HIPK: A VERSATILE REGULATOR OF MULTIPLE SIGNALING NETWORKS DURING *DROSOPHILA* DEVELOPMENT

by

Wendy Lee
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APPROVAL

Name: **Wendy Lee**

Degree: **Doctor of Philosophy**

Title of Thesis: **Hipk: a versatile regulator of multiple signaling networks during *Drosophila* development**

Examining Committee:

Chair: **Dr. Eldon Emberly**
Assistant Professor, Department of Physics

_____

Dr. Esther Verheyen
Senior Supervisor
Associate Professor of Molecular Biology and Biochemistry

_____

Dr. Nicholas Harden
Supervisor
Associate Professor of Molecular Biology and Biochemistry

_____

Dr. Nancy Hawkins
Supervisor
Assistant Professor of Molecular Biology and Biochemistry

_____

Dr. Lynne Quarmby
Internal Examiner
Professor of Molecular Biology and Biochemistry

_____

Dr. William Brook
External Examiner
Associate Professor of Biochemistry and Molecular Biology; Medical Genetics; University of Calgary

Date Defended/Approved: February 16, 2009

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ABSTRACT

Development of multicellular organisms is a dynamic process that requires cell-cell communication through the transmission of signal transduction pathways. Signaling cascades elicit transcriptional programs to synchronize discrete biological processes to pattern a multicellular organism. These signals are tightly regulated and their deregulation can lead to cancer onset and progression.

Homeodomain interacting protein kinases (Hipks) represent a family of serine/threonine kinases. Members of this group (in particular Hipk2) have been implicated as important factors in transcriptional regulation to control cell growth, apoptosis and development. These studies identified an essential requirement for the sole *Drosophila* member of this family, Hipk. Genetic and phenotypic analysis revealed novel roles for Hipk in the Notch and Wingless (Wg) pathway. Specifically, Hipk utilizes diverse mechanisms to regulate gene transcription depending on the cellular context.

In the developing eye, Hipk stimulated the early function of Notch mediated growth. Consistent with this model, genetic interaction analyses demonstrated that Hipk phosphorylates the global co-repressor Groucho (Gro) to relieve its inhibitory effect on Notch, thereby promoting the Notch signal. In addition, loss of *hipk* led to reduced expression of Notch targets, including the growth promoting factor, Eyegone (Eyg). Furthermore, overexpression of Hipk led to overgrown visual organs in both the adult and

larval tissue. A similar role for Hipk in promoting growth in additional tissues was also observed, this likely represents a general role for Hipk in growth.

Genetic and phenotypic evidence in the wing demonstrated a role for Hipk as a positive regulator in the Wg pathway. Mutant and misexpression analyses in the wing demonstrate that Hipk promotes Wg signaling through Armadillo (Arm) stability and stimulation of Wg target gene expression. Consistent with these observations, Hipk enhanced Tcf/Arm-mediated gene expression in cell culture. In addition, Hipk can bind to Arm and Tcf, and phosphorylate Arm. Using both *in vitro* and *in vivo* assays, Hipk was found to promote the stabilization of Arm. Similar molecular interactions between Lef1/β-catenin and vertebrate Hipk2 were observed, suggesting a conserved role for Hipks in promoting Wnt signaling.

These studies reveal that Hipk is a key regulator of multiple signaling networks during development.

To Mama and Papa

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CHAPTER 1
GENERAL INTRODUCTION

1.1 Signal transduction pathways in *Drosophila* development

The development of multicellular organisms is a dynamic and complex process, requiring cell to cell communication. Cells communicate with one another to form tissues that will eventually give rise to organs. Proteins play a pivotal role in intercellular communication to orchestrate major biological processes by transmitting instructions to cells in the form of signal transduction pathways. In response to stimuli, these pathways relay information from the cell surface to the cytosol and, ultimately, to the nucleus, triggering dynamic changes in cell behaviour by altering gene expression levels. Signaling can also cause changes in cell structure and architecture in response to cues, but for the purpose of this study I focus on signaling pathways which culminate in changes in gene expression.

Signaling cascades are activated upon the binding of a signaling molecule or ligand to a receptor, which in turn activates cytoplasmic components of the pathway that regulate DNA binding transcription factors. These signals can elicit both long and short range effects depending on the type of ligand that is bound to the receptor. Secreted ligands called morphogens, such as Wingless (Wg) and Bone Morphogenetic Proteins (BMPs) send both long and short range signals. Morphogens can stimulate a spectrum of biological processes since cells respond differentially to a single cue depending on their

distance from the ligand source. Expression of gene targets is induced by different signaling thresholds; hence the morphogen can activate multiple transcriptional targets in one instance through a dose dependent mechanism. Short range signals such as Notch (N), require cell-cell contact, and the N signal is transduced through the binding of the transmembrane ligand Delta (Dl) on one cell to the N receptor on an adjacent cell. Often N acts through a signaling process termed lateral induction, or lateral inhibition, which restricts the developmental program within a group of equivalent cells (equivalence group), whose members can potentially give rise to the same fates. This is a stochastic event and occurs when a slight shift in signaling capacity in one cell allows it to influence the developmental program of the other cells in the equivalence group. Thus, these signals are useful for generating cell fate diversity

Extensive knowledge in the field of development and signal transduction have been uncovered using *Drosophila melanogaster* as a model organism, facilitated by its short life cycle, ease of manipulation and vast collections of characterized mutants. The simple and highly regulated organization of many tissues, such as the wing and eye, provides a powerful system to unravel the molecular mechanisms governing developmental processes. Furthermore, findings from research studies in *Drosophila* provide invaluable insight into the mechanisms governing development in higher organisms because of functional conservation across species, including humans,

In *Drosophila*, most adult structures are derived from single layered epithelial sacs called imaginal discs. These primordial sacs are initially set aside during embryogenesis, and undergo massive growth and proliferation during the three larval stages (first, second and third larval instars) (Garcia-Bellido and Merriam, 1969). An

imaginal disc can give rise to multiple tissues. For example, the anterior region of the eye-antennal disc will form the antenna, while the posterior region will develop both the adult eye and head (Fig. 1-1) (Haynie and Bryant, 1986). Following the growth phase, the *Drosophila* eye is patterned through the progression of the morphogenetic furrow (MF), an indentation in the epithelium along the ventral-dorsal axis. The MF travels across the eye disc from the posterior margin towards the anterior, leaving differentiated photoreceptors in its wake (Ready et al., 1976; Tomlinson and Ready, 1987). The final structure of the adult eye contains 800 hexagonal ommatidia, each consisting of eight photoreceptor cells arranged in a radial pattern accompanied by accessory cells such as bristles and pigment cells (Wolff and Ready, 1993)

The *Drosophila* wing is another invaluable tissue for genetic analysis due to its stereotyped size, shape and venation pattern. The wing blade is derived from cells located in the presumptive wing pouch of the imaginal wing disc. The adult wing possesses five longitudinal veins (LI –L5) extending along the proximal to distal axis and two connecting cross veins, the anterior cross vein (ACV) and posterior cross vein (PCV) (Fig. 1-2C). The wing margin houses an organized arrangement of mechanosensory bristles and its development initiates at the dorsal/ventral boundary of the wing pouch of the imaginal wing disc (Fig. 1-2A). The adult wing is connected to the mesothorax by the wing hinge, both of which also develop from the larval wing primordium. The outer ring surrounding the wing pouch will form the foundation for the future hinge structure, while the dorsalmost compartment of the wing disc forms the mesothorax or notum.

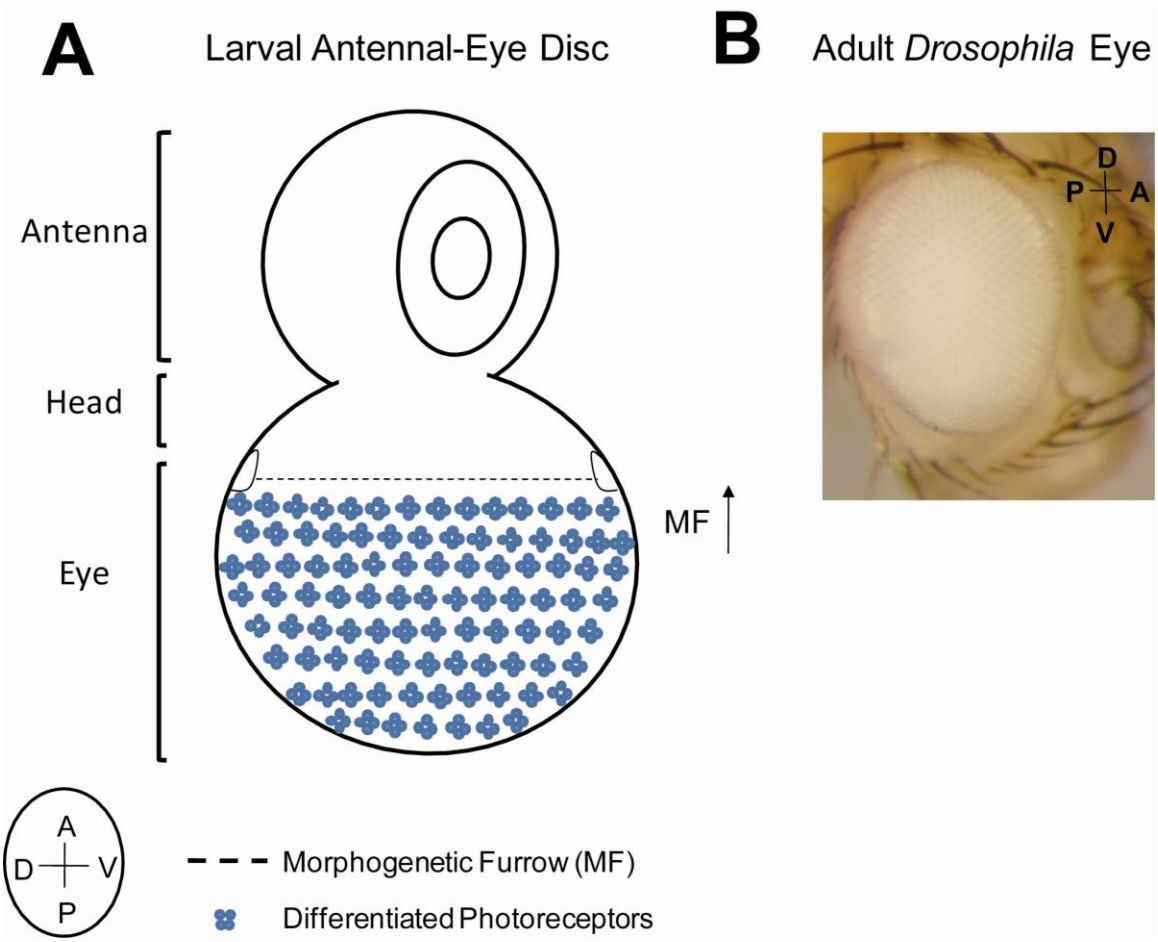

Figure 1-1 *Drosophila* eye development

(A) Schematic diagram of a larval eye-antennal disc. The smaller anterior region of the eye-antennal disc will form the adult antenna, and the posterior portion of the disc or eye disc will develop the future head and eye structures. As the morphogenetic furrow (MF) traverses from the posterior margin towards the anterior of the disc, it leaves differentiated photoreceptors behind it.

(B) The adult Drosophila eye consists of a hexagonal array of roughly 800 ommatidia, or photosensing units. The regular arrangement of these structures produces a smooth appearance. Dorsal (D)- ventral (V), and anterior (A)- posterior (P) axes are indicated at the bottom of the figure.

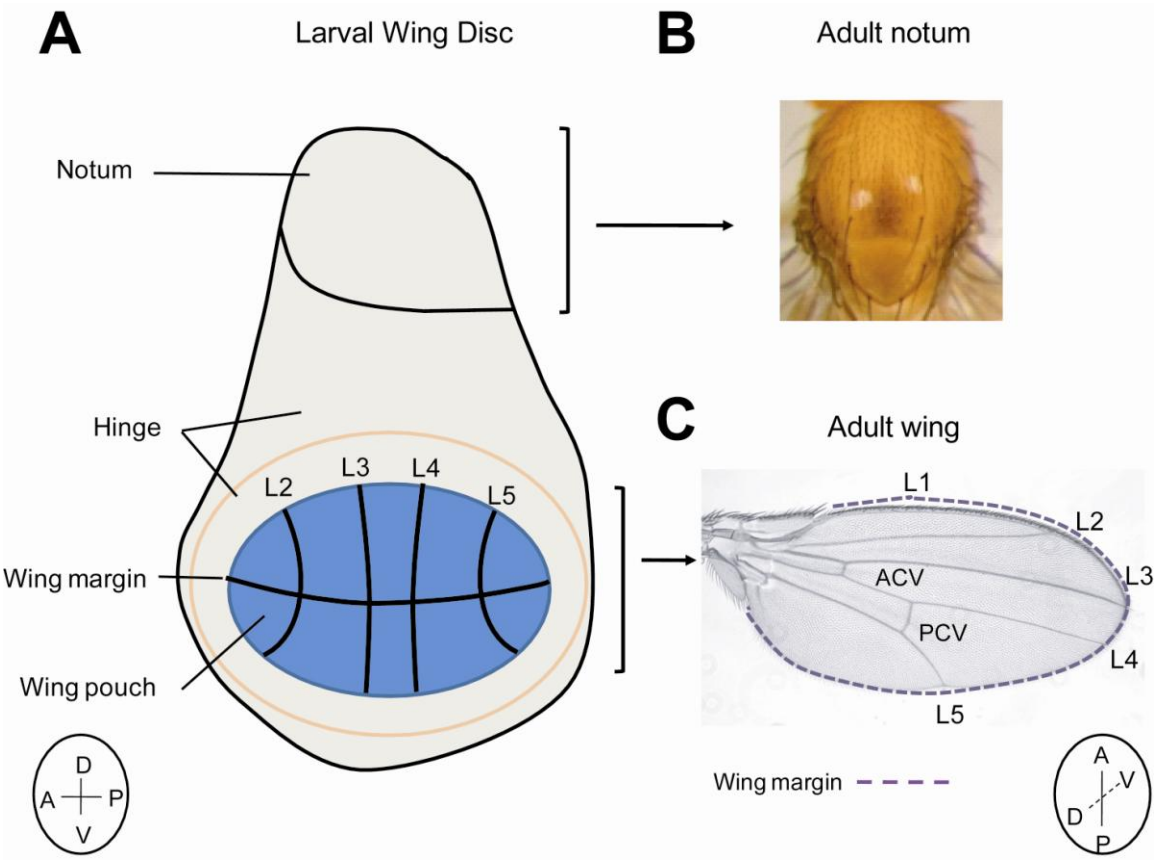

Figure 1-2 *Drosophila* wing development

(A) The anatomy of the adult wing and mesothorax (notum) are derived from the wing pouch (in blue) and the dorsal-most compartment of the wing disc, respectively. The patterning of the wing blade occurs during larval stages and the positions of the vein and wing margin primordia are indicated in the developing wing pouch.

(B) The wildtype adult notum possesses a peripheral nervous system comprised of stereotypically positioned sensory organs or bristles (macrochaetes).

(C) The wildtype adult wing possesses longitudinal veins (L1-L5) and the connecting anterior cross vein (ACV) and posterior cross vein (PCV). The wing margin is indicated by the purple dashed line, and corresponds to the dorsal/ventral boundary of the imaginal discs (shown in C)

Dorsal (D)- ventral (V), and anterior (A)- posterior (P) axes are indicated at the bottom of the figure.

During the early pupal stage, the monolayered wing disc protrudes and folds back upon itself along the future wing margin to form a bilayered tissue (Klein, 2001).

The final organization of the *Drosophila* eye and wing requires the actions of a handful of conserved signaling pathways, including N, Wg, BMP and Epidermal Growth Factor Receptor (EGFR). Although, many signaling components of these pathways have been implicated in a single sequential cascade, growing evidence is mounting that these signals can converge and be integrated to form signaling networks. Hence, the *Drosophila* eye and wing can be utilized to dissect not only the regulation of individual pathways, but may also be used to provide insight into the interplay between the signaling cascades.

1.2 Kinases and their roles in signaling networks

Complex arrays of signaling networks are collectively synchronized to achieve the proper development of all metazoans. Their activity must be tightly regulated to ensure the proper patterning and growth of tissues. Regulation of a signaling pathway can occur at any level within the pathway, from perturbation of ligand-receptor interactions to regulation of the activity of transcription factors in the nucleus. Some regulators affect the on or off state of pathways while others are involved in fine-tuning. These regulatory controls are essential and ensure that accurate and physiologically necessary levels of signaling are achieved without aberrant signaling, which can have deleterious effects. It has been well-established that the signaling pathways which direct normal development can lead to cancer onset and progression when they are misregulated in adults. Hence,

understanding how signaling pathways are regulated can be used to identify novel therapeutic targets.

Signaling components are tightly regulated by post translational modifications. In many instances, the activity of a protein is determined by its phosphorylation state. Phosphorylation reactions are catalyzed by enzymes called protein kinases and involve the transfer of a phosphate group (PO_4) from adenosine triphosphate (ATP) and its covalent attachment to a free hydroxyl group on either a serine (S), threonine (T) or tyrosine (Y) residue. These modifications are highly dynamic and are reversed by phosphatases, which function to remove the phosphate group from proteins. There are numerous families of kinases that are classified according to their target specificity. Most notably they include serine/threonine kinases, such as Mitogen Activated Protein Kinases (MAPK), tyrosine kinases and dual specificity kinases that act on all 3 types of residues. Most kinases target conserved consensus sequences within their substrates, for example MAPK phosphorylates PxS/TP motifs (Biondi and Nebreda, 2003). Kinases in turn are also regulated by post translational modification and their on and off states are dictated by their phosphorylation levels. Most MAPKs possess a conserved regulatory TxY polypeptide motif within their activation domain that is phosphorylated at both the threonine and tyrosine residues (Kultz, 1998). This phosphorylation event induces a conformation change within the kinase, permitting it in turn to bind and phosphorylate its substrate. Protein kinases also possess a conserved lysine residue within the catalytic domain that is critical for the phosphotransfer reaction to its substrate and point mutation of this residue inhibits the activity of the kinase (Carrera et al., 1993).

Protein kinases and phosphatases play a central role in many signaling pathways and are key effectors in many cellular functions. Changes in the phosphorylation status of a molecule can have widespread influences on its signaling activity. Kinases and phosphatases indirectly mediate gene expression levels, from altering the activity of a transcriptional regulator to affecting its subcellular localization or modifying its ability to bind its substrate or DNA. Phosphorylation and dephosphorylation can inhibit the function of signaling components altogether by simply targeting the substrate for degradation or conversely, elevate its signaling potential by promoting its stability.

Only a handful of conserved signaling pathways are consistently reiterated throughout development for directing cell fate. The spectrum of cellular responses outnumbers the signaling pathways that are utilized to confer the appropriate developmental program. One means of creating such diversity is by utilizing a single transcription factor that responds differentially to various signaling inputs. The transcriptional program can be further manipulated by the co-factors bound to the protein, which in turn recruit the necessary machinery to activate or inhibit transcription. Such a combinatorial system allows a single signaling component to trigger and to terminate a developmental process. Kinases can confer specificity to a transcription factor by modifying the composition of the transcriptional units, either by excluding or promoting its binding capacity to particular co-factors.

1.3 Hipk family

Homeodomain-interacting protein kinases (Hipks) are a relatively novel family of serine/threonine kinases (Kim et al., 1998). Various cellular and biochemical roles have been ascribed to the four highly conserved vertebrate homologs, Hipk1-4. With the

exception of the more distantly related Hipk4, members of this family share a common protein structure characterized by a N-terminal kinase domain, a homeoprotein interaction domain (HID), followed a PEST domain, rich in Proline, Glutamic acid, Serine and Threonine (Fig. 1-3) (Kim et al., 1998). The PEST region contains binding domains for many non homeodomain transcription factors (Rinaldo et al., 2007a). The kinase domain is well conserved, sharing sequence homology greater than 90% in Hipk1-3 and is also present in the *Drosophila* homologue. In *Drosophila*, the sole member of the family has been referred to as both Hipk (Link et al., 2007) and Hipk2 (Choi et al., 2005). In this study, I refer to the *Drosophila* protein as Hipk since it shares sequence homology with all its vertebrate counterparts, most notably with Hipk1-3 (Fig. 1-4).

Vertebrate Hipk2, the best characterized of all the Hipk members (reviewed in Calzado et al., 2007; Rinaldo et al., 2007a) was originally identified in a screen as a corepressor for the homeodomain transcription factor NKx-1.2 (Kim et al., 1998). Biochemical studies have identified Hipk2 as a co-factor for a myriad of proteins involved in transcriptional regulation, chromatin remodelling and evolutionarily conserved signaling pathways. Many of these events are mediated through phosphorylation by Hipk2. Although Hipk2 binds and phosphorylates several proteins, Hipk2 specificity for its substrate remains unclear, since Hipk2 recognizes several different consensus sequences. Depending on the cellular context, Hipk2 can also promote transcription by affecting the activity and/or stability of its targets.

Figure 1-3 Schematic representation of Hipk family protein domains

The kinase domain resides toward the N terminus, followed by an interaction domain for homeodomain transcription factors and a PEST containing region, containing binding sites for non-homeotic factors, that is followed by an autoinhibitory domain.

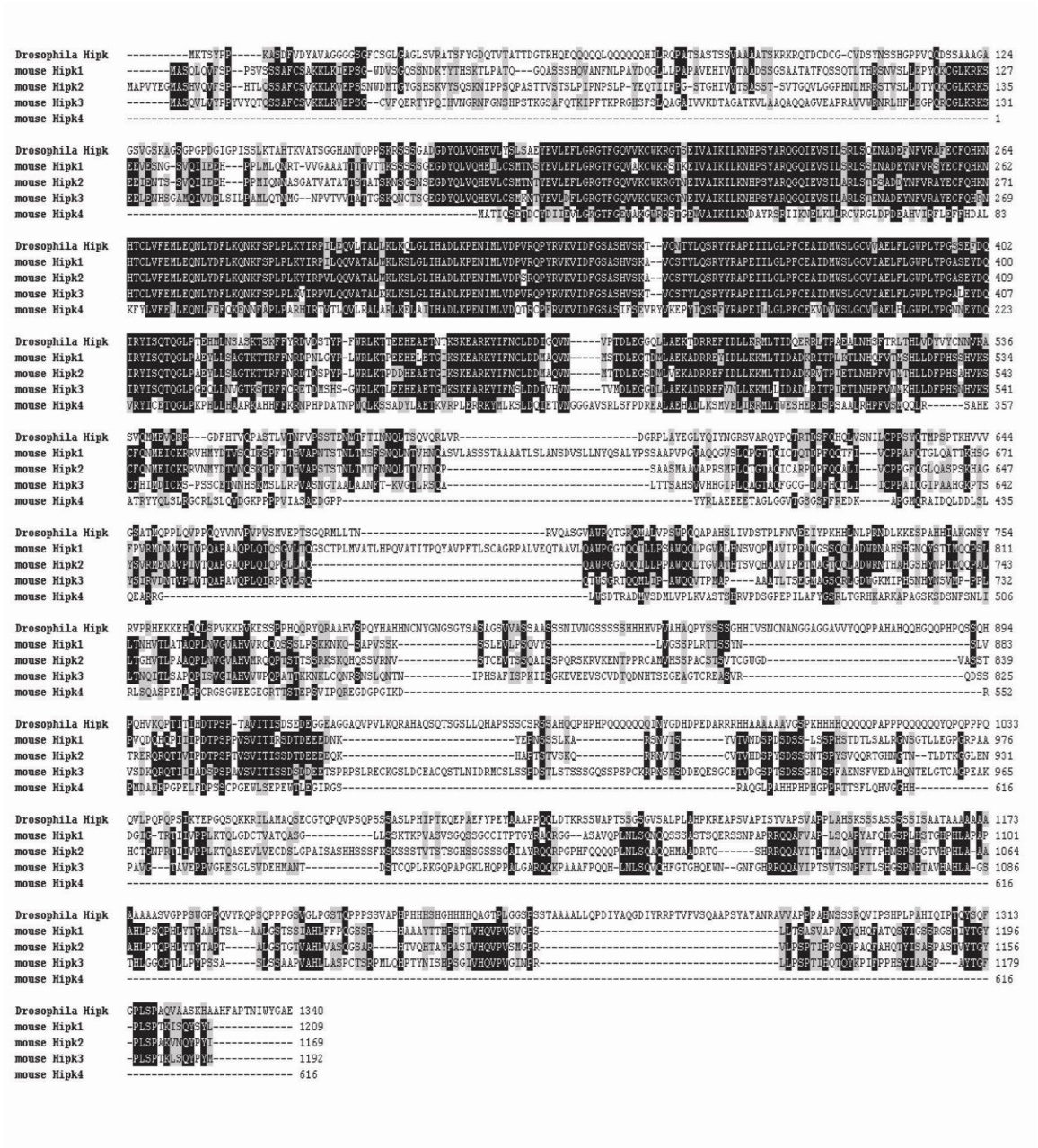

Figure 1-4 Sequence homology between *Drosophila* Hipk and vertebrate Hipks

Protein alignment showing the relationship between *Drosophila* Hipk and murine Hipk1-4. Black shading indicates conserved residues, while light grey represents similar residues.

Recent studies have begun to unravel the physiological relevance of Hipk2 through genetic loss of function analyses. Disruption of murine *hipk2* or *hipk1* produces grossly normal fertile mice, suggesting a functional redundancy between *hipk1* and *hipk2* in certain biological processes (Kondo et al., 2003; Wiggins et al., 2004). Mice mutant for both *hipk1* and *hipk2* are inviable and manifest neural tube closure defects and homeotic transformations in the axial skeleton (Isono et al., 2006). These mice fail to close the anterior neuropore due to proliferative defects in the neural folds and underlying mesoderm (Isono et al., 2006), suggesting that Hipks may regulate cell numbers during development. Furthermore, *hipk2* knock out mice exhibit an expansion of the epidermal stem cell population and the number of cells in the G_0/G_1 and S phases of the cell cycle increases in *hipk2*$^{-/-}$ keratinocytes and mouse embryonic fibroblasts (Wei et al., 2007). These observations reveal that Hipks may act as key players in growth, possibly through their regulation of cell cycle progression.

Hipk2 also controls cell numbers by modulating other biological processes such as apoptosis and cell survival. Programmed cell death is crucial during development and in degenerative conditions. The choice between cell death or survival is coordinated by differential expression of proapoptotic and prosurvival genes. In the sensory neurons, the Brn3a transcription factor promotes cell survival by upregulating the expression of prosurvival genes, including the neurotrophic receptors, such as *trkA* and members of the *bcl* family. Mice lacking Brn3a exhibit increased apoptosis and reduced expression of multiple *trk* family members, subsequently leading to sensory neuron loss (Huang et al, 1999). Hipk2 has been studied in the nervous system and in vivo and in vitro evidence suggests Hipk2 modulates the expression profile of prosurvival genes. *hipk2* knockout

mice produce extra sensory neurons in the trigeminal ganglion and overexpression of Hipk2 in cultured sensory neurons induces apoptosis leading to neuron loss (Wiggins et al., 2004; Zhang et al., 2007). Moreover, *hipk2* mutant mice display elevated expression of Brn3a targets, prosurvival genes such as *trkA* and *bcl-x*$_{L.}$ Consistent with these observations, biochemical analyses reveal Hipk2 promotes cell death by suppressing Brn3a mediated transcription in vitro (Wiggins et al., 2004). Cellular survival is also influenced by morphogenetic signals and studies indicate that Hipk2 also regulates prosurvival factors other than Brn3a. In Human hepatoma cells, Hipk2 contributes to TGFβ activated JNK (Jun-N terminal kinase)-controlled apoptosis (Hofmann et al., 2003). In this cellular context, Hipk2 induces Promyelocytic Leukaemia (PML) nuclear body breakdown, releasing the JNK effector Daxx into the cytoplasm, effectively transmitting the JNK signal (Hofmann et al., 2003). Hipk2 exerts unique effects on cell survival signals depending on the cellular context. Contrary to a proapoptotic role for Hipk2 in the sensory neurons, Hipk2 provides a survival cue in dopamine (DA) neurons. *hipk2* knockout mutants have reduced midbrain DA neurons, leading to defects in psychomotor behaviour (Zhang et al., 2007). Hipk2 promotes TGFβ-dependent survival of DA neurons through an interaction with the major effectors of the pathway, R-Smads, including Smad2 and Smad3 (Zhang et al., 2007). These studies reveal that Hipk2 regulates cell survival by acting at several branches of the signaling circuitry.

In addition to the essential role of Hipk2 during development, the protein kinase also modulates the stress-induced behaviour of cells. Under such dire circumstances, cells either arrest in the cell cycle or initiate programmed cell death (Bartek and Lukas, 2001). The p53 tumour suppressor mediates the cellular response by acting as a sensor,

monitoring the intensity of DNA damage (Jin and Levine, 2001). Irreversible DNA damage induces the phosphorylation of p53 at ser46, which in turn, activates the transcription of proapoptotic genes, ultimately triggering programmed cell death (Oda et al., 2000). The apoptotic transcriptional program is inhibited in cells upon exposure to sublethal DNA damage. p53 triggers cell cycle arrest in these cells by inducing the expression of growth arrest genes, enabling their recovery from genotoxic cues (Vousden and Lu, 2002). The p53 switch between cell death and growth arrest is mediated by post translational modification. Phosphorylation of p53 at the conserved ser46 residue is critical for the activation of proapototic genes by shifting the affinity of p53 from promoters of growth arrest targets to promoters of apoptotic genes (Mayo et al., 2005; Oda et al., 2000). Hipk2 promotes p53 mediated transcription of proapoptotic genes by phosphorylating human p53 at ser46 and its murine homologue at ser58 (Cecchinelli et al., 2006; D'Orazi et al., 2002; Hofmann et al., 2002). p53 is tightly regulated by multiple regulatory circuits, including a feedback loop mediated by its inhibitor, the E3 ubiquitin ligase Mouse Double Minute 2 (MDM2) (Harris and Levine, 2005). Non severe DNA damage induces MDM2-mediated degradation of Hipk2, inhibiting phosphorylation of p53 and enabling cells to undergo p53 mediated growth arrest (Rinaldo et al., 2007b). Hipk2 is also regulated by other factors including Axin, which promotes Hipk2 mediated cell death by forming a multimeric complex with the autoinhibitory domain of Hipk2 (Rui et al., 2004). Activation of programmed cell death is modulated by several factors, including the corepressor CtBP (C-terminal binding protein) which inhibits expression of proapototic genes. Hipk2 promotes CtBP degradation, inducing apotosis in a p53 independent mechanism (Zhang et al., 2003).

In *Drosophila*, Hipk regulates cell death of the intervening epithelium between the dorsal and ventral wing surfaces of the adult wing. *hipk* mutants and somatic mutant clones (with an allele equivalent to $hipk^4$ that was generated in my studies) have wings that progressively produce cuticular blemishes after eclosion. This phenotype is identical to those observed in mutants of proapoptotic pathways. Furthermore, reduced *hipk* levels leads to extra neurons in the embryo and extra interommatidial cells in the pupal eye, indicative of a general role for Hipk as a proapoptotic factor throughout *Drosophila* development (Link et al., 2007).

Additional studies in the *Drosophila* eye using transgenic flies misexpressing *hipk* uncovered a role for Hipk during organogenesis. Occassionally, misexpression of a constitutively active form of Hipk stimulates ectopic eye formation near the existing eye, while overexpression of a kinase inactive form of Hipk suppresses eye development. The global corepressor, Groucho (Gro) inhibits eye specification by forming a repressor complex with the master eye regulator, Ey, consequently inhibiting its transactivation activity. In vitro studies demonstrate that Hipk phosphorylates Gro, inducing the disassembly of the repressor complex, subsequently promoting Ey activity (Choi et al., 2005). Hipk2 has also been isolated in a repressor complex containing the NK-3 homeoprotein, Gro and the histone deacetylase HDAC (Choi et al., 1999). Therefore, Hipks potentially control other developmental processes through its regulation of Gro.

Recently, Hipk2 was identified as an intermediate component of a MAPK pathway in hematopoietic cells (Kanei-Ishii et al., 2004). These kinase cascades are sequentially activated and play important regulatory roles in biological processes. Wnt-1 activation relays a signal downstream to the Transforming growth factor-ß activated

kinase-1 (Tak1), a MAPKKK, which in turn activates Hipk2. Hipk2, together with its downstream substrate, the MAPK Nemo-like kinase (Nlk) promotes the degradation of c-Myb, a transcription factor involved in proliferation, differentiation and apoptosis (Kanei-Ishii et al., 2004). Inhibition of c-Myb results in cell cycle arrest and triggers differentiation. Wnt-Nlk-Hipk2 signaling cassette regulates other members of the Myb family, including A-Myb. Wnt-NLK-HIPK signal inhibits A-Myb by preventing its association with the coactivator, CBP (Creb Binding Protein) and methylating Histone H3 at A-Myb bound promoter regions (Kurahashi et al., 2005).

The canonical Wnt signal is essential during multiple processes throughout development and its deregulation has been implicated in the onset and progression of cancer. Pathway activation culminates in the stabilization of cytosolic β-catenin and its subsequent nuclear entry, forming a transcriptional complex with TCF/Lef1 DNA binding protein. NLK is a conserved antagonist of the canonical Wnt signaling pathway by modifying the activity of the β-Catenin/TCF transcriptional complex (Ishitani et al., 1999; Rocheleau et al., 1999; Zeng and Verheyen, 2004). In vertebrates, Tak1 and Nlk function together to regulate Wnt signaling. Similarly, homologous to *tak1 and nlk, lit-1* and *mom-4* in *C.elegans* are components of this conserved pathway and function together during embryogenesis to regulate asymmetric cell divisions (Rocheleau et al., 1999). It remains unclear whether Nlk-Hipk2 pathway also functions to regulate canonical Wnt signaling. Recent studies have uncovered roles for Hipks in Wnt signaling in several experimental systems. In HEK293 cells, CtBP corepressor binds the C-terminal tyrosine/histidine (YH) domain of Hipk2, and deletion of this domain suppresses Hipk2 mediated inhibition of LEF1/β-catenin controlled transcription in vitro (Wei et al., 2007).

Furthermore, Hipk1 also associates with Dishevelled (Dsh/Dvl), a cytosolic component of both the Wnt canonical and non-canonical pathways (Louie et al., 2009). In *Xenopus laevis*, Hipk1 inhibits β-catenin/LEF-1 mediated transcription during dorsal-ventral axis specification and promotes the canonical signal in the mesoderm with the onset of gastrulation. Overexpression and knock down of Hipk1 induces convergent extension defects, causing severe gastrulation and neural tube closure phenotypes (Louie et al., 2009). These observations suggest roles for Hipks in several Wnt signaling cascades including, the canonical and non canonical planar cell polarity pathway.

Although studies in cell culture have identified a growing list of Hipk2 interactors, investigation of the biological significance of these interactions in multicellular organisms has been minimal. The main goal of my studies was to elucidate the role of Hipk in *Drosophila* development and discover how kinases regulate various transcriptional programs to pattern a multicellular organism.

