Hip Hop: Hipk promotes tumourigenesis in Drosophila through JAK/STAT signaling

by

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Abstract

Signal transduction pathways are crucial for co-ordinated development and growth of multicellular organisms. Dysregulation and mutations of components in these pathways can often lead to tumourigenesis. Evolutionarily conserved Homeodomain-Interacting-Protein-Kinase (Hipk) is a strong growth regulator of many signal transduction pathways, and elevated levels of hipk lead to tumour-like masses. While many known regulators of Hipk exist, we attempted to identify novel phospho-regulators of Hipk activity. Here we present evidence that Hopscotch, a core tyrosine kinase of the JAK/STAT cascade, is a putative phospho-regulator of Hipk activity in multiple contexts. We show that modulation of Hipk expression levels modifies JAK/STAT activity in both wildtype and tumourous tissues. Finally, we show that Hipk interacts with the JAK/STAT transcriptional effector STAT92E. Thus, our work provides a role for Drosophila Hipk in tumourigenesis and regulation of the JAK/STAT cascade.

Dedication

I dedicate this to family and friends for their constant support throughout the years.

Acknowledgements

I would first and foremost like to thank my senior supervisor, Dr Esther Verheyen for her consistent support and inspiration since the beginning. Your teachings provided me with the ability to critically analyze situations and develop strategies to solve problems, in and outside of the lab. During difficult times, you were an unrelenting source of encouragement to keep going, and your passion throughout the years drove me forward. I am eternally grateful for all our discussions over the years, as they have helped shape me into a more observant, strategic, and politically active person. I would also like to thank members of my supervisory committee, Dr. Nancy Hawkins and Dr. Nicholas Harden, for their support and scientific suggestions over the years, which has contributed greatly to my scientific understanding and experimental designs.

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Finally I would like to thank my Mom and Dad, and my sisters Shannon and Julie, for their love and support throughout the years, as well as the constant reminders that I need to clean my place. Some things never change.

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List of Acronyms

Term	Initial components of the term
Act	Actin
APC	Adenomatous polyposis coli
CDK	Cyclin Dependent Kinase
CK1	Casein kinase
Dll	Distal-less
DNA	Deoxyribonucleic acid
Dpp	Decapentapalegic
Eyg	Eyegone
Flp	Flippase
FRT	Flippase recognition target
GFP	Green fluorescent protein
GSK3	Glycogen synthase kinase
Hipk	Homeodomain Interacting protein kinase
Hop	Hopscotch
Hpo	Hippo
IR	Interfering ribonucleic acid
JAK	Janus kinase
JH	Jak kinase homology
Mekk	Mitogen activated protein kinase kinase kinase
PEST	Peptide sequence rich in Proline, Glutamic Acid, Serine and Threonine
Ptc	Patched
RFP	Red fluorescent protein
RNAi	RNAi interference (interfering RNA)
Sav	Salvador
Sca	Scabrous
SCF^{Slimb}/βTrcp	Skp, Cullin, F-box containing complex/beta transducing repeats-containing proteins
SFU	Simon Fraser University
SH2	Src homology 2

SOCs	Supressors of cytokine signaling
SRC	Sarcoma
STAT	Signal transducer and activator of transcription
SUMO	Small Ubiquitin-Like Modifier
TAZ	Tafazzin
TCF	T-cell factor
UAS	Upstream Activating Sequence
Wg	Wingless
Wts	Warts
YAP	Yes-associated protein
Yki	Yorkie
Zw3	Zeste white 3

Chapter 1. Introduction

1.1. General Signaling Overview

The development of multicellular organisms is strictly governed by a complex set of signal transduction pathways. While several types of signaling pathways exist, such as intracellular, extracellular and intercellular signaling pathways, here we will focus on intercellular signaling pathways that commence with an extracellular signal. Extracellular signal transduction events initially begin when an extracellular ligand, (i.e growth factor, hormone or protein) triggers a cellular response by binding to a membranous receptor or receptor complex. Binding of ligands to their receptors can induce a conformational change in a membrane-associated protein, and in most signal transduction pathways, leads to events such as altered cellular metabolism, gene expression, cell shape or movement. As such, many of these signal transduction pathways must be tightly regulated to ensure proper development and appropriate levels of growth. Dysregulation of these signal transduction components can lead to malfunctioning of these pathways. If these pathways malfunction it can lead to disorders, the formation of tumours and in some cases the metastasis of these tumours into neighbouring tissues. This will be discussed in further detail in subsequent sections.

The conformational change that the receptor undergoes determines how the signal is propagated. In some pathways, such as Notch signaling, the receptor is cleaved at the membrane, and the internal portion can interact with other signaling proteins, enzymes, organelles or transcription factors in either the cytoplasm or it can migrate into the nucleus (Bray 2006; Kopan and Ilagan, 2009). The cellular outcome depends on the components with which this protein interacts and if specifically a transcription factor, can lead to a modification in gene expression.

In many signal transduction pathways, the signal needs to be propagated through a series of biochemical events and protein-protein interactions. One specific signal propagation event can occur if the receptor is an enzymatic protein called a kinase. Upon activation by the ligand, a kinase will induce phosphorylation, either of itself or other signaling proteins. Phosphorylation is the addition of a phosphate group (PO_4^{3-}) to a protein or organic molecule. This reaction requires adenosine triphosphate (ATP) to donate its gamma unstable phosphate group to the substrate protein and requires a protein kinase, which catalyzes the addition of the phosphate group to the substrate (Burnet and Kennedy, 1954). The products of the reaction include a phosphorylated substrate protein and adenosine di-phosphate (ADP). This reaction is present in all cells and tissues and can be responsible for activity and function of enzymes, transcription factors, and other kinases. There are several versions of phosphorylation. These include trans-phosphorylation, the phosphorylation of a kinase by a different protein kinase, as seen in mitogen-activated protein kinases (Dhillon et al., 2007), cis-autophosphorylation, the phosphorylation of a kinases active site by its own kinase domain (Lochhead et al 2005, Lochhead et al 2006) and trans-autophosphorylation, the phosphorylation of a kinase by an independent kinase of the same type, as seen with SRC kinase (Cooper and MacAuley, 1988). In eukaryotes, phosphorylation must occur on either serine, threonine or tyrosine amino-acid residues (Ciesla et al, 2011). Phosphorylated proteins can have their phosphate group removed by enzymes called phosphatases, and the reaction is termed dephosphorylation (Fischer and Krebs, 1955). Phosphorylation and dephosphorylation are among the events termed post-translational modification. Other post-translational modification include ubiquitination, SUMOylation, and glycosylation. Due to their importance in development and disease, the identification of new phosphorylation reactions continues to be extensively studied. One way that geneticists study phosphorylation reactions is with the fruit fly *Drosophila melanogaster.*

1.2. *Drosophila melanogaster* as a model organism

To study how Hipk affects signaling pathways and tissue development of multicellular organisms, geneticists used the fruit fly *Drosophila melanogaster*. For the past century, Drosophila has been used as an elaborate genetic tool to study how an

organism develops over time. Many of the genes and proteins in *Drosophila melanogaster* share some level of homology with their vertebrate counterparts, allowing fly geneticists to suggest that interactions occurring in Drosophila might be conserved in vertebrate development. Useful tissues such as imaginal discs allow us to modulate gene expression to determine which proteins and signal transduction networks are necessary for specific aspects of normal and/or tumour development, and genetic modulation of all proteins of interest can be performed with relative ease. Specific regions of the wing imaginal disc including the wing pouch, presumptive notum, and sensory organ precursors respectively give rise to adult features such as the wings, notum, and bristles of the adult fly (Bardin et al, 2001; Golzalez, 2013; Jennings, 2011) (Figure 1).

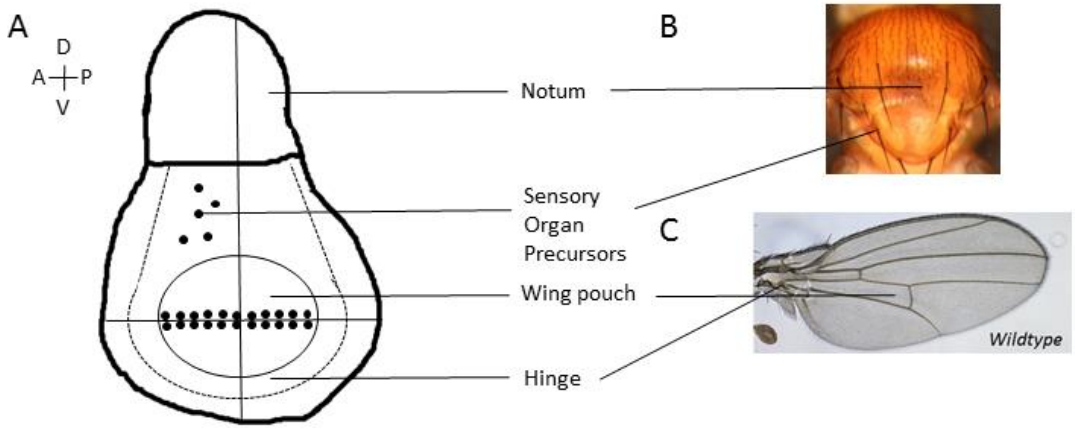


Figure 1 Imaginal and adult structures of the Drosophila wing disc.

A) Third-instar Drosophila wing disc, axis labelled to the left. Sensory organ precursors are also present along the dorsal/ventral boundary in the wing pouch. B) The most dorsal region of the wing imaginal disc gives rise to the notum (back) of the adult fly. Sensory organ precursors give rise to micro- and macrochaete bristles in adult flies. C) The wing pouch gives rise to the wide flat wing blade. The hinge connects the notum and the wing blade.

To manipulate gene expression in Drosophila, a widely-used tool is the GAL4-UAS system (see Methods). This system, originally identified in yeast, utilizes GAL4, a transcription factor, to induce transcription of GAL4 dependent target genes. GAL4 dependent target genes all have UAS (upstream-activating-sequences) that GAL4 protein recognizes and binds to induce transcription. GAL4 protein was initially inserted randomly into the genome, which resulted in GAL4 expression from numerous genomic enhancers. Now, thousands of enhancer specific GAL4 lines have been characterized, allowing

geneticists to modulate any GAL4 dependent target genes in a tissue specific manner through simple genetic crosses (Brand and Perrimon, 1993) (Figure 19A in Methods).

Another frequently used technique is the use of mitotic clones. These are used to generate patches (clones) of homozygous mutant tissue in a heterozygous animal. This technique is frequently employed when a mutation of interest causes organismal lethality. Mitotic clones are generated by Flp-*FRT* (flippase recognition target) mediated recombination, using FRT sites located near the centromere of the chromosome harbouring the mutation of interest. Mitotic recombination can be induced between one homologous chromosome bearing a mutation in addition to an FRT site, and the second homologous chromosome bearing an FRT and a marker, most often GFP or RFP. In this cross the fly also harbours a heat shock inducible flippase gene, so that following a brief heat shock, the heat shock induced flippase will mediate recombination between the FRT sites. This allows previously heterozygous cells to obtain two copies of the mutant allele. As these cells are negatively marked (lack GFP or RFP), we can easily distinguish mutant cells from neighbouring wildtype cells. As such, we can observe morphology and protein levels in homozygous mutant tissue and compare it directly to wildtype tissue within the same sample, making this technique an invaluable genetic tool (Theodosiou and Xu; 1988, Johnston, 2002) (Figure 19B in Methods).

1.3. Hipk Family Discovery

Signal transduction proteins such as kinases can signal to certain transcription factors, many of which are homeoproteins. Homeoproteins are encoded by Homeobox genes, which play immensely important roles in development and determination of cell fate in multicellular organisms (McGinnis and Krumlauf 1992, Krumlauf 1994). These genes encode sequence specific DNA-binding proteins that can act either as transcriptional activators and/or repressors, and play a significant role in controlling gene expression in a temporal and spatial manner (Mann and Chan, 1996).

The Homeodomain-interacting protein kinase (Hipk) family was identified through a yeast two-hybrid screen to identify cofactors for the NK-2 family of homeoproteins (Kim et al, 1998). In this assay, transformants from mouse embryonic (days 7 to 11)

matchmaker cDNA libraries were screened in a HF7C yeast strain containing the NK-2 bait plasmid. Two positive clones from yeast two-hybrid screen showed similar amino acid sequences and were further examined. cDNAs of these two clones were cloned, sequenced and interestingly, both sequences contained an open reading frame for a protein kinase motif (Figure 2A). As such, these genes were named Hipk1 and Hipk 2 (Homeodomain-interacting protein kinase 1, and 2). From this initial screen, Hipk3 was also cloned, a third member of the Hipk family. Several years later, another Hipk-like protein was characterized from a previously uncharacterized kinase like gene (Arai et al, 2007; Gerhard et al, 2004). This protein became known as Hipk4 (Arai et al, 2007).

Because of the baits used in the Kim et al (1998) yeast-two hybrid assay, it was clear that the Hipk family contains a homeo-interacting-domain. To determine where Hipk localizes in monkey kidney CV-1 cells, cells were transfected with GFP-Hipk2 and the fluorescence derived from GFP was primarily localized in nuclear speckles, which suggested that Hipks are nuclear protein kinases. Further deletion analysis showed that Hipk2 contained multiple nuclear localization signals and a nuclear speckle retention signal (Kim et al, 1998). Hipk can also be SUMOylated by SUMO1, and the presence of this covalent modification correlates with its localization to nuclear speckles (Kim et al, 1999). This region is followed by a PEST sequence and a C-terminal region high in tyrosine and histidine residues, which potentially serves as an auto-inhibitory domain (Figure 2A). Homeoproteins are highly phosphorylated during development (Krause and Gehring, 1989). However, the phospho-specific effect Hipk had on homeodomain transcription factors was unknown at the time. Kim et al (1998) went on to show that Hipks act as transcriptional co-repressors for NK homeodomain transcription factors and suggest that Hipk could have many other roles in the regulation of gene expression through other transcription factors.

