Homeodomain-interacting protein kinase regulates Yorkie activity to promote Drosophila tissue growth

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Abstract

The Hippo (Hpo) tumour suppressor pathway regulates tissue size by inhibiting cell proliferation and promoting apoptosis. The core components of the pathway, Hpo, Salvador, Warts (Wts) and Mob-as-tumor-suppressor (Mats), form a kinase cascade to inhibit the activity of the transcriptional regulator Yorkie (Yki). Homeodomain-interacting protein kinases (Hipks) are a family of conserved serine/threonine kinases that regulate various transcriptional factors to control developmental processes including proliferation, differentiation and apoptosis. Hipk can induce tissue overgrowth in Drosophila. Genetic interaction studies reveal that Hipk is required to promote Yki activity, overriding the negative regulation induced by the Hpo kinase cascade. Hipk neither affects Yki stability nor its subcellular localization. Moreover, hipk knockdown suppresses the overgrowth and target gene expression caused by hyperactive Yki. Hipk interacts with and phosphorylates Yki and in vivo analyses show that Hipk’s regulation of Yki is kinase-dependent. To the best of our knowledge, this is the first kinase identified to positively regulate Yki.

Keywords: Hipk; Yki; Drosophila; tissue growth
Dedication

To Dad and Mom
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Chapter 1  Introduction

1.1  The Drosophila Hippo signalling pathway

Organ size control is a fundamental and core process of development of all multicellular organisms. The Hippo kinase pathway is a highly conserved growth regulatory pathway, which has a crucial role in controlling proliferation and apoptosis in both invertebrates and vertebrates. This pathway was initially studied in Drosophila because of the large hyperplastic overgrowths found in Hippo pathway mutants, suggesting a key role in the regulation of organ size. The name of the pathway came from the mutant phenotype of one of its key signalling components, the protein kinase Hippo (Hpo) (Udan et al., 2003). Loss-of-function mutations in this gene lead to tissue overgrowth, or a “hippopotamus”-like phenotype.

The core components of the Hpo pathway, Hpo (Xu et al., 1995; Harvey et al., 2003; Udan et al., 2003), Salvador (Tapon et al., 2002; Harvey et al., 2003), Warts (Wts) (Justice et al., 1995; Xu et al., 1995) and Mob-as-tumor-suppressor (Mats) (Lai et al., 2005) form a kinase cascade (Figure 1-1). Mutation of these genes results in robust tissue overgrowth without alteration of cell fate determination or cell polarity. hpo mutant clones in Drosophila eye imaginal discs lead to induction of cell proliferation and suppression of apoptosis. Hpo activates Wts, a member of the nuclear Dbf2 related (NDR) family of protein kinases, by direct phosphorylation without affecting Wts expression (Wu et al., 2003). When the pathway is active, Hpo in complex with Sav phosphorylates Wts and its co-factor Mats to activate Wts kinase activity (Wu et al., 2003; Wei et al., 2007). The upstream components, including the two FERM-domain proteins, Expanded (Ex) and Merlin, and the WW domain-containing protein Kibra, promote Hpo activity (Pellock et al., 2007; Baumgartner et al., 2010; Genevet et al., 2010; Yu et al., 2010). The atypical cadherin Fat (ft) and its ligand Dachsous (ds) influence the subcellular
localization of the atypical myosin Dachs (D) and Ex to regulate the kinase Wts (Cho and Irvine, 2004; Bennett and Harvey, 2006; Mao et al., 2006; Reddy and Irvine, 2008). This finding presented the possibility that Hpo signaling may indeed be regulated by an extracellular signal—a ligand expressed on the surface of an adjacent cell. The effects of loss of ft on tissue growth and planar polarity were well documented but the underlying mechanism by which ft regulated growth was not known. Loss of ft in mutant clones in imaginal discs caused the induction of transcriptional targets of the Hippo pathway. Interestingly, Ft was shown to regulate Hippo pathway activity by two distinct signaling interactions. One is directly through Ex and the Hpo kinase cascade leading to a Ft-Ex-Hippo pathway (Bennett et al., 2003; Silva et al., 2006; Willecke et al., 2006). Ft-Ex-Hippo pathway activity may also be influenced by Decapentaplegic (Dpp) and Wingless (Wg) morphogen gradients (Willecke et al., 2006; Zecca and Struhl, 2007; Rogulja et al., 2008). It has been proposed that Ft activates the Hippo pathway by regulating Ex protein level and localization. (Bennett et al., 2003; Silva et al., 2006; Willecke et al., 2006; Tyler and Baker, 2007). The other mechanism showed that Ft directly binds to D, which in turn regulates the abundance of Wts (Cho et al., 2006; Feng and Irvine, 2007; Reddy and Irvine, 2008). Therefore, Ft can regulate the Hippo pathway independently of Hpo and other upstream components leading to the Fat-D-Wts pathway.

Wts becomes activated and interacts with its cofactor Mats upon Hpo-mediated phosphorylation. This protein complex regulates gene expression by phosphorylating and inhibiting the non-DNA binding transcriptional coactivator Yorkie (Yki). Wts phosphorylation of Yki on Serine 168 inhibits Yki activity by promoting the interaction with 14-3-3 proteins, which in turn causes Yki cytoplasmic retention (Oh and Irvine, 2008; Oh and Irvine, 2009; Ren et al., 2010). The TEAD/TEF family transcription factor Scalloped (Sd) has been identified as the first known transcription factor to interact with Yki, and this interaction promotes Yki nuclear
localization. The Sd-Yki interaction initiates the expression of the genes involved in inhibition of apoptosis and acceleration of growth and cell cycle progression, such as *Drosophila inhibitor of apoptosis protein 1 (Diap1)*, *cyclin E (cycE)*, the *bantam* microRNA (*ban*) and *wingless (wg)* (Cho and Irvine, 2004; Thompson and Cohen, 2006; Goulev et al., 2008; Wu et al., 2008; Zhang et al., 2008). Recently, Homothorex (Hth) was identified as a Yki partner in *Drosophila*, promoting eye growth in part by regulating *ban* (Peng et al., 2009). Moreover, the Irvine group has demonstrated that the Yki and Mad (the transcription factor of the Dpp pathway) can interact directly to form a transcription factor complex that regulates the expression of downstream target genes crucial for growth, including *ban* (Oh and Irvine, 2011). Yki is thus a growth promoter, whereas Hpo, Sav, Wts and Mats act as tumor suppressors by suppressing the growth-promoting activity of Yki.

Yki was identified in a yeast two-hybrid screen using the N-terminal domain of Wts as bait (Huang et al., 2005). Loss of Yki (named after Yorkshire Terriers, a small dog breed) in mutant clones in imaginal discs results in the development of smaller tissues, indicating that Yki is required for normal tissue growth. Overexpression of *yki* phenocopies loss-of-function mutations of *hpo* or *wts*, including elevated transcription of *cycE* and *Diap1*, increased proliferation, defective apoptosis, and tissue overgrowth (Huang et al., 2005). Hippo signaling inactivates Yki by excluding it from the nucleus via phosphorylation of a critical residue, Serine 168. Overexpressing the phosphoresistant form of Yki (Yki<sup>S168A</sup>), carrying a serine-to-alanine mutation on Yki S168, leads to overgrowth in *Drosophila* eye and wing, as well as enhances the nuclear localization (Oh and Irvine, 2008). Wts also influences the activity and nuclear localization of Yki via two additional phosphorylation sites, Serine111 and 250 (Oh and Irvine, 2009). Ectopically expressing phosphoresistant Yki<sup>S111A</sup> and Yki<sup>S250A</sup> results in milder tissue overgrowth and elevated nuclear localization, compared to Yki<sup>S168A</sup> overexpression. Even though
Yki$^{S111A}$ and Yki$^{S250A}$ show an overgrowth phenotype, 14-3-3 protein is still able to interact with Yki$^{S111A}$ and Yki$^{S250A}$ to inhibit its activity, suggesting that there are other mechanisms underlying its regulation. Yki is also regulated by phosphorylation-independent mechanisms. Direct binding between the PPxY motifs of Hpo, Wts or Ex and the WW domains of Yki can suppress Yki activity, possibly by cytoplasmic retention (Badouel et al., 2009; Oh and Irvine, 2009; Ren et al., 2010). The WW domains of Yki are not required for its phosphorylation, but instead are positively required for its activity (Oh and Irvine, 2009). Further study is needed to assess the contribution of this mode of regulation under physiological conditions as these findings relied mostly on in vitro studies.