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CHAPTER 2
HIPK IS AN ESSENTIAL PROTEIN THAT PROMOTES NOTCH SIGNAL TRANSDUCTION IN THE *DROSOPHILA* EYE BY INHIBITION OF THE GLOBAL CO-REPRESSOR GROUCHO

Wendy Lee, Bryan C. Andrews, Michael Faust, Uwe Walldorf and Esther M. Verheyen

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2.1 Abstract

To gain insight into the in vivo functions of the single *Drosophila* Hipk we characterized loss of function alleles, which revealed an essential requirement for *hipk*. We find that in the developing eye, *hipk* promotes the Notch pathway. Notch signaling acts at multiple points in eye development to promote growth, proliferation and patterning. Hipk stimulates the early function of Notch in promotion of global growth of the eye disc. It has been shown in the *Drosophila* eye that Hipk interferes with the repressive activity of the global co-repressor, Groucho (Gro). Here, we propose that Hipk antagonizes Gro to promote the transmission of the Notch signal, indicating that Hipk plays numerous roles in regulating gene expression through interference with the formation of Gro-containing co-repressor complexes.

2.2 Introduction

Groucho (Gro) is a global co-repressor that binds a diverse range of transcription factors and regulates their activity (Hasson and Paroush, 2006). It recruits the histone

deacetylase complex to DNA-bound transcriptional regulators, resulting in repression of gene expression (Chen et al., 1999). Numerous transcription factors switch from an activator to a repressor role upon association with Gro, thus regulating many conserved signaling pathways, including TGFβ, Wnt, and Notch (Cavallo et al., 1998; Roose et al., 1998). Studies in Drosophila using dominant negative and constitutively active Hipk transgenes have implicated Hipk in the promotion of eye development through the inhibition of Groucho (Gro) (Choi et al., 2005). Gro also forms a complex with the Eyeless (Ey) protein and inhibits its transactivation activity (Choi et al., 2005). Ey is a member of the Pax6 family of transcriptional regulators that plays a critical role in eye specification. In vitro, Hipk can phosphorylate Gro, causing the disassembly of the Gro/histone deacetylase complex from Ey, thereby promoting Ey transcriptional activity (Choi et al., 2005).

The Notch (N) signaling cascade is repeatedly employed throughout development to control cell fate and tissue growth (reviewed in Bray, 2006). In Drosophila, binding of the ligands Delta (Dl) or Serrate (Ser) to the N receptor induces its cleavage at multiple sites. The intracellular domain of activated N (N^{ICD}) translocates to the nucleus where it forms a complex with the DNA binding protein Suppressor of Hairless [Su(H)] to activate transcription of target genes, including the *Enhancer of split* complex [E(spl)-C] (Fig.2-1). In the N signaling pathway, Gro acts as a direct negative regulator and also interacts with the protein products of Notch target genes. In the absence of N pathway activation, Su(H) forms a repressor complex with Gro and the Hairless (H) protein to inhibit transcription of target genes (Barolo et al., 2002).

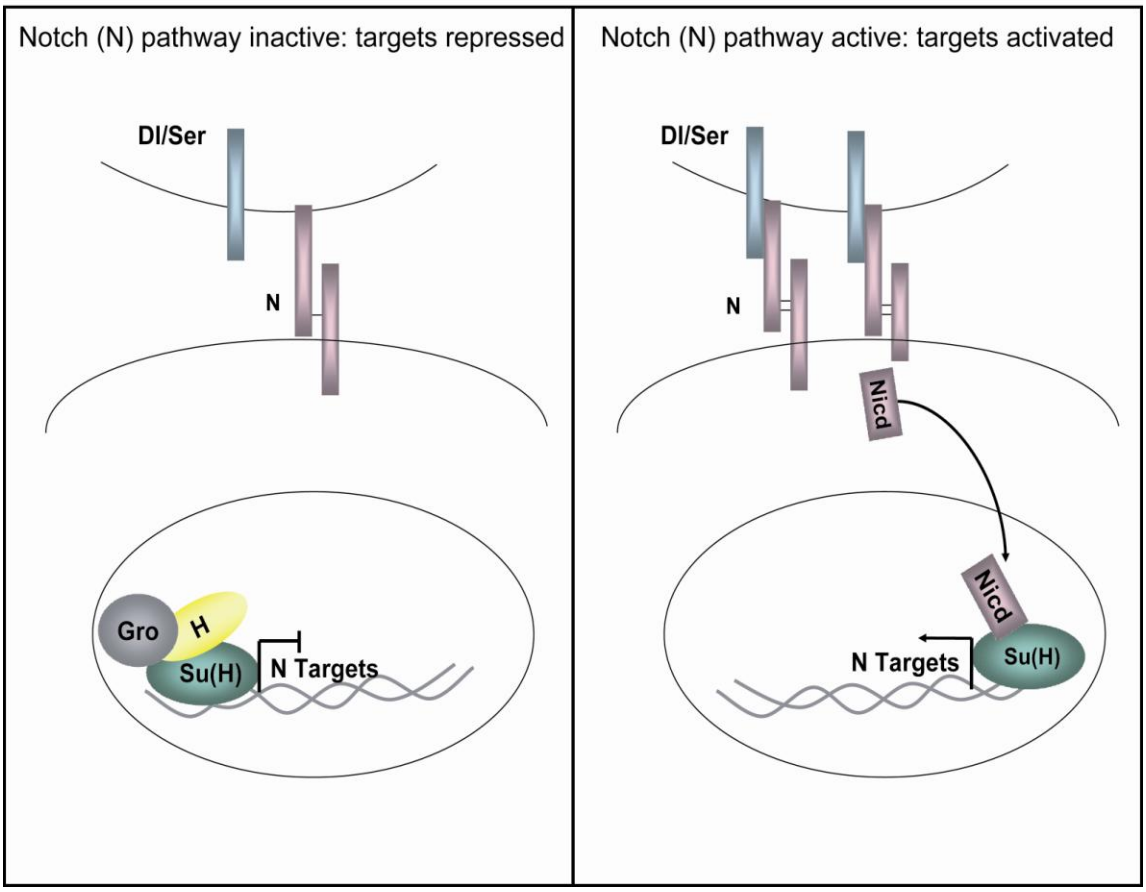


Figure 2-1 Schematic representation of the Notch pathway

In the absence of Notch (N) pathway activation, Su(H) forms a transcriptional repressor complex that inhibits activation of N responsive gene targets. Activation of the N pathway leads to the cleavage of the N receptor, resulting in the translocation ofthe intracellular domain of N (N^{ICD}) into the nucleus. Binding with N^{ICD} converts Su(H) into a transcriptional activator.

N signaling has multiple roles in the development of the Drosophila eye. Early in eye disc growth, N signaling promotes global growth of the eye disc (Dominguez and de Celis, 1998; Papayannopoulos et al., 1998). This occurs through localized activation of the N receptor at the dorsoventral (DV) midline of the disc mediated through the regulation by Fringe (Fng) (Fig. 2-2C). Fng potentiates N to respond to Dl at the D/V boundary or the boundary of Ser and Dl expressing and non expressing cells. Notch then activates expression of the *eyegone* (*eyg*) gene, which encodes a Pax transcription factor (Fig. 2-2A,B) (Chao et al., 2004; Jang et al., 2003; Jones et al., 1998; Jun et al., 1998). Eyg and Eyeless (Ey) can both promote eye development, but through different mechanisms (Dominguez et al., 2004; Jang et al., 2003). While Ey confers eye identity, Eyg is necessary for eye growth through the induction of the diffusible Jak-Stat ligand, Unpaired (Upd) (Fig.2-2A; Chao et al., 2004). Eyg activates *upd* expression at the posterior margin of the eye disc where it triggers non-autonomous growth of the eye (Fig.2-2B).

Following the growth phase, the Drosophila eye is patterned through the progression of the morphogenetic furrow (MF) across the eye disc from the posterior margin towards the anterior, leaving differentiated photoreceptors in its wake. Cells must first attain neuronal competence before they are selectively refined to achieve the final organization of retinal cells. N initially enhances proneural competence in cells anterior to the MF in the developing eye and then inhibits neural fate behind the MF (Baker and Yu, 1997; Baonza and Freeman, 2001; Li and Baker, 2001).

In this study, we reveal an essential requirement for Drosophila *hipk* and show that diminished levels of *hipk* lead to growth and patterning defects in the eye. Our findings suggest that Hipk participates in multiple processes throughout the formation of

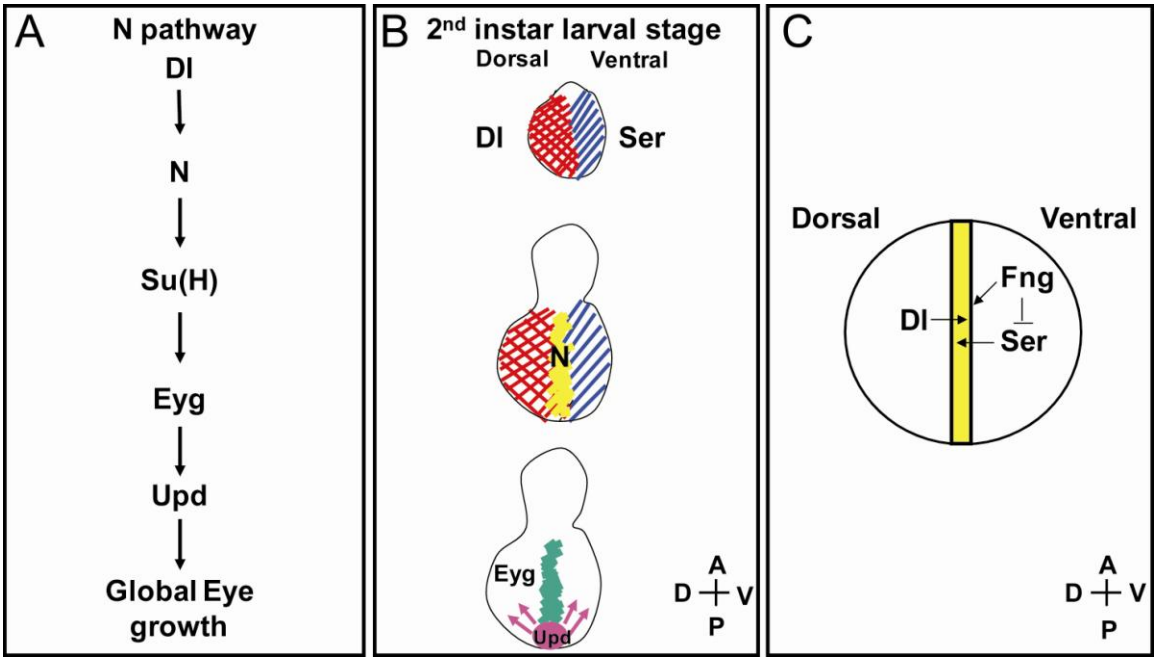


Figure 2-2 Notch promotes the global growth of the eye

(A) N signaling mediates eye growth by activating the expression of the growth promoting factor, Eyegone (Eyg), which in turn triggers the expression of the secreted Jak/STAT ligand Unpaired (Upd)

(B) By the second instar larval stage, the N ligands Dl and Ser are expressed in complementary domains of the eye disc. Localized activation of N at the Dorsal-Ventral boundary (D/V) or at the boundary of Dl and Ser expressing cells results in the activation of Eyg at the (D/V) boundary, that then activates expression of the diffusable Upd protein at the posterior margin of the eye disc leading to the global growth of the eye.

(C) The N modulator Fng ensures that N is activated along the boundary of Dl and Ser expressing cells (yellow stripe). Fng is co-expressed with Ser in the ventral cells of the early eye disc and inhibits the cells from responding to Ser. Fng mediates the localized activation of N at the boundary of ventral and dorsal cells by potentiating the ability of N to respond to Delta.

the visual organ. Genetic interactions and phenotypic similarities observed between Hipk and components of the N pathway suggest a cooperative interplay between the two. Hipk acts during early growth promotion mediated by N and Eyg, and is needed for *eyg* expression. Furthermore, N signaling is reduced in *hipk* mutant clones, as indicated by the diminished expression of the *E(spl)* target genes. We demonstrate that Hipk is required to promote the transmission of the N signal downstream of the receptor and this is mediated through its phosphorylation of Gro. This interaction represents a global mechanism through which Hipk can have a broad influence on signaling pathways.

2.3 Results and Discussion

2.3.1 *hipk* is an essential gene

To study *hipk* function in vivo, loss-of-function mutations were characterized in *Drosophila*. Deletions were generated at the *hipk* locus through imprecise excision of a transposable element (Fig. 2-3). The starting strain, $hipk^{1}$ ($P\{GT1\}CG17090^{BG00855}$), and the two excisions ($hipk^{2}$ and $hipk^{3}$) result in homozygous pupal lethality (with rare escaper adults) and trans-heterozygosity for any of these alleles and a deficiency removing *hipk* (*Df(3L)ED4177*) leads to lethality. A fourth allele, $hipk^{4}$, was generated through targeted deletion of the DNA between two transposable elements flanking the locus and this allele causes lethality prior to the 3rd larval instar. Interallelic crosses reveal an allelic series in order of weakest to strongest: $hipk^{2} < hipk^{1} < hipk^{3} < hipk^{4}$. These findings demonstrate that the single *hipk* gene in *Drosophila* is essential. Indeed, loss of both maternal and zygotic *hipk* results in embryonic lethality (data not shown).

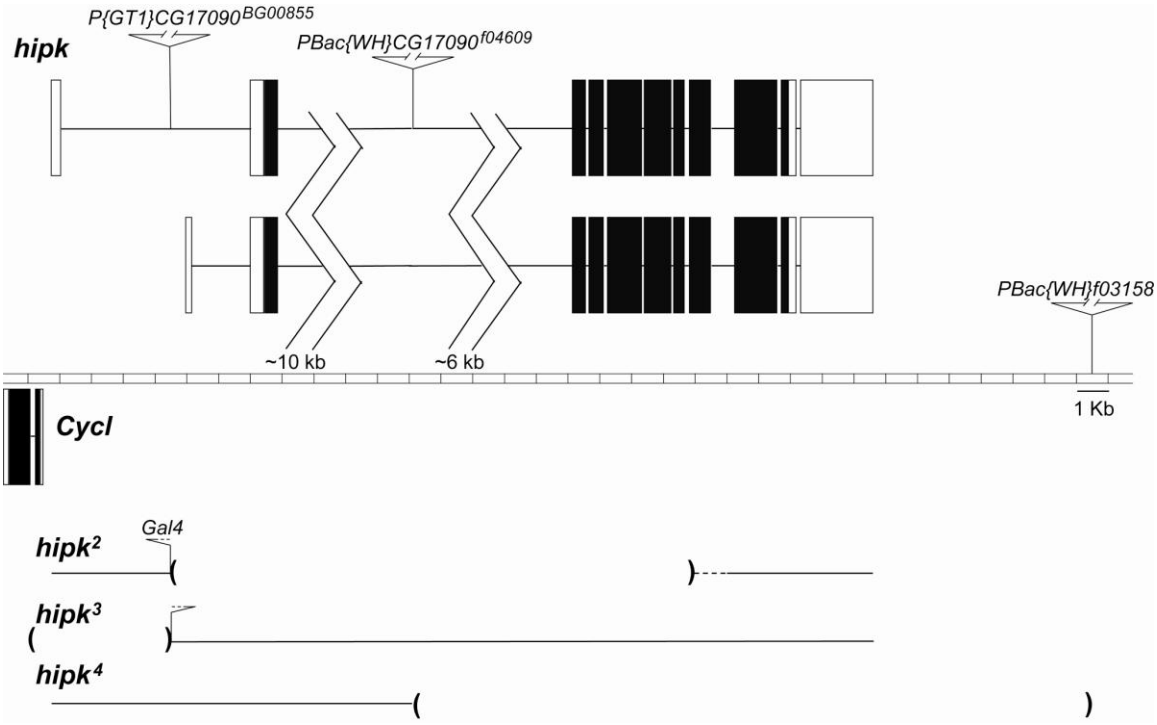


Figure 2-3 Generation of *Drosophila* hipk alleles

Schematic view of the *hipk* genomic region and organization of the *hipk* locus, *hipk* deletion alleles and the P-elements (inverted triangles) used to create the mutations. $hipk^{2}$ and $hipk^{3}$ were generated through an imprecise excision screen of $P\{GT1\}CG17090^{BG00855}$. $hipk^{3}$ also disrupts 370 bp of the neighbouring gene, *Cyclophilin-like* (*Cycl*). $hipk^{4}$ represents a targeted deletion of *hipk* through the recombination between *PBac{WH}*CG17090^{f04609} and *PBac{WH}f03158*. Boxes represent exons, and those that are filled represent the coding region. The deleted regions are denoted between the brackets and dashed lines represent unconfirmed deletions and solid black lines indicate the intact *hipk* genomic region.

2.3.2 *hipk* is required for eye development

hipk mutants consistently displayed small, rough eyes. Dissection of pharate adults from pupal cases revealed that 42% of $hipk^3$ (n= 106 eyes) homozygotes displayed a preferential loss in the ventral region, leading to a small round eye. Additional eye phenotypes include the appearance of non-retinal tissue in 25% of $hipk^3$ homozygotes (n=106). Transheterozygosity for the various *hipk* alleles displayed similar eye phenotypes to $hipk^3$ homozygotes (Fig. 2-4B, C).

Staining of neuronal cells in 3rd instar eye imaginal discs with the neural anti-Elav antibody (Robinow and White, 1991) revealed that 25% (n=30) of $hipk^3$ homozygotes display a loss of photoreceptors (Fig. 2-4G). This loss was most prominent in the lateral poles of the eye disc as the Elav-positive cells did not extend to the dorsal and ventral margins of the eye disc as is seen in wildtype (Fig. 2-4F). The loss of photoreceptors likely correlates with the loss of eye structure in adults (Fig. 2-4B, C). Further reduction of Hipk activity by generating loss of function somatic clones with a stronger allele, $hipk^4$, also led to a decrease of Elav staining (Fig. 2-4L-N, P-R). Under such conditions, neural differentiation is most sensitive to the loss of *hipk* near the MF. $hipk^4$ clones proximal to the MF displayed diminished Elav staining (Fig. 2-4M, Q). This effect is not restricted to the lateral poles, as was observed in $hipk^3$ homozygotes. While photoreceptors in clones located posterior to the MF appeared to differentiate correctly, the spacing of these cells was reduced and irregular, suggesting *hipk* is also required for patterning of cells posterior to the MF. We found that the loss of photoreceptors is likely

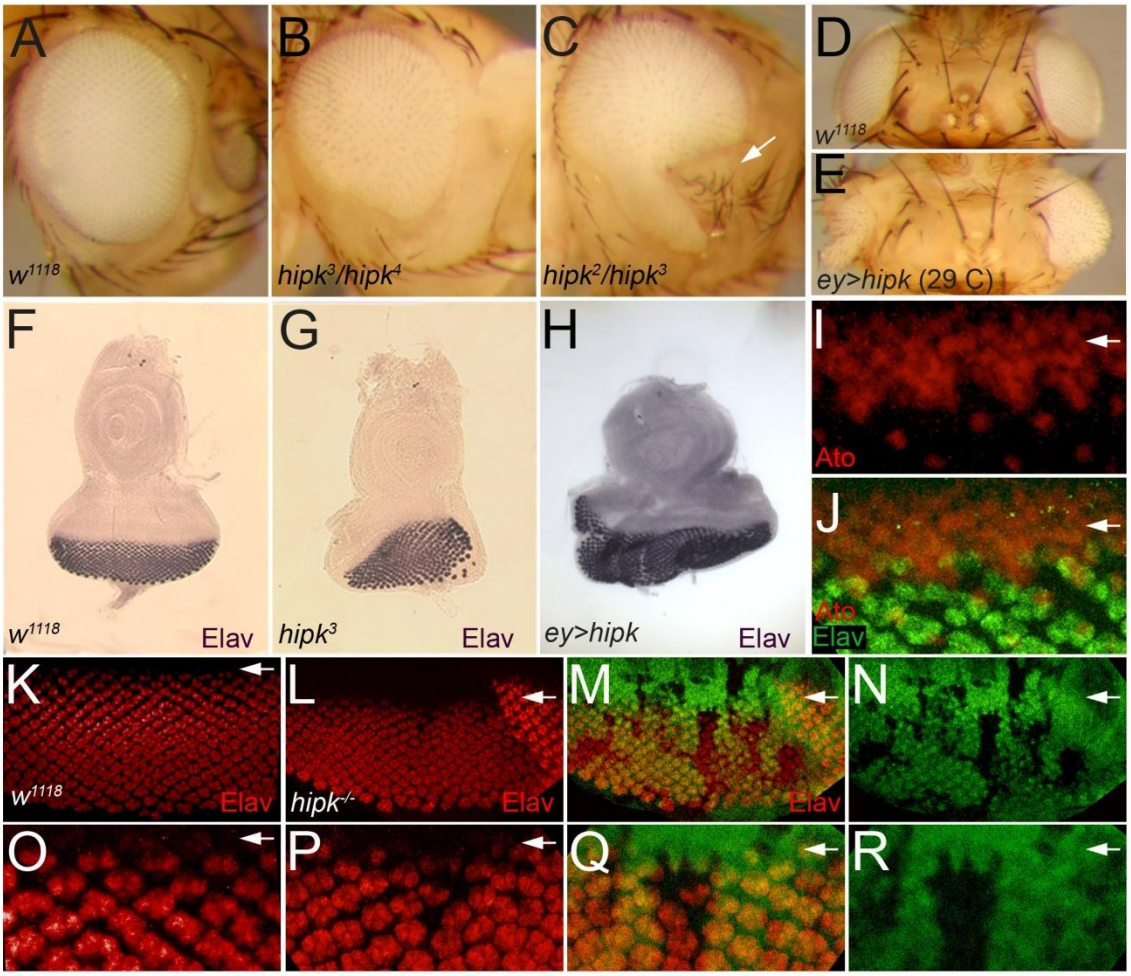


Figure 2-4 *hipk* is required for eye development

Adult eyes from (A) w^{1118} and (B) $hipk^{3}/hipk^{4}$. (C) $hipk^{2}/hipk^{3}$ eyes occasionally contain non-retinal tissue (arrow). (D-E) Dorsal view of adult head. (D) w^{1118} (E) *ey>hipk* flies develop outgrowths in the adult eye. (F-H) 3rd instar eye discs stained for the neuronal marker Elav to identify photoreceptors of the developing eye. (F) w^{1118} (G) $hipk^{3}$ homozygote (H) *ey>hipk* discs show overgrowths. Immunohistochemical staining with Atonal (Ato) antibody of w^{1118}eye discs (I, J) marks the proneural region anterior to the MF and the refinement to single Ato-positive founder cells in Elav-labeled photoreceptors (J). (K, O) w^{1118} (L-N, P-R) $hipk^{4}$ somatic clones marked by the absence of GFP (green in M, N, Q, R). Discs were stained for Elav (K-M, O-Q). (L-M) Elav expression is reduced in $hipk^{4}$ clones located near the MF and photoreceptors in posterior clones are irregularly spaced. O-R are 3x zoom images of K-N. *ey>hipk* were raised at 29°C. Adult eyes are oriented anterior to the right, dorsal side upwards. Imaginal discs are oriented ventral towards the right, anterior side upwards. In all figures, small horizontal arrow in eye disc indicates the approximate location of the MF.

not attributed to a defect in eye specification, as Ey expression is not diminished in hipk4 somatic clones (Fig. A-1).

We next determined if the loss of photoreceptors observed in *hipk* clones could be a secondary effect of altered cell cycle regulation or cell death during retinal specification. No apparent changes were observed in discs stained to visualize cell proliferation, using anti-phospho histone 3 antibody (Fig. 2-5A-D), or levels of apoptosis, as visualized by staining for the activated Drosophila ICE caspase (drICE; Fig. 2-5E-H; Fraser et al., 1997). Hence it appears that loss of photoreceptors in *hipk* mutant cells may be linked to a modification in early eye development, rather than altered cell death.

Consistent with our loss of function analyses that implicate a role for *hipk* in eye patterning, we found that ectopic expression of *UAS-hipk* also affected eye development. Using *ey-Gal4* (at 29˚C) to drive wild type Hipk expression throughout larval eye development caused abnormal rough eyes, of which 33% also displayed cuticle-like structures (n=92, Fig. 2-4E, 2-7B; Brand and Perrimon, 1993; Hazelett et al., 1998). In these flies, we also observed a novel role for *hipk* as a regulator of organ size. 39% of *ey>hipk* flies showed overgrown eyes (Figs. 2-4E; 2-7B) that are likely caused during larval development, as we also observed overgrowths in imaginal discs (Fig. 2-4H). Thus Hipk plays a role in the patterning of the eye, although the underlying mechanism is still unknown.

2.3.3 *hipk* is expressed dynamically in the eye primordium

During eye development, *hipk* is expressed in a dynamic pattern throughout the eye disc (Fig. 2-6). Antisense RNA in situ hybridization revealed that in the late second

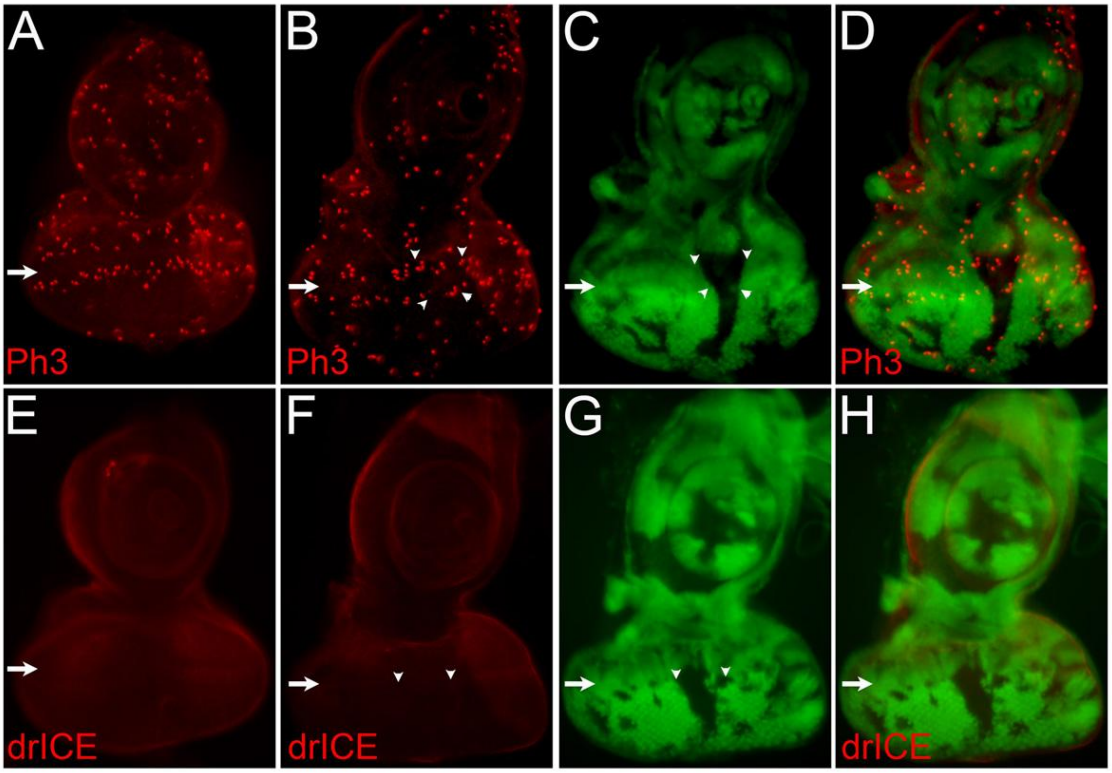


Figure 2-5 *hipk* eye phenotypes are not due to alterations in proliferation or apoptosis

(A,E) w^{1118} (B-D, F-H) $hipk^{4.}$ somatic clones, marked by absence of GFP (green) in panels C-D, G-H. Third instar eye discs stained for the mitotic marker, phospho-Histone 3 (A-D) and the apoptotic marker drICE (E-H). Loss of photoreceptors in *hipk* mutant clones are not caused by defects in proliferation or apoptosis.

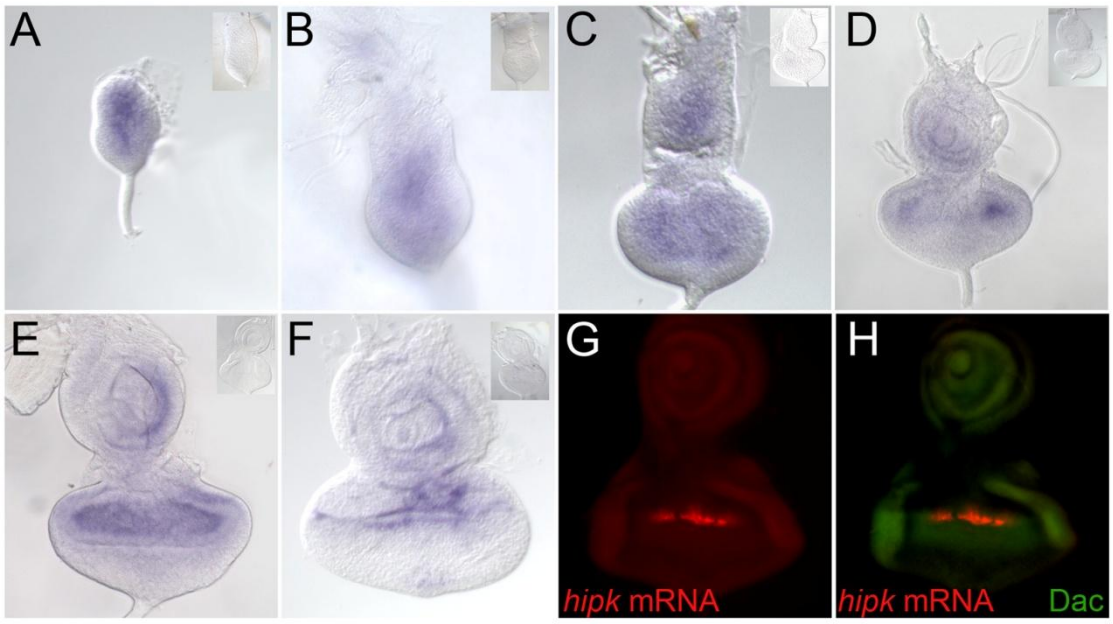


Figure 2-6 *hipk* is dynamically expressed in the developing eye

hipk RNA in situ hybridization in (A) late 2nd instar (B-D) early to mid 3rd instar and (E-F) late instar eye discs . *hipk* is uniformly expressed in the medial domain of 2nd instar eye disc and beginning in mid third instar *hipk* expression is refined to the anterior domain of the developing eye primordia. (G, H) Fluorescent in situ hybridization using an antisense *hipk* RNA probe and co-stained with anti-Dac antibody (H) in a late 3rd instar eye disc. *hipk* colocalizes with Dac anterior to the progressing MF. (A-F) Discs in small inset panels were hybridized with sense *hipk* RNA probe.

larval instar (L2) *hipk* is enriched in the medial domain of the visual primordium including the D/V boundary of the eye disc (Fig. 2-6A) and this localization persists into early third instar (Fig. 2-6B-C). Beginning in mid 3rd instar larval stage, *hipk* expression is enriched in the anterior folds of the eye discs (Fig. 2-6D) and becomes broadly expressed in the anterior region of the eye disc ahead of the MF (Fig. 2-6E). Later in late third instar, the localization varies between discs and likely reflects very dynamic changes in expression. In these discs, *hipk* is further refined to a narrow stripe covering much of the width of the disc (Fig. 2-6F). Using a combination of fluorescent in situ hybridization (FISH) and antibody staining, we observed co-localization of *hipk* and the retinal determination factor Dachshund (Dac) at the anterior-most edge of the Dac expression domain (Fig. 2-6H). This edge of Dac expression delimits the anterior boundary of the cells entering the neural program (Fig. 2-6H; Bessa et al., 2002). This dynamic pattern of expression shows that *hipk* is expressed at the D/V organizing center early and later in undifferentiated cells anterior to the MF.

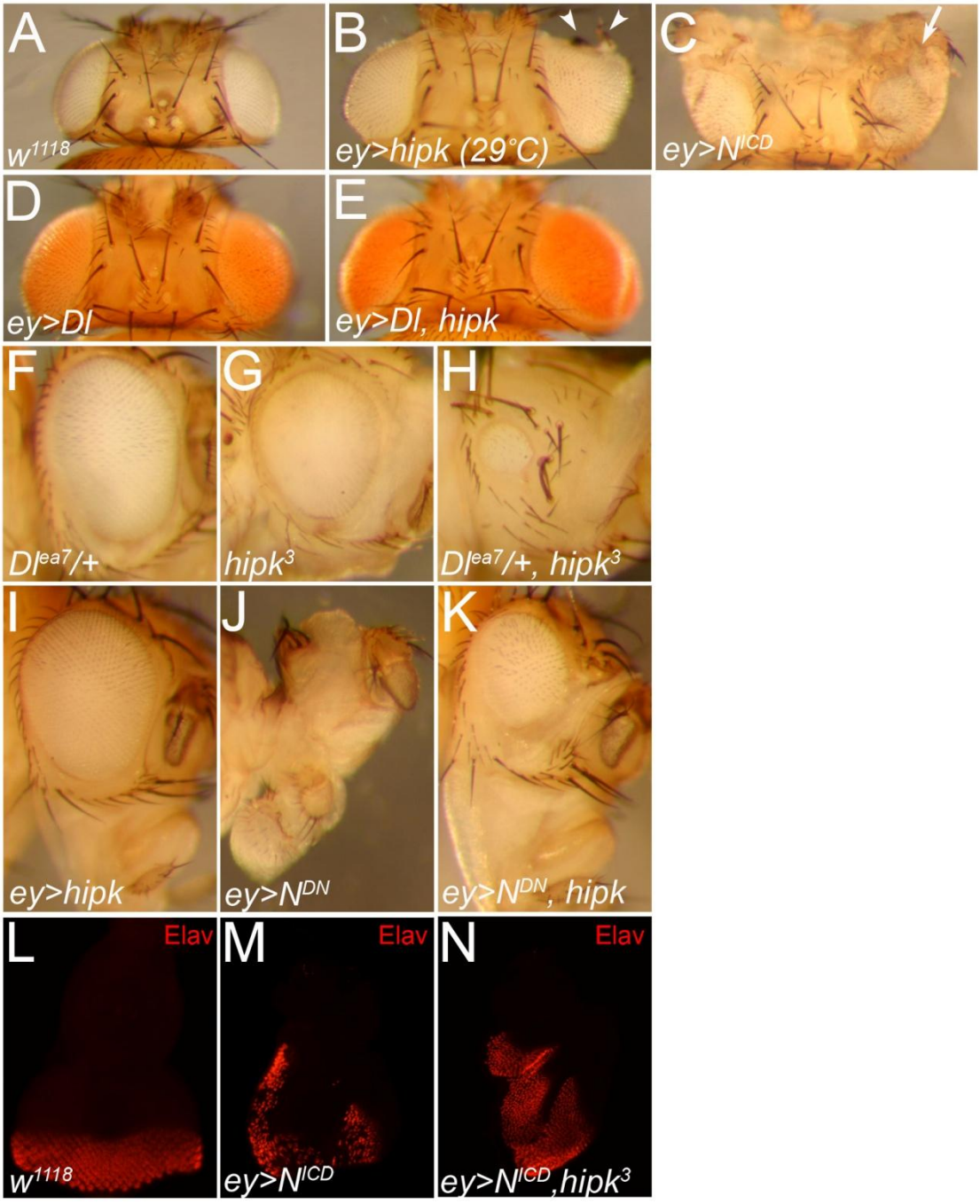
2.3.4 *hipk* interacts synergistically with components of the N pathway

N signaling controls many aspects of eye development such as proliferation and the establishment of the eye field. Loss of N signaling causes a small eye phenotype (Dominguez and de Celis, 1998; Papayannopoulos et al., 1998) and gain-of-function mutants leads to an overproliferation of the eye (Go et al., 1998; Kurata et al., 2000). In addition, the dorsal and ventral eye regions are asymmetrically regulated, as loss of the Ser regulator *Lobe* results in preferential loss of the ventral eye domain (Chern and Choi, 2002; Singh and Choi, 2003). The loss of eye tissue in *hipk* homozygous mutants (Figs. 2-4B, C) and the overgrowth defects in *ey>hipk* (Figs. 2-4E and 2-7B) resemble those

observed with modulated activity of N (Fig. 2-7C; Kurata et al., 2000) and suggest a potential role for Hipk as a mediator of N-regulated growth processes.