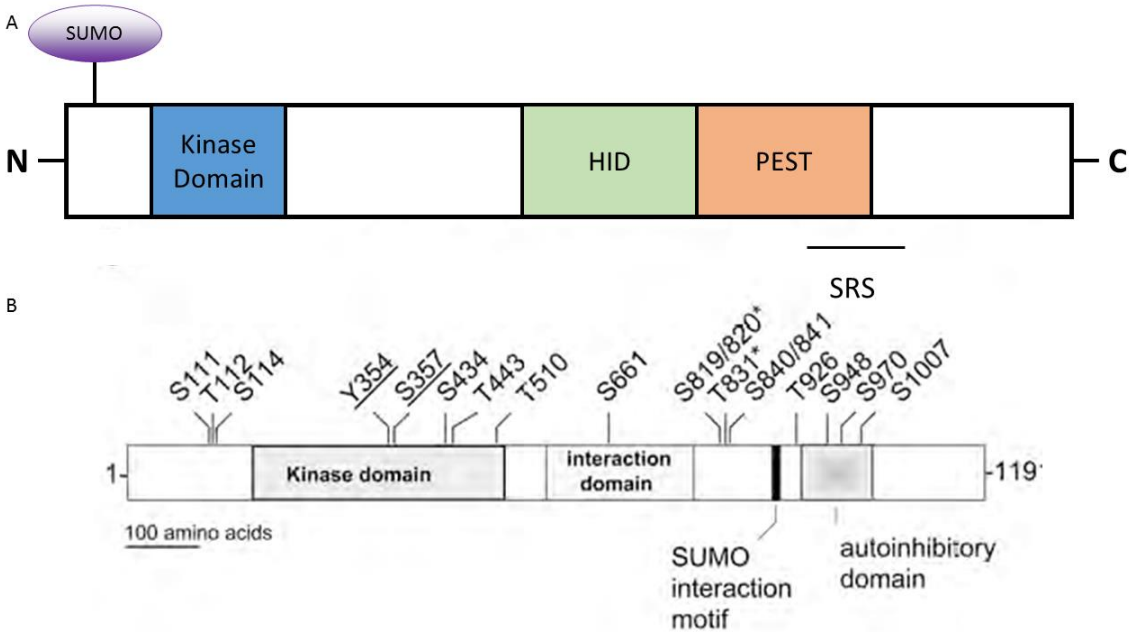


Figure 2 Schematic of the domains of vertebrate Hipk1-3.
A) HID: Homeodomain Interacting Domain, SUMO: (Small Ubiquitin-Like Modifier) binding site, PEST: Peptide sequence rich in Proline, Glutamic Acid, Serine and Threonine. SRS: Speckle Retention Signal. B) Hipk2 domains with putative serine, threonine and tyrosine phosphorylation sites. Note Y354 and S357, activation loop residues that need to be phosphorylated for full Hipk activity and proper localization (Taken from Saul et al, 2013).

Recently, the wealth of literature intended to identify Hipk functions has expanded greatly. Of particular emphasis has been how Hipk is involved in development of multicellular organisms through the regulation of signal transduction pathways required for proper normal growth (Lee et al, 2009a, Lee et al, 2009b, Swarup and Verheyen 2012, Chen and Verheyen 2012, Rinaldo et al, 2008). Due to its involvement in signal transduction pathways, many studies have investigated how Hipk proteins can promote or inhibit other transcriptional regulators (Kim et al, 1998).

Furthermore many studies have investigated how Hipk proteins can promote or prevent tumour formation and metastasis (reviewed in Saul and Schmitz, 2013; Hofmann et al, 2012). This introduction will focus on how Hipk knockdown and over-expression ameliorates the output of several signal transduction pathways, and how Hipk over-expression leads to increased proliferation of tissues and tumour formation.

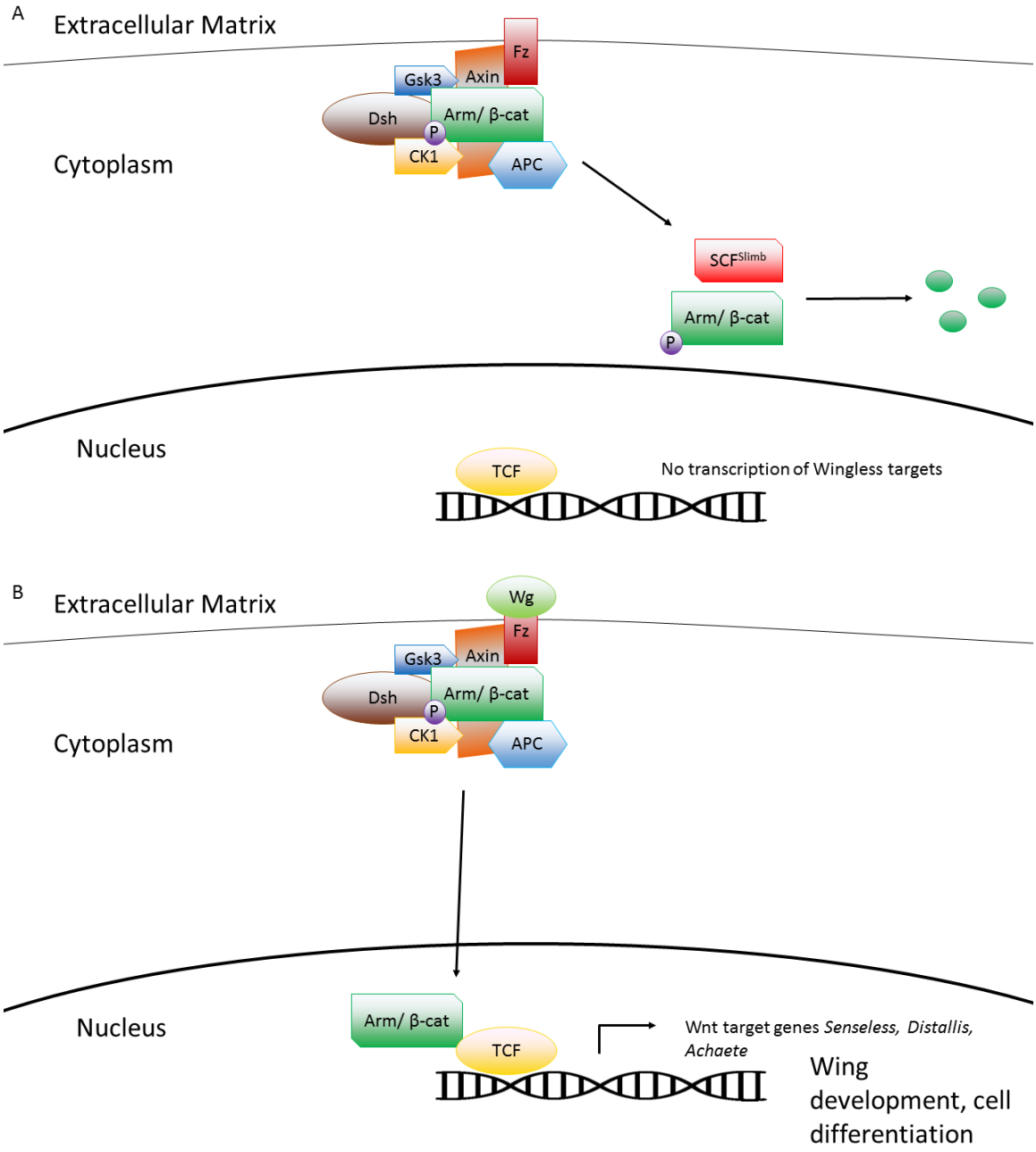
1.4. Hipk's Role in Signal Transduction

In Drosophila, there is only one Hipk, however, it shows sequence homology greater than 90% in its kinase domain with vertebrate Hipk family members. Outside of this domain, Drosophila Hipk is slightly conserved with vertebrate Hipk family members. Initially, two signal transduction pathways were identified as being modified by Hipk activity: the Wingless/Wnt pathway and the Notch pathway (Lee et al 2009a; Lee et al, 2009b). Here we will focus on Hipk activity influencing the Wingless/Wnt pathway.

The Wingless (Wg) signaling pathway has two states, on and off. In the off state, when no Wingless morphogen is bound to the Frizzled/Arrow co-receptors, cytoplasmic levels of Armadillo (Arm)/β-catenin (the transcriptional effector) are kept low by a protein destruction complex composed of Axin, APC, GSK3/Zw3 and CK1 (Figure 3A). This prevents Arm from migrating to the nucleus and interacting with a TCF transcription factor, and prevents the transcription of Wingless target genes. Binding of the Wg ligand to the Frizzled/Arrow co-receptors leads to Dishevelled-mediated inactivation of the protein destruction complex, allowing stabilized Armadillo/β-catenin to translocate to the nucleus, where it binds to the TCF transcription factor and induces transcription of its target genes (Figure 3B) (Swarup and Verheyen, 2012; Bejsovec 2006; Clevers and Nusse, 2012).

Lee *et al* (2009b) found that Hipk acts as a positive regulator of the Wingless/Wnt pathway, and showed that Hipk expression rescues loss of the Wg pathway output, and loss of *hipk* enhances decreased *wg* wing phenotypes. Furthermore, they showed that Arm-mediated gene expression is kinase-dependent, and that Hipk directly phosphorylates Armadillo. Similar molecular interactions were seen between Hipk2 and β-catenin (β-cat) in mammalian cell culture and suggests that an evolutionarily conserved role for Hipk proteins promoting Wg/Wnt signaling. Swarup and Verheyen (2012) went on to show that Hipk specifically inhibits ubiquitination of Arm/β-cat by the SCF^{Slimb}/βTrCP ubiquitin ligase. When the primary ubiquitin ligase that controls levels of β-cat is inhibited by increased levels of Hipk, Arm levels are stabilized since Arm is no longer targeted to be ubiquitinated by Slimb. Therefore, elevated Hipk ultimately prevents degradation of Arm by the proteasome. Not only does Hipk act on this part of this ubiquitin complex, it

has also been shown to bind to and increase the transcriptional activity of the Arm/TCF complex (Lee et al, 2009b) (Figure 3C).


A
Extracellular Matrix
Fz
Gsk3
Axin
Dsh
Arm/ β-cat
P
CK1
APC
Cytoplasm
SCFSlimb
Arm/ β-cat
P
Nucleus
TCF
No transcription of Wingless targets
B
Extracellular Matrix
Wg
Fz
Gsk3
Axin
Dsh
Arm/ β-cat
P
CK1
APC
Cytoplasm
Nucleus
Arm/ β-cat
TCF
Wnt target genes Senseless, Distallis, Achaete
Wing development, cell differentiation

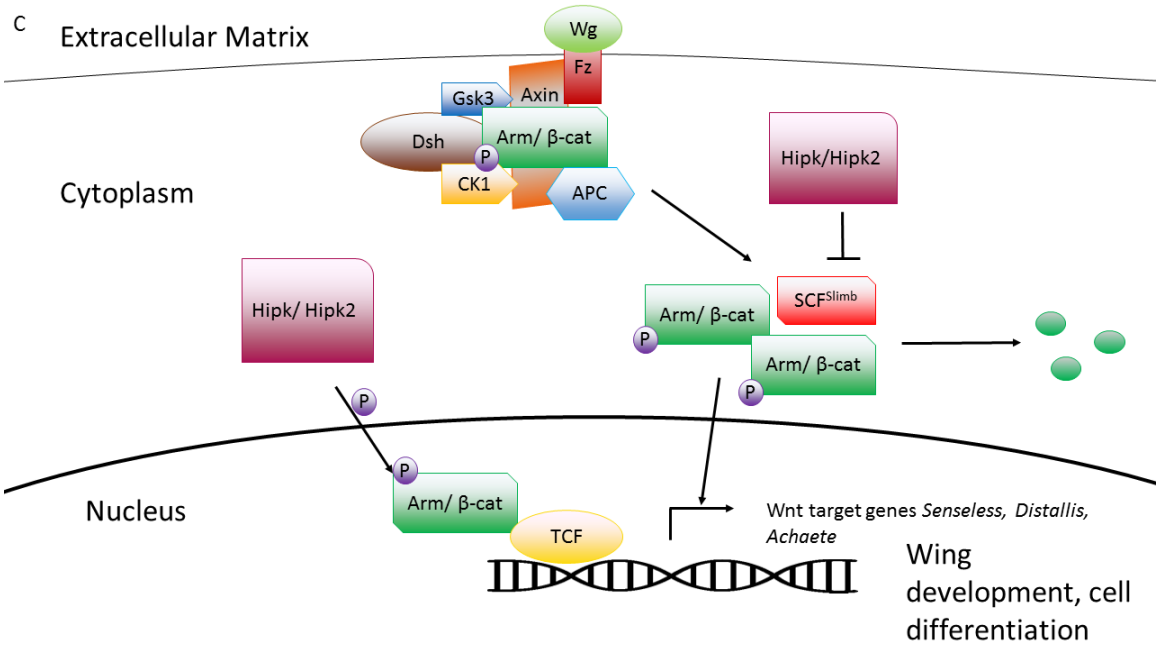


Figure 3 Hipk/Hipk2 acts on multiple components to regulate Wg/Wnt signaling.

A) Arm/β-cat levels are kept low in cells through the Axin-associated destruction complex. Arm/β-cat is phosphorylated by Gsk3, CK1 and other kinases, targeting it for degradation by the SCF^{Slimb}/βTrCP ubiquitin ligase B) With Wg present, Dishevelled mediated degradation of the destruction complex results in stabilized Arm, which can translocate to the nucleus, bind to TCF, and turn on transcription of Wg target genes. C) Drosophila and vertebrate Hipk act to inhibit functional $SCF^{Slimb,}$ allowing Arm/β-cat levels to accumulate. Hipk also phosphorylates Arm β-cat on another residue, and activates Arm/ β-cat/TCF transcription.

Hipk also modulates the Hippo pathway, which is essential for overall tissue growth. Hippo pathway components were discovered in genetic mosaic screens as tumour-suppressor genes and include *warts (wts), hippo (hpo) and Salvador (sav).* Mutations of these genes lead to severe over-proliferation of imaginal disc tissues. In this pathway, Hpo directly interacts with Sav to phosphorylate and activate a complex consisting of Wts and another core Hippo signaling protein Mats. A yeast-two hybrid screen using Wts as bait identified the transcriptional coactivator Yorkie (Yki). When Hippo signaling is active Wts directly phosphorylates and inhibits Yki via a 14-3-3 protein complex, which in turn prevents over-proliferation of cells (Figure 4) (Zhao et al, 2011). The majority of Hpo pathway members act to inhibit Yki. Yki in turn promotes the expression of growth promoting and apoptosis inhibiting genes. Thus activation of the Hpo pathway leads to reduction of Yki-regulated gene expression. Major components of the Hippo signaling pathway are conserved in vertebrates, and rather unsurprisingly, loss-of-

function mutations in vertebrate orthologs of the components *wts*, *sav* and *hpo* have been implicated in the development of mammalian cancer, as the pathway under normal conditions is responsible for restricting cell growth and proliferation, as well as inducing apoptosis (Harvey and Tapon, 2007).

Chen and Verheyen (2012) demonstrated that Hipk is required to promote Yki activity, as reducing levels of Hipk in a hyperactive Yki background supressed Yki-induced overgrowths and Yki-induced increases in target gene expression. Finally, it was shown that Hipk's kinase ability is necessary for the increase in Yki targets upon overexpression, and that Hipk directly phosphorylates Yki (Figure 4). Thus Hipk acts to inhibit the Hpo pathway, by promoting Yki activity and expression of its target genes.