Roles of the Hippo pathway in organ size determination and tumour suppression have been confirmed in the mouse model. Overexpressing YAP, the mammalian homolog of Yki, causes enlarged mouse livers (Camargo et al., 2007; Dong et al., 2007). An increase of YAP protein levels and nuclear localization have been observed in multiple human cancers (Zhao et al., 2007; Hao et al., 2008; Oka et al., 2008; Steinhardt et al., 2008). Overexpression of TAZ, a paralogue of YAP, has been found in human breast cancer samples and non-small-cell lung-cancer cell lines (Chan et al., 2008; Zhao et al., 2012). Downregulation of the Hippo pathway components Mer and Sav in mice also result in liver enlargement and tumour formation (Lee et al., 2010; Lu et al., 2010; Zhang et al., 2010). Loss of one or both copies of YAP can suppress liver expansion and tumorigenesis caused by Mer deficiency (Zhang et al., 2010). Together, these studies highlight a significant role of the Hippo pathway in organ size regulation and tumorigenesis. The studies of the mammalian Hippo pathway give great insights about cancer therapy and regenerative medicine.
The atypical cadherin Ft interacts with its ligand Ds to regulate the activity of Ex/Mer/Kibra complex. Upon the activation of Hpo signalling, the Ex/Mer/Kibra complex activates Hpo leading to Wts activation by phosphorylation. Upon inhibition of the pathway, Yki escapes from Wts-mediated phosphorylation and interacts with Sd and translocates to the nucleus in order to initiate the expression of Hpo target genes.
1.2 Hipk family

Homeodomain-interacting protein kinases (Hipks) are a family of serine/threonine kinases highly conserved across species including in vertebrates, and there are four members of the family (Hipk 1-4) in mice and humans. Hipk1-3 were originally discovered by their ability to bind homeobox-containing transcription factors (Kim et al., 1998), and Hipk4 was identified in the human genome sequence based on the high homology with the other three members (Manning et al., 2002). These kinases are involved in diverse process, such as regulating transcription, chromatin remodelling and regulating conserved signalling pathways. Members of this family have been implicated in several developmental processes, including cell proliferation, differentiation, apoptosis and homeostasis. Hipks were originally defined, based on the structure of Hipk1-3, as nuclear serine/threonine kinases characterized by the presence of a kinase domain and a sumoylation site in their N-terminal region, a homeobox-interacting domain in the central portion of the protein and a speckle-retention signal (SRS) superimposed on a PEST region (Hedley et al., 1995; Kim et al., 1998). The kinase domain and the PEST region are present in all four members of the family while the homeobox-interacting domain and the sumoylation site are absent in Hipk4, which is mainly localized in the cytoplasm (Arai et al., 2007). Several studies have analyzed the localization of Hipks upon overexpression. These experiments consistently showed mostly nuclear speckles for Hipk1–3 and a diffuse cytoplasmic staining. The nuclear localization might be regulated by the nuclear localization signals as well as by the presence of the putative SRS in the C-terminal region of the proteins (Fig. 1-2).
Figure 1-2 Schematic representation of vertebrate Hipk1-3 protein domains

The kinase domain is at the N terminus. SUMO at the N-terminus is the sumoylation site by SUMO-1. HID is homeobox-interacting domain, followed by the PEST-sequence including the SRS sequence at the C terminus of the protein. The protein sequence of the kinase domain in vertebrate Hipk is highly conserved in Drosophila Hipk (Lee, 2009).
Vertebrate Hipk1 is a novel signal transducer in tumour necrosis factor alpha (TNFα)-induced apoptosis signalling, which activates the apoptosis signal-regulating kinase 1 (ASK1)/Jun N-terminal kinase (JNK)/p38 mitogen-activated protein kinase signalling cascade (Li et al., 2005). Hipk1 and Hipk2 have been shown to phosphorylate and activate p53, resulting in the enhancement of p53-dependent transcription, cell growth regulation and apoptosis initiation upon genotoxic stress (Bitomsky and Hofmann, 2009). The Fusco group (Trapasso et al., 2009) reported that disruption of murine Hipk2 causes growth deficiency and cell cycle arrest. *hipk2* knockout (KO) mice are viable but with significantly smaller size than the wildtype mice. Mouse embryo fibroblasts (MEFs) from the *hipk2* KO mice exhibits a reduced proliferation rate that is associated with cell accumulation in the G0/G1 phase of the cell cycle and reduced level of the cell regulator cyclin D and CDK6. This defect can be rescued by re-expression of wildtype Hipk2 in KO MEFs, which supports the involvement of Hipk2 in the regulation of cell proliferation. The findings from the mammalian cell culture also shows that Hipk2 also regulates transforming growth factor (TGF)-β induced JNK activation and apoptosis (Hofmann et al., 2003). Hipk3 transduces pro-apoptotic signals by death receptors through interaction with TRADD and FADD. These findings suggest that Hipk proteins are involved in the control of cell growth in response to various extracellular and intracellular stimuli. Although several studies have shown a role of Hipks in cell growth, the functions of Hipks in the growth-regulatory pathways are not completely understood.

Previous studies have suggested that Hipks regulate transcription factors to control cell proliferation, differentiation and apoptosis. c-Ski negatively regulate the transcriptional activation induced by Smad proteins (Luo et al., 1999; Sun et al., 1999; Xu et al., 2000). Members of the Smad proteins act as transcriptional activators in the TGF-β and BMP signaling pathways. Hipk2 directly interacts with c-Ski and Smad to negatively regulate the Smad1/4-induced transcriptional
activation in BMP signalling. Carboxyl-terminal binding protein (CtBP) is a transcriptional corepressor that interacts with various cellular factors and has a role in development and cell-cycle regulation. Hipk2 downregulates CtBP through phosphorylation to promote apoptosis (Zhang et al., 2003). Taking these findings together, it has been hypothesized that Hipks regulate the activity of their interacting partners through binding and phosphorylation leading to modulation of transcriptional output of various target genes.

*Drosophila* has a single Hipk ortholog, which is conserved with vertebrate family members (Choi et al., 2005; Link et al., 2007). Based on the protein sequence alignment between vertebrate and *Drosophila* Hipks (Lee, 2009), the kinase domains in vertebrate Hipks (Figure 1-2) are highly conserved in *Drosophila* Hipk. Moreover, sequence conservation between vertebrate and *Drosophila* Hipks was also observed in the other protein domains (Lee, 2009), which leads to the possibility of functional conservation between vertebrate and *Drosophila* Hipks. The Ambram group demonstrates that hipk is essential for programmed cell death in the *Drosophila* wing epithelium to regulate proper cell number during an epithelial-to-mesenchymal transition for removing epithelial cells after eclosion (Link et al., 2007). Loss of hipk (in the hipk^DI^ mutation) leads to increase of neuronal cell numbers, such as interommatidial cells in the pupal eye and neurons in the embryonic central nerve system, through regulating cell death (Link et al., 2007). The Jiao group recently has demonstrated that regulation of Hipk by sumoylation is also a conserved feature (Huang et al., 2011). Following perturbation of sumoylation through knocking down the small ubiquitin-related modifier (SUMO) smt3, *Drosophila* Hipk, which is normally located in the nucleus, gains access to the cytoplasm to activate the JNK signaling pathway (Huang et al., 2011).