Genetic interaction studies were undertaken to investigate the interaction between the N pathway and *hipk*. Heterozygosity for the N ligand Dl, in Dl^{eA7}/+, enhances the small eye phenotype of $hipk^{3}$ mutants (Fig. 2-7H). 30% of these small eyes are half the normal size and more dramatically, 20% were a quarter of the normal eye size (Fig. 2-7H; n=65). In contrast, in $hipk^{3}$ homozygotes (Fig. 2-7G), only 4% of eyes were reduced to half the size and 2% were a quarter of the normal size (n=106), respectively. These phenotypes were much more severe than those observed with the $hipk^{3}$ homozygous mutation alone and suggested a potential synergy between *Dl* and *hipk*. This interaction was also observed with the $hipk^{2}$ allele. Similarly, the overproliferation defect observed in *ey>Dl* (Fig. 2-7D) was enhanced by the co-expression of Hipk (Fig. 2-7E).

Most strikingly, expression of dominant negative N with *ey-Gal4* led to a dramatic loss of the eye (Fig. 2-7J; Kumar and Moses, 2001) which was suppressed by co-expression of Hipk (Fig. 2-7K). Such a rescue of reduced N signaling strongly suggests Hipk acts to promote N signaling downstream of the receptor. Further support for this model is seen upon examining imaginal disc phenotypes. Overexpression of the constitutively active N^{ICD} with *ey-Gal4* leads to severely abnormal eye discs with dramatic overgrowths (Kurata et al., 2000) and reduced number of photoreceptors as a result of increased lateral inhibition (Fig. 2-7; Chao et al., 2004). Decreasing *hipk* in these discs restored the population of photoreceptors (Fig. 2-7N). These findings suggest *hipk* likely regulates a subset of N-mediated processes.


A
w1118
B
ey>hipk (29°C)
C
ey>NICD
D
ey>Dl
E
ey>Dl, hipk
F
Dlea7/+
G
hipk3
H
Dlea7/+, hipk3
I
ey>hipk
J
ey>NDN
K
ey>NDN, hipk
L
Elav
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ey>NICD,hipk3

Figure 2-7 *hipk* synergizes with the Notch pathway and functions downstream of N activation

(A) w^{1118}. (B) *ey-Gal4/UAS-hipk* grown at 29˚C displayed overgrowths. Occasionally, eyes develop ectopic cuticle (arrowheads in B). (C) *ey-gal4/+; UAS-*N^{ICD}/+ have hyperplasia of the eyes and head (arrow). (D) *ey-Gal4/+; UAS-*Dl^{17c}/+ causes an overproliferation phenotype. (E) *ey-Gal4, UAS-hipk/+ UAS-*Dl^{17c}/+ . Co-expression of *hipk* with *Dl* enhances the overproliferation defect. (F) Dl^{eA7}/+ eyes are wild type in size. (G) $hipk^3$ homozygote. (H) Dl^{eA7}, $hipk^3/hipk^3$. Heterozygosity for Dl^{eA7} enhances the $hipk^3$ small eye phenotype. (I) *ey-Gal4, UAS-hipk/+* grown at 25ºC. (J) *ey-Gal4/UAS-*N^{DN}. Loss of the N signal inhibits eye formation. (K) *ey-Gal4,UAS-hipk*/UAS-N^{DN}. Co-expression of *hipk* rescues *ey>*N^{DN} phenotype. (L) w^{1118}, (M) *ey-Gal4/+; UAS-*N^{ICD}/+, (N) *ey-gal4/+; UAS-*N^{ICD}, $hipk^3/hipk^3$ eye discs stained with Elav. All crosses were carried out at 25ºC unless otherwise noted.

2.3.5 N signaling activity is reduced in *hipk* loss-of-function cells

Our analyses suggested that *hipk* cooperates with the N pathway. To assess whether Hipk is required to promote the transduction of this cascade, N activity was measured in *hipk* mutant cells by examining the expression of the products of the *E(spl)* complex, direct targets of Su(H). Using an antibody that recognizes 4 of 7 products of the *E(spl)* complex (Fig. 2-8A,B; Jennings et al., 1994), we observed a decrease in E(spl) expression in mutant cells, most evident in cells located near the furrow (Fig. 2-8C-E). Therefore, *hipk* is required for the efficient transduction of the N signal and *hipk* mutant cells have reduced N signaling activity.

Intriguingly, clones located in the posterior of the eye disc display slightly elevated expression of E(spl), suggesting additional mechanisms through which *hipk* patterns the eye. These findings, and the complexity of the *hipk* phenotype, demonstrate that *hipk* plays multiple roles during eye development in addition to its role as a positive regulator of the N signal.

2.3.6 Hipk phosphorylates Gro on several sites

Hairless (H) is an antagonist of N that functions as an adaptor to bridge Gro and Su(H) to form a repressor complex (Barolo et al., 2002). This mechanism is utilized to inhibit N signaling in multiple developmental processes (Hasson et al., 2005). It was shown that Hipk phosphorylation could antagonize Gro function by promoting the disassembly of the repressor complex (Choi et al., 2005), so we investigated whether this may be the route through which Hipk promotes N activity. We hypothesized that Hipk may serve as a general antagonist of Gro and consequently promote Su(H)-mediated

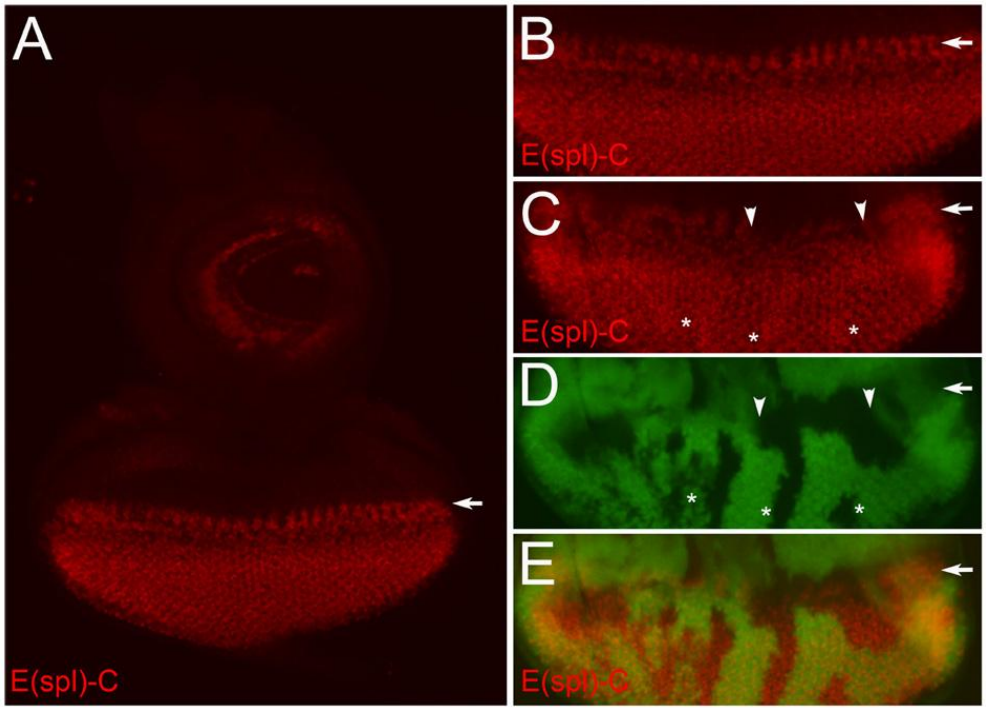

Figure 2-8 Notch signaling activity is reduced in *hipk* mutant clones

(A,B) w^{1118}. (C-E) $hipk^{4}$ somatic clones (marked by the absence of GFP, green) stained with an antibody that recognizes a subset of E(spl) proteins showed a decrease in $hipk^{4}$ clones near the MF (the clone region is flanked by white arrowheads). A mild increase in E(spl) is seen in clones located posterior to the MF (asterisks are located next to the clones of interest).

transcription by inhibiting the interactions between the repressor complex and Su(H). If such a mechanism exists, we predict that expression of a phosphomimetic form of Gro, in which Hipk phosphorylation sites are mutated to glutamic acid residues, would exert effects reminiscent of those observed by expressing wildtype Hipk.

To test this model, biochemical studies were performed to characterize the interaction between Hipk and Gro. Kinase assays were performed using purified Gro (full length and derivatives) from bacterial lysates in the presence of GST-Hipk. Hipk specifically phosphorylated the SP domain of Gro (Fig. 2-9A; data not shown). Further analyses using synthetic Gro decapeptides identified two Hipk target residues, namely S297 and T300 (Fig. 2-9B). S297 was also identified as a Hipk site by Choi et al. (2005). These sites were mutated to alanine (Gro^{AA}) to test Hipk's specificity in a kinase assay (Fig. 2-9C). While full length Gro was phosphorylated by Hipk, the Gro^{AA} variant was resistant to phosphorylation, confirming S297 and T300 as Hipk target sites. These residues are also conserved in human Hipk2, as shown in the sequence alignment in Fig. 2-9.

To generate a phospho-mimetic variant, these target residues were mutated to glutamic acid (Gro^{EE}). If Hipk can indeed repress Gro activity, then this form of Gro should be constitutively inhibited, while the Gro^{AA} variant should display constitutive activity. To test the properties of these Gro variants in vivo, transgenic fly strains expressing gro^{AA} and gro^{EE}, under control of the UAS promoter were generated. Expression of Gro^{AA} with *ey-Gal4* (Fig. 2-10I) produced a similar loss of eye phenotype to that seen in $ey{>}gro^{WT}$ flies (Fig. 2-10A). Such phenotypic similarities suggested that Gro^{AA} is functionally equivalent to wild type Gro. $ey{>}gro^{EE}$ flies (Fig. 2-10E) displayed

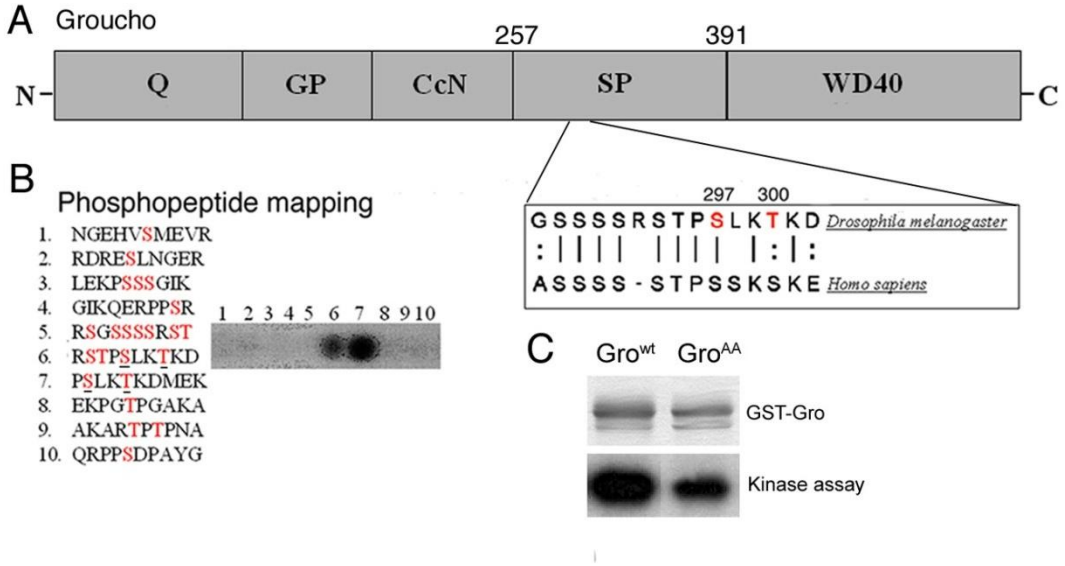

Figure 2-9 Hipk targets Groucho for phosphorylation

(A) Schematic diagram of Gro's protein domains. Gro is comprised of a N-terminal glutamine rich Q region, glycine/proline rich GP region, CcN domain containing CK2 and CDC2 phosphorylation sites and a nuclear localization signal, serine/proline rich SP region and a C-terminal WD40 domain containing 40 amino acid motifs terminating in tryptophan and aspartic acid that are sites for protein-protein interaction. Phosphorylation by Hipk was found to map to the serine and proline rich SP domain. (B) Synthetic Gro decapeptides were subjected to kinase assays and the peptide containing S297 and T300 was specifically phosphorylated. (C) In vitro kinase assays were performed with wildtype full length Gro and Gro^{AA} in which S297 and T300 were mutated to alanines.

a much less severe phenotype upon misexpression than gro^{WT}, suggesting the activity of Gro^{EE} is compromised.

Misexpression of Hipk can suppress the loss of eye phenotype caused by $ey>gro^{WT}$ (Fig. 2-10A, B; Choi et al., 2005). We addressed if this rescue occurs by inhibiting Gro's repressive activity. In contrast to the suppression of gro^{WT}, phenotypes induced by both gro^{AA} and gro^{EE} were less sensitive to elevated levels of Hipk (Fig. 2-10F, J). Co-expression of gro^{AA} or gro^{EE} with *hipk* showed a phenotype most similar to the Gro derivatives alone, indicating that these forms are not sensitive to the regulation by Hipk compared with the sensitivity seen with Gro^{WT}.

2.3.7 Transmission of N signaling relies on Hipk-mediated phosphorylation of Gro

To investigate whether Hipk can promote N activity via its regulation of Gro, a series of genetic interaction assays were carried out involving gro^{WT}, gro^{EE} and gro^{AA} in conjunction with the N antagonist H. Both inhibition of *N* (Fig. 2-7J) or ectopic gro^{WT} (Fig. 2-10A) led to loss of eye structures. Co-expression of Hipk can rescue the effect of dominant negative N (Fig. 2-7K). Our model predicts that if the rescue of N signaling by Hipk is mediated through direct inhibition of Gro through phosphorylation, then we should observe a similar rescue with the phospho-mimetic form Gro^{EE}. However, we expect that the Hipk-resistant form Gro^{AA} will phenocopy the effects of Gro^{WT}. Decreasing Notch activity via expression of the antagonist *H* caused a complete loss of eye (Fig. 2-11B), similar to that caused by expression of N^{DN} (Fig. 2-7J). Expression of *hipk* at 25°C only induced a mild rough eye (Fig. 2-11C). Co-expression of *hipk* and *H* partially restored the development of retinal tissue as observed by the presence of a small

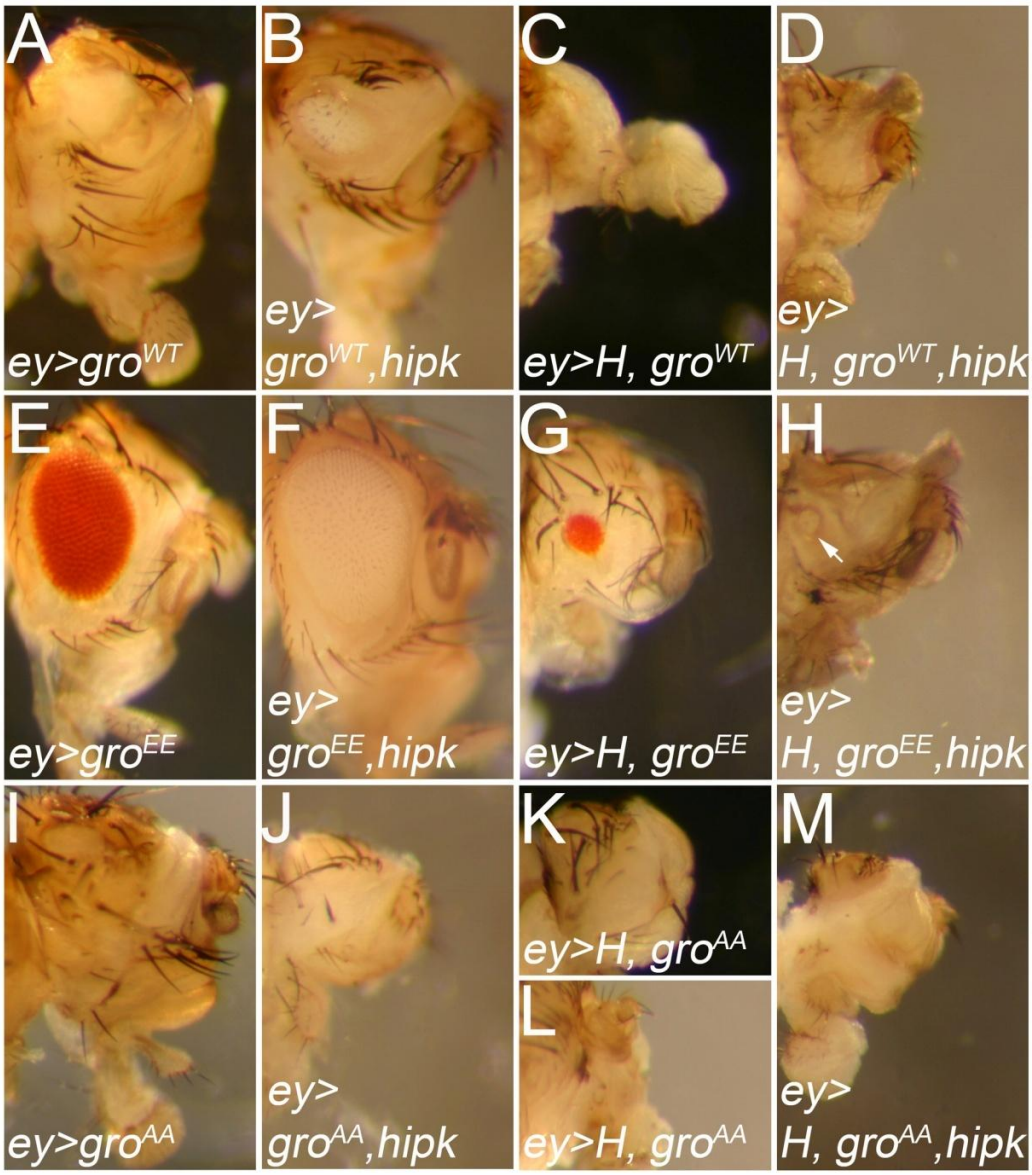

Figure 2-10 Phosphorylation of Groucho by Hipk promotes the Notch signal

The effects of various transgenes on eye and head development were assessed. In all cases, *ey-Gal4* was used to drive combinations of *Hairless* (*H*), *hipk*, *gro* and *ey* under UAS control, as indicated for each panel. Three Gro variants were expressed: wildtype (wt), gro^{AA} that is resistant to phosphorylation by Hipk, and gro^{EE}, which mimics phosphorylation of Hipk target sites. Overexpression of gro^{wt} (A) or *H* (see Fig. 2-11B) inhibits eye development. Coexpression of gro^{EE} (G), but not gro^{AA} (K, L) can antagonize the effects of H. Elevated levels of *hipk* can alleviate the repressive activity of gro^{WT} (B, D), but not that of the *gro* mutant derivatives (F, H, J and M).

eye (Fig. 2-11D). Furthermore, morphological defects in the head, including ocellar defects in the dorsal head, were also rescued, suggesting Hipk may also regulate other N-dependent processes (data not shown).

We next determined whether the mutated Gro forms could suppress the *ey>H* phenotype by mimicking the regulation seen with co-expression of Hipk. Consistent with our prediction, misexpressing gro^{EE} with *ey-Gal4* was capable of restoring eye structures in the *ey>H* background (Fig. 2-10G), phenocopying the rescue by *hipk* (Fig. 2-11D). Conversely, co-expression of gro^{AA} led to a dramatic enhancement of the *ey>H* phenotype (Fig. 2-10K, L). In these flies (n=49), the eye fails to develop and head defects are magnified as indicated by the presence of only the dorsal vertex of the head in 41%, or more severely, the entire head is lost in 29% of the flies. As expected, similar interactions were also observed with gro^{WT} (Fig. 2-10C). Taken together, these results support a model in which Hipk phosphorylates and inhibits Gro to facilitate the N pathway.

To further confirm that these effects on transduction of the Notch signal were indeed a consequence of decreasing Gro's repressive activity, we asked if the addition of Hipk could modify the interactions between the Gro derivatives and H. We predict that introducing Hipk would modify the effects of Gro^{WT} on H, but not those induced by the Gro derivatives, since these mutations would render Gro less sensitive to elevated levels of Hipk. Our results suggest that these mutations bypass the regulation of Gro by Hipk, since we observe that co-expression of *hipk, H* and gro^{WT} (Fig. 2-10D) led to a slight rescue of the very abnormal head structures seen with *ey>H,* Gro^{WT}, and suppressed the enhancement of the *ey>H* phenotype incurred by introducing *gro* (Fig. 2-10C). This

indicates that Hipk can inhibit Gro activity, thereby quenching its antagonistic effect on the N signal. However, coexpression of *H* and *hipk* in the presence of gro^{EE} or gro^{AA} did not detectably modify the phenotype seen with the combination of H and the Gro derivatives alone (Fig. 2-10H, M). These observations support the model that Hipk contributes to the propagation of the N signal by inhibiting Gro's repressive activity through phosphorylation of S297 and T300.

2.3.8 Hipk promotes eye growth

Our data support a model in which Hipk can promote N signaling during eye development through its repression of Gro. A previous report demonstrated that Hipk could promote the in vitro transcriptional activation activity of Eyeless by inhibiting Gro. In vivo data showed that Hipk could modify Gro activity and the resulting eye phenotypes were attributed to changes in Eyeless activity (Choi et al., 2005). Promotion of Ey activity would reflect a role solely in eye specification. The phenotypic consequences of modifying Hipk activity and *hipk's* dynamic expression profile both clearly suggest additional requirements for Hipk other than eye specification. To further clarify the mechanism underlying our genetic observations, we examined whether the same phenotypic rescue of *ey>H* seen with Hipk could be seen with the misexpression of Ey. If the only function of Hipk were to promote Ey activity, then we would expect a similar rescue with elevated levels of Ey. However, we found that ectopic *ey* failed to rescue the *ey>H* phenotype, and moreover, greatly enhanced it (Fig. 2-11G). Furthermore, we also found that concomitant misexpression of *hipk* mildly modified *ey>ey* phenotype (Fig. 2-11F), rather than a potent synergistic modification. These genetic interactions suggest that the ability of Hipk to rescue diminished N signaling

activity is independent of the ascribed role in promoting Ey activity (Choi et al., 2005). These findings are consistent with our genetic interaction studies and analyses of N target genes that indicate that Hipk acts to promote N signaling.

N is a recurring player in eye development, a feat that is accomplished through its unique interplay with members of the Pax6 family of transcriptional regulators. For example, N-controlled eye growth is specifically mediated via Eyg (Dominguez et al., 2004). Reducing N signaling activity induces an eye-loss phenotype, which is caused by a deregulation of organ growth through Eyg, rather than a reliance on the eye specification players Ey or Toy. Overexpression of Eyg but not Ey nor Toy reliably restored eye development in N deficient flies (Dominguez et al., 2004).

Several lines of evidence strongly suggested that Hipk promotes N-mediated eye growth. First, *ey>hipk* phenocopies the overgrowths seen with elevated levels of the N signal (Fig 2-7B-C). Second, as was seen with Eyg, simultaneous misexpression of Hipk rescues the loss of eye phenotype in a dominant negative N background (Fig. 2-7K). Furthermore, *hipk* is expressed in a region encompassing the D/V organizing growth center (Fig. 2-6A-C) where N acts to specifically control the global growth of the eye. We extended our genetic examination to confirm our model by further characterizing the interaction between Hipk and H. Specifically, we addressed whether the *ey>H* phenotype was correlated with a defect in organ growth, rather than eye specification. Consistent with such a model, overexpression of the growth regulator *eyg* (Fig. 2-11I), but not *ey* (Fig. 2-11G), restored the eye in *ey>H* adults. These suppressive effects are identical to those observed with both *hipk* and gro^{EE} transgenes (Figs. 2-11D; 2-10G).

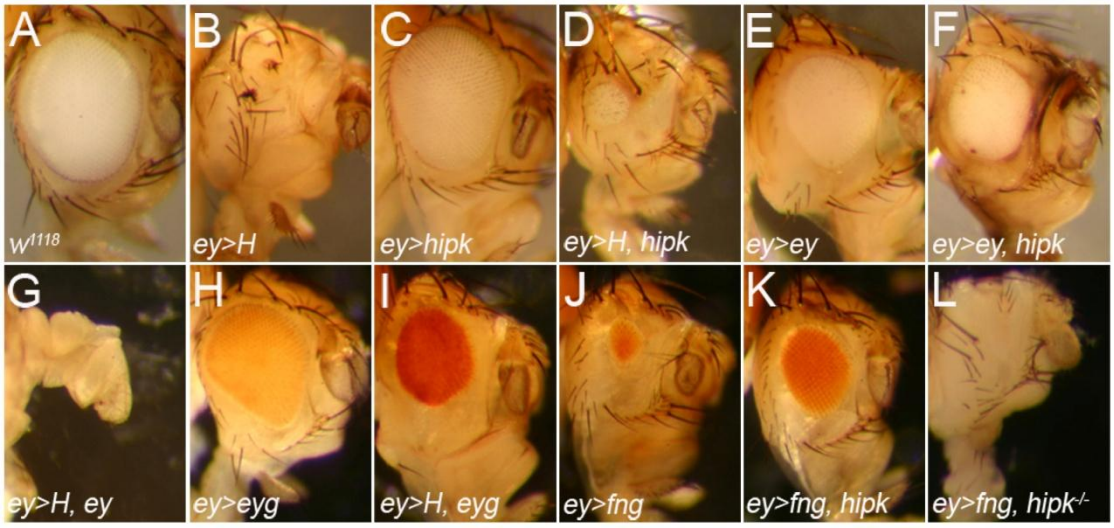

Figure 2-11 Hipk promotes eye growth

(A) w^{1118} adult eye. (B) Reducing the N signal through the misexpression of the antagonist H suppresses eye development in *ey>H* flies. Co-expression of *hipk* (D) or *eyg* (I) but not *ey* (G) rescues the loss of eye phenotype seen in *ey>H* adults. The general expression of *fng* with *ey-gal4* perturbs eye growth leading to a small eye phenotype (J), which is rescued with elevated levels of *hipk* (K). Transheterozygosity for $hipk^{3}/hipk^{4}$ enhances the effects observed in *ey>fng* pharate adults (L)

Similar to what is seen with components of the N pathway, Hipk induces pleiotropic effects throughout eye development. We sought to confirm that the genetic rescues were not attributed to a secondary effect of Hipk-mediated processes unrelated to growth. To address this, we examined the consequences of modifying Hipk levels had on the phenotype induced upon misexpression of Fringe (Fng). The small eye phenotype seen in *ey>Fng* flies (Fig. 2-11J) is attributed solely to a defect in eye growth (Dominguez et al., 2004). Thus any observed modification in this sensitized genetic background would validate a requirement for Hipk in eye growth. As predicted by our model, overexpression of *hipk* or gro^{EE} partially rescues the *ey>fng* phenotype (Fig 2-11K, data not shown). More strikingly, the eye fails to form when *hipk* activity is reduced (Fig 2-11L), a phenotype similar to what is seen with *eyg* or *Dl* mutants in an *ey>Fng* background (Dominguez and de Celis, 1998). Taken together, our genetic interactions demonstrate that Hipk promotes N-mediated eye growth.

The complementary expression domains of the N ligands Dl (Fig 2-12A) and Ser (Papayannopoulos et al., 1998) in the dorsal and ventral compartments, respectively, ensures that the N pathway is activated at the D/V boundary of the developing 2nd instar eye disc. N establishes the organizing center to mediate global growth by regulating *eyg* expression along the length of the D/V boundary (Fig 2-12A; Dominguez and de Celis, 1998). The expression of Ser (Fig 2-12C-E) and Dl (data not shown) appear normal in $hipk^{4}$ clones suggesting that the D/V center is established normally in *hipk* mutant cells. However, we observe that Eyg expression is autonomously reduced in *hipk* mutant clones (Fig. 2-12G-I). Such an effect on *eyg* expression is also observed in clones mutant for either Su(H) or Dl and Ser (Dominguez et al., 2004). Conversely, *ey>hipk* third instar

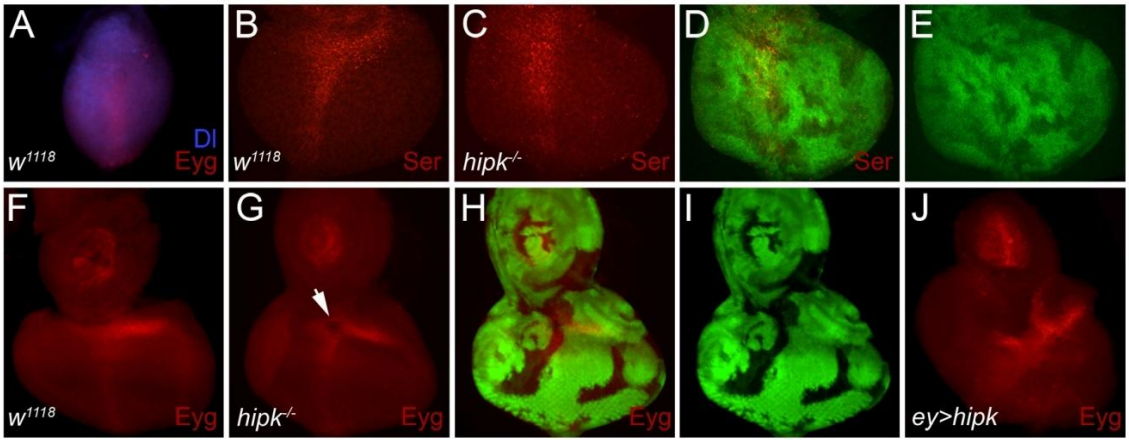

Figure 2-12 Eyg expression is reduced in *hipk* mutant clones and enhanced by ectopic Hipk

(A, B, F) w^{1118}. (C-E, G-I) $hipk^{4}$ somatic clones (marked by the absence of GFP, green). (A) Dl is enriched in the dorsal compartment of 2nd instar eye discs and partially colocalizes with Eyg at the D/V boundary. (B) Serrate (Ser) expression in w^{1118}. (C-E) In mid third instar eye discs, Ser expression is unchanged in *hipk* mutant cells. (F) Eyg expression in a w^{1118} 3rd instar eye disc (G-I) $hipk^{4}$ somatic clones (marked by the absence of GFP, green) exhibit reduced Eyg expression (arrow in G). (J) An eye disc expressing *ey>hipk* at 29°C shows overgrowths and expansion of the Eyg expression domain.

eye discs display an expanded expression domain of Eyg (Fig. 2-12J). These observations indicate that Hipk is required for activation of normal Eyg expression, and loss of *hipk* induces a growth defect.

2.4 Conclusions

The lethality of our *hipk* mutant alleles demonstrates that Hipk is an essential kinase in Drosophila that plays a critical role during eye development. Eye patterning and growth defects are observed in both *hipk* homozygous mutants and somatic clones. *hipk* mutant clones display reduced N signaling activity, as measured by the diminished expression of the N targets, E(spl) and Eyg. Therefore, our studies implicate Hipk in the positive regulation of the N signaling cascade during eye development. Although our data demonstrate that Hipk regulates N-mediated eye growth, the neural patterning defects are less severe than previously published N mutant phenotypes. We cannot exclude the possibility that these neuronal defects are due to a secondary consequence of Hipk's requirement in earlier phases of eye development or a role for Hipk in modulating additional eye patterning pathways other than the N pathway.

Our in vitro and in vivo data support a model in which Hipk phosphorylates Gro, and consequently relieves its repressive activity on the Su(H) transcriptional complex (Fig. 2-13). Overexpression of the N antagonist H results in loss of eye. We show that Hipk-mediated phosphorylation of Gro at S297 and T300 is necessary to rescue the phenotype caused by reduced N signaling. Indeed, our genetic misexpression analyses clearly demonstrate that this phosphorylation event is necessary to relieve Gro's inhibitory effect on N, thereby permitting activation of downstream N targets. Since Gro

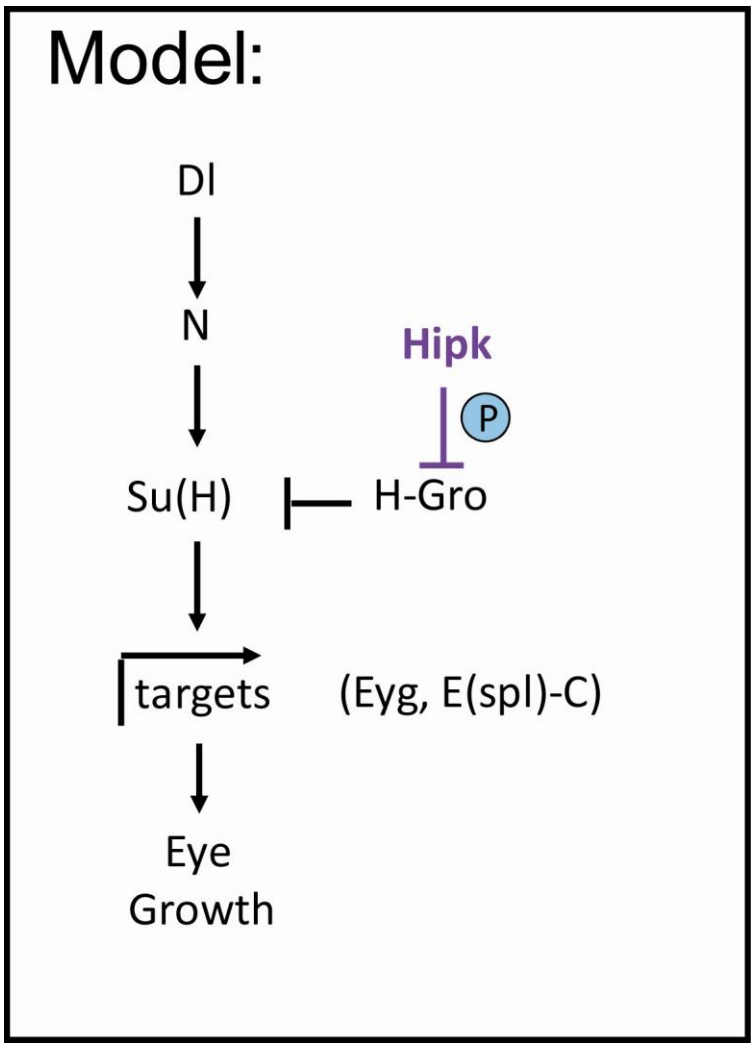

Figure 2-13 Hipk promotes Notch mediated eye growth by inhibiting Gro

Hipk interferes with the repressive activity of Gro to promote N signal transduction in the *Drosophila* eye. Hipk is required to maintain proper N signaling and to activate N targets including *E(spl)-C* and *eyg*.

is a global co-repressor, this interaction may represent a global mechanism through which Hipk can regulate gene expression during development.