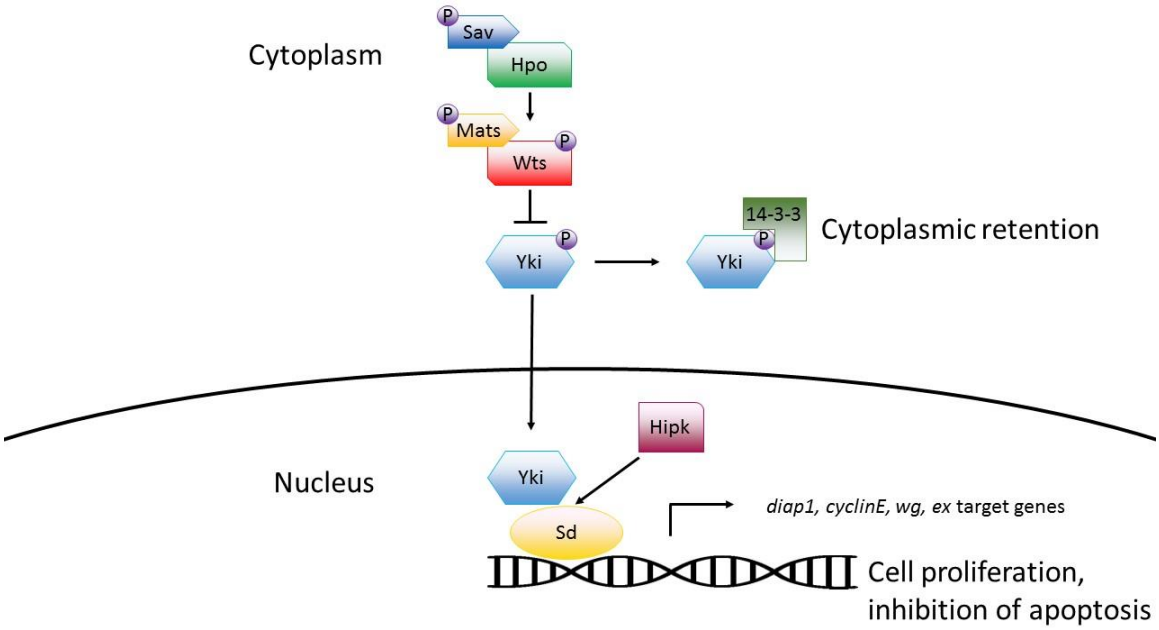


Figure 4 Hipk modulates the output of the Hippo signaling pathway.
A cascade of phosphorylation events, including Salvador phosphorylating Hippo, and Mats phosphorylating Wts, ultimately leads to the phosphorylation of Yki. Phosphorylated Yki is retained in the cytoplasm through 14-3-3. Hipk acts to phosphorylate Yki promoting its binding to Scalloped (Sd) and initiation of transcription of target genes. Yki and Hipk have been shown to co-immunoprecipitate together.

1.5. Hipk/Hipk2 Regulation

While Hipk clearly modifies many components of signal transduction pathways, a wealth of literature has also examined how Hipk levels themselves and activity are regulated. This work has primarily been performed with vertebrate Hipk2, which shares the most homology with Drosophila Hipk. Hipk regulation is very tightly controlled. Hipk2 levels in unstressed cells are kept low through ubiquitination mediated by proteins such as Siah-1 and WSB-1 (Winter et al, 2008, Choi et al, 2008). Hipk proteins are also regulated by their phosphorylation state. A previous study by Saul et al (2012) showed that vertebrate Hipk2 kinase activity is modified through the process of auto-phosphorylation. Specifically, they showed that Hipk2 is auto-phosphorylated on its activation loop residues Y354 and S327 (Figure 2B). These two residues control kinase activity, substrate affinity and localization of the kinase. Hipk2 can also be regulated by trans-phosphorylation at other phosphorylation sites outside of the kinase domain (Saul et al, 2012). Another study demonstrated that Hipk2 catalytic activity is regulated specifically by activation-loop Y354 auto-phosphorylation (Siepi et al, 2013). Saul et al (2012) mutated specific phosphorylation sites of Hipk2, and argued that differential phosphorylation of Hipk2 provides a mechanism for controlling the signal output of the kinase. Of particular interest were Hipk phosphorylation sites that were not auto-phosphorylated, suggesting that other kinases were responsible for phosphorylating these sites. This prompted the Verheyen lab to investigate if Hipk activity is modulated through trans-phosphorylation events. Previous lab members performed an *in vivo* kinase RNAi screen in an attempt to identify phospho-regulators of Hipk which is described in detail in Chapter 2.

1.6. Signal Transduction and Cancer

Initially, cancer originates because a cell acquires a mutation that leads to the loss of growth control mechanisms. This mutated cell starts to divide and subsequent mutations give rise to increased severity of uninhibited cellular proliferation. These cells form a localized tumour. Once tumours obtain certain biological properties they are able to metastasize or migrate to other tissues through the blood or hemolymph, where they can form a secondary metastatic growth. There are five consistent biological steps that

must take place in order for malignant cancerous tissues to arise. These include loss of cell adhesion, increased amounts of cellular invasiveness and motility, entry of the spreading cancerous cells into circulation, their leaving of the circulatory system into another tissue, and finally formation of a colony at the second removed site (Hanahan and Weinberg, 2000). Cancer is responsible for millions of deaths worldwide each year, and as such, much research is put into it to try and better understand the mechanisms of cancer development and treatment.

Many approaches can be used to dissect the molecular signaling mechanisms required for metastasis to occur. Obviously, because of ethical concerns, we are unable to manipulate human genomes to better understand how these processes occur. However, because of evolutionary conservation between species, we can use much simpler organisms to study the same and similar genes that exist in vertebrates. One such model organism is our favourite organism, the fruit fly, *Drosophila melanogaster*. What makes flies so great is their evolutionary conservation of signaling proteins relative to humans (Brumby and Richardson, 2005; Gonzalez, 2013; Miles et al, 2011). A wide variety of the signaling pathways such as the Hippo, Wnt and JAK/STAT pathways described in this thesis, are involved in tumour formation (Clevers and Nusse, 2012; Piccolo et al, 2013; Springuel et al, 2015). Drosophila genetic studies have identified many genes that when dysregulated or mutated, lead to tumourigenesis. With Drosophila, there is a wide array of elegant genetic techniques that can be used to study the effects of newly discovered oncogenes and tumour suppressors and their effects on cell invasion in an in vivo setting (Brumby and Richardson, 2005; Miles et al, 2011; Gonzalez, 2013). Components of these dysregulated tumour-growth-promoting pathways often crosstalk with one another, and if a common component can be identified within these pathways, then targeting that component therapeutically could be beneficial for the patient, as seen with multiple reviews discussing how the vertebrate homologs of Yki, named YAP and TAZ, are excellent choices for cancer therapy as they crosstalk with multiple signaling pathways (Piccolo et al, 2013; Zhang et al., 2015). The Hipk family is another set of proteins that regulate several signal transduction pathways. Therefore, it is possible that targeting Hipk therapeutically could be a useful treatment to slow or prevent tumor growth.

1.7. Hipk in Growth/Cancer

Several of the previously mentioned studies have demonstrated that Hipks are essential regulators of signaling pathways throughout growth and development (Lee et al 2008, Lee et al 2009, Swarup and Verheyen 2012, Chen and Verheyen 2012). Here I will focus on how Hipk influences growth and cancerous phenotypes. Hipk2 knock out mice have growth deficiencies, and while viable, are smaller (Trapasso, 2009). Furthermore, mouse embryonic fibroblasts from Hipk2 knockout mice have a reduced proliferation rate that is associated with the G1 specific cell cycle regulators CDK6 and Cyclin D (Trapasso, 2009). Interestingly, Hipk2 has a critical role in the initiation of DNA double-stranded break repair signaling, indicating it is also involved with keeping existing struggling cells alive (Chapman et al., 2012). Hipks have also been shown to regulate growth by regulating apoptosis levels in times of distress. Upon induction of genotoxic stress, Hipk1 and Hipk2 have also been shown to be involved with phosphorylating p53 at serine 46, leading to its activation (D'Orazi, 2002; Wang et al, 2001). p53 is the gatekeeper of cell division, ensuring that DNA replication and mitosis only occur in cells with healthy undamaged DNA (Levine, 1997). Hipk2 phosphorylation of p53 results in p53-dependent transcription, regulation of cell growth and ultimately cell death (D'Orazi, 2002; Wang et al, 2001). These results demonstrate not only a cell's need for Hipk to ensure proper growth of all tissue, but also the opposite, which is Hipk's ability to induce removal of dysfunctional or dying cells, highlighting the role for context specific functions of Hipk family activities.

Studies have shown that Hipk levels are elevated in several types of cancer, whereas several other studies have found Hipk to have a tumour suppressive role (Hofman et al, 2012, Saul and Schmits, 2013). Elevated Hipk levels are seen in cervical cancers, apilocytic astrynomas, kidney fibrosis, idiopathic pulmonary fibrosis, colorectal cancer cells and it is implicated in several other proliferative diseases (Al-beiti and Lu 2008, Cheng et al., 2012; Deshmukh et al 2008; D'orazi et al., 2006; Fan et al, 2011; Saul and Schmitz, 2013, Yu et al, 2009). However, several papers show decreased expression of Hipk family members are correlated with several types of cancers including thyroid and breast carcinomas, idiopathic pulmonary fibrosis, thyroid carcinomas, papillary and follicular thyroid carcinomas (Lavra et al, 2011; Pierantoni et al., 2002; Rici et al, 2012). Hipk2 deficiency has also been linked to chromosomal instability via cytokinesis failure,

increasing tumourigenicity in mouse embryonic fibroblasts (Valente et al, 2015). Furthermore, colon cancer tumours with increased expression of Hipk had a better outcome than tumours with low expression (Soubeyran et al 2011).

Previously we mentioned Hipk2's ability to phosphorylate p53 in times of gamma radiation stress and amazingly, this is seen to reduce tumour size in mouse models. Hipk2 cooperates with p53 to suppress gamma-ray radiation-induced mouse thymic lymphoma (Mao et al., 2012). This phosphorylation even turns on p53-induced target genes, which promotes apoptosis of the tumourous tissue, as described previously (D'Orazi, 2002; Wang et al, 2001). Hipk2's localization also modifies its ability to promote apoptosis by phosphorylating p53. In acute myeloid leukemia, two missense mutations in the speckle-retention signal domain of Hipk2 made the protein localize to nuclear regions with conical or ring shapes and appeared diffuse in the nucleus. Wildtype Hipk2 mostly localizes in nuclear speckles, located at interchromatin regions of the nucleoplasm (Spector and Lamond, 2010). The mutant Hipk2 proteins found in this acute myeloid leukemia had decreased activity, and were unable to interact with p53 to promote apoptosis (Li et al., 2007).

The Hipk family has a diverse regulatory role. Hipk functions in cell growth and proliferation but also promotes cell death of malfunctioning, mutant cells. Expression levels of Hipk family proteins vary widely in tumour samples derived from patients, and suggest that Hipk expression levels depend on cancer type and the patient's history. In these cancerous cells, it is certain that some of their signaling pathways components have genetic alterations that promote uncontrolled cellular proliferation. Next we will discuss one last signaling pathway, the JAK/STAT pathway, and outline the mutations prevalent in the pathway that give rise to tumour formation and metastasis.

1.8. JAK/STAT Signaling Overview

The JAK/STAT pathway is one of many signal transduction cascades essential for homeostasis and development in a wide array of organisms, from flies to mammals. In mammals, the JAK/STAT pathway is the primary signaling mechanism for several cytokines and growth factors. Across all species, JAK activation promotes cell

proliferation, differentiation, migration and apoptosis, and mutations that hyper-activate the pathway can lead to inflammatory disease, leukemia and a wide array of other diseases. As such, regulation of the JAK/STAT pathway is essential for proper development and organismal health as a whole (reviewed in Rawlings et al., 2004; Arbouzova and Zeidler, 2006; Amoyel et al., 2013; Hou et al, 2002).

Genetic analysis in Drosophila has identified the major components of the JAK/STAT signaling cascade (Figure 5). The pathway includes a JAK kinase called Hopscotch, the transcriptional effector STAT92E, the receptor Domeless, and Unpaired ligands called outstretched (Os), unpaired-2 (Upd2) and unpaired-3 (Upd3) (Agaisse et al, 2003; Binari and Perrimon, 1994; Brown et al, 2001; Chen et al, 2002; Gilbert et al., 2005; Harrison et al, 1998; Hou et al., 1996; Yan et al., 1996). The one receptor for the pathway, Domeless, shows some similarities with the cytokine-binding domains of vertebrate cytokine-receptors (Chen et al., 2002). Members of the Unpaired family can bind to Domeless to initiate dimerization of the receptor. This causes receptor-associated Hop molecules to trans-phosphorylate each other to generate SH2 docking sites for the SH2 domains of STATs. Activated Hop molecules then phosphorylate Domeless, which generates a docking site for STAT92E (Arbouza and Zeidler, 2006). Once bound, Stat92E gets phosphorylated, dimerizes via their phosphotyrosine-SH2 domain interactions and translocates to the nucleus to initiate transcription of target genes (Figure 5) (Greenlund et al., 1995). JAK/STAT signaling is essential for the development of Drosophila imaginal discs. It is required in a cell-autonomous nature in both wing and eye imaginal discs to regulate growth and patterning, as well as stem cell self-renewal (Ayala-Camergo et al., 2007; Ayala-Camargo et al, 2014, Flaherty et al., 2010) .

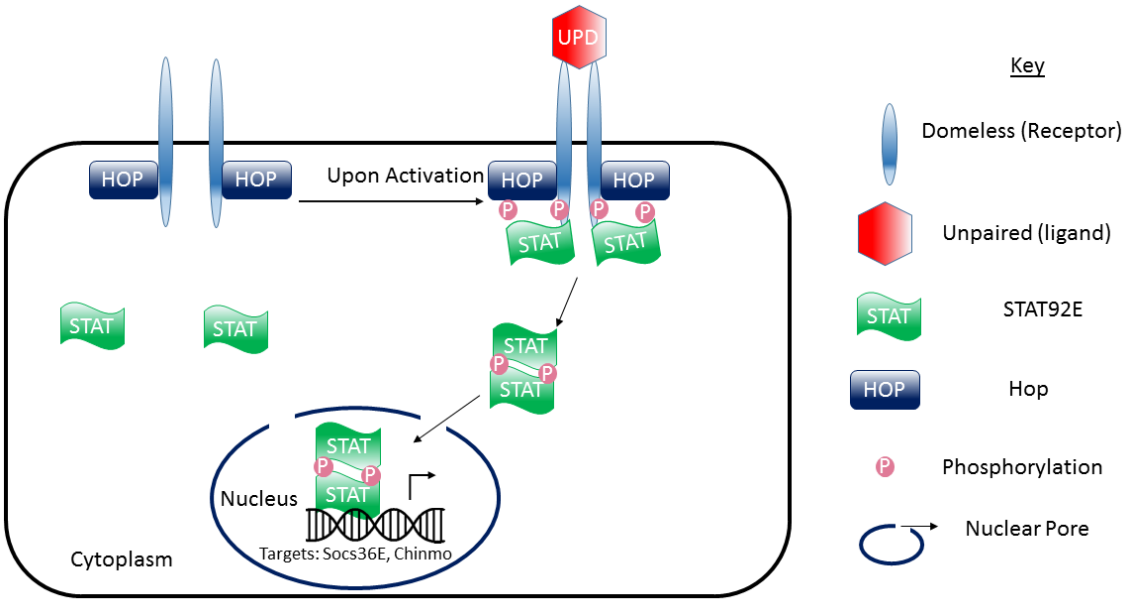


Figure 5 Canonical JAK/STAT Signaling Pathway.
Unpaired ligands binding to Dome receptor induces Dome dimerization. This allows Hop-molecules to trans-phosphorylate each-other as well as Dome, which generates binding sites for cytoplasmic STAT92E. Once bound, STAT92E molecules get phosphorylated, dimerize and translocate to the nucleus where they regulate transcription of its target genes Socs35E, Chinmo and others.