In the Verheyen lab, we have carried out a number of analyses to determine the role of *Drosophila* hipk during development, growth and signalling. The Verheyen group has generated
the hipk mutant alleles (hipk<sup>2-4</sup>) to study hipk's function in vivo (Lee et al., 2009a; Lee et al., 2009b). For my studies, I have used hipk<sup>4</sup>, which gives the most severe phenotypes based on the interallelic crosses. hipk<sup>4</sup> was generated through deletion of the DNA sequencing between two transposable elements flanking the locus, which deletes the central portion and N-terminal sequence of hipk resulting in lethality before the third-instar larval stage (Lee et al., 2009a). Viable combinations of hipk alleles give rise to adults with defective wings and smaller eyes. During <i>Drosophila</i> eye development, Hipk represses the activity of the global repressor Groucho (Gro) to promote Notch (N)-mediated eye growth (Lee et al., 2009a). As an undergraduate student in the lab, I contributed to our study on the role of Hipk in regulation of the Wingless (Wg) pathway, which plays an important role in <i>Drosophila</i> wing development. Hipk phosphorylates Armadillo (Arm), the transcriptional coactivator of the Wg pathway, and enhances Arm/dTcf-mediated transcription (Lee et al., 2009b). Later, our lab elucidated that Hipk promotes the stability of Arm and Cubitus interruptus (Ci), the effectors of Wg and Hedgehog pathways, respectively (Swarup and Verheyen, 2011). Taken together, hipk is involved in multiple pathways during <i>Drosophila</i> development.

We also found that ectopic expression of hipk leads to overgrowth in multiple <i>Drosophila</i> adult tissues, including the eye, wing (Lee et al., 2009a; Lee et al., 2009b), haltere and leg (data not shown). We therefore speculated that hipk may have a role in regulation of the signalling pathways involved in tissue growth. In addition, Wg expression is elevated upon overexpressing one copy of <i>UAS-hipk</i> by <i>omb-Gal4</i> (Figure 1-1). Interestingly, wg is the transcriptional target of the Hpo pathway, which has an important role in regulation of tissue growth. The hypothesis of my study is that hipk antagonizes Hpo signalling to regulate tissue growth. Furthermore, the main focus of my Masters degree project was to investigate the function of hipk in the Hpo pathway during <i>Drosophila</i> development.
Figure 1-3  Hipk promotes the Wg pathway in *Drosophila*

Wg signalling is activated upon the interaction between Wg ligand and its receptor, DFrizzle 2 (DFz2), and the signalling is transduced by Dishevelled (Dsh). Dsh inhibits the activity of destruction complex, including glycogen synthase kinase 3 (GSK3), Axin and adenomatous polyposis coli (APC). In the absence of Wg signal, the destruction complex causes degradation of the transcription coactivator Arm to inhibit expression of the Wg target gene. Hipk promotes Arm stability and Arm/dTCF transcriptional activity.
hipk overexpression enhances Wg expression

(A) Wildtype wing imaginal disc with anti-Wg antibody staining. (B) Overexpressing one copy of wildtype hipk with omb-Gal4, which expresses UAS-mediated gene expression in the center of the wing pouch, leads to increase of Wg expression.
1.3 *Drosophila* wing development as a model system for studying tissue growth

During development it is crucial that growth ceases when tissues or organs have attained a certain form and size. However, the regulation of final tissue size is poorly understood. The *Drosophila* imaginal discs are a well-characterized model system in which many aspects of pattern formation, growth regulation, and cell proliferation have been experimentally approached (Bryant and Simpson, 1984; Cohen et al., 1992). A growing tissue needs to recognize an endpoint in growth and stop dividing once the tissue has reached its target size. During the growth phase, cells can alter their growth programs to regenerate both cell numbers and patterning information even when a large number of cells are lost in a developing tissue. During disc growth, cell interactions that control growth are independent of cell lineage. Despite extensive experimental investigation of the imaginal discs, mechanisms underlying the determination of size remain elusive. Therefore, there is a need for models, which can explain the available data and possibly inspire novel experimental approaches.

*Drosophila* imaginal discs are epithelial structures that give rise to the adult body structures. The wing disc derives from the non-proliferating ectoderm of the embryo and proliferates extensively during the larval stages (the first, second and third instar) and the first 24-hour of pupal development (Garcia-Bellido and Merriam, 1971). The wing disc contains about 30 cells at the beginning of the first larval instar and reaches at metamorphosis, almost 4 days later a number of about 50,000 cells (Milan and Nicolas, 1991). The wing disc is formed from the embryonic ectoderm by an invagination at the intersection of a dorsal/ventral (D/V) stripe of Wg with an anterior-posterior (A/P) stripe of Decapentaplegic (Dpp). In the adult, the wing pouch gives rise to the wing blade with five longitudinal veins (L1 – L5) and two connecting cross veins [anterior cross vein (ACV) and posterior cross vein (PCV)]. The outer ring surrounding the wing
pouch forms the future hinge structure. The size of the adult wing is largely predetermined by the final size of the wing disc (Day and Lawrence, 2000).
Figure 1-5  The *Drosophila* wing

(A, B) The third instar wing imaginal disc. (A) Anti-Wg staining (red) marks the D/V boundary, separating D and V compartments in the wing pouch, and the inner (a) and outer (b) rings. The hinge, consisting of the outer ring (b), is between the wing pouch and notum, the dorsal-most compartment of the disc. (B) Anti-Dpp staining shows A/P boundary, (C) The wing pouch derives to adult wing blade, which consist of L1-L5 longitudinal veins and cross veins (ACV and PCV). The red dotted between L3 and L4 divide the wing into A and P regions.
Chapter 2   Results and Discussion

2.1 *hipk* regulates *Drosophila* wing growth by promoting cell proliferation and inhibiting apoptosis

The Verheyen lab has previously shown that excess Hipk caused overgrowth in the *Drosophila* adult eye and wing (Lee et al., 2009a; Lee et al., 2009b). While using the third-instar wing imaginal disc as a model system for the study, I generated hipk-overexpression flies and observed the wing disc morphology. Expressing one copy of *UAS-hipk* using *omb-Gal4* at the center domain of the wing pouch leads to overgrowth, and expressing two copies further enhanced the growth (Fig. 2-1 B, C). This overgrowth strikingly resembled the disc phenotype obtained when inhibiting Hippo signalling by knocking down *wts* and similarly by overexpressing *yki* (Justice et al., 1995; Huang et al., 2005; Zhang et al., 2008). Conversely, reduction of Hipk through mutation or knockdown leads to a decrease of adult eye and wing size (Lee et al., 2009a; Lee et al., 2009b). However, the cellular mechanisms that underlie Hipk’s role in growth were still unknown.