Here, we have identified Hipk as a key player in modulating growth in the eye. Given that we have also observed a similar role for Hipk in promoting growth in additional tissues, it likely represents a general role for Hipk in organ and tissue growth. Although Hipk can induce outgrowths in the wing, it does so via a Notch-independent mechanism (unpublished data). Future studies will reveal to what extent Hipk can integrate multiple signaling inputs or regulate transcriptional complexes.

2.5 Contributions

As the first author, I perfomed most of the experiments and I wrote the article with E.M.V. I contributed most of the data with the exception of mapping the Gro phosphorylation sites (Fig. 2-9) and the generation of the *gro* mutant transgenes, which were performed by our collaborators, M.F and U.W. B. C. A. generated the *hipk*^{4} allele.

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CHAPTER 3
HIPKS PROMOTE WNT/WG SIGNALING THROUGH STABILIZATION OF BETA-CATENIN/ARM AND STIMULATION OF TARGET GENE EXPRESSION

Wendy Lee, Sharan Swarup, Joanna Chen, Tohru Ishitani and Esther M. Verheyen

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3.1 Summary

The Wnt/Wingless (Wg) pathway represents a conserved signaling cascade involved in diverse biological processes. Misregulation of Wnt/Wg signal transduction has profound effects on development. Homeodomain interacting protein kinases (Hipks) represent a novel family of serine/threonine kinases. Members of this group (in particular Hipk2) are implicated as important factors in transcriptional regulation to control cell growth, apoptosis, and development. Here, we provide genetic and phenotypic evidence that the sole Drosophila member of this family, Hipk, functions as a positive regulator in the Wg pathway. Expression of *hipk* in the wing rescues loss of the Wg signal, while loss of *hipk* can enhance decreased *wg* signaling phenotypes. Furthermore, loss of *hipk* leads to diminished Arm protein levels while overexpression of *hipk* promotes the Wg signal by stabilizing Arm, resulting in activation of Wg responsive targets. In Wg transcriptional assays, Hipk enhanced Tcf/Arm-mediated gene expression in a kinase-dependent manner. In addition, Hipk can bind to Arm and dTcf, and phosphorylate Arm. Using both in vitro and in vivo assays, Hipk was found to promote the stabilization of

Arm. We observe similar molecular interactions between Lef1/β-catenin and vertebrate Hipk2, suggesting a direct and conserved role for Hipk proteins in promoting Wnt signaling.

3.2 Introduction

The evolutionarily conserved Wnt/Wingless (Wg) signaling pathway is involved in diverse biological processes, including determination, proliferation, migration and differentiation during embryonic development and adult homeostasis (reviewed in Clevers, 2006). Inappropriate activation of Wnt-dependent gene expression in mammals can lead to numerous cancers, and loss of Wnt pathway activity also profoundly affects development. The ability of this pathway to control different developmental events in a temporally and spatially specific manner requires coordination between numerous regulators. Canonical Wnt signaling controls cell fate by regulating transcription of target genes (Cadigan and Nusse, 1997). A representation of both the Wg and Wnt canonical pathways are shown in Fig. 3-1. Wingless (Wg), a secreted glycoprotein, is the best characterized of the seven Drosophila Wnt ligands, and initiates the canonical pathway by binding to the Frizzled2 (Fz2) and LRP5/6/Arrow co-receptors (Bhanot et al., 1996). This leads to the activation of Dishevelled, which then inhibits the activity of the destruction complex composed of Axin, glycogen synthase kinase 3β (GSK-3β)/Zw3 and adenomatous polyposis coli (APC) (Ikeda et al., 1998; Kishida et al., 1998; Polakis, 1997). As a result, cytosolic Drosophila β-catenin called Armadillo (Arm) accumulates and enters the nucleus to interact with a Tcf/Lef (dTcf) family transcription factor to promote target gene expression (van de Wetering et al., 1997). In the absence of Wg signaling, the Axin/GSK-3β/APC complex promotes the proteolytic degradation of Arm

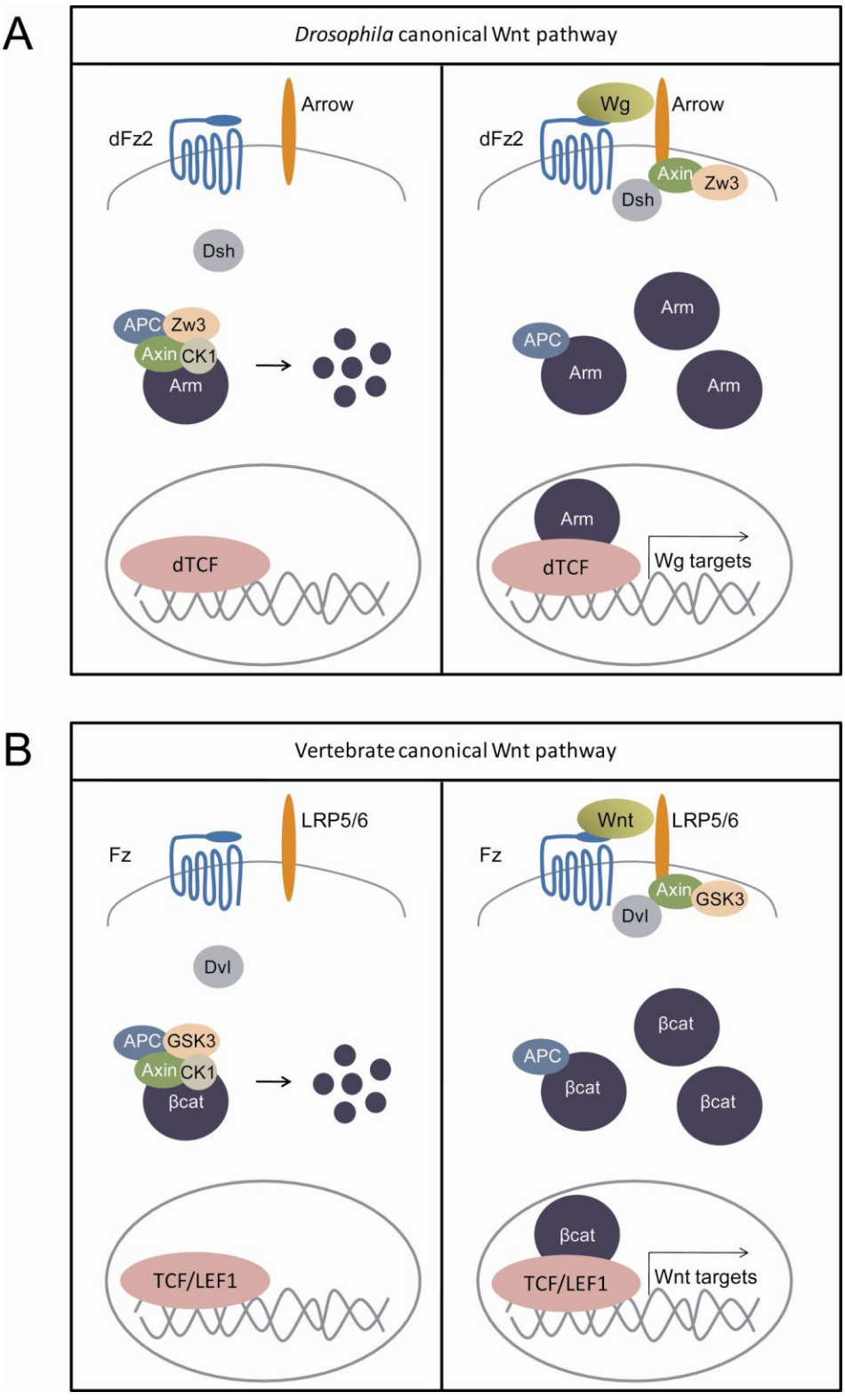
A
Drosophila canonical Wnt pathway
dFz2
Arrow
Dsh
APC
Zw3
Axin
CK1
Arm
dTCF
Wg
Arrow
dFz2
Axin
Zw3
Dsh
Arm
APC
Arm
Arm
Arm
dTCF
Wg targets
B
Vertebrate canonical Wnt pathway
Fz
LRP5/6
Dvl
APC
GSK3
Axin
CK1
βcat
TCF/LEF1
Wnt
LRP5/6
Fz
Axin
GSK3
Dvl
βcat
APC
βcat
βcat
βcat
TCF/LEF1
Wnt targets

Figure 3-1 Schematic representation of the Wg/Wnt canonical pathway

(A) In the absence of the *Drosophila* Wg signal (left panel), Arm is targeted for degradation by sequential phosphorylation events by CK1 and Gsk3 in conjunction with the destruction machinery, composed of Axin and APC. Pathway activation (right panel) occurs when the Wg ligand binds to the dFz2/Arrow receptor complex, leading to the activation of Dishevelled (Dsh), inhibition of the destruction complex and the stabilization of Arm. Arm is then free to enter the nucleus and form a transcriptional complex with TCF to activate Wg responsive targets.

(B) A representation of the inactive (left panel) and active (right panel) canonical Wnt signaling in vertebrate. Activation of the Wnt cascade leads to the recruitment of GSK3 and Axin to the cell membrane, likely inhibiting their function in β-catenin (β-cat) degradation.

(Aberle et al., 1997; Willert et al., 1999; Yost et al., 1996), while transcriptional corepressors bind to Tcf and repress transcription (Cavallo et al., 1998; Roose et al., 1998).

The Nemo-like kinase family (Nlk) of protein kinases can regulate activation of Tcf/Lef target genes (Ishitani et al., 1999; Rocheleau et al., 1999; Zeng and Verheyen, 2004). In Drosophila, Nemo (Nmo) inhibits dTcf activity, and is itself a transcriptional target of the Wg pathway (Zeng and Verheyen, 2004). Recently Homeodomain-interacting protein kinase 2 (Hipk2) was proposed to participate in a kinase cascade to activate Nlk during the regulation of the c-Myb transcription factor (Kanei-Ishii et al., 2004). We thus sought to identify whether this regulation was perhaps more general and whether Drosophila Hipk played a role in regulating Nmo, and thus also Wg signaling. We rapidly learned that Hipk exerts a positive effect on Wg signaling, distinct from Nmo, which we have more fully characterized using the developing wing as a model system.

The patterning of the adult wing blade is a tightly regulated process involving numerous essential signaling pathways, including Hh, Wg, Notch, EGFR, and TGFβ, making it an excellent tissue in which to examine regulatory and epistatic relationships between many genes involved in patterning. The adult wing blade possesses five longitudinal veins (LI-LV) that extend proximally to distally. These are connected by the anterior cross vein (ACV) and posterior crossvein (PCV). The Wg pathway acts at several stages of wing patterning and growth (reviewed in Martinez Arias, 2003). Wg is expressed along the dorsal/ventral boundary, which in imaginal discs is a stripe bisecting the wing imaginal disc, and in adult wings gives rise to the wing margin and bristles that

surround the edge of the wing blade. Loss of *wg* can lead to loss of the entire wing, to wing to notum transformations, to wing notching, or loss of bristles along the entire wing margin (Phillips and Whittle, 1993; Couso et al., 1994). Wg also promotes proliferation in the wing disc and ectopic Wg can induce outgrowths from the ventral surface of the wing (Phillips and Whittle, 1993; Diaz-Benjumea and Cohen, 1995).

In this study, we present an analysis of the function of Hipk in Drosophila canonical Wg signaling. Genetic studies show that ectopic *hipk* can rescue phenotypes due to loss of function *wg* alleles or inhibition of the pathway with a dominant negative Fz2 receptor. Immuno-histochemical studies show that *hipk* positively regulates expression of Wg targets, and that Hipk can act to stabilize cellular levels of the Arm protein in wing discs. Wnt reporter assays show that both Drosophila Hipk and mouse Hipk2 can promote the Wnt-responsive Topflash reporter. In addition, we have found that Hipk/Hipk2 can promote the stabilization of Arm/β-catenin in cell culture and in vivo. Our results suggest that Hipk is a positive regulator of the Wg pathway that refines Wg activity during wing development. Our findings suggest that these roles may be conserved across species.

3.3 Results

3.3.1 Hipk plays a role in wing patterning and other developmental processes

As a starting point in studying Hipk, we generated and analyzed a series of loss-of-function mutations (Lee et al., 2009). Loss of zygotic *hipk* resulted in pupal or larval lethality, a finding recently also described by Link et al. (2007). Whole mount in situ hybridization reveals that *hipk* is expressed broadly in a non-uniform pattern in multiple

stages of development including all imaginal discs (data not shown). Removal of the maternal contribution caused embryonic lethality characterized by twisted embryos and head holes, showing that *hipk* is an essential gene for Drosophila development (W.L., unpublished, Fig. A-2). In our analyses we focussed on the most severe allele, $hipk^4$, which causes early larval lethality, or pupal lethality in trans to $hipk^3$ (Lee et al., 2009). Given the embryonic lethality caused by loss of maternal Hipk, we speculate that maternally contributed Hipk perdures and obscures its requirement at later stages and impacts the severity of mutant phenotypes. We utilized the FLP/FRT technique to generate mutant somatic clones to examine the requirements for Hipk in patterning adult structures (Xu and Rubin, 1993).

In this study we focused on the role of *hipk* in the development of the wing. Clones of cells mutant for $hipk^4$ show ectopic veins in the anterior region of the wing blade along LII, loss of the PCV, and occasional notches in the wing margin (Fig. 3-2B). Reducing *hipk* function by expression of 2 independent Gal4-responsive *hipk*-RNAi construct in the wing pouch with *sd-gal4* (Fig. 3-2C) or *vg-Gal4* (data not shown) also caused a wing notching phenotype reminiscent of those seen upon decreased Wg signaling (Phillips and Whittle, 1993; Couso et al., 1994; Rulifson et al., 1996).

We next studied the effects of ectopic expression of *hipk* using the Gal4-UAS system (Brand and Perrimon, 1993). We observed phenotypes that suggested that *hipk* is involved in promoting Wg signaling. Expression of one copy of *hipk* in the central domain of the imaginal wing discs with *omb-Gal4* (Fig. 3-2C) induced the formation of an additional wing margin and outgrowths emanating from the distal most tip of the ventral surface of the wing (Fig. 3-2D,E). Expression of two copies of *UAS-hipk*

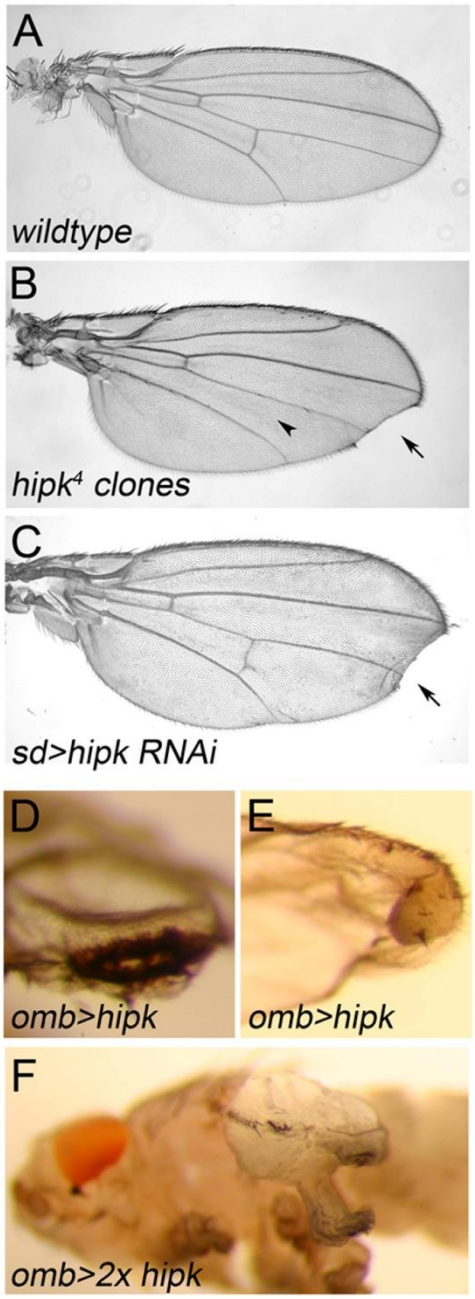

Figure 3-2 Modulation of *hipk* affects wing development

(A) Wildtype wing. (B, C) Reducing *hipk* function by generating somatic $hipk^4$ clones (B) or through expression of *UAS-hipk-RNAi* in the wing with *sd-gal4* (C) both led to the loss of the wing margin (arrow). (D-E) Overexpression of *hipk* in the wing blade with the *omb-gal4* driver caused the formation of an additional wing margin (D) and outgrowths from the ventral side of the wing (E). (F) Misexpressing 2 copies of *hipk* led to the formation of additional wing tissue outgrowths.

enhanced this phenotype and caused the outgrowths to extend further distally from the ventral plane of the wing (Fig. 3-2F). These effects phenocopied the effects observed in ectopic *wg*-expressing clones (Diaz-Benjumea and Cohen, 1995). Similarly, expression of *UAS-hipk* in the wing (Fig. 3-3B) phenocopies the ectopic venation pattern seen both upon ectopic expression of activated Arm (Arm^{S10}) with *bs-Gal4* (Fig. 3-3C) and in nmo^{DB24}/nmo^{adk2} mutants (Fig. 3-2D). Although control of wing vein patterning is not generally attributed to Wg signaling, we and others have observed ectopic venation upon elevated Wg signaling. For example, ectopic expression of constitutively active Arm by *en-Gal4* in the posterior region of the wing, or ubiquitously with *69B-Gal4* or *MS1096-Gal4*, leads to disturbed and ectopic venation (Greaves et al., 1999; Lawrence et al., 2000). Moreover, loss of function clones of *sgg/zw3* (encoding the fly homolog of GSK3β, a component of the destruction complex) induce the formation of ectopic veins (Ripoll et al., 1988). The results of these phenotypic analyses of *hipk* are surprising because they demonstrated that Nmo and Hipk did not act in concert to inhibit Wg signaling. Rather, *hipk* mutant and gain of function phenotypes suggest a role in promoting the Wg pathway.

3.3.2 Hipk can rescue inhibition of Wg signaling in the wing

To explore whether Hipk is a positive regulator of the Wg pathway, as suggested by our initial analyses, genetic interaction studies were performed. During early wing development, Wg specifies the wing and disruption of signaling during the second larval instar induces a wing-to-notum transformation (Fig. 3-3E; Morata and Lawrence, 1977; Ng et al., 1996). Only 14% of wg^{1}/wg^{1-17} transheterozygotes (n=72) develop a normal pair of wings and misexpression of *hipk* in this genetic background suppressed the

formation of the ectopic notum and restored the structure of the wing (Fig. 3-3F) in 41% of flies (n=39). Expressing a dominant negative form of the Fz2 receptor *Dfz2N33* ubiquitously in the wing with *69B-Gal4* caused a severe loss of wing margin phenotype in both the anterior and posterior regions of all wings examined (Fig. 3-3G; n=41; Zhang and Carthew, 1998). This phenotype was entirely rescued with 100% penetrance (n=47) upon *hipk* co-expression (Fig. 3-3H). Altogether, these interactions indicated that *hipk* can counteract the effects of inhibiting the Wg pathway, and more importantly, that elevating the levels of Hipk can compensate for the reduced Wg signal.

Consistent with these observations, we find that removal of one dose of *hipk* can enhance phenotypes due to inhibited Wg activity. Expression of the *Dfz2N33* along the anterior-posterior boundary using *dpp-Gal4* induced a mild wing notching phenotype (Fig 3-3I, 47%, n=15). Heterozygosity for $hipk^4$ enhanced the loss of wing tissue as seen by the formation of moderate (27.8%, n=36) to severely truncated wings (Fig 3-3J, 30.6%,), which were rarely observed in *dpp>Dfz2N33* adults (6.7%, n=15). Similarly, heterozygosity for $hipk^3$ enhanced the severity of the wing notching induced by expression of Axin with *sd-Gal4* (Fig. 3-3K, L). We next determined if these interactions represented a general antagonism between Hipk and negative regulators of the pathway. Overexpression of *nmo* with *sd-Gal4* phenocopied the wing notching phenotype of *wg* loss of function mutants (Zeng, 2004) (Fig. 3-3M; 61%, n=51). *sd>nmo* wing notches were suppressed upon the co-expression of *hipk* (Fig. 3-3O; 5.6%, n=53). On the other hand, ectopic veins along LV seen in *sd>hipk* (Fig. 3-3N; 100%, n=63) were suppressed by elevated levels of *nmo* (Fig. 3-3O. 32.1%, n=53). These results suggest a mutual antagonism between Nmo and Hipk. In summary, these genetic interaction studies with

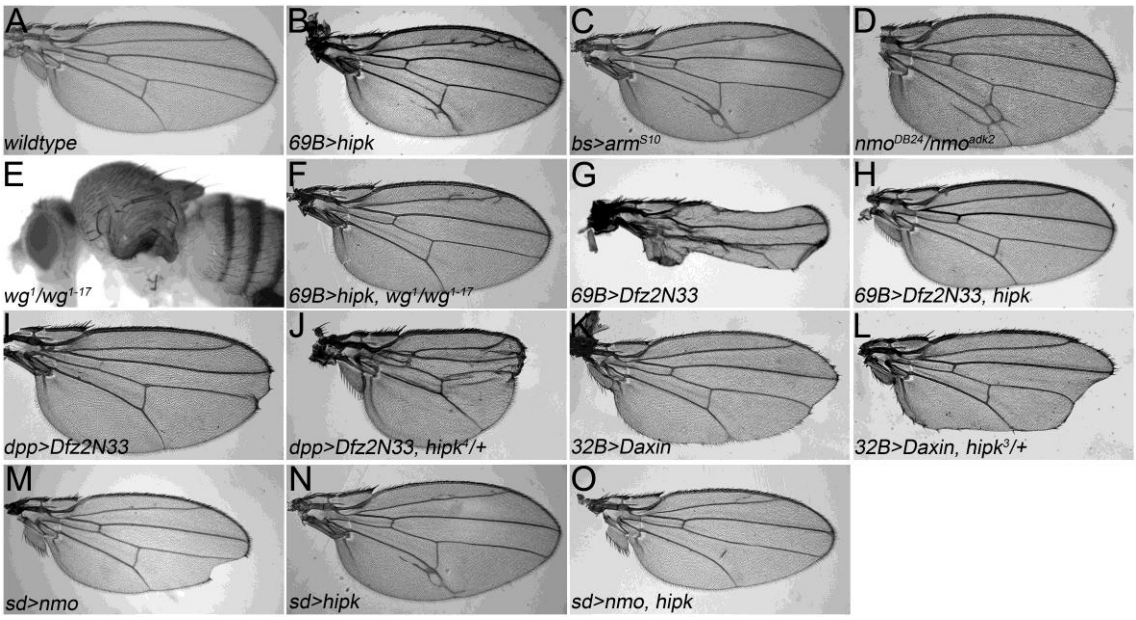

Figure 3-3 Increasing the levels of Hipk can compensate for the loss of the Wg signaling

(A) Wildtype wing. (B) *UAS-hipk/69B-Gal4*. Misexpression of *hipk* produced a mild ectopic vein phenotype similar to what is seen with *UAS-arm*^{S10}*/+; bs-gal4/+*(C) or *nmo*^{DB24}*/nmo*^{adk}(D). (E) The hypomorphic *wg* allelic combination, *wg*^{1}*/wg*$^{1-17}$, caused a wing-to-notum transformation, which was rescued by coexpression of *hipk* (F). (G) *UAS-DFz2N33/+; 69B-Gal4/+* causes a severely notched wings. (H) *UAS-DFz2N33/+; 69B/UAS-hipk*. Simultaneously misexpressing *hipk* rescued the loss of wing tissue caused by ectopic expression of DFz3N33. (I) *UAS-DFz2N33/+; dpp-Gal4/+*. (J) *UAS-DFz2N33/+; dpp-Gal4/hipk*^{4}. (K) *sd>DAxin* causes mild notches and loss of posterior margin bristles which are enhanced by loss of one copy of *hipk* in *32B>Daxin, hipk*^{3}*/+* (L). Decreasing the Wg signal by overexpressing the Wg antagonist *nmo* leads to a wing notching phenotype (M), which is suppressed by coexpression of *hipk* in *sd>nmo, hipk* (O).

either a loss or a gain of *hipk* demonstrates that Hipk plays a positive role in transmission of the Wg signal.

3.3.3 Hipk can promote Wg target gene expression

Our genetic observations suggested that Hipk promoted the Wg pathway. To assess whether modulation of *hipk* levels could affect Wg signaling activity, we examined the expression of the Wg targets, *distalless* (Dll), *achaete* (Ac) and *senseless* (Sens) in both *hipk* RNAi discs and in discs ectopically expressing Hipk. Expression of a *hipk* RNAi construct (*sd>hipk RNAi*) led to reductions in Dll (Fig. 3-4B), Sens (Fig. 3-4E) and Ac (Fig. 3-4H) in the wing pouch. Conversely, ectopic Hipk (*omb>hipk*) enhanced and expanded the expression domains of Dll (Fig. 3-4C), Sens (Fig. 3-4F) and Ac (Fig. 3-4I) in the wing pouch.

3.3.4 Hipk can promote Arm stabilization

In the absence of Wg pathway activation, the destruction complex targets Arm for degradation. Upon Wg signaling, Arm is stabilized and accumulates in the cytoplasm, serving as a measure of Wg activity. In wildtype wing discs, cytoplasmic Arm is stabilized in cells adjacent to the D/V boundary in which Wg is active (Peifer et al., 1994; Fig. 3-5A). We determined the status of stabilized Arm in *hipk* mutant clones. We found that Arm is reduced in *hipk* clones at the D/V boundary (arrows in Fig. 3-5B-D). These mutant cells showed a reduction of cytoplasmic Arm (Fig. 3-5B''-3-5D''), while the adherens junction pool of Arm appeared normal. A similar decrease in Arm is seen in discs expressing *hipk RNAi* (Fig. 3-5E).

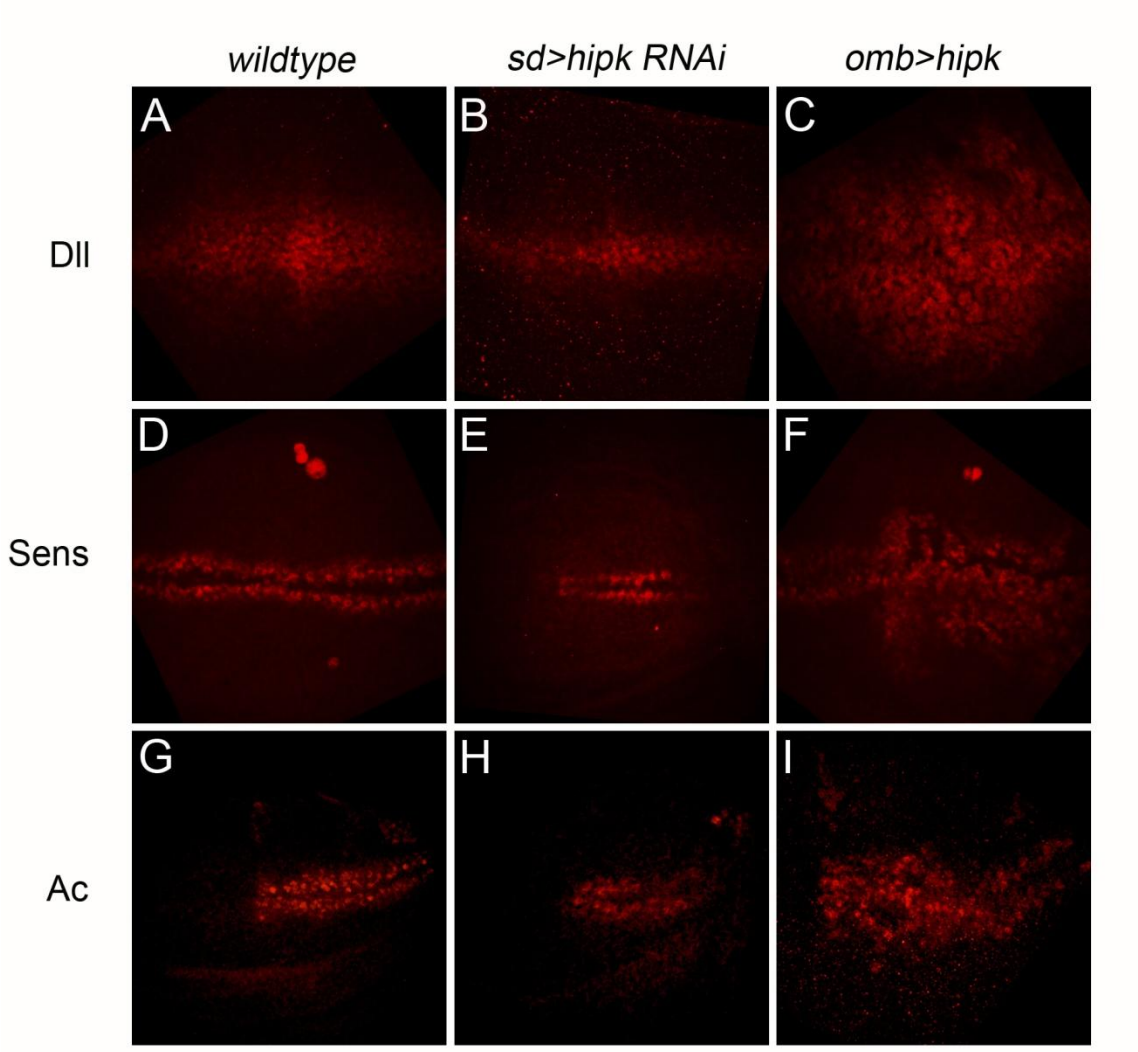

Figure 3-4 Hipk promotes Wg target gene expression

Antibody staining for Wg targets was performed in w^{1118}, *sd>hipk RNAi* and *omb>hipk* discs. (A-C) Dll protein. (D-E) Sens protein. (G-I) Ac protein

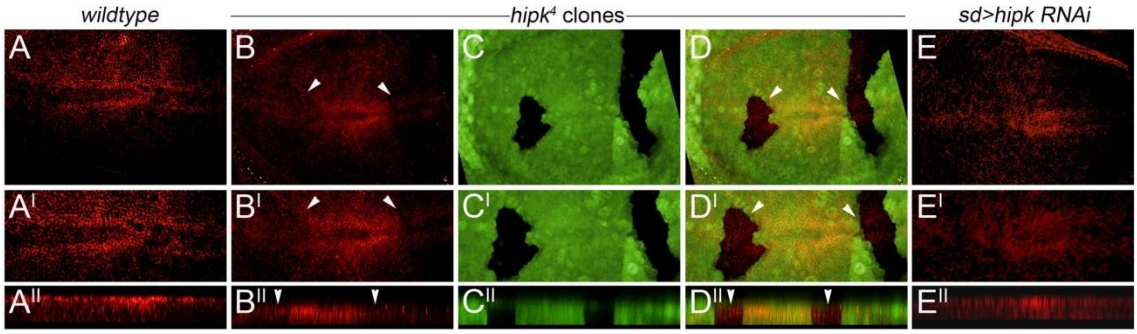

Figure 3-5 Reduction of *hipk* results in loss of stabilized Arm protein

The expression of stabilized Arm was examined in wing discs bearing *hipk*^{4} mutant clones. (A) Wildtype third instar wing disc. A' is a magnification of A, and A'' is a Z-section through the disc. (B-D) *hipk*^{4} somatic clones were marked by the absence of GFP (green in C, D). Arm protein levels (red in B, C) were reduced in *hipk* somatic clones (arrowheads in B, D). B' -E' show higher magnification views of discs, and B''-E'' show Z-sections to reveal the subcellular localization of Arm. In *hipk* mutant cells, Arm levels were normal in the adherens junctions (arrowheads in B''). (E) Expressing *sd>hipk RNAi* reduces overall Arm levels.

We then assessed whether Hipk could promote Arm stabilization by ectopically expressing Hipk using *omb-gal4*. Endogenous Arm protein levels are expanded in *omb>hipk* wing discs (Fig. 3-6B), compared to wildtype (Fig. 3-6A). Consistent with these observations, Western blot analysis of *omb>hipk* imaginal discs revealed higher levels of Arm than lysates obtained from wildtype larvae (Fig 3-10A). These results suggested that elevated levels of Hipk can promote more Arm stabilization even in regions of the wing disc receiving lower levels of Wg signaling and that the stabilized Arm is active for Wg signaling, as it can induce target gene expression.

3.3.5 Hipk inhibits the degradation of Arm

To further address the role of Hipk in Arm stabilization, the consequences of reducing *hipk* were assessed on the stability of a series of *UAS-Arm* constructs expressed throughout the wing pouch in $hipk^{3}/hipk^{4}$ mutant wing discs. The Arm constructs, $UAS\text{-}Arm^{S2}$ and $UAS\text{-}Arm^{S10}$ (Pai et al., 1997), have been used to dissect the regulatory mechanisms of Arm localization and stability in the embryo (Tolwinski and Wieschaus, 2001) and wing (Bajpai et al., 2004). $UAS\text{-}Myc\text{-}Arm^{S2}$ (encoding Myc-tagged full length Arm) was expressed in a broad domain in the central portion of the wing pouch and in the ventral periphery using *omb-gal4* (Fig.3-6C). Wing discs stained with anti-Myc antibody showed stabilization of Myc-Arm along the D/V boundary and the dorsal hinge primordia, similar to what was seen with endogenous Arm (Fig. 3-6D). In *hipk* mutant discs expressing $omb>myc\text{-}Arm^{s2}$, we observed a failure to accumulate Arm protein (arrow in Fig. 3-6E), suggesting that in these discs Arm protein is not efficiently stabilized due to the reduction of Hipk.