In mammals, there are four JAK kinases: JAK 1, JAK 2, JAK 3, and Tyk2. Structurally, JAK kinases are subdivided into so called JAK kinase homology (JH) domains. The JH1 domain is the tyrosine catalytic domain. The “pseudokinase” JH2 domain is a kinase-like domain that is defective in that it lacks a functional ATP binding site, however this domain is still required for full activity (Leonard and O’Shea, 1998). Generally, ligand-mediated activation of the receptor causes the JAKs to move closer together, allowing trans-phosphorylation to occur. These now activated JAKs dually phosphorylate their receptors, which generates a SH2 binding site for JAKs major substrate, STATs (Arbouza and Zeidler, 2006; Aaronson and Horvath, 2002; Shuai et al. 1993). STATs are found in the cytoplasm until they are activated by JAKs. There are seven mammalian STATs, each of which contains a conserved tyrosine residue that is phosphorylated by JAKs. However, there is sufficient diversity in STAT amino acid sequences and their tissue-specific distributions give mammalian STAT proteins diverse functions when responding to extracellular signaling proteins. In mammals, STAT3 and STAT5 share the greatest homology with Drosophila STAT92E (Arbouza and Ziedler,

2006). The phosphorylation event on the tyrosine activation residue allows for the dimerization of STATs, and transcription of target genes described previously (Aaronson and Horvath, 2002, Shuai et al., 1993).

JAK STAT signaling is heavily regulated to ensure proper function. There are three major types of regulations. The first includes protein tyrosine phosphatases (PTP). Researchers found that RNAi targeted against one such phosphatase PTP61F lead to both increases of STAT-responsive reporters and the levels of tyrosine-phosphorylated HOP and STAT92E protein, indicating that it normally acts as a negative regulator of the pathway (Baeg et al, 2005). The second group includes SOCS (suppressors of cytokine signaling). Active JAK/STAT signaling will induce the transcription of *socs36E*, which is a potent pathway repressor. Socs36E inhibit STAT phosphorylation by binding and inhibiting JAKs or competing with STATs for phosphotyrosine binding sites on cytokine receptors., As such JAK/STAT transcription regulates itself through a negative-feedback loop (Callus et al, 2002; Karsten et al, 2002; Krebs et al, 2001).The third type of regulator are the PIAS proteins (protein inhibitors of activated STAT), which bind to dimerized STAT molecules and degrade them via SUMOlation (Kotaja et al., 2002). Like many signaling pathways necessary for development, the dysregulation of the JAK/STAT pathway can have severe consequences. Next I will discuss how in Drosophila and vertebrates mis-regulated JAK/STAT components contribute to melanotic tumour formation and human cancers such as leukemia.

1.9. JAK/STAT Signaling in Cancer:

Before oncogenic mutations in JAK/STAT signaling were discovered in humans, early studies linked JAK/STAT signaling to blood cell tumours, and the phenotype was termed “fly leukemia.” (Corwin and Hanratty, 1976; Hanratty and Ryerse, 1981). Drosophila blood consists of three cell types. Plasmatocytes formed from hemocytes in the larval lymph gland function as phagocytes throughout the hemolymph to remove bacteria and apoptotic cells (Evans et al, 2003). STAT92E is required for plasmatocyte differentiation (Minakhina et al, 2011). Tumourous lethal (Tum-l) is a temperature sensitive dominant mutation in *hop* that causes over-proliferation of hemocytes and the formation of melanotic tumours in flies (Hanratty and Dearolf, 1993; Hanratty and Ryerse 1981).

Melanotic tumours are black masses of hemocytes that have invasive properties and promote cell death. Hop^{Tum-l} is caused by a G341E substitution in the JH4 domain (Luo et al., 1995). A second tumour-inducing Hop mutation is T42 and is caused by an E695K substitution in the JH2 domain (Lou et al, 1997). Both mutations lead to a hyper-activated kinase, which continuously phosphorylates and activates STAT92E to associate with DNA (Luo et al, 1997). Overexpressing wildtype or Hop^{Tum-l} in the lymph gland can induce the formation of melanotic tumours (see Figure 11 A-B) (Harrison et al, 1995). Overactive JAK/STAT in eye imaginal discs results in large increases in the number of mitotic cells. (Figure 6 C-D) (Bach et al, 2003).

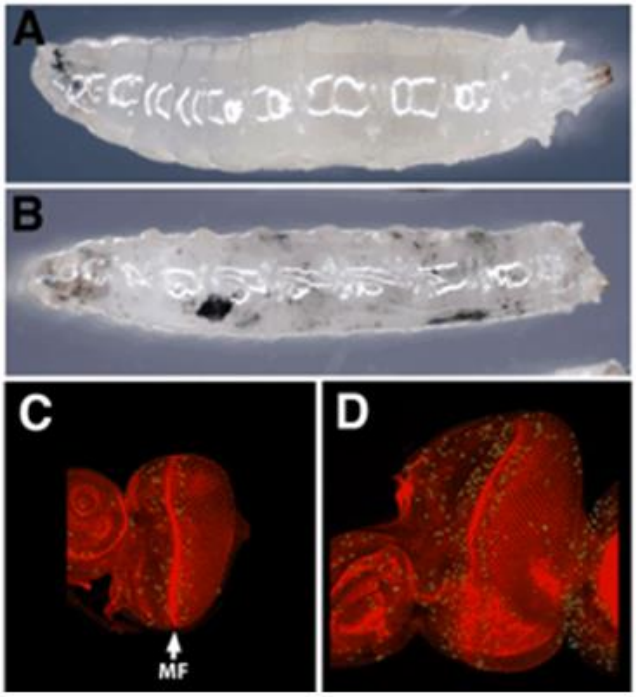


Figure 6 JAK/STAT mis-expression results in melanotic tumours and cellular over-proliferation.

A) Wildtype late third instar larvae B) Hop gain-of-function JH2 mutation leads to proliferation of lamellocytes, which ultimately form lethal melanised tumours. Taken from Lou et al, 1997. C) Wildtype late third instar eye imaginal disc (MF=morphogenetic furrow) D) Unpaired expression increases mitotic cells (green), with increased density seen in the eye disc. Taken from Bach et al, 2003.

In vertebrates, activating mutations in JAK/STAT signaling are a causal event in human leukemia, myeloproliferative neoplasms (MPNs) and solid tumours (Lacronique et al, 1997; Jones et al, 2005; Kravolics et al, 2005; Levinge et al, 2005; Moriggl et al, 2005). Many of these tumour and cancer samples have elevated levels of JAK2, which similarly

to Drosophila Hop^{Tum-l} mutations, results in a constitutively-activated kinase, and highlights the usefulness of using Drosophila as a model for diseases.

In Chapter Three of the results section, I describe our evidence that Hipk can induce abnormal levels of proliferation and tumour-like structures commonly seen upon mis-expression of JAK/STAT pathway components. These data lead to our hypothesis that Hipk may be inducing these tumor-like formations through JAK/STAT signaling. We show that Hipk directly effects the output of the Drosophila JAK/STAT pathway using the JAK/STAT reporter 10X STAT92E-GFP. Next, we attempted to elucidate if Hipk interacted with components of the JAK/STAT pathway to induce its effects. Epistasis experiments showed that Hipk mis-expression ameliorates JAK/STAT signaling in parallel to Hopscotch. Finally I show that Drosophila Hipk acts directly on STAT92E, as detected by a proximity ligation assay.

Chapter 2. Identifying Phospho-Regulators of Hipk function

Jessica Blaquiere and Rubia Chung performed the initial screen (Figure 7A-E) and Nathan Wray followed up on the initial screen data (Figures 7(F-J)-11).

Verheyen lab members carried out an RNAi screen to assay whether knockdown of any particular kinase could genetically modify a phenotype caused by overexpressed Hipk. This *in vivo* kinase screen was designed to specifically analyze bristles derived from sensory organ precursors on the notum of the adult fly. Scabrous-Gal4 (*sca-Gal4*) was used to express UAS-transgenes or RNAi directed at specific genes of interest in the sensory organ precursor cells in the wing imaginal disc (Figure 2). Using *sca-Gal4* to express a HA-tagged form of Hipk (called Hipk 1M based on its insertion site), and denoted as *sca>Hipk1M-HA,* leads to a strong shortening in bristle size compared to wild type (Figure 7A-B). To determine if Hipk activity could be ameliorated by knock down of a regulator, RNAi was used to knock down 307 kinases whilst expressing Hipk. The goal was to identify putative phospho-regulators, using the Hipk over expression bristle phenotype in the notum region as a marker for Hipk activity. Knock down of the collection of kinases resulted in a variety of effects. Upon knockdown, some kinases caused an enhancement or worsening of the baseline *sca>Hipk1M-HA* phenotype (Figure 7C-C''), whereas upon knockdown of other kinases, the baseline *sca>Hipk1M-HA* phenotype was suppressed or rescued (Figure 7D-D''). In addition, many kinases had no effect (labelled as no change) (Figure 7E). In some cases there were inconsistent results; knockdown of the same kinase using different RNAi lines yielded both enhancement and repression results (labelled as mixture) (Figure. 7E) A representation of primary results of the 307 kinases tested is shown in Figure 7E**.** We were most interested in kinases, which upon knockdown, resulted in a suppression phenotype. This is because if we assume that phosphorylated Hipk represents the active state of the protein, then removal of a potential activator should yield a repression of the baseline shortened bristle phenotype. Furthermore, in genetic modifier screens like the one we performed, it is substantially easier to make an enhanced phenotype versus a suppressed phenotype. As such, any kinase knockdown that repressed Hipk activity would indicate that the kinase is normally required for full Hipk activity. Thus, the kinase we knocked down could possibly interact

with Hipk. The screen showed that knock down of the protein kinases cyclin-dependent-kinase 8 (CDK8), Hopscotch (Hop) and mitogen-activated-protein kinase kinase kinase 1 (MEKK1), among others, suppressed the baseline overexpressed Hipk phenotype in the most substantial manner in the initial screen. I joined the project at this point and began by validating the kinase hits to see if repression effects were also seen when expressing another cDNA for Hipk, termed Hipk3M-HA (Figure 7 F-J). Two of the above hits, Hop^{RNAi} and $Cdk8^{RNAi}$ consistently modified the *sca>GFP;Hipk3M-HA* phenotype in different tissues (Figure 7 I-J). However, $Mekk1^{RNAi}$ was unable to rescue the baseline over-expression phenotype in other contexts, and investigation into this kinase as a regulator of Hipk was later discontinued (Figure 7H).

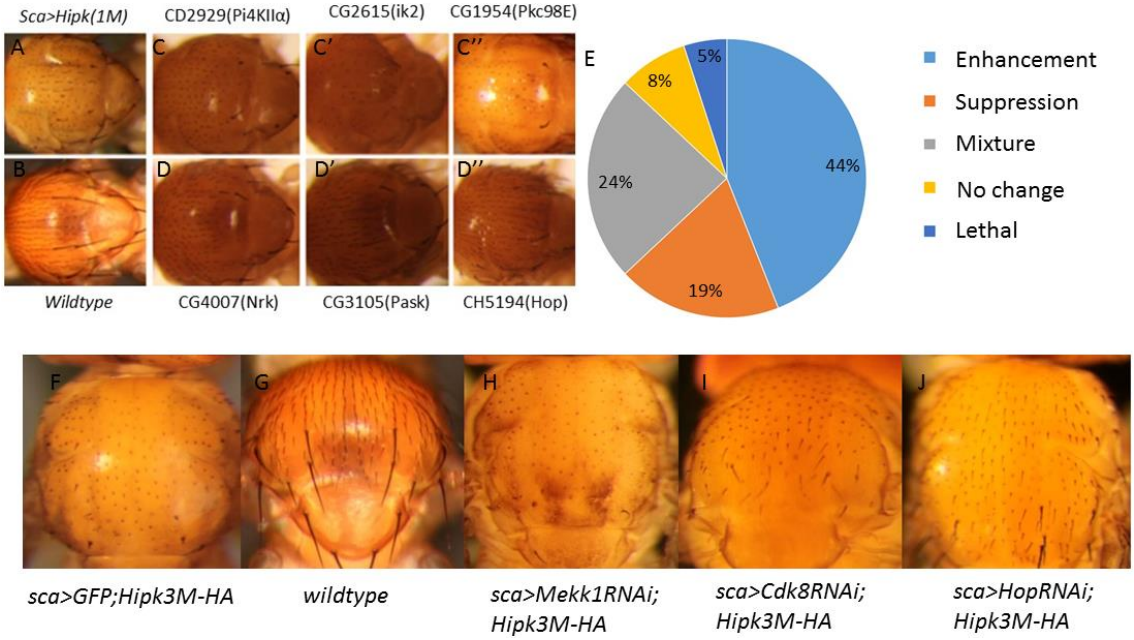


Figure 7 Multiple Kinases are required for full Hipk activity.
A) *sca>Hipk1M-HA* results in severe shortening of notum bristles compared to wild type (B). C-C'') Kinase knockdown enhancing background *sca>Hipk1M-HA* phenotype. D-D'') Kinase knockdown repressing *sca>Hipk (1M)* phenotype. E) Primary Screen results of all 307 RNAi lines (see legend; F) *sca>GFP;Hipk3M-HA* leads to severe shortening of bristle phenotypes compared to wild type (n=153) (G). H) *sca>Mekk1RNAi; Hipk3M-HA* does not rescue baseline *sca>GFP;Hipk3M-HA* phenotype (n=56). I) *sca>Cdk8RNAi; Hipk3M-HA* rescues the baseline *sca>GFP;Hipk3M-HA* phenotype (n=79). J) *sca>HopRNAi;Hipk3M-HA* rescues the baseline *sca>GFP;Hipk3M-HA* phenotype (n=89).

Because of the rescue phenotypes seen with these two kinases using *scabrous-GAL4*, I next decided to test if these kinases repressed Hipk activity using other drivers

that are expressed in the notum. To do this I used *eyegone-Gal4 (eyg). eyg>GFP;Hipk* caused a severe cleft in the notum of the adult fly compared to wildtype (Figure 8A-B). *hop* knockdown had the greatest effect on Hipk activity in the notal region. Of the *eyg> HopRNAi; Hipk3M-HA* flies scored, 12.6% resembled wildtype (Figure 8C'''), 59.8% had a substantial reduction in cleft size, indicating a suppression of the Hipk phenotype (Figure 8C''), 12.6% showed a moderate Hipk phenotype (Figure 8C') and 3.4% were severely affected and resembled the positive control *eyg>GFP, Hipk3M* (Figure 8C). *Cdk8* knockdown had some effect on Hipk activity in the notal region as well. Although *eyg>Cdk8RNAi;Hipk3M-HA* flies never fully resembled wildtype, 44.6% were slightly affected by Hipk activity, thus suppressed from the positive control phenotype (Figure 8D), 47.3% were moderately affected (Figure 8D'), and 14.9% were still severely affected by Hipk activity (Figure 8D). *eyg>Mekk1RNAi;Hipk3M* flies always resembled the positive control and were severely affected due to Hipk activity, thus showing no rescue in this context (Figure 8E). Percentages of the level of rescue across all the described genotypes for all *eygGal4*-based experiments can be seen in Figure 8F.

