-suppressor gene fat controls tissue growth upstream of expanded in the hippo signaling pathway. Curr Biol *16*, 2081-2089.

Simon, M.A., Xu, A., Ishikawa, H.O., and Irvine, K.D. (2010). Modulation of fat:dachsous binding by the cadherin domain kinase four-jointed. Curr Biol *20*, 811-817.

Smith-Bolton, R.K., Worley, M.I., Kanda, H., and Hariharan, I.K. (2009). Regenerative growth in Drosophila imaginal discs is regulated by Wingless and Myc. Dev Cell *16*, 797-809.

Sopko, R., Silva, E., Clayton, L., Gardano, L., Barrios-Rodiles, M., Wrana, J., Varelas, X., Arbouzova, N.I., Shaw, S., Saburi, S., *et al.* (2009). Phosphorylation of the tumor suppressor fat is regulated by its ligand Dachsous and the kinase discs overgrown. Curr Biol *19*, 1112-1117.

Staley, B.K., and Irvine, K.D. (2010). Warts and Yorkie mediate intestinal regeneration by influencing

stem cell proliferation. Curr Biol *20*, 1580-1587.

Sun, G., and Irvine, K.D. (2011). Regulation of Hippo signaling by Jun kinase signaling during compensatory cell proliferation and regeneration, and in neoplastic tumors. Dev Biol *350*, 139-151.

Tapon, N., Harvey, K.F., Bell, D.W., Wahrer, D.C., Schiripo, T.A., Haber, D.A., and Hariharan, I.K. (2002). salvador Promotes both cell cycle exit and apoptosis in Drosophila and is mutated in human cancer cell lines. Cell *110*, 467-478.

Thompson, B.J., and Cohen, S.M. (2006). The Hippo pathway regulates the bantam microRNA to control cell proliferation and apoptosis in Drosophila. Cell *126*, 767-774.

Tyler, D.M., and Baker, N.E. (2007). Expanded and fat regulate growth and differentiation in the Drosophila eye through multiple signaling pathways. Dev Biol *305*, 187-201.

Udan, R.S., Kango-Singh, M., Nolo, R., Tao, C., and Halder, G. (2003). Hippo promotes proliferation arrest and apoptosis in the Salvador/Warts pathway. Nat Cell Biol *5*, 914-920.

Vita, M., and Henriksson, M. (2006). The Myc oncoprotein as a therapeutic target for human cancer. Semin Cancer Biol *16*, 318-330.

Watt, F.M., Frye, M., and Benitah, S.A. (2008). MYC in mammalian epidermis: how can an oncogene stimulate differentiation? Nat Rev Cancer *8*, 234-242.

Wei, X., Shimizu, T., and Lai, Z.C. (2007). Mob as tumor suppressor is activated by Hippo kinase for growth inhibition in Drosophila. EMBO J *26*, 1772-1781.

Willecke, M., Hamaratoglu, F., Kango-Singh, M., Udan, R., Chen, C.L., Tao, C., Zhang, X., and Halder, G. (2006). The fat cadherin acts through the hippo tumor-suppressor pathway to regulate tissue size. Curr Biol *16*, 2090-2100.

Willecke, M., Hamaratoglu, F., Sansores-Garcia, L., Tao, C., and Halder, G. (2008). Boundaries of Dachsous Cadherin activity modulate the Hippo signaling pathway to induce cell proliferation. Proc Natl Acad Sci U S A *105*, 14897-14902.

Wilson, A., Murphy, M.J., Oskarsson, T., Kaloulis, K., Bettess, M.D., Oser, G.M., Pasche, A.C., Knabenhans, C., Macdonald, H.R., and Trumpp, A. (2004). c-Myc controls the balance between hematopoietic stem cell self-renewal and differentiation. Genes Dev *18*, 2747-2763.

Wu, S., Huang, J., Dong, J., and Pan, D. (2003). hippo encodes a Ste-20 family protein kinase that restricts cell proliferation and promotes apoptosis in conjunction with salvador and warts. Cell *114*, 445-456.