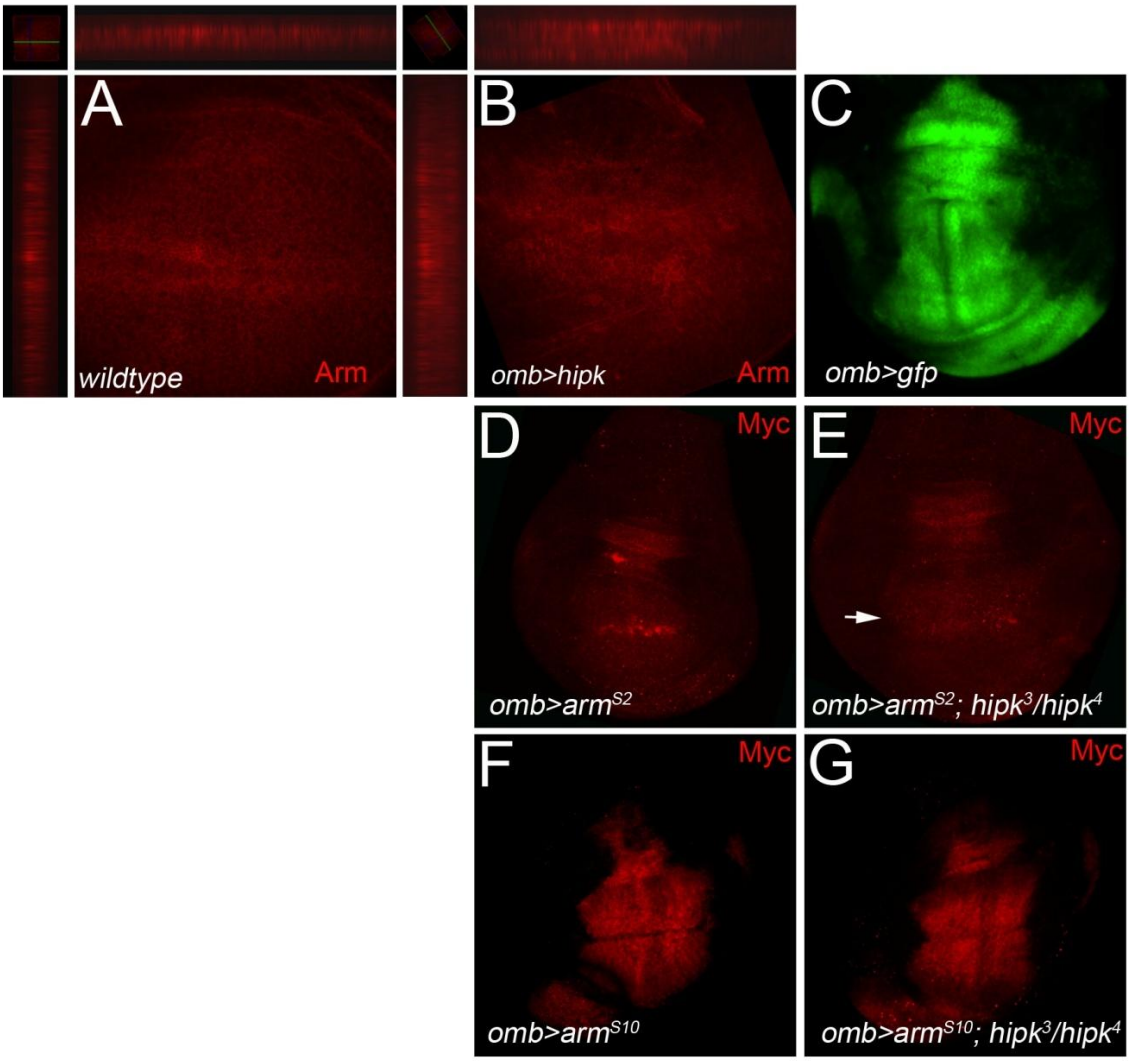

Figure 3-6 Hipk is required to stabilize Arm in vivo

The expression of Arm protein was examined in third instar wing discs. (A) wild type. Small panels beside and above show Z-sections through the disc. (B) omb-Gal4/+; UAS-hipk/+ discs show expansion of the Arm domain. (C) omb-gal4/+; UAS-GFP/+ staining shows that omb-gal4 expression domain. (D) omb-gal4/+; UAS-Myc-ArmS2 wing discs were stained with anti-Myc antibody to monitor the stabilization of exogenous wild type Arm. Myc-ArmS2 is stabilized in response to high Wg activity. (E) This effect was suppressed in hipk mutant discs, omb-gal4/+; UAS-Myc-ArmS2, hipk3/hipk4 . (F) omb-gal4/+; UAS-Myc-ArmS10/+. Expression of a degradation resistant form of Arm was visualized with an anti-Myc antibody. Myc-ArmS10 was stabilized throughout the omb-Gal4 expression domain. (G) omb-gal4/+; UAS-Myc-ArmS10/+; hipk3/hipk4. Reducing hipk activity did not affect the stabilization of constitutively active Arm.

Expression of a *UAS-Myc-Arm*^{S10} construct lacking the amino terminal target sites of the destruction complex induced a *wg* gain-of-function phenotype characterized by ectopic bristles within the wing blade (Couso et al., 1994; data not shown). *omb>UAS-Arm*^{S10} discs show stabilized Myc-tagged Arm throughout the *omb* expression domain (Fig. 3-6F). The accumulation of the degradation-resistant form of Arm is unchanged in discs lacking *hipk* (Fig. 3-6G). These data show that Hipk promotes the stabilization of wild type Arm, while degradation resistant Arm bypasses the need for Hipk-promoted stabilization.

3.3.6 Hipk promotes Wg signaling through a Gro independent mechanism

The Notch (N) signal functions upstream of the Wg pathway to induce *wg* expression at the D/V boundary of the developing wing discs (Neuman and Cohen, 1996). We have found that during *Drosophila* eye development, Hipk regulates Gro to promote N mediated growth (Lee at al, 2008), thus we sought to examine whether Hipk promotes Wg indirectly as a consequence of its interaction with N. To explore this possibility, we examined the expression of several N targets, such as *wg*, Cut, the *m8* member of the Enhancer of split complex (*E(sp)l-C)*, and the N ligand, Delta (Dl), which is activated in a positive feedback loop during wing vein development (Huppert et al., 1997). We found no changes in the expression of *E(spl)m8*, Dl, nor *wg* at the D/V boundary of the wing disc (Fig. A-3). Hipk may regulate a subset of N targets as Cut expression is reduced in *hipk* somatic clones (Fig. A-3M-D). Although, *wg* expression is reduced in *hipk* clones located in the outer ring of the wing pouch (Fig. A-3J-L), Hipk does not regulate *wg* expression at the D/V boundary to promote Arm stability. Taken

together these findings strongly suggest that Hipk's effect on the Wg pathway is not mediated indirectly through Notch.

In the absence of the Wg signal, Tcf interacts with the global corepressor, Gro, forming a transcriptional repressor complex to inhibit the activation of Wg responsive genes (Cavallo et al., 1998). It has previously been shown that Gro is antagonized by Hipk-mediated phosphorylation (Choi et al., 2005, Lee et al., 2008). We therefore sought to determine if Hipk contributes positively to the Wg signal by downregulating Gro and consequently, inhibiting the repressor function of Tcf. Overexpression of Gro with *vg-Gal4* inhibits wing formation, as observed by the formation of a small wing. We found that the *vg>Gro* phenotype was not suppressed upon coexpression of *hipk*, which would be expected if *hipk* antagonizes *Gro* during wing development. These effects are not specific to *vg-Gal4* and similar findings were observed with various *Gal4* drivers, including *69B-Gal4, sd-Gal4* and *omb-Gal4*. These results suggest that Hipk does not downregulate Gro activity during wing development.

To further discern whether Hipk may promote the Wg signal by inhibiting the activity of the Tcf/Gro repressor complex, a series of genetic interaction studies were performed with a dominant negative form of TCF, *UAS-dTcf*Δ*N*, which contains an amino terminus deletion, preventing the protein from binding to Arm, but retaining the ability to bind to DNA (van de Wetering et al., 1997). Expression of this dominant negative form of Tcf in the wing elicited the repressive effect of Tcf and mimics the wing to notum phenotype of *sd>Daxin* adults. Coexpression of *hipk* failed to re-establish wing development, and this was not specific to *sd-Gal4*, as similar results were also obtained with *vg-Gal4* (not shown). Taken together, these results suggest that elevated levels of

Hipk cannot overcome the repressor function of the Gro/Tcf complex, therefore Hipk likely promotes the Wg pathway via a Gro independent mechanism.

3.3.7 Hipk forms a complex with Tcf and Arm

Since Hipk could modulate Arm stability and expression of Wg target genes, we examined if Hipk interacted with the core components of the transcriptional complex. In HEK293T cells transfected with HA-tagged Hipk and Myc-tagged dTcf, Hipk was co-immunoprecipitated in a complex with dTcf (Fig 3-7A). Myc-Hipk and HA-tagged Arm also formed a complex in HEK293T whole cell lysates (Fig 3-7B). These complexes were also seen in HeLa cell lysates transfected with mouse Flag-tagged Hipk2 and β-catenin or Human T7-tagged Lef1 (Fig 3-7C).

We next investigated whether β-catenin was required for the interaction between Lef1 and Hipk2. Hipk2 binds a Lef1ΔN mutant, lacking the β-catenin binding domain, suggesting that β-catenin is not required for the interaction between Lef1 and Hipk2 (Fig 3-7C, lane 4). Furthermore, Hipk coimmunoprecipitated with Lef1ΔC, a construct lacking the conserved DNA binding HMG box, suggesting this region was also dispensable for the Lef1/Hipk2 interaction (Fig 3-7C, lane 6). While the β-catenin and Hipk2 interaction does not require Lef1, we observe that complex formation is enhanced in the presence of Lef1 (Fig 3-7C, lanes 8,9).

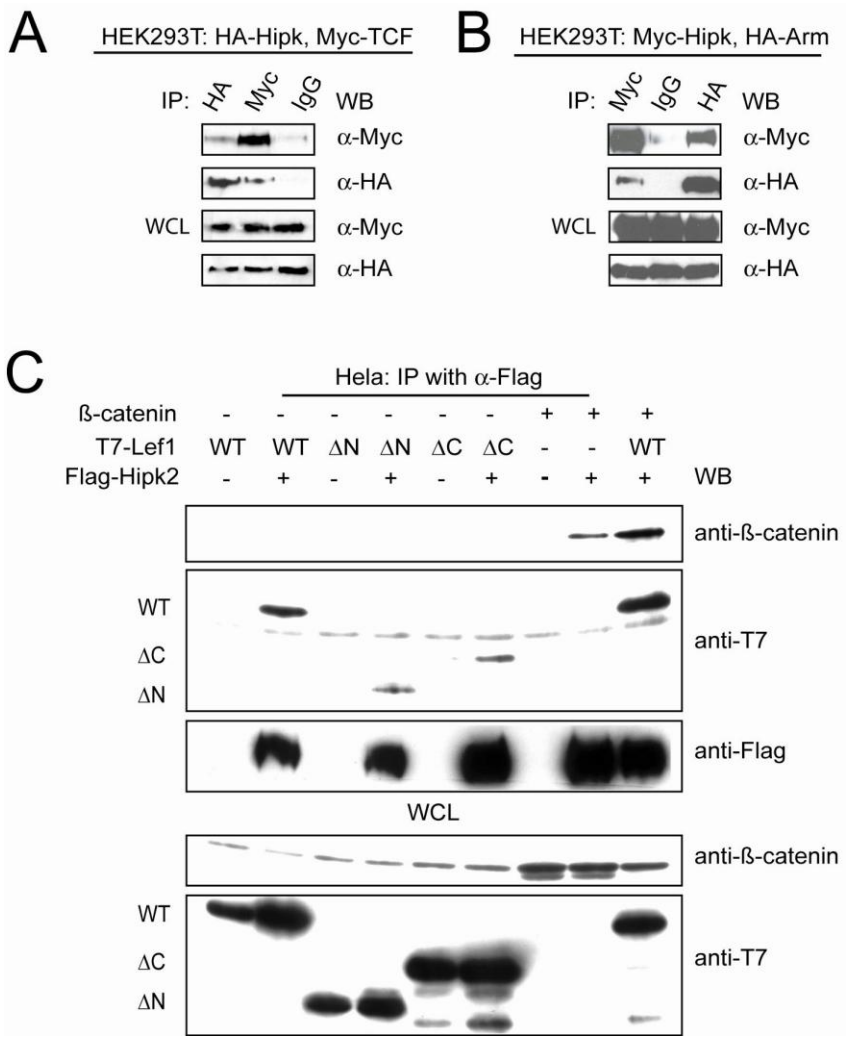

Figure 3-7 Hipk proteins can bind to Tcf/Lef1 and Arm/β-catenin

(A) HEK293T cells were cotransfected with HA-Hipk and Myc-Tcf. Cell lysates were immunoprecipitated (IP) with anti-HA, anti-Myc or IgG (control) antibodies and extracts were visualized by Western blotting (WB) using anti-HA or anti-Myc antibodies, for Hipk and Tcf, respectively. (B) Myc-Hipk and HA-Arm plasmids were cotransfected into HEK293T cells. Lysates were incubated with anti-HA, anti-Myc or IgG (control) antibodies and immunoprecipitates were detected through WB with anti-HA or anti Myc, for Arm and Hipk, respectively. (C) Mammalian Hipk2 interacts with both β-catenin and Lef1. Flag-Hipk2 and T7-Lef1 or β-catenin were cotransfected into HeLa cells. Cell lysates were immunoprecipitated with indicated antibodies and protein complexes were visualized by immunoblotting with Flag, T7 and β-catenin. Hipk2 bound to T7-Lef1ΔN and T7-Lef1ΔC, deletion mutants lacking the β-catenin and HMG binding domains, respectively.

3.3.8 Hipk phosphorylates Arm

In vitro kinase assays were performed to determine if Hipk phosphorylates components of the transcriptional complex. We found that Hipk phosphorylates Arm, but not dTcf (Fig. 3-8A, Lane 1; data not shown). The kinase activity was critical for this event, as a kinase dead Hipk protein (Hipk KD) was unable to phosphorylate Arm (Fig. 3-8A, lane 2). We next tested the ability of Hipk to phosphorylate Arm truncations (Fig. 3-8A, lanes 4, 11; Fig. 3-8B). We find that Hipk can phosphorylate a number of Arm truncations, namely Arm-N (encoding only the N-terminus, Fig 3-8A, lane 4), Arm ΔN (missing the N-terminus, data not shown) and Arm ΔC (missing most of the C-terminus, Fig 3-8A, lane 11) but not Arm-R (composed of just the central repeat region, data not shown) suggesting that phosphorylation sites map to both the N and C termini of Arm. Hipk proteins are known to phosphorylate numerous targets on their substrates, and further detailed analyses will reveal the functional significance of each of these phosphorylation events.

3.3.9 Hipk enhances Arm/Tcf-mediated transcription

We examined if Hipk affected expression of the Tcf-responsive Topflash transcriptional reporter (Korinek et al., 1997). Transfection of HEK293T cells with Hipk enhanced the transcriptional activity induced by dTcf/Arm nearly 10 fold compared to transfection with dTcf and Arm alone (Fig. 3-9A, lanes 2,3). This effect was not observed upon transfection with Hipk KD (Fig. 3-9A, lane 4), suggesting that the kinase

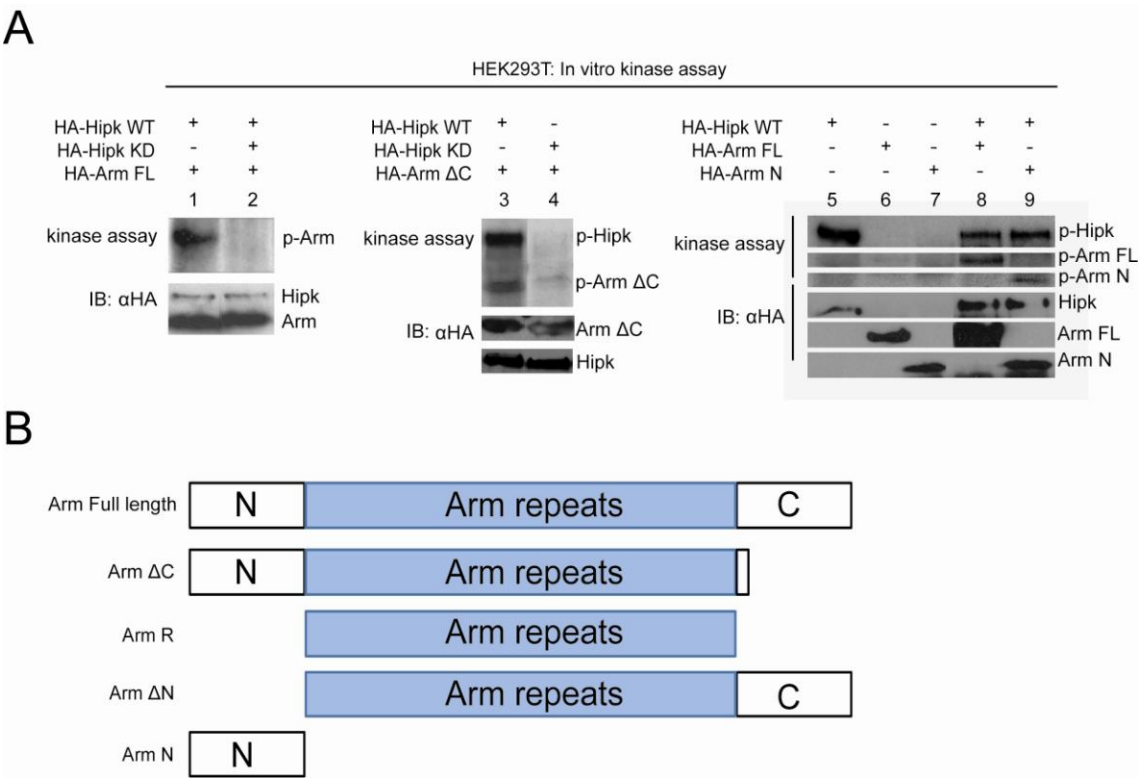

Figure 3-8 Hipk phosphorylates Arm.

(A) HEK293T cell lysates expressing the indicated constructs were immunoprecipitated with appropriate antibodies and the purified proteins were subjected to in vitro kinase assays. Arm is phosphorylated in the presence of Hipk WT (lane 1), but not with Hipk KD (lane 2). Relative levels of protein used in the kinase assay were visualized by immunoblotting (IB) with indicated antibodies. (Lanes 3-11) Indicated truncations of Arm were subjected to kinase assays and loading controls are indicated. (B) Schematic of the Arm truncations used in the study.

activity of Hipk is required for this effect. We also assessed the combined effect of Nmo and Hipk on Topflash. As expected, expression of the Tcf antagonist Nmo suppressed Topflash activation (Fig. 3-9A, lane 5). This inhibitory effect was partially relieved when HEK293T cells were co-transfected with Hipk and Nmo (Fig. 3-9A, lane 6).

Next we examined the ability of Hipk to promote Topflash through interactions with the endogenous proteins (Fig. 3-9C) in Drosophila S2R+ cells that express the Fz2 receptor, which can be activated with Wg-conditioned media. Transfection of Hipk resulted in activation of Topflash in the absence of Wg-conditioned medium (Fig. 3-9C, lanes 1, 2). Upon addition of Wg-conditioned media, cells transfected with Hipk showed a robust induction of Topflash (Fig. 3-9C, lanes 4, 5).

3.3.10 Hipk and Hipk2 are functionally conserved

While Hipk enhances Topflash in HEK293T cells and genetically promotes Wg signaling, transfection of Hipk2 led to an inhibition of Topflash in HEK293T cells (data not shown; Wei et al., 2007). This effect of Hipk2 may be attributed to additional mammalian Hipk homologues or to the cellular context. To investigate this, we performed similar assays with Hipk2 in HeLa cells and Drosophila S2R+ cells. Transfection of Hipk2 in HeLa cells led to an enhancement of Topflash compared to control cells that were solely treated with Wnt-3a (Fig. 3-9B, lanes 5-6) or Wnt-1 conditioned media (Fig. 3-9B, lanes 9-10). Hipk2 KD did not cause an increase in the transcriptional response, indicating this effect is kinase dependent (Fig. 3-9B, lanes 7,11). We also found that Hipk2 enhanced the Wnt response in a kinase dependent manner in SW480 colon carcinoma cells, a cell line in which β-cat is constitutively stabilized.

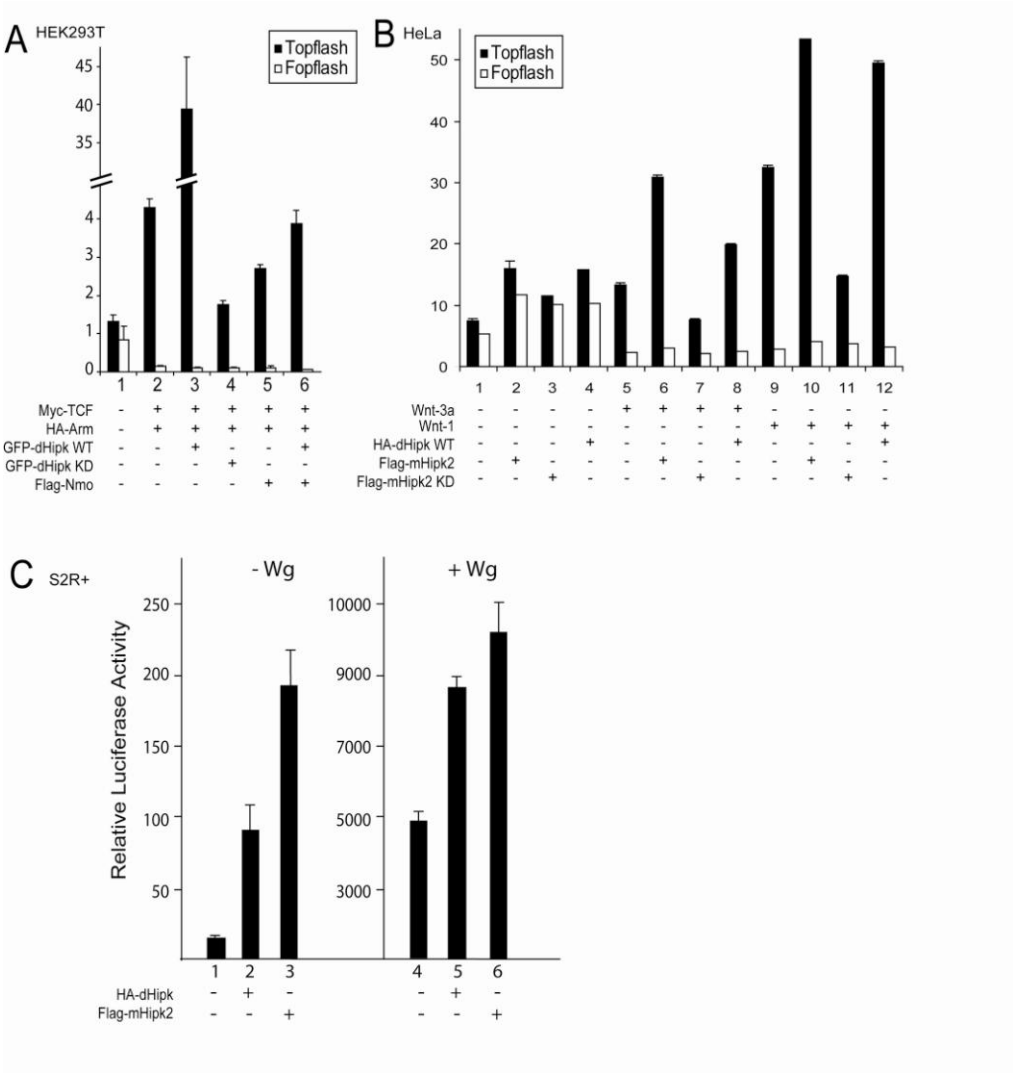

Figure 3-9 Hipk/Hipk2 enhance Wg/Wnt responsive transcription in vitro

(A) Topflash assays in HEK293T cells showed promotion of dTcf/Arm-dependent transcription by Hipk in a kinase-dependent manner. Topflash values are indicated on the left in black columns. These values were from the average of three independent transfection experiments. Vectors used for each experiment are as indicated in the figure. The negative control Fopflash values are given on the right in white columns. (B) Hipk2 promotes Lef1-mediated transcription in a kinase-dependent manner. Hipk can also stimulate Topflash in Hela cells. Transcriptional assays were performed with vertebrate homologues in HeLa cells. Indicated values represent the average of two independent transfection experiments. Results are labeled according to those described in (A). (C) Topflash assays were performed in Drosophila S2R+ cells in the absence (lanes 1-3) or presence of Wg-conditioned media (lanes 4-6). Both Hipk (lanes 2, 5) and Hipk2 (lanes 3, 6) enhanced Topflash under both conditions.

Strikingly, addition of fly Hipk to mammalian HeLa cells also enhanced the transcriptional response (Fig 3-9B, lane 8 and 12). Consistent with the HeLa cell data, Hipk2 was able to induce Topflash in the presence or absence of Wg-conditioned media in Drosophila S2R+ cells (Fig. 3-9C, lane 3 and 6). These experiments revealed that Hipk2 and Hipk perform conserved functions in multiple cellular contexts.

3.3.11 Hipk promotes Arm stability in vivo and in vitro

Our data suggested that Hipk is required to stabilize both endogenous and overexpressed full length Arm. We confirmed this by western blotting of protein extracts from *omb>hipk* wing discs and observed dramatically elevated levels of Arm protein (Fig. 3-10A), consistent with the elevated levels of Arm seen in discs in Fig. 3-6B. Arm protein levels are also increased in S2R+ cells (Fig. 3-10B) and HeLa cells (Fig. 3-10C) transfected with Hipk2.

Further cell culture assays were performed to confirm this role. Degradation of Arm was assessed in HEK293T cells expressing HA-Arm that were treated with the protein synthesis inhibitor, cycloheximide (Chx; Abou Elela and Nazar, 1997). In these cells, addition of Hipk prolonged the stability of Arm (Fig. 3-10D). Similar results were obtained in Hela cells (data not shown). The presence of HA-Hipk KD partially promoted the accumulation of Arm levels, but not to the extent seen with Hipk WT.

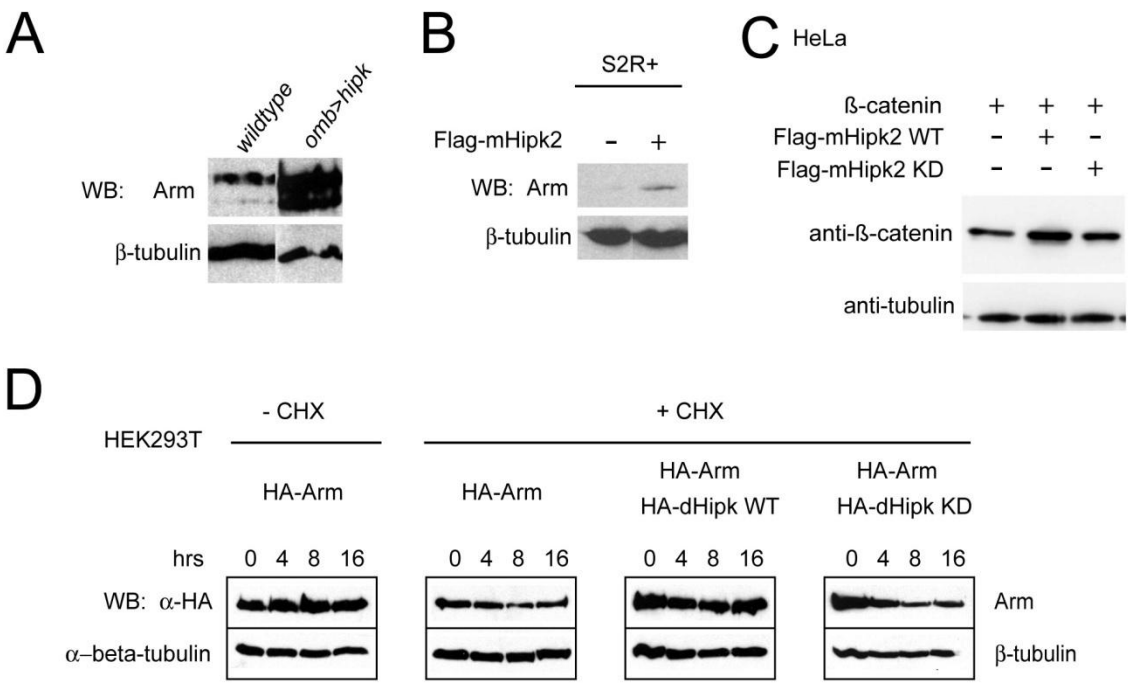

Figure 3-10 Hipk enhances the stability of Arm

(A) Cell lysates from discs expressing *omb>hipk* showed elevated Arm protein as compared to wildtype. (B) Lysates from S2R+ cells transfected with Hipk2 show elevated Arm, compared to control. (C) Protein lysates from Hela cells transfected with Hipk2 and β-catenin show elevated levels of β-catenin compared to control and after transfection with mHipk2 KD. (D) HEK293T cells expressing the indicated constructs were treated with the protein translational inhibitor cycloheximide (Chx). Whole cell lysates were collected over several time points after treatment and analyzed by Western blot. Arm levels were visualized by immunoblotting with anti-HA antibody. Coexpression of Hipk WT, and to a lesser degree Hipk KD enhanced the stability of Arm. β-tubulin was used as a loading control.

3.4 Discussion

3.4.1 Hipk promotes Wg signaling

The Wg/Wnt pathway is crucial for the initiation and maintenance of developmental programs in multicellular organisms across the animal kingdom. Alterations in signaling activity can have dire consequences on cell fate, in the most severe circumstances, the initiation and progression of tumorigenesis. In this study, we reveal that Hipk possesses an intrinsic ability to promote Wg pathway activity and this regulatory function for Hipk is conserved in both *Drosophila* and mammalian cells. Through a combination of genetic and biochemical analyses, our data reveal that Hipk proteins promote Tcf/Lef1-mediated transcription. Additionally, Hipks enhances the stabilization of Arm/β-catenin in several cell lines and *hipk* mutant clones in the wing disc have diminished Arm protein levels. Overexpression of Hipk induces a broader domain of stabilized Arm, suggesting Hipk is required to maintain the signaling pool of cytosolic Arm. We propose a model in which Hipk promotes the Wnt/Wg signal via its regulation of Arm stabilization (Fig. 3-11).

3.4.2 Hipk and Nmo exert differential effects on Wg signaling

Nlk is a conserved antagonist of the Lef1/β-catenin transcriptional complex. Our research has shown that Nmo is also an inducible antagonist of the Wg signal in the developing Drosophila wing (Zeng et al., 2004). It was previously reported that Wnt-1 induces the activation of a putative Tak1-Hipk2-Nlk kinase cascade to promote the degradation of the c-Myb transcription factor (Kanei-Ishii et al., 2004). We sought to

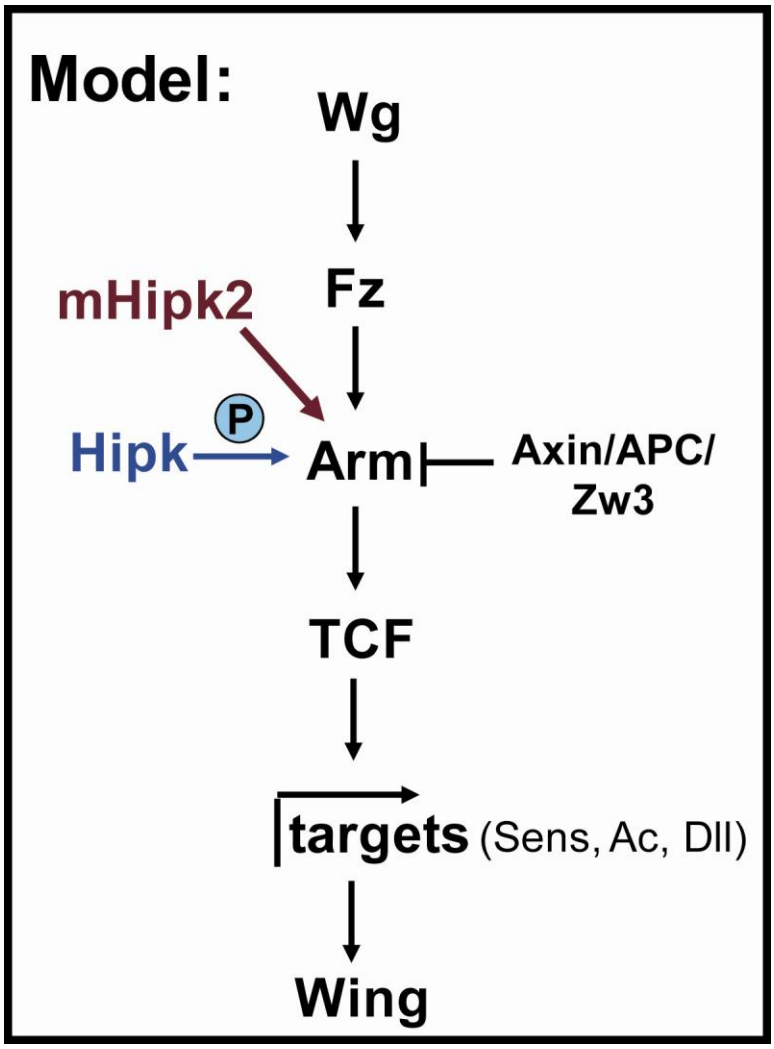

Figure 3-11 Hipk promotes Wg signal transduction

Hipk can phosphorylate Arm and promote its cytoplasmic and nuclear accumulation, leading to the expression of Wg targets such as Dll, Sens and Ac, which are necessary for wing development. Hipk proteins may share a conserved function in promoting Wnt/Wg signaling, possibly through their role in Arm stabilization, as murine Hipk2 can also enhance β-catenin protein levels.

delineate the physiological relevance of this potential kinase cascade, specifically we determined if these interactions played a role in the regulation of the Wg pathway. Our data reveal that in Drosophila Nmo and Hipk do not form a kinase cascade in this context, rather they exert opposing effects on the same pathway, likely through distinct mechanisms.

3.4.3 Hipk proteins promote Tcf/Lef1-mediated transcription

The Wg morphogen can bring forth a spectrum of biological processes. Sustaining maximal levels of signaling could be accomplished through the amplification and enhancement of the signal in the wing margin. In support of this model, we found that overexpression of Hipk expands the expression of Wg targets such as Dll, Sens and Ac. Transcriptional assays reveal that both *Drosophila* Hipk and mouse Hipk2 enhance the transcriptional activity of Tcf and Lef1, respectively, in a kinase-dependent manner. These findings strongly suggest that Hipk and Hipk2 function to enhance the activity of the transcriptional complex to promote the Wg/Wnt signal.

3.4.4 Hipk promotes Wg signaling through a Gro independent mechanism

In the absence of pathway activation, several transcription factors of conserved signaling pathways such, as N and Wg/Wnt, adopt a repressor function upon binding to Gro (Cinnamon and Paroush, 2008). In the instance of Wnt signaling, Gro competes with β-catenin for binding to Lef/Tcf and formation of Lef/Tcf-Gro complexes transcriptionally silences Wnt target genes (Daniels and Weis, 2005; Cavello et al., 1998). Here, we identified a novel role for Hipk in Arm stability in the wing, which may shift the molecular stoichometry in favour of the Tcf/Arm interaction, further alleviating

the repressive effect of Gro on the Wg signal. My studies cannot exclude the possibility that Hipk may also stimulate Wg pathway activity through the phosphorylation of Gro. It remains to be determined if the phosphorylated Gro isoform reduces the molecule's binding affinity with Tcf, similar to the observed inhibitory effects on the formation of the repressor complex composed of Gro and the Pax6 homolog, Ey (Choi et al., 2005). However, this seems unlikely in the wing given that several lines of evidence suggest that Hipk promotes Wg signaling in a Gro independent manner. Past studies also indicate that Tcf does not likely act as a default repressor in the absence of Wg signaling in the wing, since *tcf* somatic clones failed to derepress the expression of Wg responsive targets (Schweizer et al., 2003). Furthermore, overexpression of Hipk is insufficient to rescue the wing phenotype induced by elevated levels of Gro, which would be expected if Hipk antagonizes Gro during wing development. However, these studies have not excluded the possibility that Hipk may downregulate the repressive capacity of Gro to promote Wg signaling in other tissues, such as the embryo where Tcf forms a complex with Gro to silence Wg targets. Future studies addressing the role of Hipk in other Gro mediated processes would begin to resolve the extent that Hipk utilizes such a regulatory mechanism to control development.