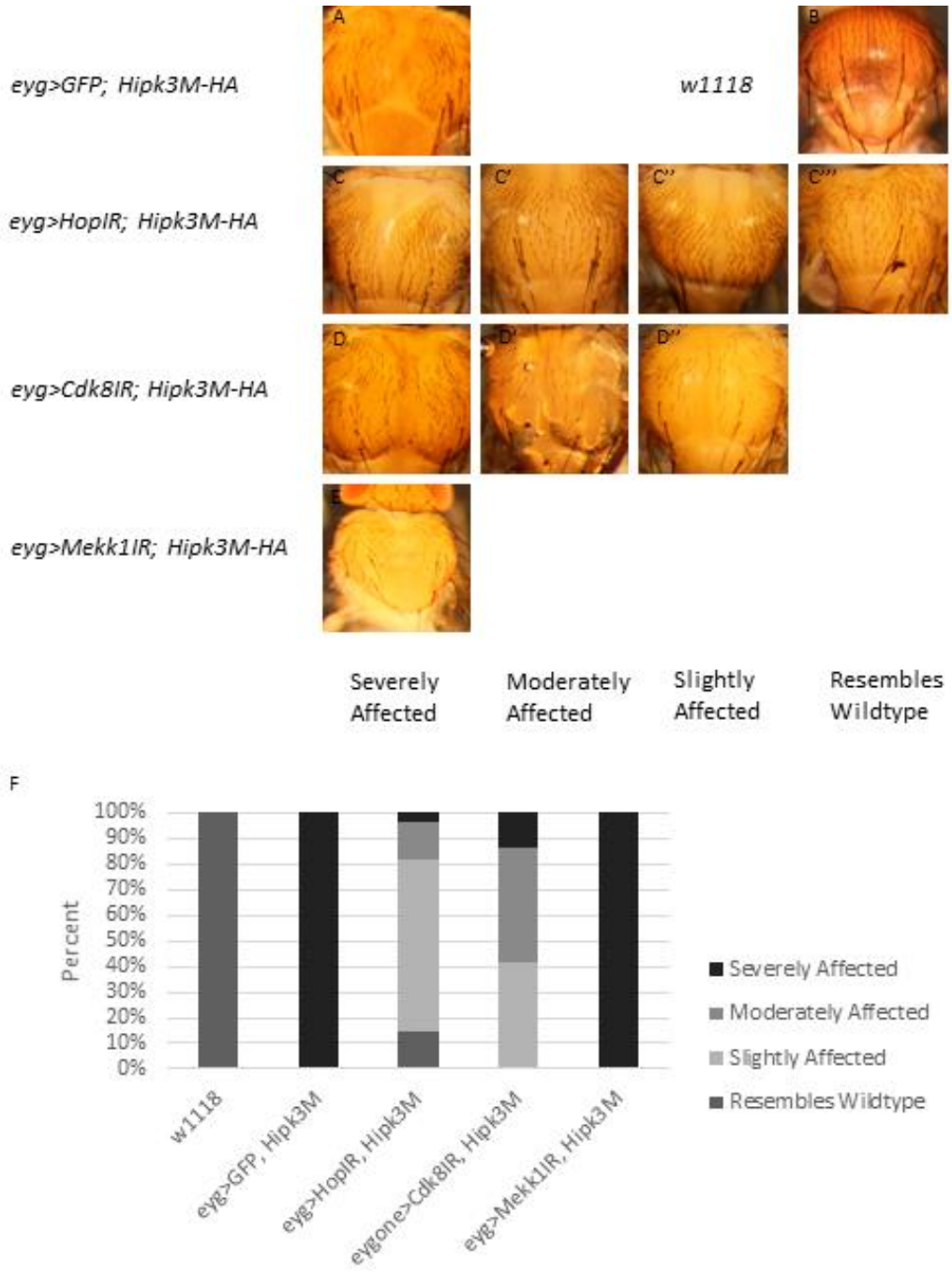
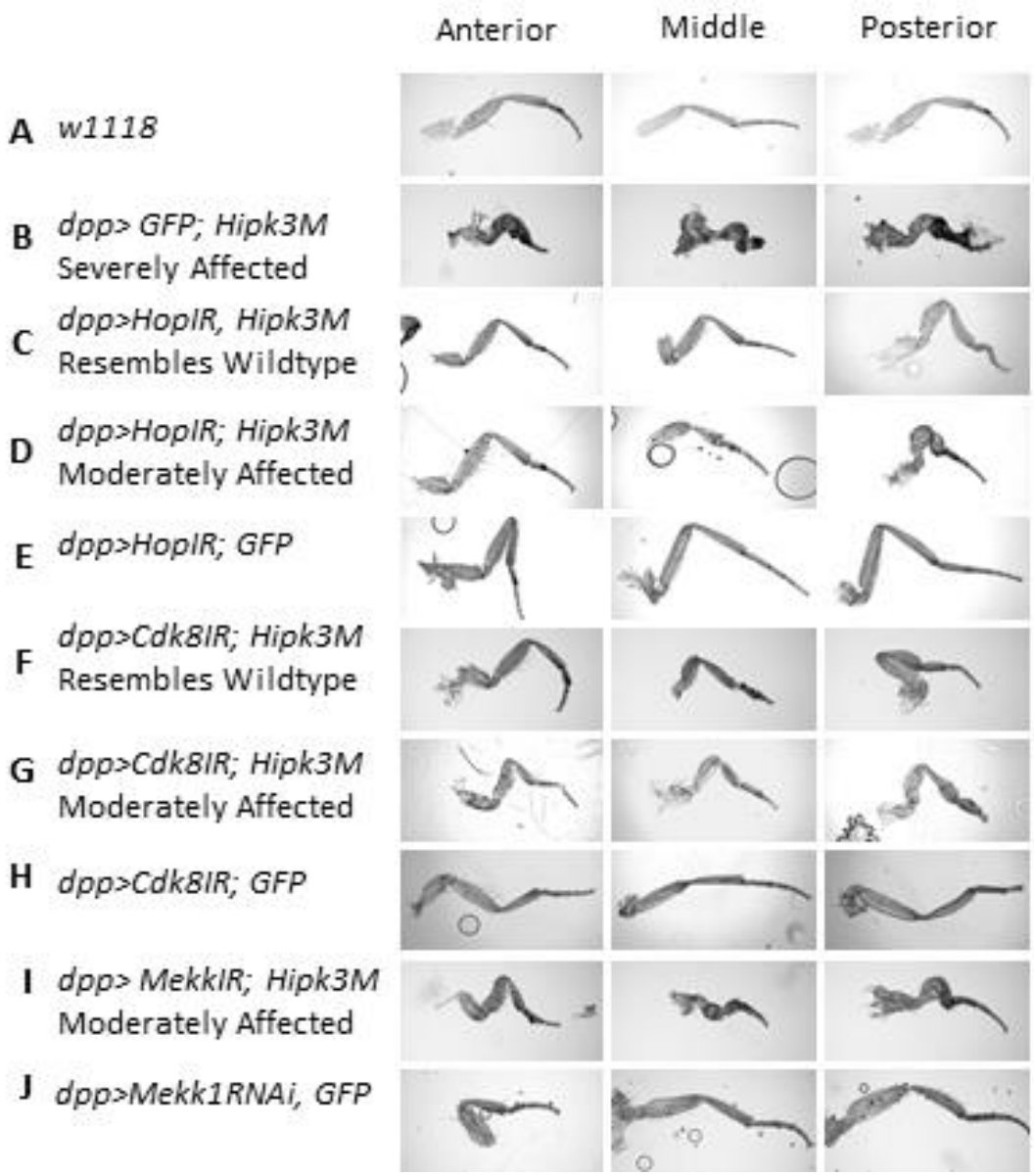


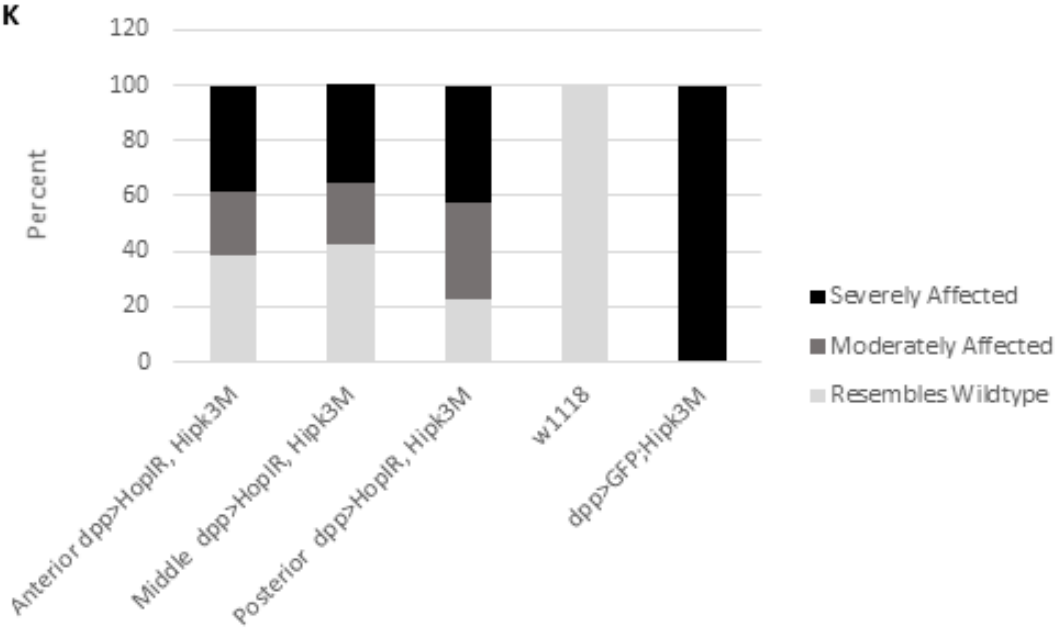
Figure 8 Hop and Cdk8 knockdown modulates Hipk activity in the Drosophila notum.

A) *eyg>GFP;Hipk3M-HA* (n=67) results in a cleft where the notum fuses on the back of the adult fly compared to wildtype (B). C-C''') Hop knockdown reduces the severity of the *eyg>GFP;Hipk3M-HA* baseline phenotype in the majority of flies scored (n=87). D-D''') Cdk8 knockdown reduces the severity of the *eyg>GFP;Hipk3M-HA* baseline phenotype in the majority of flies scored (n=79). E-E'') Mekk1 knockdown does not reduce the severity of the *eyg>GFP;Hipk3M-HA* baseline phenotype in the majority of flies scored (n=70). F) Quantification of level of rescue for all described genotypes.

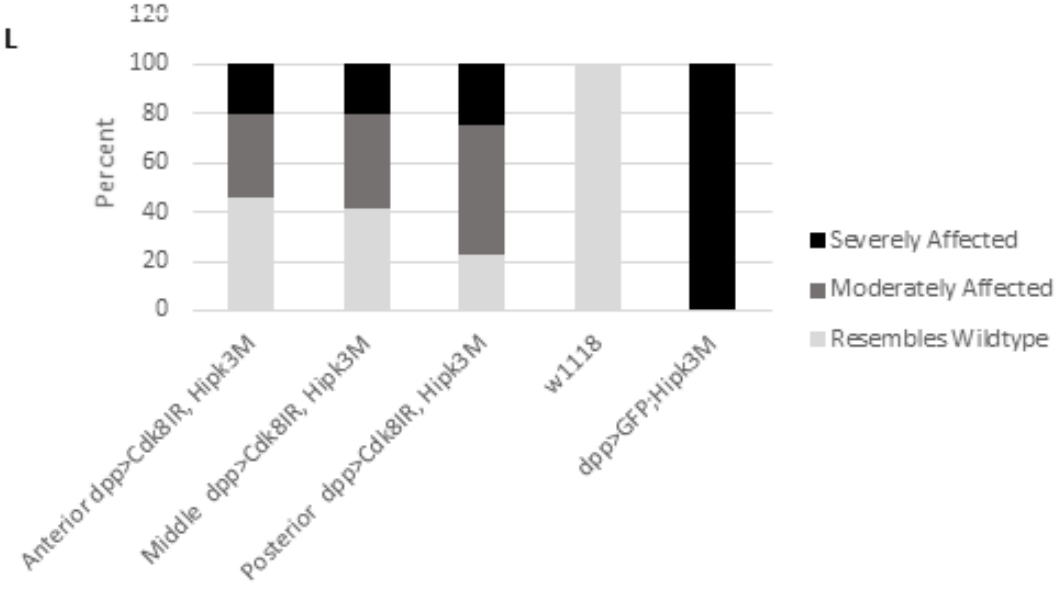
From here I decided to look at other adult Drosophila structures, specifically leg tissue. Driving expression of Hipk using *dpp-GAL4* (*dpp>GFP;Hipk3M-HA*) led to a severe effect on leg tissue, resulting in a gnarled, fattened phenotype compared to wild type legs (Figure 9A-B). In this context, both Cdk8 and Hop knockdown led to suppression of Hipk-induced defects in leg tissue (Figure 9C, F). Posterior, middle and anterior legs of all genotypes were scored and placed in the following categories: severely affected, mildly affected, and legs that resembled wildtype. Hop knockdown had a strong effect on Hipk overexpression phenotypes, with 34.51% of all (posterior, anterior and middle) legs resembling wildtype (Figure 9 C, K), and 26.67% displaying moderate levels of Hipk activity (Figure 9D, K). Interestingly, Cdk8 knockdown had the strongest effect on Hipk activity in the leg, with 36.73% of legs resembling wildtype (Figure 9F, L), and 41.50% showing slight levels of Hipk activity on average (Figure 9G,L). Similar to what was seen in the notum, Mekk1 knockdown only slightly rescued the baseline *dpp>GFP;Hipk3M-HA* phenotype with only 15.48% of showing moderate Hipk activity levels, and zero percent resembling wildtype (Figure 9I, M). Hop, Cdk8 and Mekk1 knockdown on their own had no effect on adult Drosophila leg structure (Figure 9E, H, J).