Tissue overgrowth can result from enhanced cell proliferation, decreased apoptosis or both. To determine whether *hipk* has a role in these processes, I performed immunohistochemistry with anti-phospho-histone 3 (PH3) and anti-cleaved Caspase 3 (Casp3) antibodies, markers for mitosis and apoptosis, respectively. Ectopic Hipk induced an increase in PH3-positive cells (Fig. 2-1 E, E’), implying that elevated Hipk levels enhanced cell proliferation. Conversely, increased Casp3 staining was observed in wing discs in which *hipk* was knocked down by using a UAS-driven RNAi construct (Fig. 2-1 G, G’), indicating that reducing Hipk function led to increased cell death. Thus, Hipk promotes cell proliferation and inhibits apoptosis to control the wing size.
Figure 2-1  *hipk* promotes wing overgrowth by enhancing cell proliferation and inhibiting apoptosis

(A, D and F) Control wing imaginal disc. (B-C) One or two copies of HA-tagged *UAS-hipk* are expressed by using the *omb-Gal4* driver. (D-E’) Anti-PH3 staining indicates cells undergoing mitosis in the control (D) and *omb>hipk* disc (E, E’). (B, C, E’) Anti-HA staining reveals *omb-Gal4* expression domain. (F-G’) Anti-Casp3 staining reveals cell death in the control (F) and *sd>hipkRNAi; GFP* discs. (G’) GFP expression pattern indicates region in which *UAS-hipkRNAi* is expressed with *sd-Gal4* driver.
2.2 *hipk* modulates Hippo target gene expression

Since the genetic studies demonstrated that *hipk* enhances cell proliferation to promote wing growth, I next examined whether Hipk regulates Hpo-mediated tissue growth by affecting expression of the Hpo target genes in a *hipk* gain- or loss-of-function background. Overexpressing *hipk* using *ptc-Gal4*, which drives UAS-mediated expression along the anterior-posterior (A/P) boundary, led to ectopic expression of both *Diap1-lacZ* (Fig. 2-2 B,B’) and CycE (Fig. 2-2 D,D’). This result suggests that the overgrowth phenotype observed with elevated Hipk level phenocopies the effect of ectopic *yki* expression.

To test target expression in the *hipk* mutant background, I generated loss-of-function clone by using the flippase (FLP)/FLP recognition target (FRT) system and a GFP marker. FLP will only induce mitotic recombination at homologous FRT sites on the chromosome, in this case, by heat-shock induced expression of FLP. The GFP marker is on the sister chromatid, which marks wildtype cells after mitotic recombination occurs. Thus homozygous mutant patches of *hipk* cells are marked by the absence of GFP. I examined the transcription of *ex* and *wg* in *hipk* mutant somatic clones by using *ex-lacZ* and *wg-lacZ*, respectively. In *hipk* mutant somatic clones, *ex-lacZ* expression was abolished due to loss of *hipk* function (arrows in Fig. 2-2 F, F’). I also found a dramatic increase in *ex-lacZ* upon *hipk* overexpression in the wing (Fig. 2-7 D, D’). Loss of *hipk* also eliminated *wg-lacZ* expression in the proximal region of the wing pouch (arrows in Fig. 2-2 H, H’) where its expression is regulated by the Hpo pathway (Cho et al., 2006), but not at the D/V boundary where its expression is Hpo-independent (marked by an asterisk in arrows in Fig. 2-2 H, H’). Given that the Hpo pathway is involved in cell growth, both CycE and Diap are general markers for cell survival and cell cycle progression, and *wg* is also a transcriptional target of the JNK pathway (Ryoo et al., 2004), which plays a role in cell death. Although *hipk* is able to modulate the expressions of these general cell proliferation markers (Fig. 2-2 B, B’, D, D’, H,
H’), it also regulates transcriptional level of ex (Fig. 2-2 F, F’ and Fig. 2-7 D), which is a Hpo component playing a role in the negative feedback of the pathway. These results show that hipk can regulate the expression of several Yki transcriptional targets to control wing growth.
Figure 2-2  *hipk* regulates expression of Hpo target genes

Hpo target genes (Control expression patterns shown in A, C, E, G) are examined following *hipk* overexpression (B, B', D, D') or *hipk* loss-of-function clones, marked by the absence of GFP (F', H'), eliminated *ex* or *wg* transcription, respectively (shown by arrows). (H, H') Asterisk indicates unaffected *wg* transcription at the D/V boundary.
2.3 *hipk* genetically antagonizes Hpo-mediated growth inhibition

After finding that Hipk affects the transcriptional readout of the Hpo pathway, I performed genetic interaction analyses to determine its role in the pathway. I used *sd-Gal4* to express various UAS-controlled RNAi lines targeted against Hpo pathway components in the wing pouch and measured the adult wing size. Increasing Yki activity by knocking down *ex, mats* or *wts* enhanced wing growth (Fig. 2-3 E, G, I, M). Reducing Hipk in these genetic backgrounds significantly suppressed excess wing growth (Fig. 2-3 F, H, J), suggesting that reducing Hipk blocks the growth induced by Yki activation. These suppression results imply that Hipk counteracts the Hpo-mediated growth inhibition during *Drosophila* wing development. Next, I examined the interaction between Yki and Hipk in the tissue growth. Reducing Yki level, as activating Hpo signalling, greatly inhibits wing growth (Fig. 2-3 K). Conversely, expression of *sd>hipk<sup>wt</sup>* causes a statistically significant increase in wing size (Fig. 2-3 D, M). Knocking down *yki* in a *hipk*-overexpression background prevents the growth induced by Hipk, suggesting that Yki is required for Hipk-mediated growth (Fig. 2-3 L, M). In addition, co-expressing *yki* and *hipk* prompted massive overgrowth (Fig. 2-8 B) in comparison to expressing *yki* alone (Fig. 2-8 A), indicating that Hipk potentiates Yki activity to induce growth. These genetic findings imply that Hipk can influence Hpo-mediated wing growth, and Hipk requires Yki for its function in promoting growth.
Figure 2-3  *hipk* influences Hpo-mediated wing growth

(A-L) Adult female wings from the indicated genotypes. *UAS-hipk* indicates expression of wildtype *hipk*. (M) The average of adult wing sizes (n=50) from A-L is measured and plotted in columns labeled by panel letter. The error bars show standard error of the mean. Paired student’s t-test is used to determine significance in size differences between genotypes as indicated by asterisks (**** *P*<0.0001).
2.4 Hipk is required for Yki transcriptional activity

My genetic interaction analyses demonstrated that knockdown of hipk suppressed the overgrowth caused by knockdown of Yki negative regulators, such as ex, mats and wts. Moreover, knocking down Yki suppressed the large wing phenotype caused by ectopic Hipk. Therefore, I speculated that Hipk may have a role in regulation of Yki activity to control tissue growth. Since Wts is the immediate upstream regulator of Yki, I next examined Hippo target gene expression in the double knockdown or mutant background of hipk and wts to further investigate Hipk’s position in the pathway.

The upregulation of ex-lacZ seen with wts knockdown (Fig. 2-4 B), which enhanced Yki transcriptional activity, was prevented by simultaneous knockdown of hipk (Fig. 2-4 C). In addition, hh>wtssRX4i wing disc showed an increase in size of the posterior compartment (Fig 2-4 B, by comparing to the control wing disc in Fig 2-4 A), and hipk knockdown suppressed this wing phenotype (Fig. 2-4 C). This suppression suggests that Yki requires Hipk in order to activate the target expression and promote tissue growth. To test whether the role of hipk in regulation of Yki transcriptional activity is not tissue specific, I also examined CycE expression in the Drosophila eye imaginal disc. CycE is normally expressed in a narrow stripe of cells posterior to the morphogenetic furrow (Fig 2-4 F). The wts mutation enhanced CycE expression as a dramatic broadening of the expression domain (Fig 2-4 G-G’). Strikingly, enhancement of CycE expression observed in the wts clone was suppressed in the hipk, wts double mutant clone (Fig. 2-4 H, H’). Thus in this context activated Yki requires Hipk for its ability to initiate target gene expression and to promote tissue growth. Furthermore, overexpressing hipk did not rescue loss of ex-lacZ expression caused by excess Wts (Fig. 2-4 D, E). The overexpression study suggests that Hipk and Wts regulate the same component in the Hpo pathway, which is Yki in
this case. These findings suggest that Hipk can influence the expression of Yki transcriptional targets.
**Figure 2-4.** *hipk* promotes Yki transcriptional activity