3.4.5 Hipk stabilizes Arm

Accumulation of stabilized Arm is paramount to effective Wg signaling. Failure to escape the destruction complex results in Arm degradation and inhibition of Tcf-mediated gene activation. Thus understanding the regulation of Arm is central to the global understanding of how the Wg signal is modulated. In our studies, we reveal a role for Hipk in Arm stabilization. This feature is highlighted by the loss of stabilized Arm in

hipk mutant clones. Additionally, in *hipk* mutant discs, overexpressed wildtype Arm fails to accumulate, despite its expression in domains of high Wg signaling. These findings demonstrate that Hipk plays an important role in Arm stabilization. Hipk may reduce the ability of Arm to interact with destruction complex components or may increase the nuclear retention of Arm. In the absence of Hipk, either of these scenarios would give the destruction complex more access to Arm. In agreement with such a model, we find that increasing Hipk activity in the wing surpasses the inhibitory effects of the degradation machinery and expands the perimeter of stabilized Arm. Furthermore, we have found that the presence of Hipk or Hipk2 in cell culture stabilizes Arm/β-catenin. Thus the enhanced transcriptional activity is likely due to the elevated availability of Arm protein.

3.4.6 Mechanisms governing Arm/β-catenin stability

It is crucial for normal development to maintain the proper amounts of β-catenin, as elevated levels of β-catenin can lead to cancer (reviewed in Clevers, 2006). Elaborate regulatory networks in the cytoplasm and nucleus are vital to maintaining appropriate levels of β-catenin. It is well documented that phosphorylation in the amino terminus of β-catenin is critical for its negative regulation (reviewed in Daugherty and Gottardi, 2007). A chain of phosphorylation events begins when Casein Kinase I (CKI) primes β-catenin for successive modifications by GSK3β (Amit et al., 2002; Liu et al., 2002; Yanagawa et al., 2002). Central to this event is Axin, which provides a scaffold for APC, CK1, GSK3 and β-catenin (Amit et al., 2002; Hart et al., 1998; Hinoi et al., 2000; Ikeda

et al., 1998). N-terminally phosphorylated β-catenin is ubiquitinated by βTrCP ubiquitin ligase and targeted for degradation via the proteasome.

Wnt signaling promotes the accumulation of β-catenin, however some of the mechanisms governing this process remain enigmatic. While overexpression of wildtype β-catenin/Arm is unable to overcome the effects of the degradation machinery (Mohit et al., 2003; Pai et al., 1997), Wnt stimulated β-catenin can resist the activity of the destruction complex. While achieving stabilized pools of β-catenin represents the core goal of the Wnt pathway, high levels of β-catenin are not always coupled with elevated transcription (Guger and Gumbiner, 2000; Staal et al., 2002). For example, in Xenopus, alanine substitution of one of the GSK3 target residues, leads to elevated β-catenin levels, without causing an increase in Tcf mediated transcription (Guger and Gumbiner, 2000). Thus further posttranslational modifications of β-catenin are necessary to potentiate its signaling activity.

Furthermore, phosphorylation can affect β-catenin stability by affecting protein-protein interactions that regulate protein turnover and activity. Phosphorylation of β-catenin by Cdk5 inhibits APC binding to β -catenin (Munoz et al., 2007; Ryo et al., 2001) while phosphorylation by CK2 promotes β-catenin stability and transcriptional activity (Song et al., 2003).

3.4.7 Regulation of β-catenin/Arm-mediated transcription

Recent advances have begun to unravel the molecular complexity that controls β-catenin-mediated transcription within the nucleus. Upon pathway activation, Tcf recruits Arm to the enhancers of Wg-responsive genes where Arm forms multiple transcriptional

complexes along its length (Hoffmans et al., 2005; Kramps et al., 2002; Thompson et al., 2002; Hecht et al., 1999; Mosimann et al., 2006). Formation of these transcriptional units is needed for the transmission of the Wg/Wnt signal. Recent studies have shown that phosphorylation (distinct from the N-terminal phosphorylation that triggers β-catenin destruction) may modulate its ability to recruit these cofactors (reviewed in Daugherty and Gottardi, 2007). We have found that the Hipk-dependent stabilized form of Arm is transcriptionally active and induces the expression of Wg targets, suggesting modification by Hipk may promote protein interactions.

3.4.8 Models for the role of Hipk in Wg/Wnt signaling

APC and the cell adhesion molecule E-cadherin compete with Tcf for overlapping binding sites on β-catenin, as shown in a schematic diagram in Fig. 3-12 (Hulsken et al., 1994; von Kries et al., 2000). Competition between proteins may play an important role in the regulation of the Wnt signaling pathway (reviewed in Xu and Kimelman, 2007). We propose that Hipks may promote the stability of Arm/β-catenin by excluding further interactions with other proteins, including those that antagonize Arm/β-catenin. Given that Hipks can also bind to Tcf/Lef1, we predict that these proteins may act synergistically to displace the inhibitory partners of β-catenin. In agreement with such a role, we observe that Lef1 enhances the interaction between Hipk2 and β-catenin and these interactions may insulate β-catenin from components of the degradation machinery.

While Hipk phosphorylates Arm, the functional significance of this modification has yet to be determined. Hipk might facilitate the interactions between Arm and its transcriptional cofactors, as Hipk2 phosphorylation has been shown to affect gene

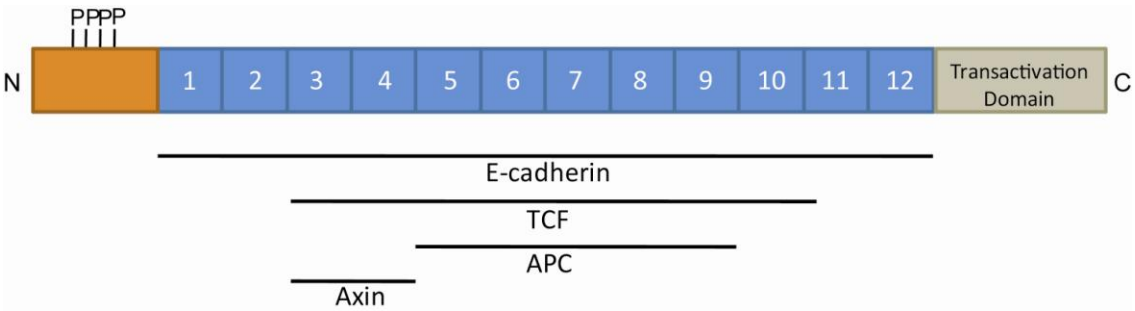

Figure 3-12 β-catenin protein domains

β-catenin (β-cat) is composed of 12 so-called Arm repeats and an N terminus that is targeted for phosphorylation by GSK3 and CK1 (as shown by P). The C terminus houses the transactivation domain. Many binding partners share overlapping binding sites for β-cat in the Arm repeats(as indicated by the black lines).

regulation by modifying the composition of various transcriptional complexes (reviewed in Calzado et al., 2007). Hipk may also enhance the formation of the β-catenin/Tcf transcriptional complex by inducing a conformational change and/or reducing the affinity of possible inhibitors for β-catenin through the phosphorylation of β-catenin. Recently it was reported that Hipk2 could antagonize β-catenin/Tcf-mediated transcription in a kinase-independent manner (Wei et al., 2007). While these data appear in conflict with our findings, we have observed that the effect of Hipk2 on transcription is very cell type- and target gene-dependent (Fig. A-4), suggesting Hipk2 function is affected by its cellular context, most likely due to the availability of targets and co-factors.

The dynamic localization of Hipk2 in the nucleus, nucleoplasm and in cytosolic speckles suggests that the protein may carry out distinct roles in each site (reviewed in Calzado et al., 2007; Rinaldo et al., 2007). Given the growing list of interacting proteins, it is tempting to speculate that specific Hipk function is determined in part through its particular localization. It is also possible that Hipk/Hipk2 may act as a scaffolding protein, bringing together multiple binding partners. Ongoing biochemical studies will further uncover the molecular significance of these interactions. Hipk proteins are emerging as important components of multiple signaling networks. Our studies describe the roles of Hipk and Hipk2 as Wnt/Wg regulators and shed light on the regulatory mechanisms governing this conserved pathway.

3.5 Contributions

As the first author, I performed most of the experimental design, and I wrote the article with E.M.V. I generated the data presented in Fig. 3-3, 3-6, 3-9A and contributed

equally to the immunohistochemical analysis in Fig. 3-4 and 3-5 with J.C. S.S and I contributed to the data presented in Fig. 3-8 and Fig. 3-10A. S.S generated the biochemical data on the role for Hipks in Arm stability in vitro and transcriptional assays in S2R+ shown in Fig. 3-9C, 3-10B and D. T.I contributed the data with mHipk2 in HeLa cells in Fig. 3-7C, 3-9B and 3-10C. The phenotypic characterization of *hipk* in the wing (Fig. 3-2) and initial genetic characterization of Hipk in the Wg pathway shown in Fig. 3-3B, G and H were performed by J.C.

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CHAPTER 4
CHARACTERIZATION OF *DROSOPHILA* NEMO IN PERIPHERAL NERVOUS SYSTEM DEVELOPMENT

4.1 Summary

My Ph.D research uncovered novel roles for Hipk in *Drosophila* development through its regulation of both the N and Wg signal transduction pathway. However, during the initial stages of my studies, I also addressed how the protein kinase, Nemo (Nmo) contributes to the specification of the *Drosophila* peripheral nervous system (PNS).

Patterning of the PNS is a highly complex process that occurs in a sequential series of events. Sensory organ (SO) development begins with the selection of a single sensory organ precursor (SOP) from a group of equivalent cells in proneural clusters. Each cell within the cluster can potentially give rise to the SOP. Complex signaling networks between cells limit the number of neural precursors and control its subsequent fate in the SO lineage. *nmo* encodes a MAPK-like kinase, an important regulator of conserved signaling pathways such as BMP and Wg. I found that modifying Nmo levels disrupts SO patterning in the adult notum suggesting that Nmo is necessary for proper patterning of the PNS. Elevated levels of Nmo inhibit the expression of the proneural protein Achaete (Ac), leading to a failure to form proneural clusters and a subsequent loss of neural fate. In addition to a role for Nmo as an inhibitor of Ac expression, genetic

interaction studies also reveal a function for Nmo in promoting neural fate. These studies reveal a dual role for Nmo in the developing PNS and this likely reflects its function at several stages of SO development to refine the patterning of the PNS of the adult notum.

My recent phenotypic analysis of Hipk suggested a requirement for Hipk in adult PNS development. I performed further genetic interaction analysis between the two kinases in the developing PNS and observed an antagonistic interaction between Nmo and Hipk, similar to what was characterized during wing development (Fig. 3-3O; Lee et al., 2009b). Therefore, an antagonistic interplay between Nmo and Hipk also extends to the patterning of the PNS, indicating a general antagonism between the two kinases in multiple developmental processes

4.2 Introduction

An organism's response to external stimuli is vital for an individual's survival. The adult peripheral nervous system (PNS) relays information to the brain to trigger the appropriate physiological response. The *Drosphila* PNS comprises several types of sensory organs (SO), including the external SOs, and their stereotypical arrangement provides a powerful system to dissect the genetic circuitry governing neural development. The SOs of the notum, or back of the fruitfly, are a series of mechanosensory structures or bristles called macrochaetae (Fig. 4-1). The SO is composed of a neuron with a glial and sheath cell and two external structures, the socket and shaft. The SO is derived from a sensory organ precursor (SOP), initially specified in the dorsalmost compartment of the imaginal wing disc which will develop into the adult notum (Fig. 4-1).

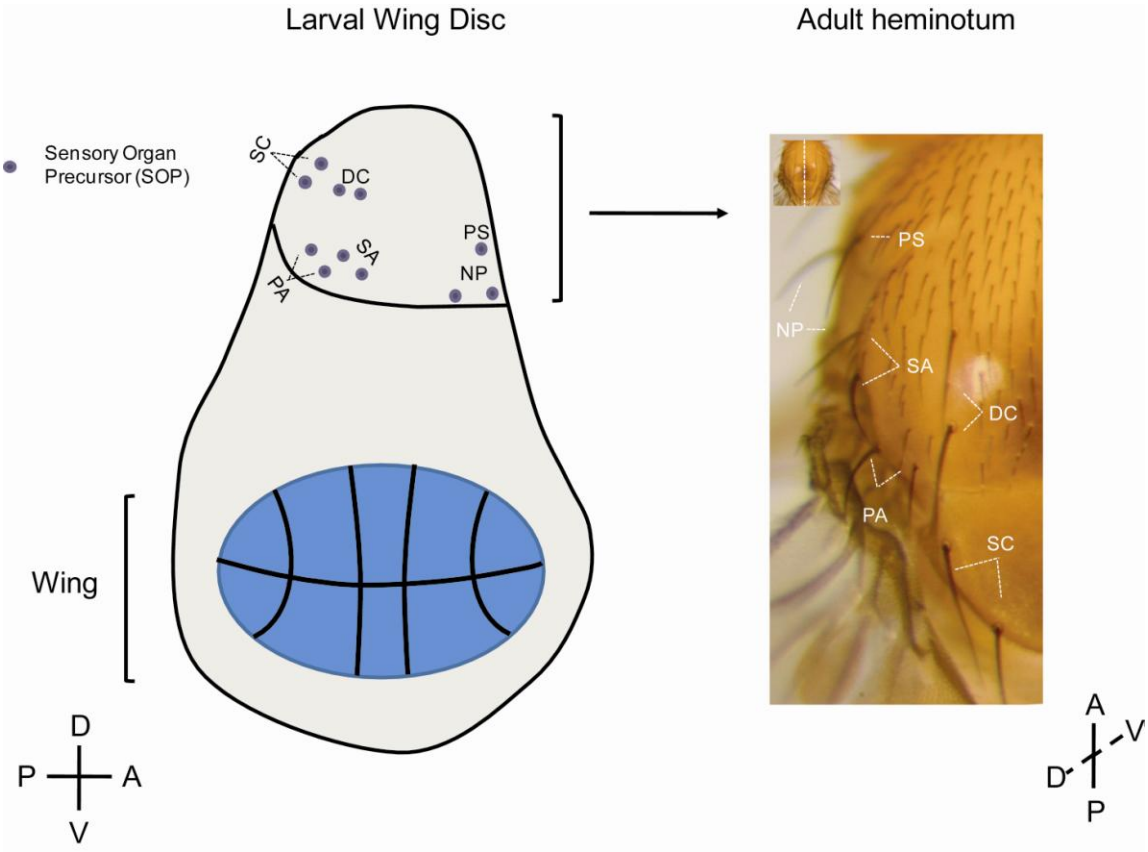

Figure 4-1 Generation of the adult peripheral nervous system of the *Drosophila* notum

On the Drosophila notum, the adult peripheral nervous system (PNS) is a series of sensory organs (SO) or bristles arranged in a stereotypical pattern. The SO is derived from a sensory organ precursor (SOP), initially specified in the dorsalmost compartment of the imaginal wing disc which will give rise to the adult notum. Abbreviations for macrochaete type: (PS) presutural, (NP) notoplerual, (SA) supraalar, (PA) postalar, (DC) dorsocentral, (SC) scutellar. Shown is the dorsal view of the adult heminotum.

The process of SO development occurs in a sequential and step-wise manner (Fig. 4-2). Neural specification is demarcated by the expression of the proneural genes, *acheate* (ac) and *scute* (sc) within a small group of ectodermal cells. The presence of Ac and Sc endows these cells with the ability to form proneural clusters. *ac* and *sc* are members of the *ac-sc complex* (AS-C) and encode transcription factors of the basic helix-loop-helix (bHLH) family (Campuzano and Modolell, 1992). Each cell within the proneural cluster can potentially give rise to the neural fate. Through the process of lateral inhibition, a single cell will accumulate sufficient levels of the proneural determinants, and will be selected to become the SOP. The neighbouring cells expressing lower levels of Ac and Sc will adopt the epidermal fate. Once the SOP is selected by lateral inhibition, its developmental progression requires the activation of *neuralized* (*neu*), *senseless* (*sens*), *hindsight* (*hnt*) and *asense* (*ase*) and the downregulation of the proneural genes (Cubas et al., 1991; Nolo et al., 2000). The SOP will undergo three rounds of asymmetric divisions to produce an adult SO comprised of its five different cell types (Hartenstein and Posakony, 1990) . The mother SOP will divide to produce two secondary daughter cells, pIIa and pIIb (Hartenstein and Posakony, 1990). pIIa will generate the mechanosensorys' external structures, the shaft and the socket (Hartenstein and Posakony, 1990) Meanwhile, pIIb will undergo another round of division to produce a glial cell and a pIIIb precursor cell (Gho et al., 1999). The latter will divide once more to produce the internal components of the SO, the neuron and the sheath cell.

The choice between neuronal and epidermal specification is tightly regulated through the efforts of key signal transduction pathways such as the Notch (N) pathway.

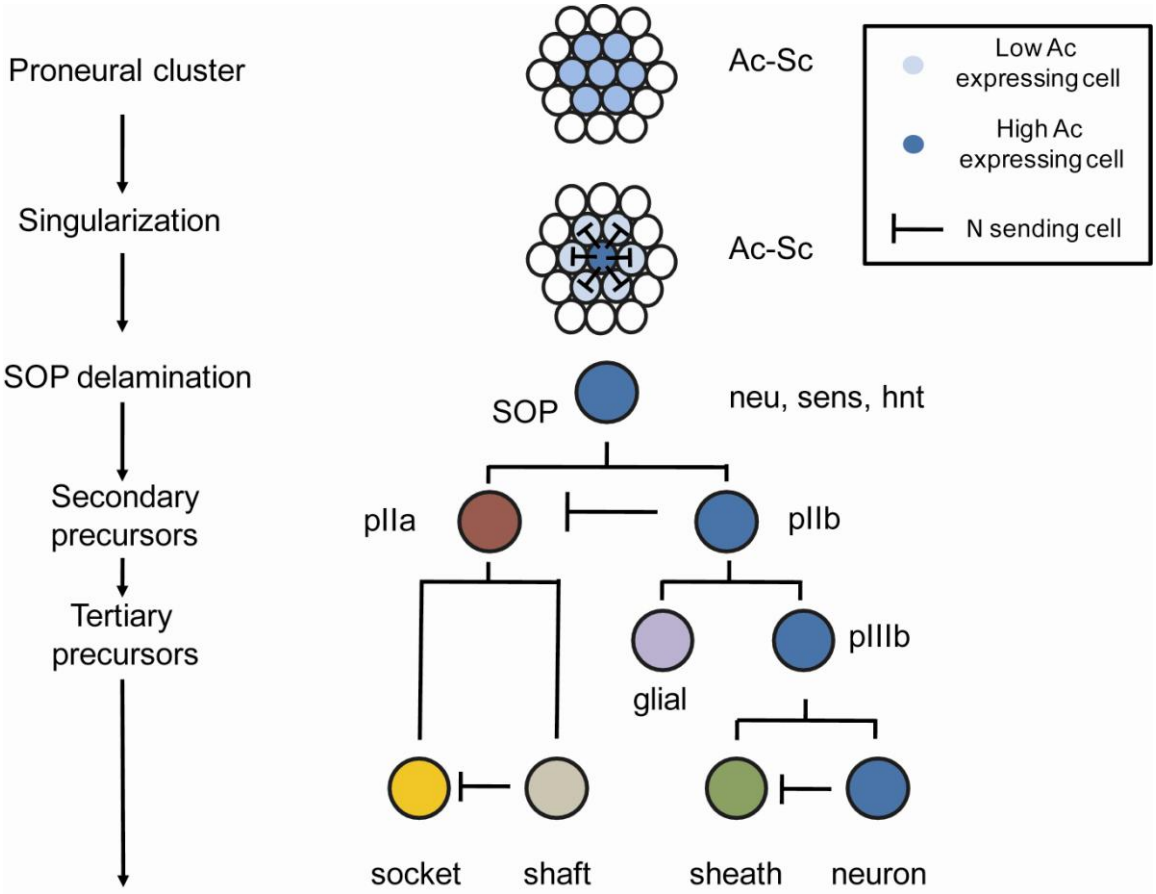

Figure 4-2 Sensory organ development

The process of sensory organ (SO) development occurs in a sequential and step-wise manner, beginning with the formation of proneural clusters, which are determined by the presence of Ac and Sc within these cells. Through the process of N-mediated lateral inhibition, a single cell will accumulate high levels of Ac-Sc, and will be selected to become the SOP and begin to express neu, sens, and hnt. After a series of divisions, the SOP will give rise to the bristle and its associated structures. N signaling occurs between the various cell types to ensure that each cell adopts a unique fate.

N functions at multiple stages in SO development. Initially N acts within the proneural clusters to refine neuronal specification through the process of lateral inhibition (Heitzler et al., 1996). Levels of the N ligand, Delta (Dl) are highest in the SOP relative to its neighbouring cells (Heitzler et al., 1996; Schweisguth, 1995). From the SOP, Dl sends a N mediated inhibitory signal to the neighbouring cells. In these epidermal cells, N is active and the intracellular domain of the receptor is cleaved and forms a transcriptional complex with the DNA binding protein Suppressor of Hairless (Su(H)) leading to the activation of downstream targets, including the *Enhancer of split Complex* (*E(spl)-C*) (Fig. 2-1). E(spl) prevents the actions of proneural proteins and hence inhibits SOP development in epidermal cells (Heitzler et al., 1996; Oellers et al., 1994; Ohsako et al., 1994). Once the SOP is specified, N also acts during the division of the neuronal precursor to inhibit neuronal development of the pIIa daughter cell. N sends an inhibitory signal from the PIIb precursor cell to pIIa thereby promoting the bristle and socket fate (Hartenstein and Posakony, 1990). N also sends a signal from the bristle to the socket cell, as well as from the neuron to sheath cell to diversify the cells types. N signaling in the SO lineage directs signal sending cells to a distinct fate from those receiving the signal (Koelzer and Klein, 2003). Dynamic roles of N signaling in each stage of SOP development is evident when *N* and *Dl* temperature sensitive mutants produce unique phenotypes depending on when they are shifted to the restrictive temperature. Removal of N inhibition during SOP selection results in excessive SOP recruitment (Hartenstein and Posakony, 1990). The subsequent loss of N in the SOP induces a symmetric division of the precursor cell to generate only pIIb progeny and ultimately, produce a naked notum with excessive neurons at the expense of the other

fates (Hartenstein and Posakony, 1990; Parks and Muskavitch, 1993). Reducing N in the SOP lineage can transform the socket to a shaft fate, and produces bristles with duplicated shafts (Barolo et al., 2000; Zeng et al., 1998).

Additional tiers of regulation other than N exist to control SOP numbers. Neural specification is further controlled through the interactions between the bHLH proteins Ac-Sc and the HLH protein, Extramacrochaetae (Emc). The helix loop helix protein domain is present in many transcriptional regulators and enables proteins to form homo and heterodimers, while the basic region confers the ability to bind to DNA (Murre et al., 1989). Emc inhibits transcription of genes essential for the neural program by binding with proneural bHLH proteins such as Ac and Sc (Martinez et al., 1993; Van Doren et al., 1991; Van Doren et al., 1992). Formation of these inactive heterodimers prevents the bHLH proteins from binding to DNA and hence, inactivates transcriptional targets required for neural differentiation. These various repressive mechanisms establish a tightly controlled system to limit the number of neuronal precursors.

Nemo (Nmo) belongs to a novel family of serine/threonine protein kinases most similar to MAPKs (Choi and Benzer, 1994). Nmo was initially identified in *Drosophila* to play a role in the rotation of photoreceptor cells in the developing ommatidium (Choi and Benzer, 1994). Recently, a role for Nmo in eye specification during the earliest stages of visual development was uncovered (Braid and Verheyen, 2008). In addition to its role in the eye, Nmo functions in multiple developmental processes including embryogenesis, wing vein patterning and apoptosis (Mirkovic et al., 2002; Verheyen et al., 2001). It has been demonstrated that Nmo plays a vital role in development through its regulation of multiple conserved signaling pathways including bone morphogenetic

protein (BMP) and Wingless (Wg) (Zeng et al., 2007; Zeng and Verheyen, 2004). Previously, genetic interactions were observed between *nmo* and the N antagonist, *Hairless* (*H)* in the adult PNS, indicating a possible role for Nmo in the regulation of the N pathway (Verheyen et al., 2001).

Genetic and phenotypic analyses were undertaken to elucidate the role of Nmo in the patterning of the adult nervous system. Modifying Nmo levels led to a disruption of bristle patterning suggesting that Nmo is likely necessary for the proper patterning of the PNS. Genetic interaction studies between *nmo* and components of both the N pathway and known neuronal regulators such as *ac* and *emc* suggest that Nmo likely acts to refine the patterning of the PNS of the adult notum. Phenotypic analysis of Homeodomain-interacting protein kinase (Hipk), an antagonist of Nmo during wing development (Lee et al., 2009b), suggested a requirement for Hipk in adult PNS development. An antagonistic interaction between Nmo and Hipk also extends to the patterning of the peripheral nervous system, indicating a general antagonism between the two kinases in multiple developmental processes. In summary, these studies indicate that Nmo likely functions at multiple stages during the development of the adult peripheral nervous system.

4.3 Results and Discussion

4.3.1 Nmo plays a role in sensory organ patterning

Several lines of evidence suggested a putative role for Nmo during the patterning of the peripheral nervous system of the developing notum. It has been demonstrated that *nmo's* expression in the presumptive notum overlaps with the pattern of SOP emergence

(Zeng and Verheyen, 2004). Furthermore, genetic interaction studies revealed that *nmo* can enhance the loss of bristles phenotype induced by a mutation in the N antagonist, H (Verheyen et al., 2001).

Further phenotypic analyses using both mutant and misexpression studies were performed to assess whether *nmo* functions in the notum to organize adult SOs. nmo^{adk2} and nmo^{DB24} homozygotes displayed a notal bristle phenotype, in which a subset of macrochaetae were duplicated. Nmo affected two different types of macrochaetes. I observed bristle duplication in 17% and 13% of the anterior dorsalcentrals (aDC) and 20% and 31% of the anterior postalar (aPA) in $nmo^{ad\ k}$ (n=96) and nmo^{DB24} (n=39) mutants, respectively. In wildtype adults (n=251), duplicated aDC and aPA were observed at a low frequency of 0.8% and 2%, respectively. The presence of duplicated bristles may be induced by the selection of additional cells to SOP fate. Such defects could also reflect abnormalities in subsequent asymmetric divisions of the SOP. These alterations can lead to a conversion of the SOP lineage, such as the formation of two pIIa precursor cells at the expense of the pIIb fate. nmo^{adk2} adult wings also develop fewer mechanosensory bristles along the wing margin. Staining of neuronal progenitor cells in 3rd instar wing discs with the SOP marker, *neuralized* (*A101-LacZ*) revealed that nmo^{adk2} homozygotes display fewer SOPs in the anterior wing margin (data not shown). The loss of wing margin bristles in the adult wing likely correlates with a reduction in the number of SOPs. Neuronal defects are not limited to the sensory organs of the wing discs. Defects in other tissues are also observed during embryogenesis (Andrea Uetrecht, unpublished), indicating a general role for Nmo in neural development.

Consistent with Nmo participating in neuronal patterning processes, misexpression of *nmo* in the notum also led to a PNS phenotype. Elevating levels of Nmo in proneural clusters with *scabrous-Gal4* (*sca-Gal4*, n=40) led to a reduction of a subset of macrochaetes (Table 4-1). Specifically four bristle types were affected, I observed a loss of 10% of the posterior notopleural (pNP), 30% of the posterior supraalar (pSA), 10% of the anterior postalar (aPA), and 10% of the anterior dorsocentral (aDC) in *sca>nmo* adults. Overexpressing 2 copies of *nmo* with *sca-Gal4* enhanced the loss of bristle phenotype by increasing the frequency of the phenotype, as well as affecting a wider range of macrochaete types in the notum (Table 4-1). When Nmo was misexpressed in broad domains of the notum with either *apterous-Gal4* (*ap-Gal4*; Fig. 4-3B-C) (Zeng and Verheyen, 2004), or *pannier-Gal4* (*pnr-Gal4*; Fig. 4-3D*)* or the ubiquitous driver, *c765-Gal4*, it also led to a loss of macrochaetae phenotype (Table 4-1). To determine if elevated levels of *nmo* inhibited the process of sequestering cells into the neural program, *ap>2x nmo* wing discs were stained with the SOP specific markers, Hnt and Sens. In these discs, the loss of bristle phenotype was associated with a reduction of neural precursor cells, as seen with fewer Hnt (Fig. 4-3H) and Sens (Fig. 4-3F) expressing cells. To assess whether the loss of SOPs was induced by changes in the expression of proneural determinants, Ac expression was examined in these specimens. *ap>2x nmo* imaginal wing discs stained with an anti-Ac antibody revealed a reduction in Ac protein levels, which suggests that proneural clusters failed to form properly. Therefore, Nmo likely functions during the early stages of SO formation to inhibit the formation of proneural cluster.

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Genotype	Gal4 Expression pattern	n	Macrochaete Type										
			PS	aNP	pNP	aSA	pSA	aPA	pPA	aSc	pSc	aDC	pDC
sca>nmo	proneural clusters	40	1	1	**0.9 (10%)**	1	**0.7 (30%)**	**0.9 (10%)**	1	1	1	**0.9 (10%)**	1
sca>2x nmo		40	1	**0.8 (20%)**	1	1	**0.2 (80%)**	**0.8 (20%)**	1	0.7	0.8	**0.4 (60%)**	**0.8 (20%)**
c253>nmo	late in proneural clusters	40	1	1	1	1	1	1	1	1	1	1	1
c253>2x nmo		40	1	1	1	1	0.9	1	1	1	1	1	1
A101>2x nmo	SO and SOP	40	1	1	**0.9 (10%)**	1	1	1	1	**0.5 (50%)**	**0.9 (10%)**	1	1
309>nmo	SOPs	40	1	1	1	1	1	1	1	1	1	1	1
309>2x nmo		92	1	1	1	1	**0.9 (10%)**	1	1	1	1	1	1
109-6-8>nmo	SO lineage	88	1	1	1	1	1	1	1	1	1	1	1
c765>nmo	weak ubiquitous expression	42	1	1	1	1	1	1	1	**1.1 (10%)**	1	1	1
c765>2x nmo		30	1	1	1	1	**0.5 (50%)**	1	1	**1.1 (10%)**	1	**0.83 (17%)**	1

Table 4-1 Elevated levels of Nmo induce bristle loss in the adult notum

Sensitivity to increased levels of Nmo varies with the type of bristle. Results represent average number of macrochaetes present. A value of 1 indicates the normal number of bristles, and values <1 or >1 represent bristle loss or duplicated bristles, respectively. Percentage reduction are indicated in parenthesis and n is the number of heminota analyzed. Abbreviations for macrochaete type: (PS) presutural, (NP) notoplerual, (SA) supraalar, (PA) postalar, (DC) dorsocentral, (SC) scutellar. (a) anterior, (p) posterior.

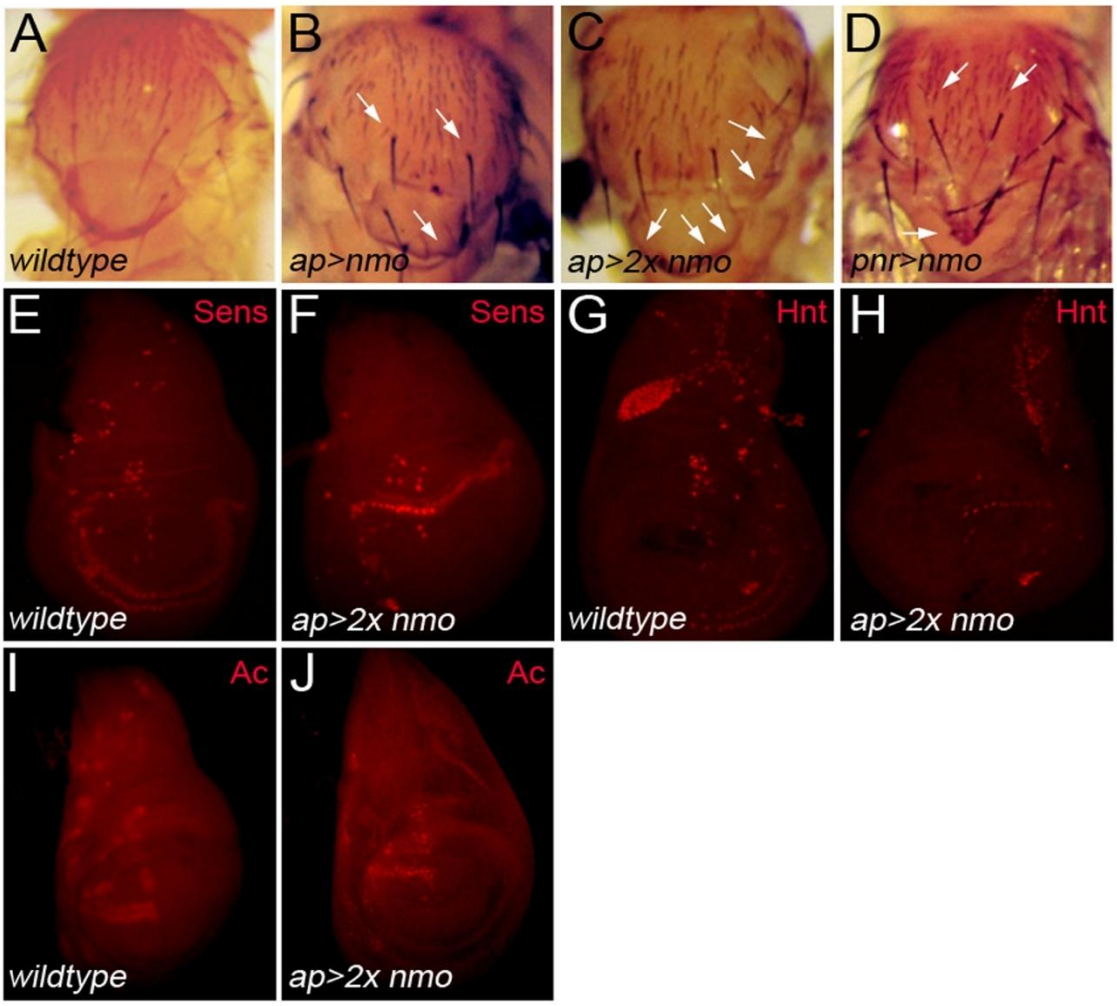

Figure 4-3 Elevated levels of Nmo inhibits SOP formation

(A) Wildtype notum. (B) *ap>nmo*. (C) *ap>2x nmo.* (D) *pnr>nmo*. Misexpression of Nmo produced a loss of bristle phenotype on the notum (arrows in B-D). Elevated levels of Nmo inhibit Ac expression and SOP selection. Antibody staining for SOP markers and Ac expression were performed in w^{1118} and *ap>2x nmo* wing discs. (E-F) Sens protein (G-H) Hnt protein (I-J) Ac protein.

To identify the precise developmental stages during which Nmo functions to pattern the bristles, Nmo was also misexpressed with various GAL4 drivers at each step of SOP development (Table 4-1). Overexpression of Nmo in the SOP using *A101-Gal4 or 309-Gal4*, respectively led to a milder effect compared to the earlier proneural cluster driver, *sca-Gal4* (Table 4-1). Hence, elevated levels of Nmo in the SOP daughter cells also have the potential to modify the developmental program once the neural progenitor cell is selected. Based on these observations, one cannot exclude the possibility that Nmo is repeatedly utilized for the division of the SOP mother cell that will subsequently give rise to the sensillum structure. It is not uncommon for a particular protein to serve multiple functions. The existence of such a regulatory mechanism results in a synchronized and rapid method utilized by the cell to adapt to changes within its developmental program. Nevertheless, both mutant and misexpression phenotypic analyses strongly suggest that Nmo plays a role in the formation of the PNS.