Anterior
Middle
Posterior
A w1118
B dpp> GFP; Hipk3M
Severely Affected
C dpp>HopIR, Hipk3M
Resembles Wildtype
D dpp>HopIR; Hipk3M
Moderately Affected
E dpp>HopIR; GFP
F dpp>Cdk8IR; Hipk3M
Resembles Wildtype
G dpp>Cdk8IR; Hipk3M
Moderately Affected
H dpp>Cdk8IR; GFP
I dpp> MekkIR; Hipk3M
Moderately Affected
J dpp>Mekk1RNAi, GFP

K


120
100
80
60
40
20
0
Percent
Anterior dpp>HopIR, Hipk3M
Middle dpp>HopIR, Hipk3M
Posterior dpp>HopIR, Hipk3M
w1118
dpp>GFP;Hipk3M
Severely Affected
Moderately Affected
Resembles Wildtype

L


120
100
80
60
40
20
0
Percent
Anterior dpp>Cdk8IR, Hipk3M
Middle dpp>Cdk8IR, Hipk3M
Posterior dpp>Cdk8IR, Hipk3M
w1118
dpp>GFP;Hipk3M
Severely Affected
Moderately Affected
Resembles Wildtype

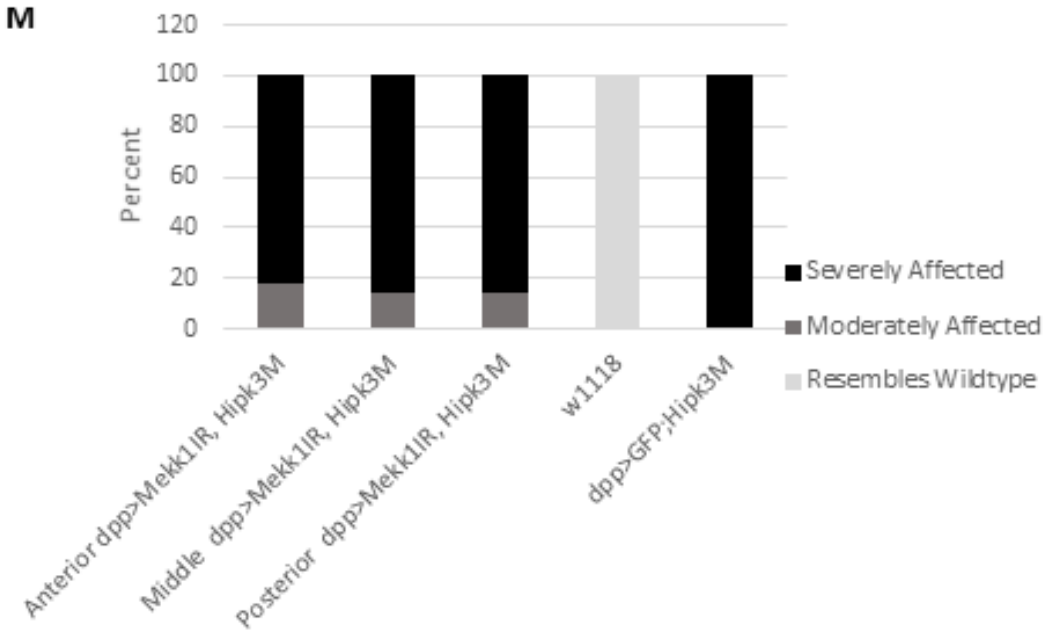


Figure 9 Hop and Cdk8 knockdown modulates Hipk activity in the Drosophila leg tissues.

A) Wildtype legs: Anterior, Middle, Posterior in columns. B) *dpp>GFP,hipk* induces overgrown, knarled limbs, adult flies are unable to eclose, and are labelled as "severely affected". C, D) Hop knockdown reduced the amount of severely affected legs, and were categorized into Moderately Affected and Resembles wildtype, quantified in K (n=85). E) Hop knockdown with titration control had no effect on leg phenotype. F, G) Cdk8 knockdown reduced the amount of severely affected legs, and were categorized into previously described categories, quantified in L (n=98). H) Cdk8 knockdown with titration control had no effect on leg tissue development. I) Mekk1 knockdown slightly reduced the amount of Hipk-induced severely affected legs, and were categorized into Moderately Affected and Resembles wildtype, quantified in M (n=56). J) Mekk1 knockdown with titration control had no effect on adult leg phenotype.

During Wnt/Wg signaling, Drosophila and vertebrate Hipk family members act to inhibit functional $SCF^{Slimb,}$ allowing Arm/β-cat levels to accumulate. Hipk also phosphorylates Arm β-cat on another residue, and activates Arm/β-cat/TCF driven transcription (Figure 3; Swarup and Verheyen, 2012, Lee et al, 2009a). Upon overexpression of Hipk with *ptc-Gal4,* we can see increased stabilization of Armadillo in 86% of wing imaginal disc at the dorsal ventral boundary (Figure 10B-B'') compared to wildtype (Figure 10A). When assessing the Wg target gene *distal-less* (using *dll-lacZ*) (see Figure 10F for wildtype), there were increased *dll-lacZ* levels in 73% of the discs within the *dpp* domain compared to wildtype (Figure 10G). Levels of Senseless were also

examined, but due to poor antibody quality, it was impossible to determine what effect Hipk and Hop were having on this Wingless target protein.

Consistent with the observations seen in adult structures where we knocked down Hopscotch and observed reduced Hipk activity, when Hop was knocked down while simultaneously expressing Hipk3M-HA, the percentage of discs with stabilized Arm and increased Dll was significantly reduced compared to Hipk3M-HA expression alone (Figure 10C, G H). Indeed, 25.8% of *ptc>HopIR, Hipk3M* discs had increased Armadillo, and 19% had increased levels of *dll-lacZ,* compared to 0% increases of Arm and *dll-lacZ* seen in wildtype wing imaginal discs (Figure 10I). To ensure that Hop knockdown alone was not affecting levels of Arm stabilization required for the Wg target transcription, we assessed levels of Arm and found there was a very slight decrease in levels of Arm in the hinge region. However, there was no effect on stabilization of Armadillo at the dorsal ventral boundary, indicating that Hop knockdown was not likely modulating Arm-induced transcription (Figure 10D). To visualize Arm destabilization for a negative control, we knocked out Hipk using mitotic clones and observed complete disruption in levels of stabilized Arm along the dorsal ventral boundary (Figure 10E-E' and Figure 11). From these data, there is slight evidence that Hop knockdown modifying Hipk's modulation of the Wingless signaling pathway. However future experiments would need to be performed to confirm these results. (Figure 10I).

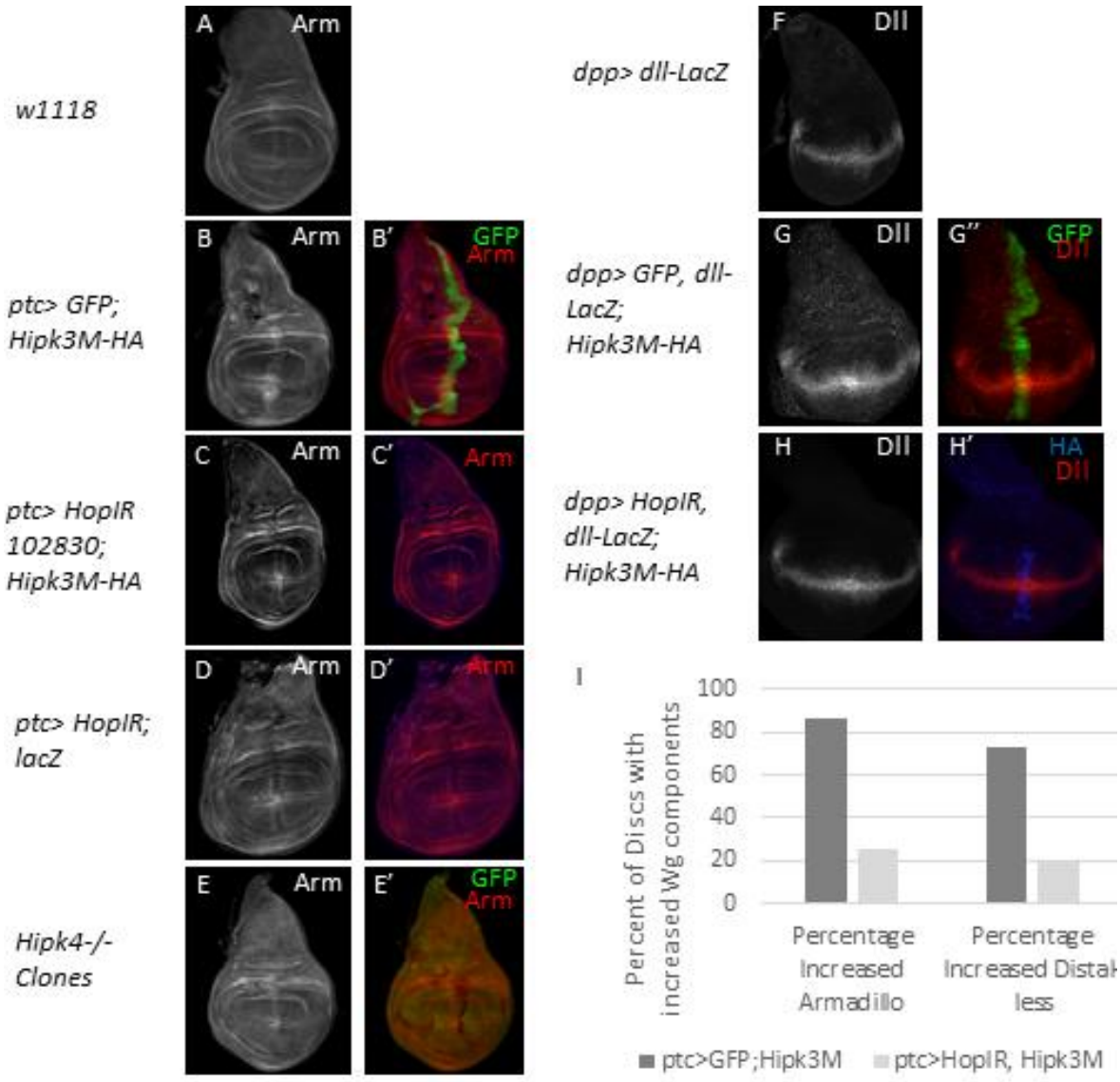


Figure 10 Hop knockdown modulates Hipk's activity on the Wingless signaling pathway:

A) Wildtype Armadillo in late-third instar wing imaginal disk B-B') *ptc>GFP;Hipk3M-HA* leads to increased, stabilized levels of Armadillo within the ptc domain (n=36). C-C') *ptc>HopIR;Hipk3M-HA* reduces hipk-activity-induced stabilization of Arm (n=31). E-E') *hipk* loss-of-function clones disrupt levels of stabilized Arm (n=9). F-F'') Dll- lacZ in late third instar wing imaginal discs. G-G') *dpp> GFP;Hipk3M-HA* leads to increases in Dll expression (n=34). H-H') *dpp>HopRNAI;Hipk3M-HA* reduces Hipk activity on the Wg target gene Dll (n=41). I) Comparison of the percentages of discs where we see increases in levels of Wingless components and targets compared to wildtype levels.

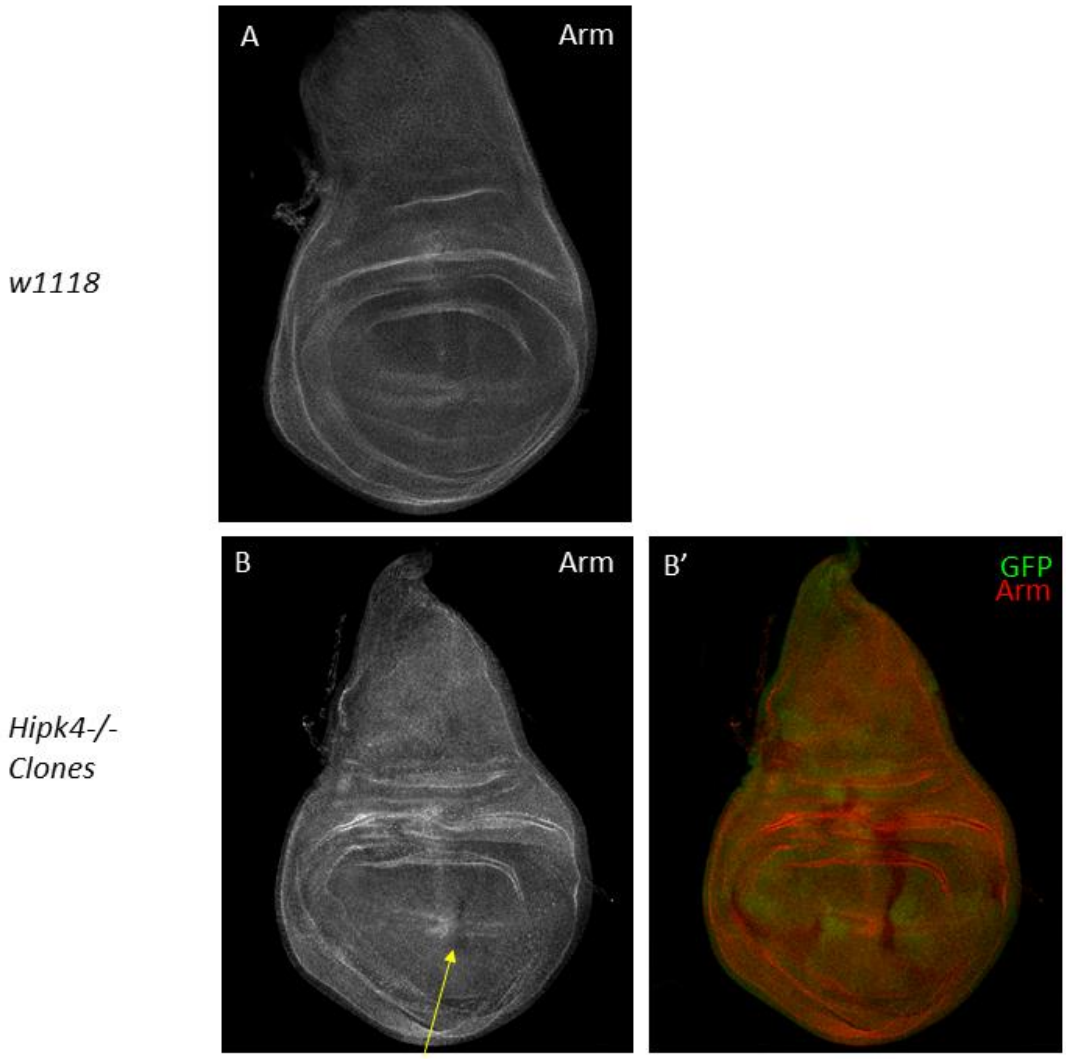


Figure 11 *hipk* loss of function disrupt levels of stabilized Armadillo
A) Wildtype Arm stain in the third instar wing imaginal disc B-B') *hipk* loss-of-function clones marked by the absence of GFP leads to cell autonomous decreases in levels of Arm within the dorsal ventral boundary (arrow).

The next pathway I decided to assess was the Hippo signaling pathway. As mentioned earlier, the Hippo pathway is primarily involved in tissue growth, and Hipk plays a role in this pathway by directly phosphorylating Yki, keeping Yki stable to bind to its co-transcriptional activator Scalloped, and able to induce transcription of its target genes, wingless, localized to the pouch and CyclinE (Chen and Verheyen, 2012).