Expression of *ex-lacZ* in control (A), *hh>* *wts^RNAi* (B), *hh>* *wts^RNAi*, *hipk^RNAi* (C), *hh>* *wts^ws* (D), *hh>* *wts^wt*, *hipk^wt* (E) wing discs. *hh-Gal4* expression in the posterior compartment is indicated by an asterisk, to the right of the dotted line indicating the A/P boundary (as detected by anti-Ci staining, not shown). Expression of CycE in control (F), *wts^xt* clones (G, G'), *hipk^4*, *wts^xt* clones (H, H') in eye discs. (F, G, H) The arrowheads mark the location of morphogenetic furrow. (G, H) The range of CycE expression is greatly increased in *wts^xt* clones (H) and is suppressed in *hipk^4*, *wtsx^1* clones (H) (comparing the size of the white brackets within the clones).
2.5 Hipk does not promote Yki activity through inhibition of Slmb

Recently, the Guan group reported that Yes-associated protein (YAP, a vertebrate ortholog of Yki) phosphorylated by Lats (Wts in Drosophila) at Serine 381 is subsequently ubiquitinated by the SCFβTrCP E3 ubiquitin ligase, resulting in YAP degradation (Zhao et al., 2010). Our lab has recently shown that Hipks inhibit the ability of Slmb (Slmb)/βTrCP to ubiquitinate Arm/β-catenin and Ci/Gli to promote Wg/Wnt and Hedgehog signalling, respectively (Swarup and Verheyen, 2011). Therefore, I investigated whether Hipk inhibits Hippo signalling by promoting Yki stability through Slmb. Though the absence of a βTrCP-recognition site in Drosophila Yki argues against such a model, we sought to confirm this further (Zhao et al., 2010).

To examine whether Drosophila Slmb has an effect on Yki protein levels, I used UAS-slmbRNAi to generate the genetic mutant background. I observed elevated Ci, a well-known Slmb substrate for degradation, in the dorsal compartment of ap>slmbRNAi wing discs, verifying the effectiveness of this transgene (Fig. 2-5 A-B’). Expressing this UAS-slmbRNAi with en-Gal4 did not alter Yki protein levels, indicating that Slmb does not regulate Yki stability (Fig. 2-6 B, B’). Moreover, slmb knockdown did not affect the expression of ex-lacZ or Diap1-lacZ (Fig. 2-5 C-F’). Therefore, Slmb does not appear to regulate Hippo signalling by affecting Yki stabilization. In addition, overexpressing hipk did not noticeably change Yki protein levels (Fig. 2-6 C-C’), implying that Hipk does not counteract Hippo signalling by stabilizing Yki.
Figure 2-5  *slmb* does not regulate Hippo target gene expression

The protein level of Ci, the Slmb degradation target, is examined in control (A) and *ap>slmb^{RNAi}, GFP* discs (B, B’) by the Ci antibody staining (red). Area of elevated Ci stain is indicated by the arrow in B. *Diap1-lacZ* expression was assessed in control (C) and *en>slmb^{RNAi}, GFP* (D, D’) discs. *ex-lacZ* expression was examined in control (E) and *hh>slmb^{RNAi}, GFP* (F, F’) discs. (B’ and D’) The GFP expression indicates region of disc in which transgenes were expressed. (F’) *hh-Gal4* expression domain is in the posterior compartment of wing disc, marked as absence of green. The anti-Ci staining (green) indicated the anterior compartment.
Figure 2-6  Slmb and Hipk do not affect Yki protein level and subcellular localization

(A-E') Wing discs stained with anti-Yki.  (A', B', C', D', E') en-Gal4 is used to drive UAS transgenes in the posterior of the disc as indicated by expression of UAS-GFP (green).  (A, A') Control disc.  (B, B') en>slmbRNAi.  (C, C') en>hipkwt.  D and E are the high-magnification images of A and C, respectively, showing Yki subcellular localization with respect to DAPI staining (blue).  The arrowheads in D and E indicate the D/V boundary.
2.6 Hipk does not regulate Hpo signalling by stabilizing Arm

Several recent studies have demonstrated an interaction between Hpo and Wg/Wnt signalling (Hergovich and Hemmings, 2010; Varelas et al., 2010; Zecca and Struhl, 2010; Xin et al., 2011; Imajo et al., 2012). The Attisano group (Varelas et al., 2010) demonstrated that Hpo signalling modulates Wg target expression. However the mechanism by which Wg signalling regulates Hpo pathway in Drosophila is still unclear. Therefore, I asked whether Hipk might regulate Hpo signalling solely through its role in stabilizing Arm protein in the Wg pathway, as has been shown by our lab previously (Lee et al., 2009b; Swarup and Verheyen, 2011).

Ectopically expressing wildtype Arm (arm\textsuperscript{S2}) under the control of hh-Gal4 neither induced wing growth nor ex-lacZ expression (Fig. 2-7 B, B’). However, increased wildtype Arm (Arm\textsuperscript{S2}) is constitutively degraded by the GSK3/APC/Axin destruction complex. Therefore, I expected that excess wildtype Arm would not influence the tissue growth and Hpo target expression. Expressing constitutively active, stabilized Arm (arm\textsuperscript{S10}), which has a deletion of the GSK3 target site and resists degradation, did cause overgrowth, as seen with increased folding of epithelial layers at the posterior domain of the wing disc (Fig. 2-7 C, C’), resulting from inducing Wg signalling to promote the wing growth. However, I did not observe any changes of ex-lacZ expression in hh>arm\textsuperscript{S10} wing discs, suggesting the overgrowth is due to Hpo-independent mechanisms. In contrast, overexpressing hipk caused both upregulated Hippo target expression and induced wing growth (Figs. 2-7 D, D’). Therefore, I conclude that Hipk does not promote Yki activity via its role in stabilizing Arm.
Figure 2-7  Hipk enhances Hippo target gene expression, but wildtype or hyperactive Arm does not

(A-D’) ex-lacZ expression is examined in control (A), hh>UAS-armS2 [a wildtype form of Arm] (B, B’), hh>UAS-armS10 [a hyperactive stabilized form of Arm] (C, C’) and hh>hipk^wt (D, D’) discs. (C) A bracket indicates the wing pouch. (B’, C’, D’) hh-Gal4 expression domain is indicated by absence of anti-Ci staining (absence of green).
2.7 Hipk regulates the nuclear activity of Yki

When Hpo signalling is activated, Wts phosphorylates Yki at Serine 168. As a result, Yki interacts with protein 14-3-3, leading to Yki cytoplasmic retention (Cho et al., 2006; Oh and Irvine, 2008). Upon inhibition of the pathway, Yki interacts with Sd to promote Yki nuclear localization and target gene expression (Goulev et al., 2008; Wu et al., 2008; Zhang et al., 2008). My genetic studies implied that Hipk antagonizes Hpo-mediated growth and enhances Yki transcriptional targets. Therefore, I speculated that Hipk may regulate Yki activity by affecting its subcellular localization - enhancing Yki nuclear localization. Surprisingly, ectopic expression of hipk does not affect endogenous Yki localization (Fig. 2-6 C, C’, E, E’). Moreover, the activity of Yki, as measured by overgrowth phenotypes, is enhanced by excess Hipk, yet I found that the localization of exogenous Yki following modulation of Hipk is unaltered (Fig. 2-8 C, C’, D, D’).