4.3.2 Nmo displays complex interactions with the N signaling pathway in the peripheral nervous system

N signaling refines SO development by controlling many aspects of the neural program. Initially, in proneural clusters, N acts during lateral inhibition to promote epidermal fate rather than SOP fate, and subsequently in daughter cells to suppress neural identity (Hartenstein and Posakony, 1990). Additionally, N also sends an inhibitory signal in the SO lineage, in particular from the shaft to the socket cell, as well as from the neuron to sheath cell to ensure each cell adopts a unique fate (Koelzer and Klein, 2003) . Reduced N signaling results in the selection of all cells of the proneural cluster giving rise to the SOPs, then these cells will generate neurons at the expense of the other fates

(Hartenstein and Posakony, 1990). The presence of duplicated bristles in *nmo* homozygous mutants and the loss of proneural clusters with elevated Nmo levels, suggest that Nmo likely regulates the SOP selection process, possibly by promoting N-mediated lateral inhibition. Genetic evidence has previously shown that *nmo* can modify N associated phenotypes in both the eye and the adult notum (Verheyen et al., 2001; Verheyen et al., 1996). Specifically in PNS patterning, reducing Nmo function can enhance the loss of notal bristle phenotype induced by the H^1 mutation (Verheyen et al., 2001), which would suggest a possible role for Nmo as an antagonist of the N pathway. To further characterize the role for Nmo in N signaling during sensory development, a series of genetic interaction studies were performed. Reducing the Notch signal by overexpressing a dominant negative form of the N ligand, Dl with *sca-Gal4* led to an increase in macrochaetes (Fig. 4-4E)**.** The lack of lateral inhibition in *sca*>Dl^{DN} produced clusters of macrochaetes throughout the body, including the head, notum and abdomen (Fig. 4-4E). Simultaneously misexpressing Nmo partially suppressed the *sca>*Dl^{DN} phenotype (Fig 4-4F), therefore suggesting that elevated levels of Nmo can compensate for a decrease in the N mediated lateral inhibition. Similar results were also observed with *ap-Gal4* driver, which can also induce a lateral inhibition defect in *ap>*Dl^{DN} adults (Fig. 4-4C), while misexpressing Nmo alone results in the absence of a subset of bristles (Fig. 4-4B). Coexpressing Nmo not only suppresses the *ap>*Dl^{DN} phenotype, but also results in a predominantly naked notum (Fig. 4-4D). The observed interaction between *nmo* and Dl^{DN} with *ap-Gal4* likely reflects the increased strength and expression pattern of *ap-Gal4*, which differs from *sca-Gal4*. *ap-Gal4* is expressed during the earlier stages in the wing disc in a broader domain than *sca-Gal4*, specifically in all cells comprising

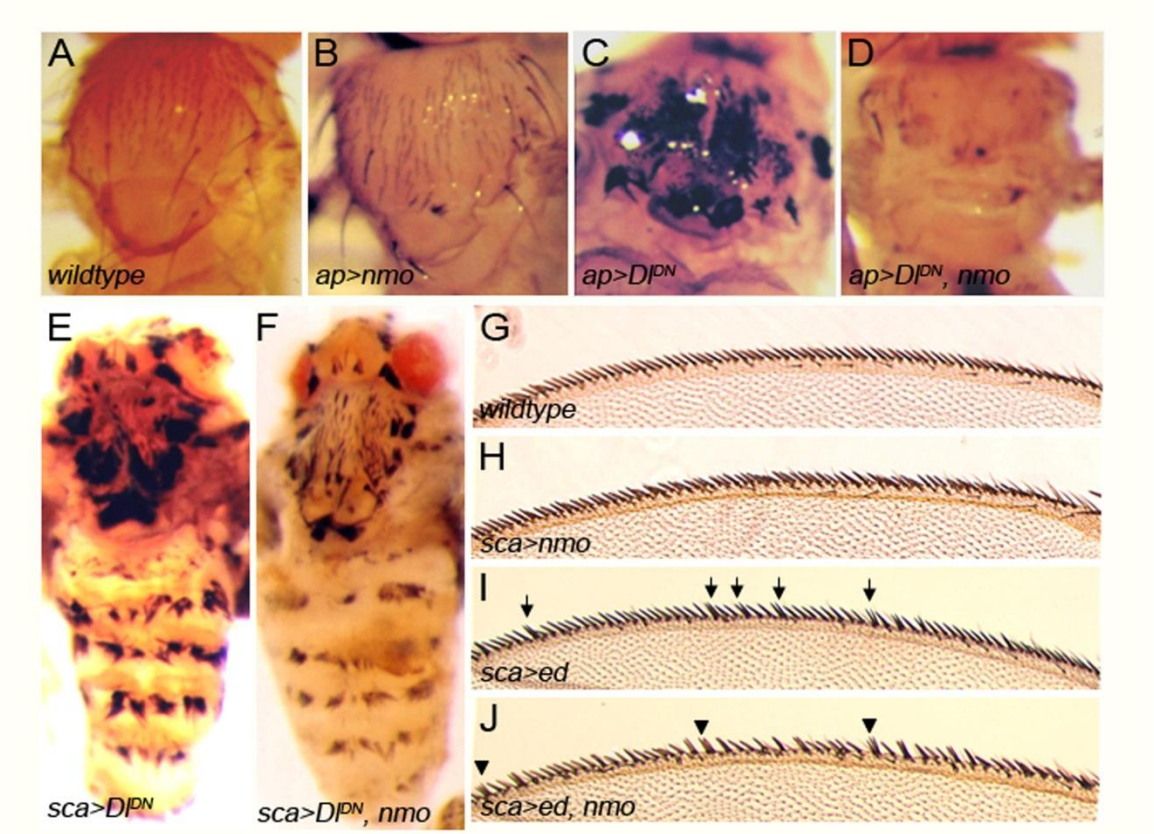

Figure 4-4 Nmo interacts with the Notch pathway in the peripheral nervous system

(A) Wildtype. (B) *ap>nmo*. (C,E) Overexpression of a dominant negative form of Dl produced clusters of macrochaetes, which were suppressed with coexpression of Nmo (D,F). (G) Wildtype wing margin. (H) *sca>nmo.* (I) *sca>ed*. Overexpression of Ed leads to duplicated bristles from a single socket (arrows), which was enhanced with elevated levels of Nmo (arrowheads in J).

the dorsal compartment of the wing primordium. Taken together, these results indicate that Nmo can modify the effects of reducing N signaling activity and Nmo may promote N mediated lateral inhibition, subsequently suppressing neural identity.

To assess whether Nmo could also act in other N mediated processes, specifically in cells that have already been selected as an SOP, additional genetic interaction studies were performed with *echinoid* (*ed*). Ed is a positive facilitator of N signaling (Ahmed et al., 2003; Escudero et al., 2003; Rawlins et al., 2003) and elevating Ed levels with *sca-Gal4*, induced the formation of duplicated shafts from a single socket (Fig. 4-4I). Overexpression of full length Ed can generate a dominant negative effect and hence phenocopy the effects of reducing the N signaling (Barolo et al., 2000; Escudero et al., 2003). This phenotype resembles defects associated with a conversion of the SO lineage. I tested whether Nmo can modify the *sca>ed* phenotype. *sca>nmo* adults display a wild type wing margin (Fig. 4-4G, H), and simultaneously increasing Nmo levels modified the *sca>ed* phenotype. In these wing margins, Nmo elevated both the frequency and quantity of ectopic bristles emerging from a single socket (Fig. 4-4J). The appearance of 3 or more bristles from a sole socket is observed in *sca>ed, nmo* wings (Fig. 4-4J), which was never observed in *sca>ed* flies (Fig. 4-4I). Therefore in the SO lineages, Nmo can also interact with the N pathway in the sensory lineage to specify the sensillum structures. In summary, these genetic interaction studies demonstrate that Nmo functions in multiple N controlled processes to properly pattern the peripheral nervous system.

4.3.3 Elevated levels of Nmo can modify the expression of N responsive targets

Since elevated levels of *nmo* suppressed the effects of *sca>Dl*^{DN} suggested that *nmo* may cooperate with the N pathway, I next addressed whether the observed genetic interactions between *nmo* and N pathway components were triggered by Nmo's ability to stimulate N signaling activity. N mediated transcription was measured in *sca>nmo* wing discs by examining the expression of the products of the *E(spl) C* which are direct targets of Su(H). Ectopic expression of Nmo led to a loss of bristles phenotype, which was caused by a failure to form proneural clusters during the early stages of SOP development. Expression of *E(spl)m8* is necessary for N-mediated lateral inhibition, and it is expressed in all cells of the proneural cluster except for the SOP. Strikingly, in *sca>nmo* wing discs (Fig. 4-5C), *E(spl)m8* expression was reduced compared to wildtype (Fig. 4-5A). Despite lower levels of *E(spl)m8* in *sca>nmo*, coexpression of Nmo did not enhance the ectopic macrochaetae phenotype of *sca>Dl*^{DN} , rather fewer bristles were observed compared to *sca>Dl*^{DN} alone (Fig. 4-4E-F). This could be due to a conversion of the SO lineage, specifically, a switch from the pIIa (socket and shaft) to pIIb (neural fate). Additional immunohistochemical analysis of the various progenitor and neural markers are necessary to elucidate the mechanism of this interaction.

To determine if elevated levels of Nmo can generally inhibit the N signal, *E(spl)-m8* levels were assessed in *ap>nmo* discs. *ap-Gal4* is expressed during the earlier stages in the wing disc in a broader domain than *sca-Gal4*, which is expressed only in the proneural clusters. In *ap>nmo* wing discs (Fig. 4-5B), *E(spl)m8* expression appeared similar to *w*^{1118} (Fig. 4-5A). Therefore Nmo's differential effect on the N pathway is highly dependent on the cellular context. I observed genetic interactions between *nmo*

and the N pathway suggesting a role for Nmo as both a positive and negative regulator of N signaling. This may be attributed to differences in the genetic contexts that were tested and further genetic assays in the appropriate setting are essential before any conclusions can be drawn. Future studies with *N* temperature sensitive alleles can be used to delineate *nmo*'*s* function during different stages of SO development.

It is possible that Nmo may exert its effect on other N responsive targets or Nmo may exert its effect on N via the regulation of additional signaling cascades. *ac* expression is regulated by several signaling inputs including Wg. Activation of the Wg pathway promotes the production of notal bristles, which is opposite to the effects of the N pathway (Phillips et al., 1999; Riese et al., 1997; Simpson and Carteret, 1989). Overexpression of Nmo in the wing primordium leads to a reduction in Wg gene targets, including Distalless (Dll) and genetic studies also revealed that Nmo can antagonize the Wg pathway to pattern the SOs of the notum (Zeng and Verheyen, 2004). Therefore, Nmo may modify N associated phenotypes indirectly by regulating other signaling networks, such as Wg to inhibit Ac expression and subsequently, neural specification. Additionally, it cannot be excluded that Nmo may function in SOP commitment, after *E(spl)m8* expression is initiated and the SOP has been selected from the proneural clusters. These studies clearly present evidence that Nmo functions at multiple branches during PNS development and I cannot exclude the possibility that Nmo may also play a role by regulating the activity of proneural proteins.

4.3.4 Nmo function is required to refine neural patterning

Several tiers of regulation exist to control the population of neural precursors. SOP selection and commitment is controlled through interactions between the proneural

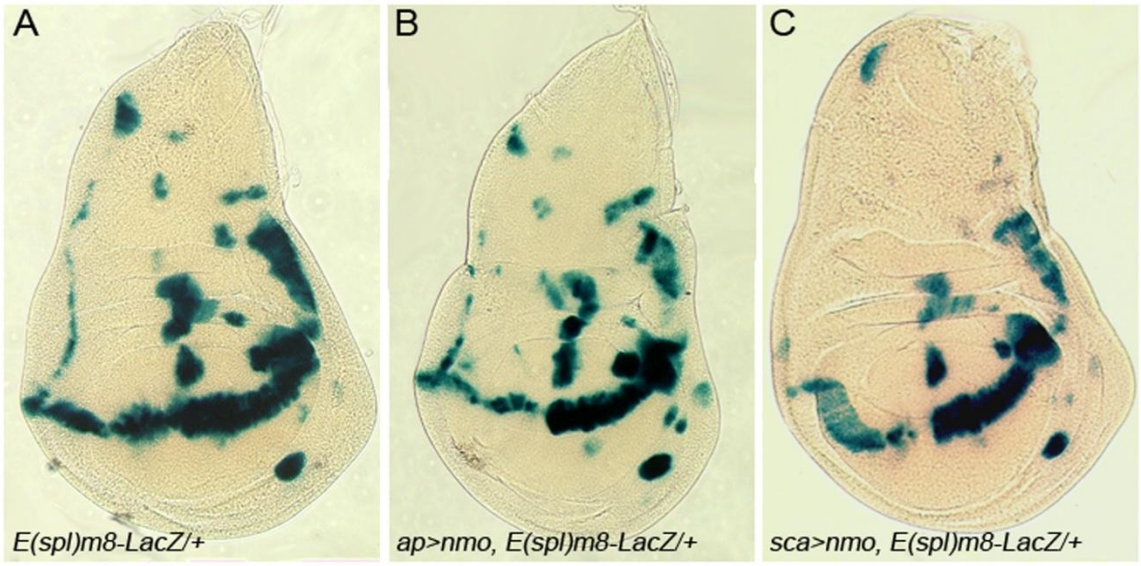

Figure 4-5 Modifying levels of Nmo has varying effects on *E(spl)m8* expression levels

The expression of the N target, *E(spl)m8-LacZ* was examined in (A) wildtype (B) *ap>nmo* and (C) *sca>nmo* wing discs. Misexpression of Nmo with *sca-Gal4*, but not *ap>Gal4* reduces *E(spl)m8-LacZ* expression in proneural clusters in the dorsalmost part of the wing disc.

bHLH proteins, Ac,Sc and Daughterless (Da) (Cabrera and Alonso, 1991; Caudy et al., 1988). These heterodimers activate the transcription of targets involved in SOP commitment. Sustaining high levels of Ac-Sc in the neural precursor is also critical for neural development (Skeath and Carroll, 1991). Sc expression within the SOP occurs through a self-stimulatory loop that is independent of the mechanisms controlling its expression in proneural clusters (Cubas et al., 1991; Culi and Modolell, 1998; Skeath and Carroll, 1991). Other than N, several repressive systems such as Emc, also act to limit SOP development. Emc interferes with the transactivation activity of proneural proteins by forming inactive heterodimers and preventing their ability to bind to DNA, hence suppressing the transcription of their targets (Cabrera et al., 1994; Martinez et al., 1993; Van Doren et al., 1991; Van Doren et al., 1992). These various regulatory mechanisms establish a tightly controlled system to properly pattern the nervous system.

To examine whether Nmo can regulate the activity or interactions of proneural proteins, genetic interaction studies were performed between *nmo*, *ac*, and *emc*. Heterozygosity for the dominant gain of function mutation, ac^{Hw49c} induced the formation of extra sensory organs (Fig. 4-6D). Homozygosity for nmo^{adk2} partially suppressed the dominant ac^{Hw49c} phenotype and inhibited the formation of a subset of ectopic bristles (Fig.4-6E). These results suggest that Nmo is also necessary for Ac activity and it may be a bimodal regulator of Ac depending on the cellular context. Namely, these interactions suggest that Nmo promotes Ac in cells normally destined for the epidermal fate or non-SOP cells. To confirm whether Nmo positively regulates Ac activity, I then addressed whether Nmo could also suppress the effects induced by decreasing the activity of Ac antagonists, such as Emc. Similar genetic modifications are expected if Nmo promotes

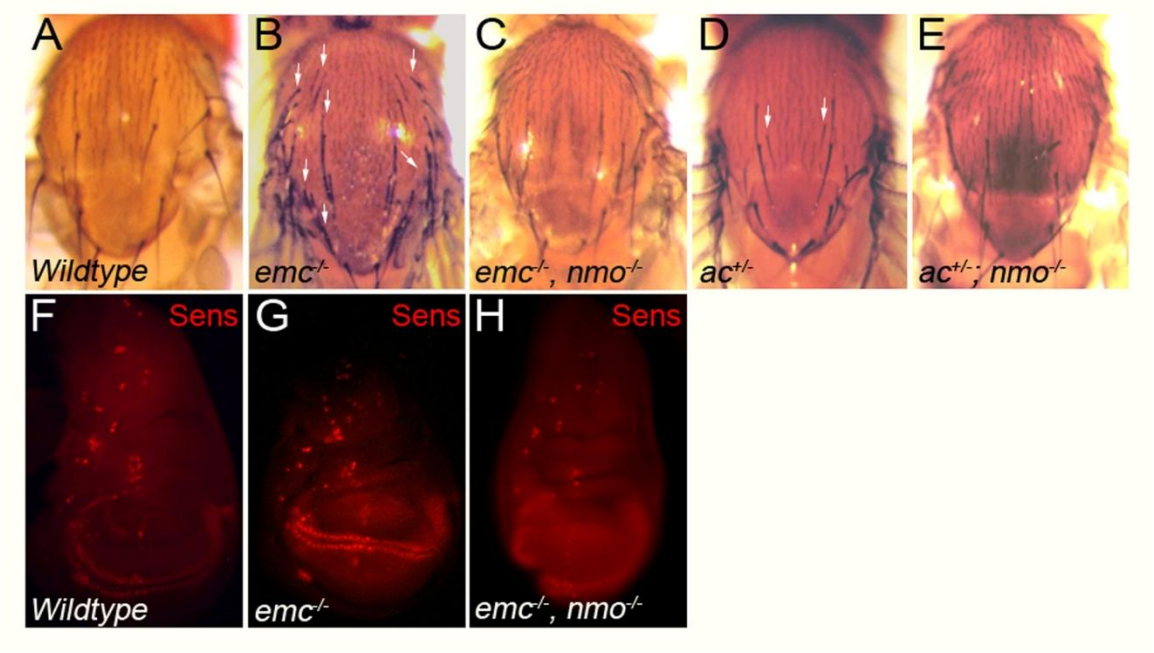

Figure 4-6 Nmo is required to promote Ac activity in epidermal cells

(A) Wildtype notum. Increasing Ac activity in either (B) emc1/emcpel or through (D) heterozygosity for the acHw-49c gain of function allele, leads to the formation of ectopic bristles, which are suppressed with loss of nmo (C, E). (F) Wildtype, (G) emcpel/emc1 and emcpel, adk1/emc1,adk1(H) wing discs were stained with the SOP marker, Sens. Homozygosity for nmoadk1 suppresses the formation of ectopic SOPs of emc mutants.

Ac activity directly or by positively regulating the formation of proneural transcriptional complexes. Similar to ac^{Hw49c} mutants (Fig. 4-6D), *emc* transheterozygous mutants also produce ectopic notal bristles (Fig. 4-6B). This phenotype results from an increase in SOP cells. emc^{1}/emc^{pel} wing discs stained with the SOP marker, Sens, display an increase in Sens expressing cells (Fig. 4-6G). Reducing *nmo* levels suppresses the ectopic bristles of *emc* transheterozygous adults (Fig. 4-6C), furthermore in these wing discs, the number of SOP cells is restored to near wild type levels (Fig.4-6F,H). These results suggest that Nmo antagonizes Emc to promote the specification of SOP cells in epidermal cells. Given Nmo's ability to suppress the ectopic bristles caused by elevated levels of Ac, either by increasing Ac activity or reducing Emc function, suggests that Nmo may be functioning outside of the SOPs, within the epidermal cells to promote the neural fate. In summary, these genetic interactions support a role for Nmo in refining the patterning of the peripheral nervous system.

4.3.5 Hipk antagonizes Nmo

We found that Hipk can antagonize Nmo in the wing (Lee et al., 2009b). Overexpression of Nmo in the wing can lead to mild wild notching and vein patterning defects that are suppressed by misexpressing Hipk (Fig.3-3M-O). In an attempt to delineate the regulatory interactions between these two kinases, further genetic studies were undertaken in other tissues. While *hipk* mutant clones and misexpression analysis provided evidence for a potential role for Hipk in specifying the mesothorax bristles, the underlying mechanism of its role has not yet been established. Reducing or elevating levels of Hipk both led to a loss of bristle fate (Fig. 4-7B,C). As well, loss of *hipk* also induced the formation of ectopic sockets (Fig 4-7B). It is tempting to speculate that Hipk

may stimulate N activity, similar to its role in the eye (Lee et al. 2009a). Overexpression of Hipk phenocopies the effects induced by elevating N signaling in the developing notum. Increasing levels of Hipk by misexpressing 2 copies of Hipk with *sca-Gal4* led to the absence of neural fate in the notum primordium as revealed by preliminary immunohistochemical staining with the neuronal marker, anti-sens antibody (data not shown). These adults produce a nearly naked notum lacking most of the mechanosensory organs (Fig. 4-7C). I found that coexpressing Nmo partially suppresses *sca>2x hipk* neuronal phenotype, resulting in the restoration of bristle like structures (Fig. 4-7E). Therefore Nmo and Hipk also exert opposing effects during the patterning of the peripheral nervous system.

Biochemical studies will begin to unravel the mechanism underlying the inhibitory effects of these kinases on one another's activity. Given that overexpression of Nmo suppresses the effects of elevated Hipk across tissues, it is likely that Hipk may directly regulate Nmo activity or vice versa. It has been demonstrated that *Drosophila* Hipk and Nmo bind in coimmunoprecipitation assays in HEK293 cells (Maryam Rahnama, unpublished). It will be noteworthy to perform further biochemical assays, such as in vitro kinase assays to determine if Nmo is a substrate for Hipk mediated phosphorylation. Identification of these sites will be crucial to examine the physiological relevance of this phosphorylation event.

We cannot exclude the possibility that Hipk and Nmo may invoke different cellular responses through its differential regulation of the same targets. This may be processed through phosphorylation which would modify the binding capacity of the transcription factors to their respective co-activators and repressors. Therefore, the

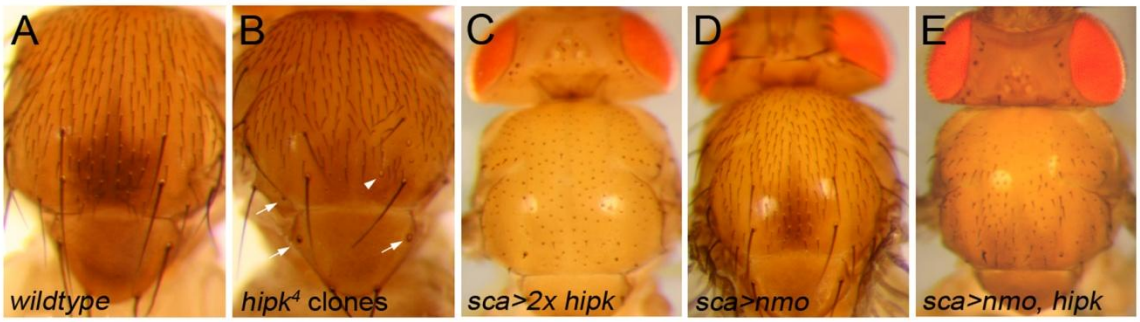

Figure 4-7 Hipk antagonizes Nmo in the adult peripheral nervous system

(A) Wildtype notum. (B) Bristle loss (arrows) and ectopic sockets (arrowhead) are present in adults bearing *hipk*^{4} clones. (C) Overexpressing 2 copies of *hipk* with *sca-Gal4* leads to a naked cuticle phenotype, which is suppressed with the coexpression of Nmo.

unique composition of the transcriptional complexes may be altered by the activity of each of these kinases.

4.4 Conclusions

These studies uncovered a dynamic role for Nmo during the development of the adult PNS. Nmo is essential to maintain the population of neural precursors during the early stages of SO development, as reducing *nmo* levels led to duplicated bristles, while its misexpression inhibits SOP selection by downregulating Ac expression levels. Genetic interaction studies also demonstrate that Nmo has multiple functions in the PNS, since loss of *nmo* is sufficient to suppress the effects of elevated proneural activity. These results suggest that Nmo is also required to sustain and promote Ac-Sc activity. Depending on the cellular context, Nmo can exert varying effects on the neural program, possibly through its dual regulation of Ac. The multifunctional nature of Nmo during PNS development reflects its complex interactions with the N pathway. In summary, Nmo acts at multiple stages of SO development to refine the patterning of the nervous system.

4.5 Contributions

I contributed to all data presented in this chapter.

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CHAPTER 5
GENERAL CONCLUSIONS

5.1 Conclusions

Development of a multicellular organism is a precisely regulated process, requiring the synchronized interplay between several key signal transduction pathways. Investigation into how these signals are regulated is vital to the global understanding of normal development. Deciphering the developmental circuitry has broader implications, since deregulation of conserved signaling cascades can lead to cancer onset and progression. My research uncovered Homeodomain-interacting protein kinase (Hipk) as a key component of a complex regulatory network, crucial for mediating signaling events during *Drosophila* development.

Members of the Hipk family, most notably Hipk2, are implicated as critical factors in transcriptional regulation to control cell growth, apoptosis and development (Calzado et al., 2007; Rinaldo et al., 2007). Hipk2 in cell culture studies has emerged as an interactor for a growing list of proteins, however it is fundamental that these biochemical analyses are complemented with studies *in vivo* to assess the physiological significance of these interactions. In hematopoietic cells, the Nemo-like kinase functions in a kinase cascade downstream of its activator Hipk2 (Kanei-Ishii et al., 2004). To address whether this pathway is conserved in *Drosophila*, I generated a series of *hipk* alleles, which revealed an essential requirement for *hipk*. These alleles also uncovered a role for Hipk in patterning events throughout the life cycle of the fly. Contrary to what

was initially expected, I have found that Hipk antagonizes Nmo in several tissues. The project has led me to discover novel roles for Hipk in both the Notch (N) and Wingless (Wg) pathways. My research has revealed that Hipk utilizes unique mechanisms to regulate gene transcription depending on the cellular context. Two of these methods are through its interaction with components of the transcriptional complexes or through its role in protein stability. Paramount to these studies is the revelation that aspects of these regulatory mechanisms discovered in *Drosophila* are functionally conserved in the mammalian system.

Futhermore, I identified Hipk as a key player in organ and tissue growth in *Drosophila*. In the developing visual organ, Hipk promotes global growth by promoting the transduction of the N pathway through the inhibition of the global corepressor Groucho. Hipk can also induce outgrowths in the wing, although the underlying mechanism is still not known. Studies within the wing revealed that Hipk also controlled the signaling output of the Wg pathway by enhancing the stability of Arm, a fundamental effector of the pathway. Moreover, a cross-species conservation of Hipk and mHipk2 was found and both proteins can be used interchangeably to activate Tcf/Lef1-mediated transcription. Observations that Hipks could also downregulate Wnt signaling (Wei et al., 2007) suggest that the effects of Hipks on signal transduction is cell type and target gene dependent. These discrepancies may be attributed to the availability of transcription factors and co-factors in the experimental systems tested. Furthermore, I have also observed similar dual roles for Hipk with respect to the N pathway. While Hipk promotes N signaling activity in undifferentiated and differentiating cells near the morphogenetic furrow, *hipk* mutant cells in photoreceptors clusters located more

posteriorly in the *Drosophila* eye disc can also lead to the upregulation of the N target E(spl)-C. These observations highlight the multifaceted nature of Hipk: that the protein kinase may be used to both trigger and terminate an identical developmental program. Utilizing a single regulator for such an event ensures that discrete biological processes are precisely synchronized to properly achieve the appropriate fate. These findings raise the question of how Hipk specificity and function is regulated. Analyses within the eye disc would be valuable given that cell populations can be monitored simultaneously as they progress through different stages of the same developmental program. Progression through each phase requires changes in the transcriptional profile, which are highly dependent on dynamic signaling pathway activities, hence the response of Hipk to the signaling networks can be monitored. Although many substrates of the Hipk family have been discovered, the mechanisms underlying Hipk regulation remains unclear. Future studies are necessary to identify key regulators of this kinase and address how Hipk responds differentially to various signaling input to specify its function.

Hipk may elicit a variety of effects on the same process by numerous mechanisms. Hipk phosphorylates both the N and C termini of Arm, thus Hipk may exert multiple functions by phosphorylating multiple target residues on a single substrate. Identification of the Hipk phosphorylation sites will be invaluable to deciphering how Hipk proteins affect the signaling capacity of the Wg/Wnt effectors. Also, given the dynamic localization of Hipks in the nucleus, nucleoplasm and in cytosolic speckles (Kim et al., 1998), it is tempting to speculate that the specific functions of Hipks are determined in part through its subcellular localization.

Hipk proteins are emerging as important components of multiple signaling networks, and another dimension of Hipk2 function is through its ability to function in a kinase independent manner. Therefore, it is also possible that Hipk/Hipk2 may act as a scaffolding protein, bringing together multiple binding partners and this may allow it to integrate multiple signaling inputs to achieve the proper cellular response. Further biochemical studies and transgenic studies with a kinase dead version of Hipk will begin to unravel the molecular significance of the unique functions of Hipk

The ability of Hipk to control different developmental events in a temporally and spatially specific manner requires coordination between numerous regulators. However, the identity of these key regulators have remain to be identified, most studies to date have focused on the regulation of Hipk in response to DNA damage (Sombroek and Hofmann, 2009). It is essential to expand the examination of the mechanisms controlling Hipk activity to other cellular scenarios.

Here, I describe a role of Hipk as a regulator of both the Wg and N signal and shed light on the regulatory mechanisms governing these conserved pathways. Given that Hipk is a critical participant in several signal transduction pathways, the protein kinase is likely vital for the efficient integration of multiple signaling pathways, possibly through its regulation of Gro. In addition, several *Drosophila* Receptor Tyrosine kinases (RTK) signals reduce the repressive capacity of Gro by inducing MAPK-directed phosphorylation of Gro at sites distinct from Hipk (Cinnamon et al, 2008; Hasson, et al., 2005). It is well known that downregulation of Gro by MAPK impinges on the transcriptional output of other pathways (Hasson et al., 2005; Orian et al. 2007), therefore, Gro is poised at the junction between signaling pathways to mediate crosstalk.

Potentially, phosphorylation of Gro by Hipk may represent a widespread mechanism to control gene expression.

Given that multiple members of the Hipk family exist in higher organisms, the existence of a sole *Drosophila* Hipk protein makes this a powerful model to study the role of Hipk proteins in conserved signaling circuitry.

5.2 References

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CHAPTER 6
MATERIALS AND METHODS

6.1 Fly stocks

Fly strains used in the various crosses were *hipk*^{1} (*P{GT1}CG17090*^{BG00855}), *UAS-fng*^{22}, *ey-Gal4*, *omb-Gal4*, *69B-Gal4*, *ap-Gal/Cy*o:*TM6B,* UAS-GFP, *pnr-Gal4*, *UAS-Dl*^{DN}, *ac*$^{Hw-49c}$, *emc*^{1}, *ey-flp, N; GFP FRT79/TM6B*, (Bloomington Drosophila Stock Center), *UAS-N*^{act}, *UAS-N*$^{[DN]}$ and *UAS-H*, *sca-Gal4* (Go et al., 1998), *Dl*^{eA7} (Verheyen et al., 1996), *UAS-Dl*^{17c} (Doherty et al., 1996), *UAS-ey*, *UAS-gro*^{WT} (*UAS-gro*^{PH1}*;* Hasson et al., 2005) and *UAS-eyg* (Aldaz et al., 2003; Jang et al., 2003), *sd-Gal4* (*sd*$^{SD29.1}$), *bs-gal4* (also referred as *1348-gal4;* Huppert et al., 1997) *UAS-Arm*^{S2}*/Cyo* and *UAS-Arm*^{S1r0} (Pai et al., 1997), *UAS-Daxin*$^{A2-4}$ (Willert et al., 1999), *UAS-Dfz2N33/Cyo* (Zhang and Carthew, 1998), *emc*^{Pel} (from A. Garcia-Bellido, Cubas et al., 1991), *E(spl)m8-LacZ* (from H. Bellen), *UAS-ed* (Escudero, et al., 2003), *UAS-nmo*$^{C5-1e}$ (Verheyen et al., 2001), *UAS-hipk RNAi* (Vienna Drosophila RNAi Center, National Institute of Genetics), *nmo*^{DB24} (Zeng et al., 2004), *nmo*^{adk2} (Verheyen et al., 2001), *hs-Flp22*; *GFP, FRT79/TM6B, hipk*^{3}*/TM6B, hipk*^{4}*, FRT79/TM6B.* All wild-type flies are *w*^{1118}, and all crosses were performed according to standard procedures at 25°C. In assays examining interaction between two UAS transgenes, control crosses were performed with *UAS-lacZ*, to rule out suppressive effects due to titration of Gal4.

6.2 Generation of *hipk* alleles

hipk^{2} and *hipk*^{3} were generated through the imprecise excision of *hipk*^{1} (*P{GT1}CG17090*^{BG00855}), a P-element located within the first intron of *hipk* (Fig. S1). *P{GT1}CG17090*^{BG00855} is homozygous lethal and transheterozygosity with *Df(3L)ED4177* also results in lethality. The precise excision of the P-element results in the reversion of this phenotype, demonstrating that the insertion of this P-element within the *hipk* locus is responsible for the lethality. To generate *hipk* deletion alleles, *w; P{GT1}CG17090*^{BG00855}*/TM6B* females were crossed to *w; Δ 2-3, Sb/TM6B,e* males to mobilize the P-element. Single *w; P{GT1}CG17090*^{BG00855}*/Δ 2-3, Sb* mottled eyed males were selected and crossed to *w; TM3/TM6B* virgins. Single *w; P{XP}***/TM6B* white eyed male were crossed back to *w; TM3/TM6B* virgins to create stable *w; P{XP}*/TM6B* lines (P{XP}* represents the excised P-element). To genetically test for the presence of a deletion mutation in the *hipk* locus, *P{XP}*/TM6B* males from each stock were crossed to *w; P{GT1}CG17090*^{BG00855}*/TM6B* and non *TM6B* flies were tested for lethality.

In wild type flies, 5BGF (TCTGTCCACGACGCAAGTTTTCCTCAG) and 5BGR (CGCTTGTTCTTGCCGCTGTTATTGTCC); 3BGF (AAAAACCCCAATGCAAGCCAACTGAGT) and 3BGR (ACGGCGCGTGTGTGATAACGATAACTC) primer pairs generate a 1.0 Kb and 750 bp PCR product, respectively. These primers were used to initially characterize the excision event in our mutant lines by looking for smaller or absence of PCR products compared to wildtype. Primers spanning the interval of the deletion were designed and used to generate a PCR product that was then sequenced to determine the extent of the deletions.

hipk^{2} is a deletion spanning at least 16.5 kb downstream of the P-element, including the first 6 coding exons. The Gal4 coding sequence of the P-element is retained in *hipk*^{2}.