Hipk over-expression had noticeable effects on Hippo targets CyclinE and Wingless in the wing pouch compared to wildtype (Figure 12A-B). Hipk overexpression caused increases of levels of CyclinE and Wingless in 45.0% and 91.0% of discs respectively (Figure 12G). Unlike modulating Hipk's role on the Wingless pathway, Hop knockdown did not affect Hipk-induced phenotypes seen when analyzing targets of the Hippo pathway. Knockdown of Hop did not modulate Hipk-mediated increases of CyclinE and Wingless (Figure 12D, G). Similar to what is seen with Hipk expression alone, simultaneous Hop knockdown and Hipk expression lead to 48% of discs having increased levels of CyclinE, and 91.0% of discs showing increases in levels of Wingless (Figure 12G). Hop knockdown alone had no effect on levels of CyclinE or Wingless (Figure 12E-E''). Hipk knockout using mitotic clones does not affect levels of CyclinE, but potently reduces levels of inner ring Wingless expression, a ring of Wingless expression that appears in the proximal hinge region during late-third instar development (Baker, 1988) (Figure 12F-F' and Figure 13B).

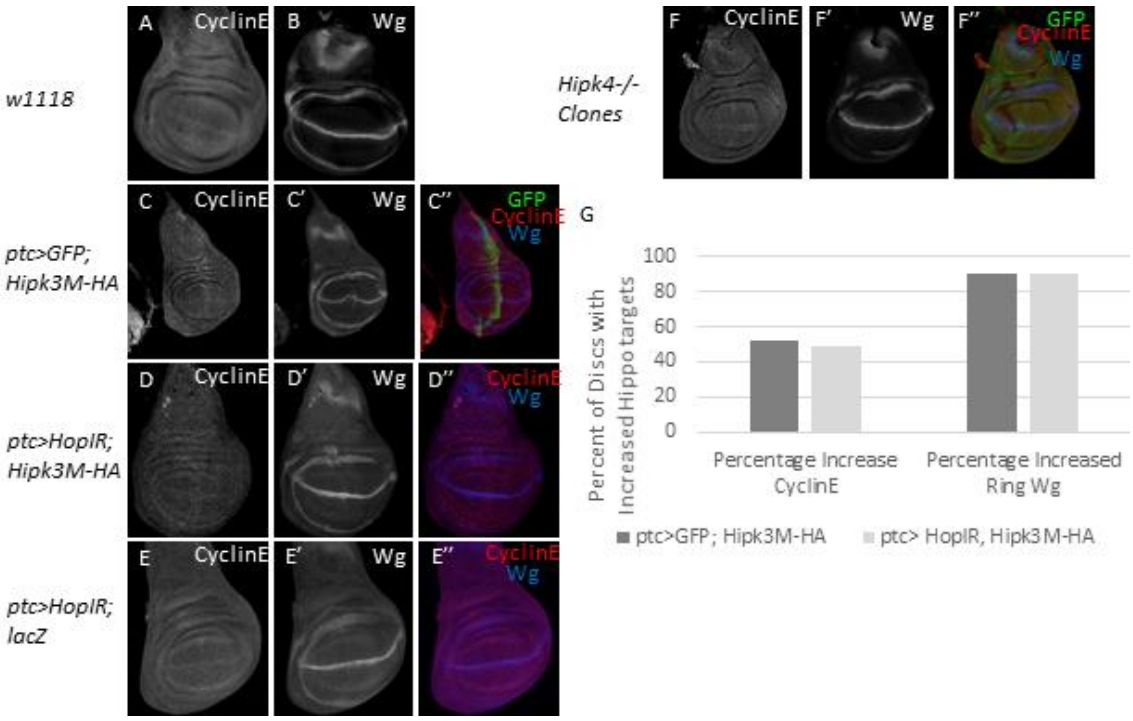


Figure 12 Hop knockdown does not modulate Hipk activity in the Hippo signaling pathway.

A) Wildtype CyclinE in late third instar imaginal discs. B) Wildtype wingless staining. C-C'') *Ptc>GFP; Hipk3M* leads to increases in levels of Cyclin E and Wingless localized to the wing hinge (n=54). D-D'') Knockdown of Hop does not modulate Hipk activity with respect to Hippo targets CyclinE (n=30). E-E'') Knockdown of Hop does not modulate Wg or CyclinE levels (n=42). F-F'') Knockdown of Hipk in clones in the imaginal disc resulted in severe disruptions of Wg localized to the hinge, but did not modulate levels of CyclinE (n=11). G) Quantification of increase in Hippo targets comparing over-expressed Hipk, and Hop knockdown with over-expressed Hipk.

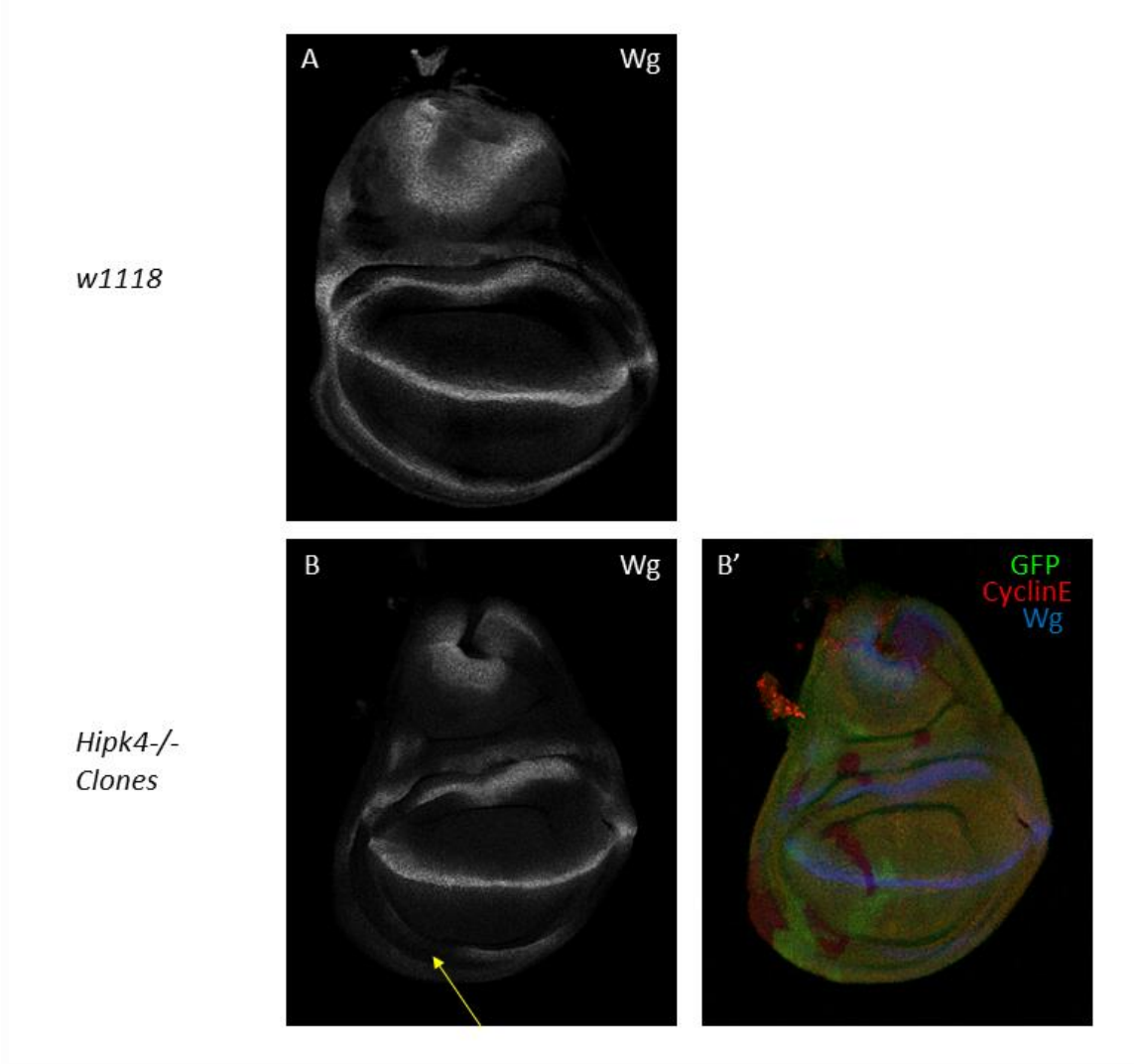


Figure 13 *hipk* loss of function disrupts Wingless in the hinge
A) Wildtype Wingless stain in the third instar wing imaginal disc. B-B') *hipk* loss-of-function clones marked by the absence of GFP leads to cell autonomous decreases in levels of Wingless localized to the hinge (arrow).

Together, these data show multiple adult and larval contexts in which Hipk activity is modulated by *hop* knockdown, suggesting a possible interaction between these two proteins. Interestingly, data from my colleague Jessica Blaquiere showed a genetic interaction with Hipk in the JAK/STAT pathway, further suggesting a possible interaction between Hipk and Hop. In Chapter 3 I will describe further experiments intended to elucidate how Hipk- and Hop interacted on the JAK/STAT cascade. What we ultimately discovered was unexpected, however, it was incredibly interesting.

Chapter 3. Interaction of Hipk with the JAK/STAT pathway

Jessica Blaquiere generated the data in Figures 14, 15A-E, and Nathan Wray generated data in Figs. 15F-H, 16-20.

As described previously, cancer can begin with a genetic alteration that drives unregulated proliferation in epithelial tissues (Hanahan and Weinberg, 2000). We used the UAS/GAL4 system to assess Hipk expression in imaginal and adult tissues to address if Hipk's role in signaling pathways affected tumour growth of epithelial tissues. Co-expression of Hipk and GFP with *dpp-Gal4* lead to excessively overgrown wing discs compared to wildtype (Figure 14C, D). This appeared similar to the proliferation seen in eye imaginal discs from previous studies (Bach et al, 2013) (Figure 6D, 14D).

Furthermore, expression of Hipk lead to tumour-like formations in whole Drosophila larvae (Figure 14B). These formations were similar in appearance to the melanotic tumours induced by constitutive JAK STAT signaling (Figure 6B) (Luo et al, 1997). Melanotic tumours form the over-proliferation of cells in the myeloid lineage. These data, along with evidence for Hipk in the Hippo pathway, and *hipk* overexpression inducing severely overgrown leg tissues (Figure 9B), suggests that genetically elevating Hipk leads to overgrowths and tumour-like formations.

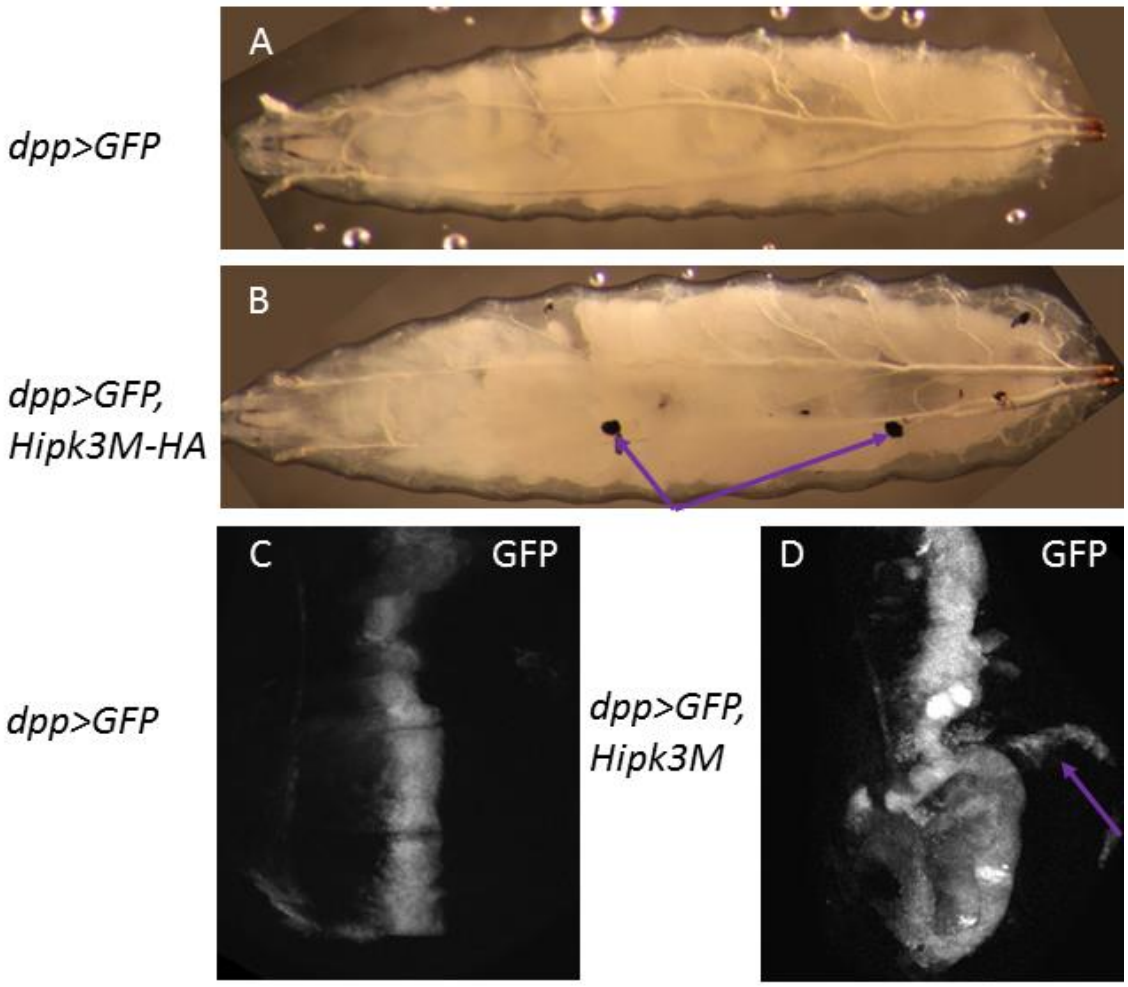


Figure 14 Hipk induces overgrowths and tumour-like formations.
A) *dpp>GF*P whole larvae. B) *dpp>GFP;Hipk3M-HA* induces formation of tumour-like formations throughout Drosophila larvae (arrows). C) *dpp>GFP* late third instar control larvae. GFP marks dpp-GAL4 expression domain D*) dpp>GFP;Hipk3M-HA* greatly expands the *dpp* expression domain (arrow).

Since observing Hipk's role in the formation of tumour-like structures similar in appearance to those caused by $Hop^{Tum\text{-}l}$ gain-of-function flies, we assessed if removal of one copy of *hipk* could modulate the lethality of flies with constitutively active JAK/STAT signaling. $Hop^{Tum\text{-}l}$ animals develop melanotic tumours at larval stages (Figure 15A), which usually results in early pupal death (Figure 15B). Removal of one copy of *hipk* extended the lifespan of the $Hop^{Tum\text{-}l}$ flies, with some flies reaching the eclosion stage (Figure 15D). To demonstrate quantitatively that *hipk* removal affected $Hop^{Tum\text{-}l}$ lethality we performed a $Hop^{Tum\text{-}l}$ lethality assay. This assay scored the stage at which lethality occurred including

early lethality (represented in Figure 15B), late pupal lethality (Figure 15D) and eclosed adults. 91% of $Hop^{Tum\text{-}l}$ flies were early pupal lethal. However when one copy of *hipk* was removed, the percentage of early pupal lethality was sharply reduced to 23%. Only 9% of the $Hop^{Tum\text{-}l}$ flies reached the late pupal stage, compared to 76% of $Hop^{Tum\text{-}l}$ flies with only one copy of *hipk*. Even more amazingly, 1% of $Hop^{Tum\text{-}l}$*;*$Hipk^{4}$*/TM6B* flies were able to fully eclose.