Wu, S., Liu, Y., Zheng, Y., Dong, J., and Pan, D. (2008). The TEAD/TEF family protein Scalloped mediates transcriptional output of the Hippo growth-regulatory pathway. Dev Cell *14*, 388-398.

Xu, N., Wang, S.Q., Tan, D., Gao, Y., Lin, G., and Xi, R. (2011). EGFR, Wingless and JAK/STAT signaling cooperatively maintain Drosophila intestinal stem cells. Dev Biol *354*, 31-43.

Xu, T., Wang, W., Zhang, S., Stewart, R.A., and Yu, W. (1995). Identifying tumor suppressors in genetic mosaics: the Drosophila lats gene encodes a putative protein kinase. Development *121*, 1053-1063.

Yan, Y., Denef, N., Tang, C., and Schupbach, T. (2011). Drosophila PI4KIIIalpha is required in follicle cells for oocyte polarization and Hippo signaling. Development *138*, 1697-1703.

Yang, C.H., Axelrod, J.D., and Simon, M.A. (2002). Regulation of Frizzled by fat-like cadherins during planar polarity signaling in the Drosophila compound eye. Cell *108*, 675-688.

Yu, J., Zheng, Y., Dong, J., Klusza, S., Deng, W.M., and Pan, D. (2010). Kibra functions as a tumor suppressor protein that regulates Hippo signaling in conjunction with Merlin and Expanded. Dev Cell *18*, 288-299.

Yue, T., Tian, A., and Jiang, J. (2012). The Cell Adhesion Molecule Echinoid Functions as a Tumor Suppressor and Upstream Regulator of the Hippo Signaling Pathway. Dev Cell.

Zanet, J., Pibre, S., Jacquet, C., Ramirez, A., de Alboran, I.M., and Gandarillas, A. (2005). Endogenous Myc controls mammalian epidermal cell size, hyperproliferation, endoreplication and stem cell amplification. J Cell Sci *118*, 1693-1704.

Zecca, M., and Struhl, G. (2010). A feed-forward circuit linking wingless, fat-dachsous signaling, and the warts-hippo pathway to Drosophila wing growth. PLoS Biol *8*, e1000386.

Zhang, J., Ji, J.Y., Yu, M., Overholtzer, M., Smolen, G.A., Wang, R., Brugge, J.S., Dyson, N.J., and Haber, D.A. (2009a). YAP-dependent induction of amphiregulin identifies a non-cell-autonomous component of the Hippo pathway. Nat Cell Biol *11*, 1444-1450.

Zhang, L., Ren, F., Zhang, Q., Chen, Y., Wang, B., and Jiang, J. (2008). The TEAD/TEF family of transcription factor Scalloped mediates Hippo signaling in organ size control. Dev Cell *14*, 377-387.

Zhang, X., Milton, C.C., Humbert, P.O., and Harvey, K.F. (2009b). Transcriptional output of the Salvador/warts/hippo pathway is controlled in distinct fashions in Drosophila melanogaster and mammalian cell lines. Cancer Res *69*, 6033-6041.

Zhang, X., Milton, C.C., Poon, C.L., Hong, W., and Harvey, K.F. (2011). Wbp2 cooperates with Yorkie to drive tissue growth downstream of the Salvador-Warts-Hippo pathway. Cell Death Differ *18*, 1346-1355.

Zhao, B., Li, L., Lei, Q., and Guan, K.L. (2010). The Hippo-YAP pathway in organ size control and tumorigenesis: an updated version. Genes Dev *24*, 862-874.

Zhao, B., Wei, X., Li, W., Udan, R.S., Yang, Q., Kim, J., Xie, J., Ikenoue, T., Yu, J., Li, L., *et al.* (2007). Inactivation of YAP oncoprotein by the Hippo pathway is involved in cell contact inhibition and tissue growth control. Genes Dev *21*, 2747-2761.

Ziosi, M., Baena-Lopez, L.A., Grifoni, D., Froldi, F., Pession, A., Garoia, F., Trotta, V., Bellosta, P., and Cavicchi, S. (2010). dMyc functions downstream of Yorkie to promote the supercompetitive behavior of hippo pathway mutant cells. PLoS Genet *6*.