The *hipk*^{3} deletion extends 4.25 kb from the 5' end of the P-element. This disruption spans 3.73 kb of the *hipk* locus and 370 bp of the neighbouring gene, *Cyclophilin-like* (*cycl;* Fig. S1). At least 4.34 kb of the P-element is retained in this mutation.

hipk^{4} was generated via recombination between *Pbac{WH}CG17090*^{f04609} and *Pbac{WH}*^{f03158} (Thibault et al., 2004). Targeted deletion was made as described by Parks et al. (2004) and led to the elimination of 19.9 kb of the *hipk* genomic region, which includes exons 4 to 13. PCR amplification with appropriate primers spanning the various deletions was performed to confirm the extent of the deletions. Putative deletions were crossed to *hipk*^{1} and *hipk*^{3} and lethality was tested for the presence of the deletion of *hipk.*

6.3 Generation of *hipk* transgenic lines

The C-terminus of *hipk* was PCR amplified from the LD08329 partial cDNA clone (Drosophila Genome Research Center) using the HipkR primer, GAATTCCTACTCAGCCCCATACCATATG and the T7 primer, TAATACGACTCACTATAGGG. LDO8329 was lacking the 169 bp N-terminal fragment which was amplified from genomic DNA using the following primers: HipkF, GAATTCAAATGAAAACGTCCTACCCCCC and Ex3R, GTTTTGACGTTTTCGCTTGCTGGTTGCAGCAG. Both PCR products were inserted

into pDrive and digested with EcoRI and MluI, then ligated into the EcoRI sites of pCMV-HA. The full length *hipk* cDNA and the HA tag was then subcloned into the Xba I site of pUAST. Transgenic lines were created by BestGene Inc.

6.4 Mapping of Gro phosphorylation sites

To analyze the phosphorylation of Gro, recombinant Gro proteins were incubated with recombinant GST-Hipk in 20 µl kinase buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 10 mM $MgCl_2$) and 3 µCi $^{32}P\gamma ATP$ for 3 hours at room temperature. The reaction was stopped by the addition of sample loading buffer. Proteins were heated for 10 min at 95^{o}C, separated through SDS PAGE electrophoresis and visualized by autoradiography.

Synthetic Gro decapeptides were synthesized and immobilized on a cellulose membrane according to the SPOT method (Frank, 2002) with a partially automated synthesizer (Abimed Auto-Spot Robot ASP 222) as recommended by the manufacturer (Abimed GmbH, Langenfeld, Germany). The membrane was incubated for 1 hour in methanol and 1 hour in kinase buffer supplemented with 1% BSA. Peptides were then subjected to a kinase assay by incubating the membrane for 3 hours at room temperature in 1 ml kinase buffer with recombinant GST-Hipk and 20 µCi $^{32}P\gamma ATP$. The membrane was then washed three times for 10 minutes with kinase buffer + 1M NaCl. Phosphorylated peptides were visualized by autoradiography.

6.5 Generation of *gro* transgenic lines

We obtained a *gro* cDNA clone from A. Nagel (Nagel et al., 2005). The site-directed mutagenesis of serine 297 and threonine 300 was performed using the Quick Change site-directed mutagenesis kit (Stratagene), using the following primers:

GroAA 5´ GTTCGTCACGTTCCACACCCGCTCTCAAGGCTAAAGATATGGA

GroAA 3´ TCCATATCTTTAGCCTTGAGAGCGGGTGTGGAACGTGACGAAC

GroEE 5´ GTTCGTCACGTTCCACACCCGAACTCAAGGAAAAAGATATGGA

GroEE 3´ TCCATATCTTTTTCCTTGAGTTCGGGTGTGGAACGTGACGAAC

The mutated *gro* cDNAs were cloned into pUAST and transformed into flies using standard methods.

6.6 Mosaic analysis

Somatic clones in the eyes were generated by crossing *hipk*^{4}*, FRT79/TM6B* to *ey-flp, N; GFP FRT79/TM6B* females. Somatic clones in the wing were generated by crossing *hsflp.22; GFP FRT79/TM6B* females to *hipk*^{4} *FRT79/TM6B* males and progeny were collected for 24 hr and heat shocked at 38°C for 90 minutes at 48 AEL. Imaginal discs were dissected from wandering late third-instar larvae for immunohistochemistry as described below and adult flies were collected for phenotypic analyses.

6.7 Adult wing mounting

Adult wings were dissected in 100% ethanol followed by mounting in Aquatex (EM Science).

6.8 Immunostaining of imaginal discs

Antibody and X-gal staining was carried out according to standard protocols. The following primary antibodies were used: rat anti-Elav (1:100; DSHB), rabbit anti-β-galactosidase (1:2000, Cappel), rabbit anti-atonal (1:1000; Jarman et al., 1994), mouse anti-E(spl) mAb323 (1:10; Jennings et al., 1994), rabbit anti-phospho-histone 3 (1:1000,

Upstate Biotechnology), rabbit anti-drICE (1:2000, Yoo et al., 2002), guinea pig anti-Eyg (1:200; Dominguez et al., 2004), rat anti-Ser (1:1000; Papayannopoulos et al., 1998), mouse anti-Dac (1:75, DSHB), 1:50 mouse-anti Achaete (concentrated supernatant;DSHB), 1:200 mouse anti-Armadillo (concentrated supernatant;DSHB), 1:400 mouse anti-Distalless (Duncan et al., 1998), and 1:1000 mouse c-myc (Sigma-Aldrich) Secondary antibodies (Jackson Laboratories) were used at 1:200. The secondary antibodies were used as follows: anti-mouse CY3 (Molecular Probes), anti-rabbit CY3 (Jackson Immunolabs), anti-mouse HRP (Jackson Immunolabs). All secondary antibodies were used at 1:200 dilution. Imaginal discs were mounted in 70% glycerol.

6.9 in situ hybridization

RNA probes were generated using the Roche DIG RNA transcription kit and fluorescent in situ hybridization (FISH) hybridization was performed according to Hughes and Krause (1999).

6.10 Generation of Arm truncation constructs

Kpn I or Bgl II restriction sites were generated in pCMV-HA-Arm full length by site directed mutagenesis with QuikChange XL II site directed mutagenesis kit (Stratagene). Mutagenized plasmids were linearized and religated to generate Arm deletion constructs. For Arm ΔC, full length Arm was cut with Tth III and Kpn I and then religated after blunting the ends. Mutagenesis primers used were:

Arm N term/repeat 1 F1.Bgl II (n465) : ccaggacgacgctgagatctcaaccagggccatacc

Arm N term/repeat 1 R1.Bgl II (n465) : ggtatggccctggttgagatctcagcgtcgtcctgg

Arm N term/repeat 1 F1. Kpn I (n468): cgacgctgagctgggtaccagggccatacc

Arm N term/repeat 1 R1.Kpn I (n468): ggtatggccctggtacccagctcagcgtcg

Arm repeat end F2. Kpn I (n=1938): cgagattatcgagcaggtacccgccactgggccgctga

Arm repeat end F2. Kpn I (n=1938): tcagcggcccagtggcgggtacctgctcgataatctcg

Construct	Primers	Restriction enzyme(s)	Size (amino acids)
1. Arm ΔN	**Arm N term/repeat 1 F1/R1.Bgl II (n465)**	Bgl II	155-843
2. Arm-N	**Arm N term/repeat 1 F1/R1. Kpn I (n468)**	Kpn I	1-156
3. Arm ΔC	**n/a**	Tth III, Kpn I	1-730
4. Arm-R	* Used template from construct 1 **Arm repeat end F2/R2. Kpn I (n=1938)**	Kpn I	155-646

6.11 Cell culture and in vitro biochemical assays

HEK293T cell culture, protein expression, immunoprecipitations and kinase assays, with *Drosophila* Hipk, were performed according to Zeng et al., (2007). The Hipk kinase dead construct (HA-Hipk-KD, EGFP-Hipk KD (Choi et al., 2005) contains a K221R mutation within the catalytic ATP-binding site. For experiments using expression plasmids encoding mammalian cDNA, HeLa cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. Cells were then transiently transfected with Polyethylenimine MW 25000 (Polysciences, Inc.). For co-immunoprecipitation assay, cells in 100-mm-diameter plates were transfected with the expression plasmids encoding mammalian cDNA (12 μg). For reporter assays, cells in 6

well 35-mm-diameter plates were transfected with the expression plasmids encoding mammalian cDNA (1 μg). Details on generation of truncation constructs provided in supplementary materials.

6.12 Transcription assays

HEK293T and HeLa cells were cultured in 6 well (35 mm diameter) plates and transiently transfected with Polyfect (Qiagen) or Polyethylenimine MW 25000 (Polysciences, Inc.) according to manufacturer's manual. Cells were transfected at 24 h after seeding with the TOPFLASH or FOPFLASH reporter gene plasmids along with each expression vector as indicated. *Drosophila* S2R+ cells were seeded (1×10^6) in 6-well plates and maintained at 25°C in Schneider's *Drosophila* medium supplemented with 10% heat-inactivated FCS (Invitrogen) and 125 μg/ml Hygromycin B (Sigma). Before transfection, cells were re-seeded in S2 conditioned media or Wingless-conditioned Schneider's medium. Cells were transfected with 40 ng of dHipk, 0.4 μg of mHipk, using the Effectene transfection reagent (Qiagen) according to the manufacturer's instructions. 8 h post transfection, the induction of genes under the control of the metallothionein promoter was performed by supplementing the medium with $CuSO_4$ at a final concentration of 0.5mM. For both mammalian and *Drosophila* cells, total DNA concentration was kept constant by supplementation with empty vector DNAs. Luciferase assays were performed with the Dual Luciferase Reporter assay system (Promega) according to manufacturer's instructions and as described in Korinek et al. (1997). The renilla luciferase pRL-CMV or pRL-EF vector was used for normalizing transfection efficiencies. The values shown are the average of one representative experiment in which each transfection was performed in duplicate or triplicate as noted.

6.13 Protein stability Assay

For examination of protein stability, HEK293T and HeLa cells were transfected in 60 mm plates with 1.5-2 μg per construct and empty vector was added to maintain a constant concentration of DNA. Cells were treated with 25 μg/mL cyclohexamide after 24 hr of transfection and harvested at the indicated times after treatment. Whole cell lysates were analyzed by SDS PAGE electrophoresis and immunoblotted with appropriate antibodies.

6.14 References

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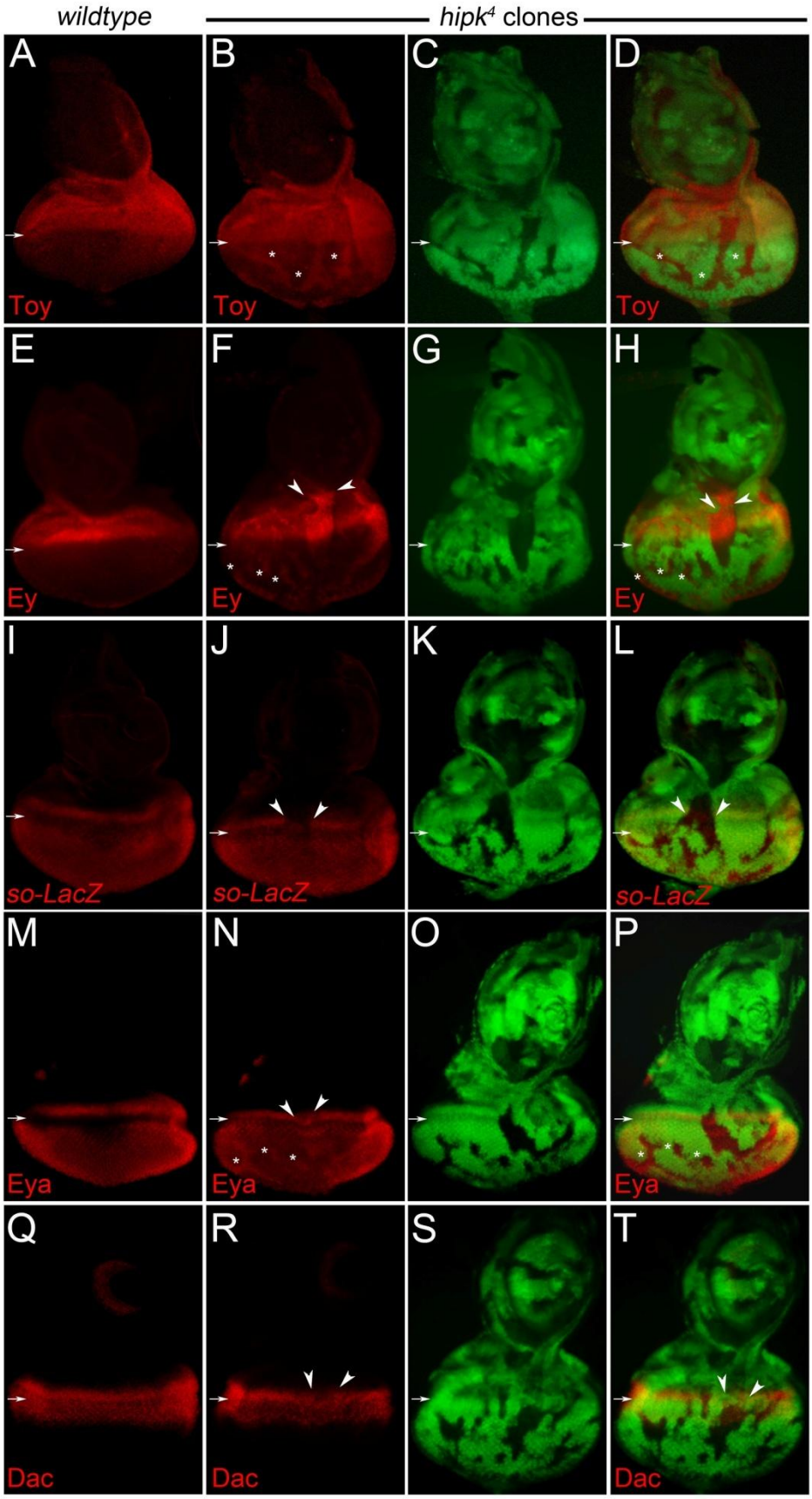
Appendix:


wildtype
hipk4 clones
A
B
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D
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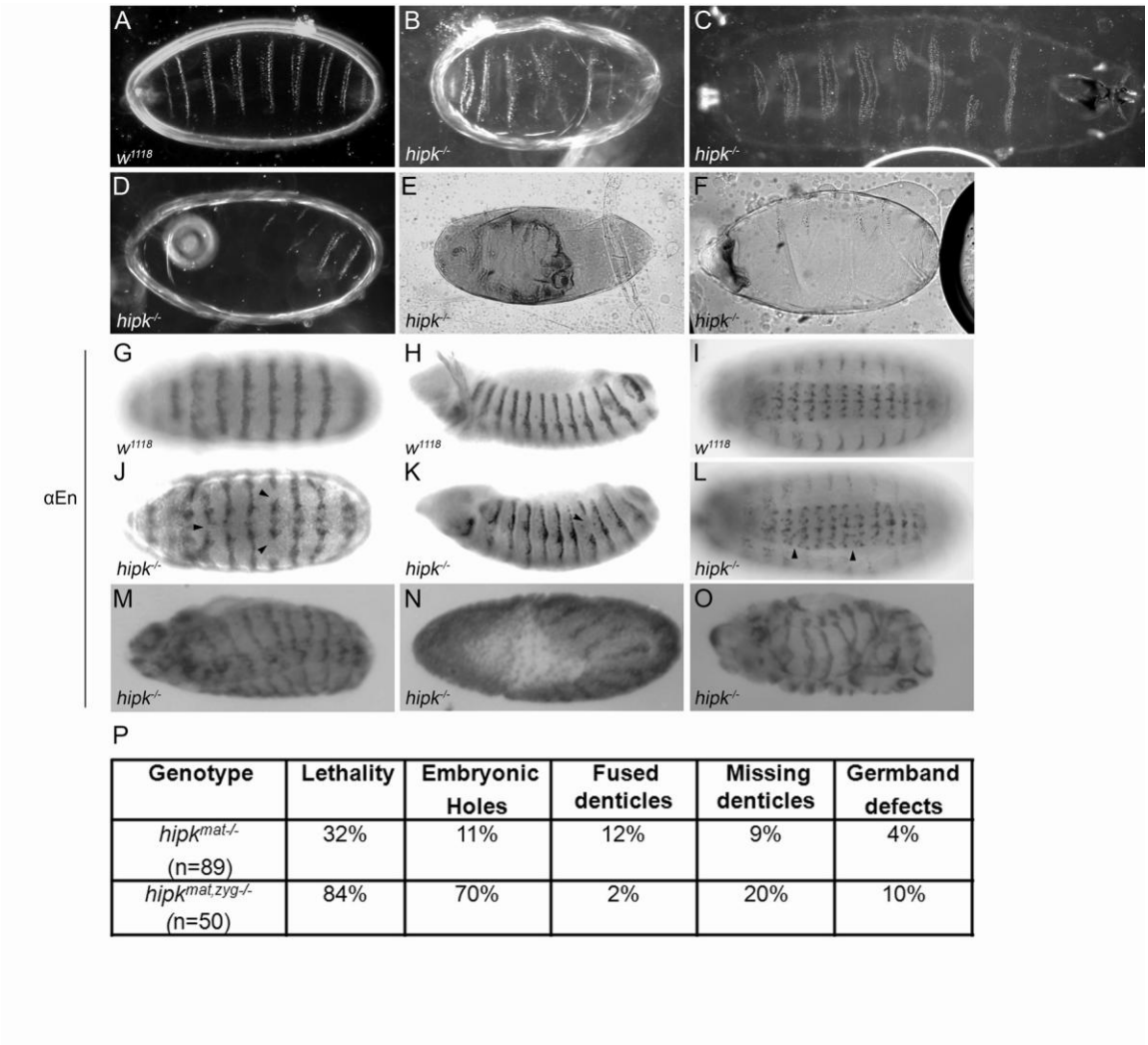
Dac

Figure A-1 Hipk modulates the expression of members of the Retinal Determination Gene Network (RDGN)

hipk^{4} mutant cells display elevated expression of the PAX6 homologs, Twin of Eyeless (Toy; B-D) and Eyeless (Ey; F-H), as shown by the arrowheads and asterisks. Expression of downstream targets of Ey including, *sine oculis* (*so*; J-L) Eyes absent (Eya; N-P), and Dacshund (Dac; R-T) are reduced in *hipk* somatic clones (arrowheads) near the morphogenetic furrow (MF). Posterior to the MF, *hipk* mutant cells exhibit elevated Eya expression (asterisks in M, O). Arrows mark the morphogenetic furrow.



P

Genotype	Lethality	Embryonic Holes	Fused denticles	Missing denticles	Germband defects
hipk$^{mat-/-}$ (n=89)	32%	11%	12%	9%	4%
hipk$^{mat,zyg-/-}$ (n=50)	84%	70%	2%	20%	10%

Figure A-2 Hipk is essential for embryogenesis

All embryos shown are derived from mothers bearing *hipk*^{4} germline clones crossed to either *w*^{1118} or *hipk*^{4}*/TM3, twi-GFP* males. Non GFP expressing *hipk*$^{mat/zyg-/-}$ embryos were selected for further analysis. Reducing both maternal and zygotic *hipk* induces multiple classes of embryonic phenotypes including denticle patterning defects (B, C), epidermal holes (E, F), and aberrant morphology induced by anomalies during gastrulation or germband extension and retraction processes (D and M-O). (G-O) Embryos were stained with an antibody against the segment polarity protein, Engrailed (En). Reduced *hipk* activity modified En expression throughout embryogenesis (arrowhead in J-O). Percentages of each phenotypic class are summarized in P.

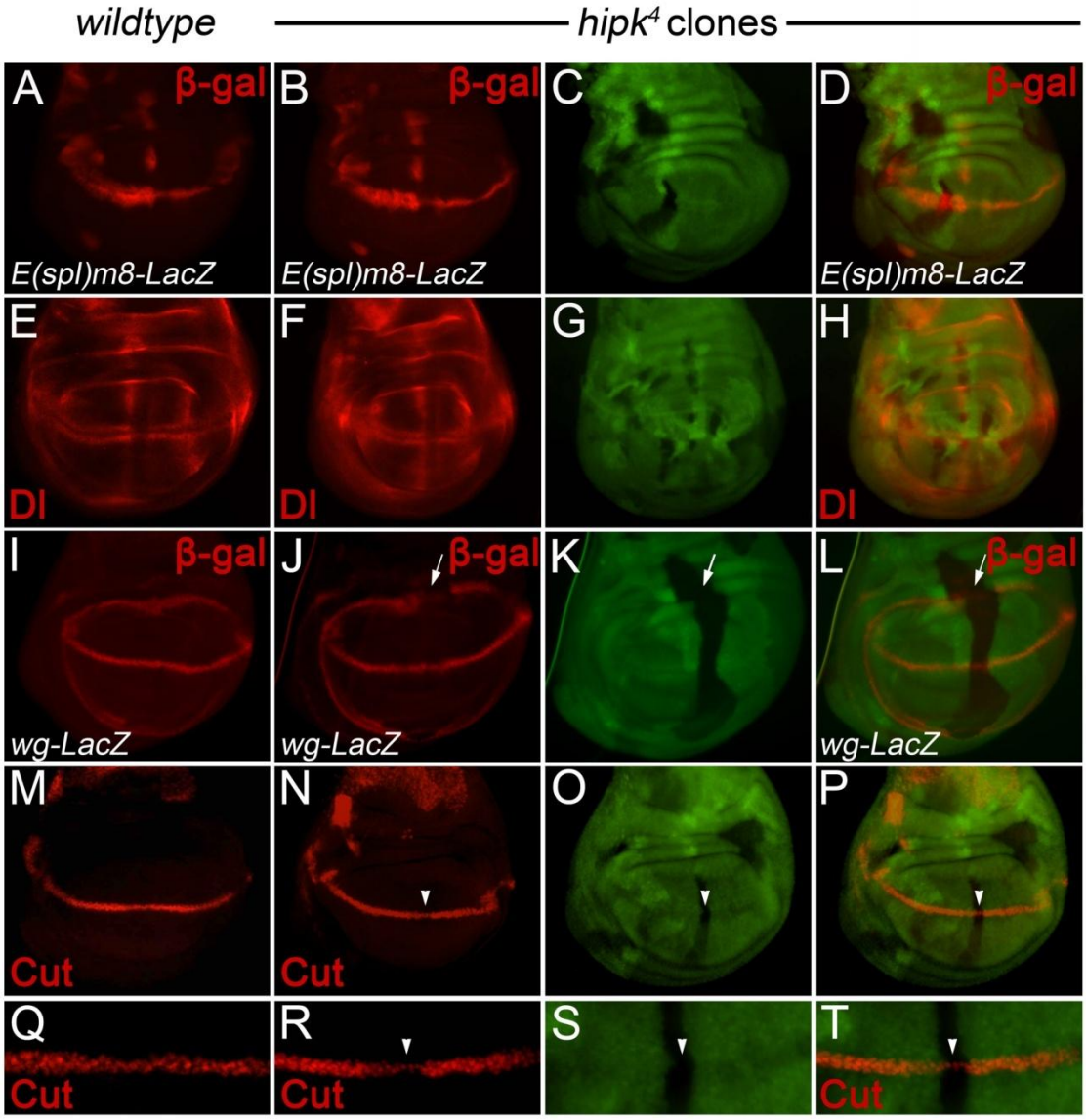


Figure A-3 Hipk regulates Cut expression and Notch signaling activity appears normal in *hipk* mutant cells in the wing

Antibody staining for Notch targets was performed in w^{1118} and $hipk^{4}$ mosaic wing discs. *(E)spl-m8-LacZ* (B-D) and Delta (Dl; F-H) expression was not altered in *hipk* mutant cells. *wg-LacZ* expression was reduced in the outer ring (arrow in J-L), and appeared normal in the wing margin primordium. Cut expression is slightly reduced in $hipk^{4}$ clones (arrowhead in N-P). (Q-T) Higher magnification of discs shown in M-P.

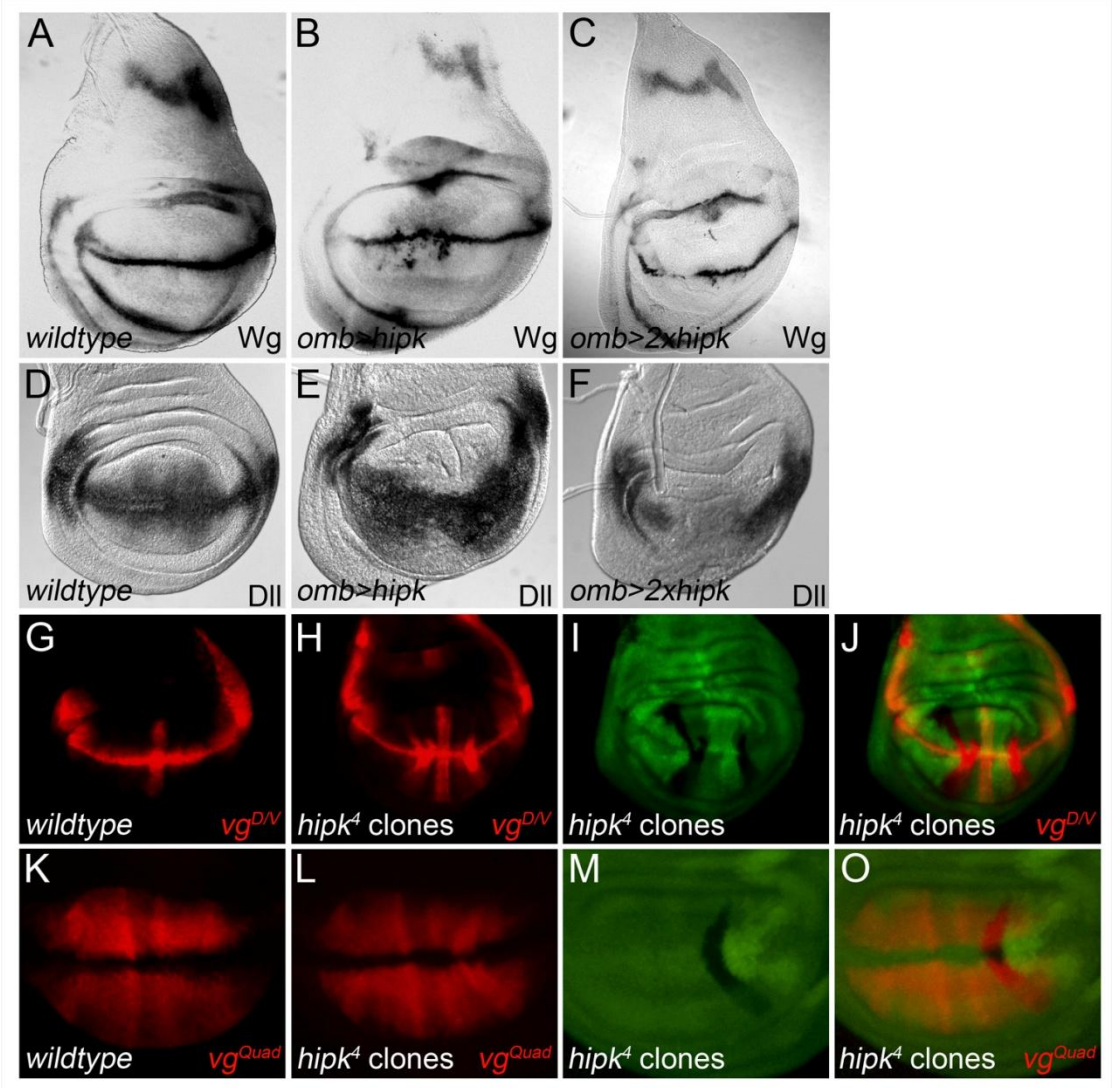


Figure A-4 Hipk regulates Wg and Vestigial expression

Wing discs were stained with the indicated antibodies. Overexpressing 1 copy of UAS-hipk expands Wg and Dll expression (B and E), while misexpressing 2 copies of UAS-hipk inhibits their expression at the intersection of the dorsal-ventral and anterior-posterior axes (C,F). Both vgQuad-LacZ and vgD/V-LacZ expression is upregulated in hipk mutant clones (H-J and L-O).

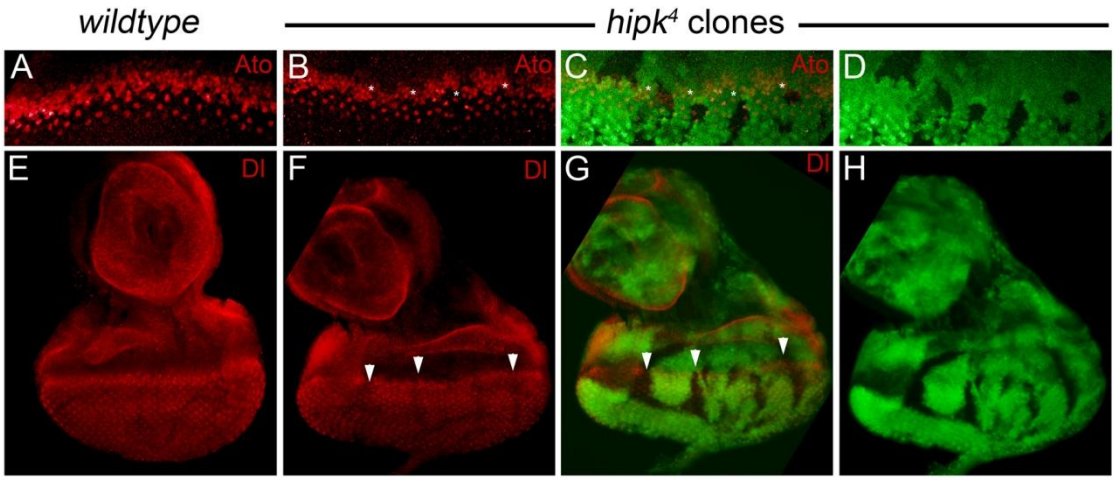


Figure A-5 Hipk regulates Atonal and Delta expression in the developing eye disc

(A, E) w^{1118}. (B-D, F-H) $hipk^{4}$ somatic clones. Decreasing *hipk* activity reduces the expression of the proneural marker, Atonal (Ato; asterisks in B-D) and the N ligand, Delta (Dl; arrowheads in F-H).

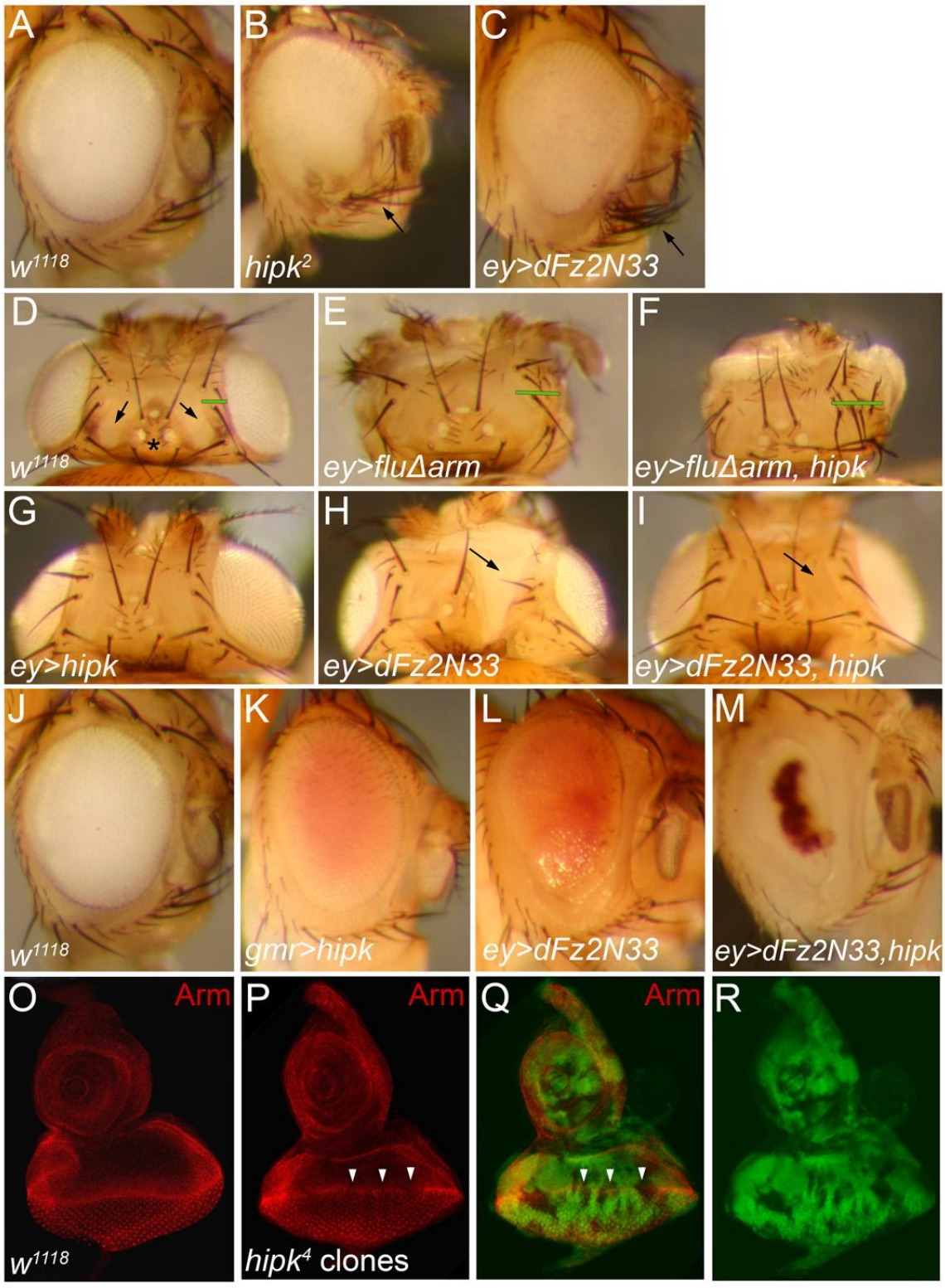

A
w1118
B
hipk2
C
ey>dFz2N33
D
w1118
E
ey>fluΔarm
F
ey>fluΔarm, hipk
G
ey>hipk
H
ey>dFz2N33
I
ey>dFz2N33, hipk
J
w1118
K
gmr>hipk
L
ey>dFz2N33
M
ey>dFz2N33,hipk
O
Arm
w1118
P
Arm
hipk4 clones
Q
Arm
R

Figure A-6 *hipk* synergizes with Wg signaling to promote head fate and Arm protein levels in the developing eye

hipk homozygotes produce extra macrochaetes on the ventral orbital cuticle (arrow in B), similar to the effects induced by misexpressing a dominant negative form of the Fz2 receptor (C). (D) The region of the head between the compound eyes is called the head vertex, consisting of structural landmarks subdivided along the medial-lateral axis. The head vertex consists of the lateral orbital cuticle (green line) with their organized array of bristles, the dorsal frons cuticle (arrows), composed of parallel ridges devoid of bristles and the medial region (asterisks) houses the ocelli and its surrounding sensory organs. Wg specifies the mediolateral region of the head and elevated levels of the signal expand the lateral orbital structures (green line in E) relative to wildtype (D). Elevated levels of *hipk* enhances the *ey>fluΔarm* head phenotype (F). Reducing the Wg signal by misexpressing a dominant negative form of the Fz2 receptor leads to the loss of frons cuticle (arrow in H), which is rescued with Hipk coexpression (I). Wg promotes apoptosis of the peripheral ommadia during mid-pupation. (K) *gmr>hipk* adults display a rough eye phenotype. (L) DecreasingWg signaling activity produces a small, glossy eye phenotype. (M) Simultaneously misexpressing hipk enhances the small eye phenotype caused by ectopic expression of DFz2N33. (P-R) *hipk* somatic clones in the eye disc display reduced Arm protein levels.