The Kim group (Kim et al., 1998) reported that vertebrate Hipks localize to nuclear speckles and can act as transcriptional co-repressors for NK homeodomain transcription factors. The function of Drosophila Hipk related to its subcellular localization is not fully understood since the SRS sequence of vertebrate Hipks is not conserved in Drosophila Hipk. Nevertheless, I have observed nuclear speckles after staining for Hipk in hh>hipk:HA wing discs (Fig. 2-9), indicating its nuclear activity/function is likely due to its localization. Thus, I hypothesized that Hipk regulates Yki transcriptional activity at the nuclear level. To test this model, I used a hyperactive form of Yki carrying a serine-to-alanine mutation on Serine 168 (Yki<sup>S168A</sup>:GFP), which loses its ability to interact with 14-3-3 thus resulting in enhanced nuclear localization and transcriptional activity (Dong et al., 2007; Oh and Irvine, 2008). Expression of UAS-<i>yki</i><sup>S168A</sup>:GFP using <i>ptc-Gal4</i> induced Wg expression in the proximal region of the wing pouch (arrows in Fig. 2-10 A, A’), as well as promoted overgrowth in the wing disc (Fig. 2-10 A), eye (Fig. 2-10 D) and haltere (data not shown). Remarkably, knocking down hipk in this genetic context rescued
the overgrowth phenotype in all tissues examined (Fig. 2-10 B, B’, E and data not shown) as well as blocked Wg induction (Fig. 2-10 B, B’), suggesting that Hipk may act as a general regulator of Yki. As stated in the introduction, Hpo, Wts or Ex can negatively regulate Yki in a phosphorylation-independent manner by promoting cytoplasmic retention via protein-protein interactions (Badouel et al., 2009; Oh and Irvine, 2009; Ren et al., 2010). The rescue phenotype might reflect an unknown direct regulation of Hpo, Wts or Ex by Hipk. Therefore, to further examine this possibility, I examined YkiS168 localization in the hipk knockdown background. Hipk reduction did not affect subcellular localization of YkiS168A::GFP (Fig. 2-11 C, C’) or total Yki (Fig. 2-11 D, D’), but did cause a reduction in cell proliferation, as seen by a decrease in number of YkiS168A::GFP-expressing cells (Fig. 2-11 D, D’). These findings support the model that Hipk directly promotes Yki transcriptional activity within the nucleus, instead of inhibiting the cytoplasmic retention activity of Yki negative regulators.
Hipk enhanced Yki-mediated wing growth but does not affect Yki subcellular localization.

sd>yki:V5 (A, C, C') and sd>hipk, yki:V5 (B, D, D') wing discs stain with anti-V5 antibody to detect subcellular localization of Yki:V5. (C', D') Nuclei are stained with DAPI (blue). (B) The overgrowth phenotype caused by overexpressing yki:V5 is further enhanced by co-expressing yki:V5 and hipk.
Figure 2-9  Hipk is localized to nuclear speckles in the wing disc

Anti-HA antibody is used to detect exogenous HA-tagged Hipk in *hh>hipk:HA* wing disc. (B, B’) A close up of the posterior region of the *hh>hipk:HA* disc with anti-HA staining for Hipk:HA (green) and DAPI to indicate nuclei (blue in B’).
Hyperactive Yki\textsuperscript{S168A} requires Hipk to promote overgrowth and target gene expression.

(A, A’) Expressing the hyperactive form of Yki (Yki S168A:GFP) causes induction of Wg expression and an elongated wing disc with folding observed in the proximal region. (B, B’) hipk knockdown rescues the Yki S168A:GFP disc phenotype and Wg induction. (A’, B’) ptc-Gal4 is expressed along the A/P boundary as shown by the GFP expression. Adult eyes from control (C), ptc>yki\textsuperscript{S168A}:GFP (D) and ptc>hipk\textsuperscript{RNAi}; yki\textsuperscript{S168A}:GFP (E). hipk knockdown (E) also rescues the eye overgrowth resulted from expressing Yki S168A:GFP (D).
**Figure 2-11** Hipk reduction does not affect Yki^{S168A} subcellular localization

*ptc-Gal4* is used to drive expression of *UAS-Yki^{S168A}:GFP* (A, A', B, B') and *UAS-hipk^{RNAi}, UAS-Yki^{S168A}:GFP* (C, C', D, D'). (C, C') Hipk reduction does not affect localization of Yki^{S168A} shown as GFP expression with nuclear staining by DAPI (blue) (D, D') Anti-Yki stain (red, detects all cellular Yki) shows that *hipk* knockdown does not change subcellular localization of either Yki^{S168A} or endogenous Yki. Noticeably, reducing Hipk function (C, C', D, D') decreases the number of cells expressing Yki^{S168A}:GFP in comparison to the number of cells seen expressing *UAS-Yki^{S168A}:GFP* alone (A, A', B, B')
2.8 Hipk regulation of Yki activity is kinase dependent

Since regulation of Yki activity is through protein-protein interactions and phosphorylation events, I performed co-immunoprecipitation and *in vitro* kinase assays to determine the mechanism(s) by which Hipk modulates Yki activity. In HEK293T cells transfected with Myc-tagged *Drosophila* Hipk and HA-tagged Yki, Hipk was co-immunoprecipitated in a complex with Yki (Lane 2 in Fig. 2-12). While the Hpo pathway is activated, Wts-mediated phosphorylation inhibits Yki activity. Given that Hipk is a serine/threonine kinase, it raises the possibility that Hipk positively regulates Yki through phosphorylation. I performed *in vitro* kinase assays and found that Yki is phosphorylated in the presence of wildtype Hipk (Lane 4 in Fig. 2-13), but not in the presence of kinase-dead (KD) Hipk (Lane 5 in Fig. 2-13). To further investigate whether the kinase function of Hipk is required to promote Yki activity, I used a KD form of *hipk* (*UAS-hipk*<sup>KD</sup>) to examine Yki target expression in wing discs. Compared to the potent effects seen with overexpressing wildtype *hipk* (Fig. 2-14 A, C), *hipk*<sup>KD</sup> was not able to induce *ex-lacZ* or *Diap1-lacZ* expression, or to promote tissue overgrowth (Fig. 2-14 B, B’, D, D’). These data indicate that Hipk requires its kinase function to positively regulate Yki transcriptional activity and to counteract the inhibition of Yki caused by Hpo pathway members. Hipk is the first kinase identified that inhibits Hpo signalling by phosphorylating Yki.
Figure 2-12  Hipk forms a complex with Yki

(Lane 1,2) pCMV-Hipk-Myc and pCMV-Yki-HA are co-transfected into HEK293T cells. (Lane 2) Cell lysates were immunoprecipitated with anti-HA antibodies, and extracts are visualized by western blotting by using anti-Myc or anti-HA antibodies to detect Hipk or Yki, respectively. (Lane 3 and 4) pCMV-Hipk-Myc and pCMV-HA are co-transfected into HEK293T cells. The cell lysate was used for the negative control of the assay. (Lane 3, 4) The lysates were immunoprecipitated with anti-HA antibodies, and extracts are visualized by western blotting by using anti-Myc to detect Hipk.
In an *in vitro* kinase assay using radiolabeled ATP, Yki is phosphorylated in the presence of wildtype (WT) Hipk (Lane 4) but not in the presence of kinase-dead (KD) form of Hipk (Lane 5). WT Hipk is phosphorylated due to autophosphorylation (Lane 1 and 4), but KD Hipk is not (Lane 2 and 5).

**Figure 2-13**  Hipk phosphorylates Yki

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Lane 1 (p-Hipk) and Lane 2 (p-Yki) show phosphorylation of Hipk and Yki, respectively. Lane 3 shows the absence of phosphorylation in the absence of ATP. Lane 4 and Lane 5 show phosphorylation in the presence of radiolabeled ATP (+[γ-32P]ATP).
The kinase activity of Hipk is required to modulate the Hpo target genes in vivo.

The KD form of Hipk is not able to induce the Hippo target expression. (A-B') Diap1-lacZ expression in ptc>hipk\textsuperscript{wt} (A) and ptc>hipk\textsuperscript{KD}.HA (B, B') wing discs. (C-D') ex-lacZ expression in hh>hipk\textsuperscript{wt} (C) and hh>hipk\textsuperscript{KD}.HA (D, D') wing discs. (B', D') Expression domain of hipk\textsuperscript{KD} is shown by anti-HA staining (green).
Chapter 3  Conclusions

My findings indicate that Hipk promotes Yki transcriptional activity to regulate tissue growth during Drosophila development (Fig. 3-1). To the best of my knowledge, this is the first kinase identified to positively regulate Yki by phosphorylation. The immunohistochemical studies reveal that hipk regulates cell proliferation and apoptosis to promote Drosophila wing growth. The genetic interaction studies support that Hipk bypasses Yki inhibition by the upstream components to modulate the target expression. Importantly, Hipk requires its kinase function to regulate Yki activity at the nuclear level.

As Hipk functions in multiple signalling pathways, I cannot completely eliminate the possibility that Hipk regulates Hpo signalling and Yki via multiple pathways, however I believe I have ruled out a regulation through Wg. Furthermore, I show that Hipk phosphorylates Yki, implying a direct regulatory role of Hipk on Yki. One recent study indicates two serine residues at +1 and +4 positions relative to Ser168 (Serine169 and 172) of Drosophila Yki are conserved among Yki and YAP (Oh and Irvine, 2009). Interestingly, these two residues are not Wts phosphorylation sites. The phosphomimetic mutations at Yki S169 and S172 (YkiS169D, S172D) reduce phosphorylation on Yki S168, as well as cause an increase in adult wing size and its nuclear localization, suggesting that phosphorylation of these two sites induces Yki activity (Oh and Irvine, 2009). To examine whether these two residues are Hipk phosphorylation sites, I performed in vitro kinase assay for GST-Hipk and GST-YkiS169A, S172A, phosphoresistant mutations at Yki S169 and S172. In the presence of GST-Hipk, GST-YkiS169A, S172A is phosphorylated (data not shown), which is similar as I observed from the kinase assay for GST-Hipk and GST-Yki. These findings suggest: 1. Yki S169 and S172 are not Hipk phosphorylation sites. 2. Hipk phosphorylates multiple sites on Yki, which includes these two residues. Given that Hipk does not promote Yki nuclear localization, Yki S169 and S172 are mostly likely not the
Hipk target sites since Yki$^{S169D, S172D}$ enhances its nuclear localization. To further investigate the kinase function of Hipk on regulation of Yki activity, it is important to identify Yki phosphorylation sites and subsequently to generate phosho-mimetic and phosphoresistant forms of Yki. These mutant proteins will be evaluated for their ability to affect Hpo activity in biochemical assays *in vitro*, and also *in vivo* through the generation of transgenic flies.

Although Hipk promotes Yki by phosphorylation, the functional significance of this modification has yet to be further investigated. Given that Hipk exerts its effects on nuclear Yki, it may play an important role to facilitate the interactions between Yki and its transcriptional co-factor Sd. The Irvine group found that *Diap1* expression is specifically activated in response to Sd-Yki activity, and we showed that *hipk* overexpression enhances *Diap1* expression. In our analyses, we have applied the same genetic criteria and obtained similar results as were used to demonstrate that Sd was an essential factor for Yki-mediated growth (Goulev et al., 2008; Wu et al., 2008; Zhang et al., 2008). The Irvine group recently reported that Mad, the transcription factor of the Dpp pathway, directly interacts with Yki to form a transcription factor complex that regulates expression of Hpo target *ban* (Oh and Irvine, 2011). I found that loss of *hipk* eliminates expression of the Dpp reporter Vg(QE)-lacZ, which requires Mad activation for its expression, suggesting that *hipk* inhibits Mad-mediated transcription (data not shown). Interestingly, *hipk* overexpression reduces expression of br-C12-lacZ, a specific reporter for a Yki-Mad responsive enhancer (data not shown). This finding indicates that *hipk* has different roles in regulation of the Hpo transcriptional factors to control the signalling. Recently, the Cohen group demonstrates that the EGFR pathway regulates *ban* expression by inhibiting the transcriptional repressor Capicua (Herranz et al., 2012). Capicua acts as a transcriptional repressor though interaction with Gro (Jimenez et al., 2000; Ajuria et al., 2011). Since Hipk antagonizes Gro to promote *Drosophila* eye growth, Hipk may also regulate *ban* expression by inhibition of Gro activity. In addition, Hth
interacts with Yki to regulate expression of *ban* during *Drosophila* eye development. Given that *hipk* plays a role in regulation of the eye growth, we cannot eliminate the possibility that *hipk* is involved in Hth-Yki transcriptional activity to regulate *ban* expression in the eye. These findings suggest that the mechanism of Yki regulation with its various cofactors and transcriptional targets is complex and that Hipk may exert diverse effects at different target sites in different tissues.

In vertebrates, the LIM domain protein Zyxin regulates Hipk2 protein level (Crone et al., 2011). In *Drosophila*, D promotes interaction between Zyxin and Wts resulting in Wts degradation. (Rauskolb et al., 2011). Altogether, it raises the possibility that Drosophila Zyxin promotes Hipk stability, which in turn enhances Yki transcriptional activity. Given that *ex* is the component and transcriptional target of the Hpo pathway, it is important to determine whether the transcriptional level, protein localization, activity and stability of *hipk* are regulated by the Hpo signalling since Hipk regulation is not fully understood.

Given that vertebrate Hipk inhibits β−TrCP activity, it raises the possibility that Hipk regulates Hippo signalling through stabilizing Yap in mammalian system. I have found elevation of Yap expression and phosphorylation on Yap S127 (equivalent to Yki S168 ) caused by Hipk2 overexpression in the HEK293T cells (data not shown). Although Hipk2 induces an increase in Yap protein level, we still cannot conclude that Hipk2 regulates Yap activity by promoting its stability since Yap phosphorylation is also increased upon Hipk2 overexpression. In COS7 cells transfected with Hipk2, we did observe that exogenous Hipk2 co-localizes with endogenous Yap in the nucleus. Further studies should entail whether this growth regulation by Hipk family members is evolutionarily conserved. Several studies on vertebrate Hipks report its role in cell proliferation and apoptosis, suggesting that Hipks may also promote YAP activity and/or inhibit Hpo signalling to regulate growth in vertebrates. My studies demonstrate that Hipk promotes the
activity of oncogene Yki to regulate *Drosophila* tissue growth and shed light on the role of Hipks in biological control and cancer development
Figure 3-1  Hipk promotes Yki transcriptional activity

Hipk counteracts the Hpo-mediated growth inhibition to regulate Yki activity. Hipk interacts with and phosphorylates Yki to initiate Yki target gene expression, including \textit{ex, wg, DIAP1} and CycE. The kinase function of Hipk is required to promote Yki transcriptional activity at the nuclear level.
Chapter 4  Materials and Methods

4.1  Fly strains and crosses


The following RNAi lines obtained from Vienna Drosophila RNAi Center (Dietzl et al., 2007) were: *hipk* (vdrc108254), *hipk* (vdrc 32854), *slimb* (vdrc 34273), *ex* (vdrc 109281), *wts* (vdrc 9928), *mats* (vdrc 108080) and *yki* (vdrc 40497).

All wildtype flies are *w^{1118}* and all crosses were performed according to standard procedures at 25°C. Unless indicated, the vdrc108254 *UAS-hipk^{RNAi}* line was used. In assays examining interaction between two UAS transgenes, control crosses were performed with UAS-GFP or UAS-lacZ to eliminate suppressive effects caused by titration of Gal4. To generate *hipk* mutant clones, *hsflp.22; GFP, FRT79/TM6B* females were crossed to *hipk^{4}, FRT79/TM6B* males and progeny were collected for 24 hours and heat shocked at 38°C for 90 minutes at 48 AEL. Wing imaginal discs were dissected from late third-instar larvae for immunohistochemistry, and female adult flies were collected for phenotypic analyses.
4.2 Generation of transgenic lines

The pUAST-hipk\textsuperscript{KD}-HA construct was generated by Molecular Biology Service Center (Simon Fraser University). Arginine 221 in full-length \textit{hipk} (from pCMV-Hipk-Myc) was mutated to alanine (\textit{hipk} R221A) by site-directed mutagenesis. The EcoRI site in pUAST was mutated to a Sme site, and \textit{hipk} R221A and HA-tag were cloned into the Sme site. Transgenic lines were created by BestGene Inc.

4.3 Wing mounting and measurement

Female adult wings were mounted in Aquatex (EM Science). By using Photoshop CS4, the width of the adult wing was measured as the length between the posterior most point of L5 and the anterior wing margin, when measured perpendicular to the posterior wing margin. Standard errors about the mean and t tests were calculated using Microsoft Excel 2008.

4.4 Immunohistochemistry

Antibody staining was performed according to standard protocols (Lee et al., 2009). The following primary antibodies and dilutions were used: 1:50 anti-Ci, 1:100 anti-Wg (Developmental Studies Hybridoma Bank), 1:1000 mouse anti-\textit{β}-galactosidase (Promega), 1:200 anti-PH3, 1:100 anti-cleaved Casp3 (Cell Signaling Technology), 1:100 anti-CycE (Santa Cruz), 1:1500 rabbit anti-Yki (gift from Dr. Irvine), 1:1000 mouse anti-HA and 1:200 rabbit anti-HA (Sigma-Aldrich). Discs were mounted in Vectashield with DAPI (Vector Laboratories) and oriented with their anterior to the left.
4.5 Plasmid constructs

pGEX4T1-Yki (gift from Dr. McNeill) was used to produce GST-Yki protein. Yki was subcloned from pGEX4T1-Yki into pCMV-HA (Clontech).

pcDNA3.1-Hipk2-Flag and pCMV-Hipk-Myc are described in Lee et al., 2009a and Lee et al., 2009b.

4.6 Cell Culture and in vitro biochemical assays

HEK293T cells were cultured at 37 °C in DMEM supplemented with 10% FBS (Invitrogen). Cells were transiently transfected at 24 hours after seeding with pCMV-Hipk:Myc, pCMV-Hipk:HA (Lee et al., 2009), pCMV-Yki:HA or pCMV-HA (Clontech) by using Polyfect reagent (Qiagen) according to the manufacturer's instructions. The cells were harvested 48 h after transfection by using lysis buffer supplemented with protease inhibitors (Cell Signaling Technology). For co-immunoprecipitation, the cell lysates were incubated with mouse anti-HA antibody (Sigma-Aldrich) or mouse IgG (Santa Cruz) at 4 °C overnight. The PGS beads (Sigma-Aldrich) were added to the lysate mixture and incubated for 2 hours at 4°C followed by SDS/PAGE/Western blotting and detection with mouse anti-HA antibody and mouse anti-Myc antibody (Sigma-Aldrich).

4.7 In vitro kinase assay

Hipk:HA and Yki:HA pulled down from the cell lysate with mouse anti-HA antibody and PGS beads were incubated in kinase assay buffer (Cell Signaling Technology) and ATP [32P] (Perkin Elmer) at 30 °C for 30 min followed by SDS/PAGE/Western blotting and detection with mouse anti-HA antibody or autoradiography.
Chapter 5  References


Appendices
Appendix I Vg, V(Q)-lacZ and V(D/V)-lacZ expression in the hipk \(^4\) mutant clone

Figure I Loss of hipk induces Vg, V(Q)-lacZ and V(D/V)-lacZ expression

(A, C, E) wild type wing disc. (B, B’, D, D’, F, F’) wing disc with hipk \(^4\) somatic clone, marked by absence of GFP in B’, D’ and F’. (A) Anti-Vg antibody staining of the wildtype wing disc from Bajpai et al., 2004. hipk mutation leads to induction of Vg (arrow in B and B’), Vg(Q)-lacZ (arrow in D and D’) and Vg(D/V)-lacZ (F, F’) expression.
Appendix II  
Fat expression and subcellular localization in the $hipk^d$ mutant clone

Figure II  
Hipk does not affect Fat expression and subcellular localization in the wing disc

(A-B’) the third-instar wing disc with $hipk^d$ somatic clone, marked by absence of GFP (green) in A’ and B’. The top panels show the XY-plane image, and the bottom panels show the Z-section image. anti-Fat stain (red) in the mutant clone located in the wing pouch (A, A’) and in the peripheral region of the wing disc (B, B’). (A’, B’) The lines indicate the region that the Z-section image is taken from the XY plane.
Appendix III  Expression of bantam reporter in the *hipk* overexpression background

Figure III  Hipk overexpression causes a decrease of expression of bantam reporter

(A) Control disc. (B, B’) Overexpressing wildtype Hipk leads to reduction of bantam reporter [br-C12-lacZ (Oh and Irvine 2011)], a specific reporter for Yki-Mad responsive enhancer. Anti-HA stain (green) indicates *hipk*-overexpression domain. Since *hipk* can modulate expression of Vg, Vg(Q)-lacZ and Vg(D/V)-lacZ, which are considered as Dpp targets (Fig. I), *hipk* may suppress Dpp signalling by inhibiting Mad activity, leading to inhibition of br-C12-lacZ expression.
Appendix IV  Vertebrate Hipk2 has an effect on Yap protein level and phosphorylation

Figure IV  Hipk2 overexpression leads to increase of Yap protein level and phosphorylation

Hipk2-Flag is transfected into HEK293T cells. Cell lysates are visualized by western blotting by using anti-Yap and anti-YapS127P (detecting phosphorylation site on YAP Serine 127 by Lats) to detect endogenous Yap protein. Yap and YapS127P protein levels are increased in the presence of Hipk2 overexpression.
Appendix V  Hipk2 co-localized with Yap in the nucleus

Figure V  Hipk2 and Yap are co-localized in the nucleus.

COS7 cells without (A-B’) or with (C’D’) Hipk2-Flag transfection. B and B’ show that endogenous Yap is present in both cytoplasm and nucleus. (C-D’) In the presence of Hipk2-Flag (green in C), endogenous Yap is co-localized with Hipk2-Flag in the nucleus. (B’, D’) Nuclei are stained with DAPI (blue).
Appendix VI    Materials and Methods

VI.I: Antibodies

1: 1000 rabbit anti-fat (gift from Dr. McNeill) was used for the antibody staining in the wing imaginal disc.

1:1000 rabbit anti-Yap and anti-YapS127P (Cell Signaling) were used to detect endogenous Yap protein in COS7 cells.

VI.II: Fly strains

\(V_g(Q)-lacZ\), \(V_g(D/V)-lacZ\) are obtained from Bloomington Drosophila Stock Center. \(br-C12-lacZ\) is the gift from Dr. Irvine (Oh and Irvine 2011),
Appendix VII  References