As Hipk knockdown modified the $Hop^{Tum\text{-}l}$ level of lethality, we next assessed if Hipk directly affected the JAK/STAT pathway. To do this, we used the JAK/STAT reporter 10xSTAT92E-GFP, which recapitulates the endogenous pattern of pathway activation (Bach et al, 2013). In wing imaginal discs, JAK/STAT signaling is primarily localized to the wing hinge (Figure 2B, 15F). When Hipk is expressed in the *dpp* expression domain, we observed non-autonomous increase in JAK/STAT reporter activity, which could have been due to tissue deformation (Figure 15G-G''). Importantly, when we knocked out *hipk* using mitotic clones as described earlier, we observed a cell autonomous reduction in levels of JAK/STAT reporter activity (Figure 15H-H''). Together, this data suggests that Hipk is required for JAK/STAT signaling and prompted us to unravel where and how Hipk acted within the canonical JAK/STAT pathway.

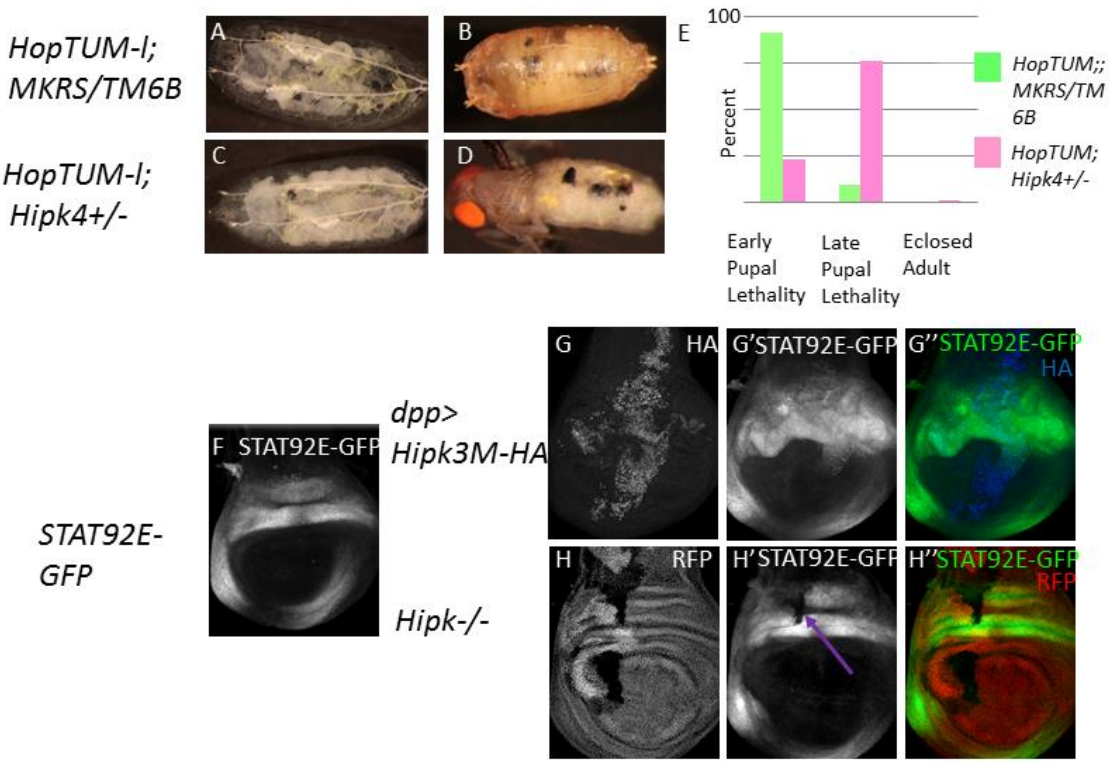


Figure 15 *hipk* is required but not sufficient for JAK/STAT signaling.
A, B) At 29°C *Hop*$^{Tum-1}$ induces larval tumour formation (A) and leads to larval/pupal lethality (B). C-D) Removing one copy of hipk suppresses tumour frequency (C) and early larval/pupal lethality (D). E) Lethality assay. Flies were categorized into early pupal lethal (see 13B), late pupal lethal (D) and fully eclosed adults. Flies with one less copy of *hipk* were more likely to reach late pupal stage before dying. F) Control wing disc displaying 10X-STAT92E-GFP expression domain. G-G'') *dpp>Hipk3M* leads to increases in levels of 10X-STAT92E-GFP reporter. Hipk over-expression is seen by the presence of human influenza hemagglutin (HA) tag. H-H') *hipk4* mutant clones, marked by absence of RFP, give a cell autonomous reduction in 10X-STAT92E-GFP levels (arrow).

We designed RNAi-based pseudo-epistasis experiments and used heat-shock-induced RFP-positive "flip-out" clones along with the previously mentioned 10xSTAT92E-GFP reporter to assess at which point in the pathway Hipk might act. Upon heat shock, the heat-induced flippase enzyme can induce the FRT-mediated excision of a stop codon that was preventing transcription of *actin GAL4*. In cells in which this recombination/excision occurs the event is clonally inherited, generating a patch of cells expressing GAL4. The *actin GAL4* would induce expression of our transgenes and RNAi of interest, as well as RFP. As such, all cells marked with RFP were of the described genotype.

Hop knockdown lead to sharp reductions in levels of the JAK/STAT reporter within the clones (Figure 16C-C''). When over-expressed Hipk was introduced into this reduced Hop-background it lead to two distinct observations. First, RFP-marked *Act>HopRNAi;Hipk3M-HA* clones had cell autonomous reductions in JAK/STAT reporter activity. Second the size of the overall disc was drastically increased (Figure 16D'D''). This is interesting in itself, because previously, we saw that Hop knockdown did not seem to modulate Hipk activity in the Hippo pathway (Figure 12), a signaling pathway mainly involved with tissue growth. Initially this suggested that Hipk could not modulate JAK/STAT activity in the absence of Hop.

Hipk overexpression in clones, similar to Hipk overexpression with *dpp-Gal4*, resulted in increased levels of JAK/STAT reporter activity, possibly due to tissue deformation (Figure 16E-E''). *hipk* knockdown had similar effects as seen in $hipk^{-/-}$ mitotic clones, as *hipk* knockdown sharply reduced JAK/STAT reporter activity (Figure 16F-F'').

Overexpression of wild type Hop in clones was lethal as heat shock induction for the same amount of time as used for the other genotypes resulted in no clones, indicating that any RFP-labelled flip-out mis-expression clones being formed were immediately extruded by the surrounding wildtype cells. To circumvent this issue, we altered our protocol and induced clones 72 hours after egg laying. Under these circumstances, tiny clones were able to form, however, these clones were also immediately extruded by surrounding wildtype tissue (Figure 16G-G''). Surprisingly, when *hipk* was knocked down in the Hop-WT background, the clones were able to grow larger (Figure 16H-H''). The above results were also found in eye imaginal discs (Figure 17). Two of the described pseudo-epistasis experiments gave us insight into how Hipk mediated its effects on the JAK/STAT pathway. First, *Act>HopRNAi;Hipk3M-HA* clones had cell autonomous reductions in JAK/STAT reporter activity, indicating that Hipk could not modulate JAK/STAT activity in the absence of Hop. Second, *Act>HipkRNAi;Hop-WT* repressed the lethality of Hop-WT clones, suggesting that full Hipk activity is required for Hop-WT induced lethality. However, we were unable to place Hipk within the canonical JAK/STAT pathway and prompted us to ask if Hip was mediating its response on the JAK/STAT pathway by some other means.

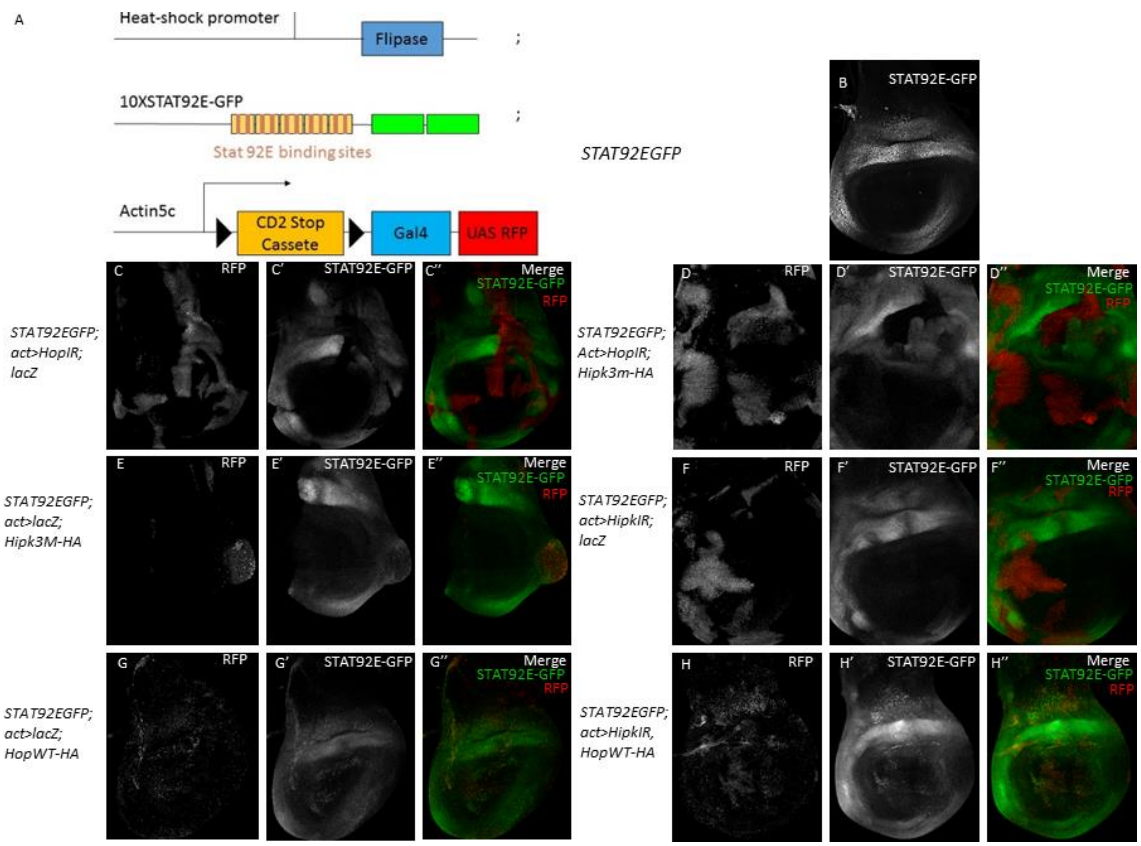


Figure 16 Hipk acts on the JAK/STAT pathway

A) Schematic of experiment design, there was a heat shock induced flippase component on the first chromosome and a 10xSTAT92E-GFP reporter on the second. The actin promoter would induce expression of the GAL4 only when the STOP codon was removed when heat shocked. Cells where RFP was expressed indicate the genotype to the left B) Wildtype Stat in the Wing imaginal disc C-C'') Hop knockdown severely decreased levels of JAK/STAT activity (n=31). D-D'') Hop knockdown with Hipk expression lead to decreased STAT within the clone, with Hipk expression severely affecting wing disc size (n=41). E-E'') Hipk overexpression leads to increased ectopic JAK/STAT activity (n=36). F-F'') Hipk knockdown also reduces levels of JAK/STAT reporter activity in the wing imaginal disc (n=35). G-G'') Hop overexpression was lethal at previous conditions used, and therefore larvae were heat shocked 72 hours after egg laying. After doing this, any clones found were immediately removed by nearby wildtype imaginal tissue. H-H'') Hop overexpression with Hipk knockdown resulted in clones that were also killed by apoptosis, however, the clones were able to get much bigger relative to Hop knockdown alone wing disc size (n=29).

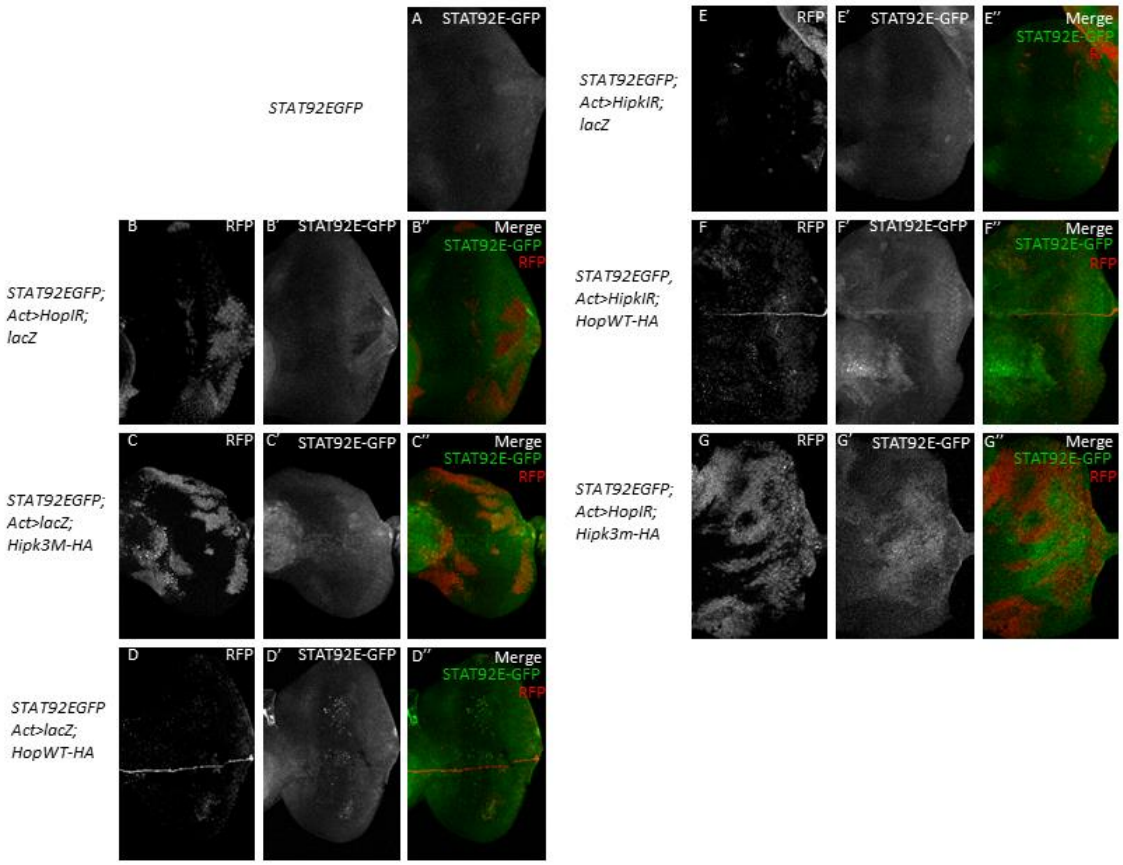


Figure 17 Hipk acts on the JAK/STAT pathway
A) Wildtype Stat in the Wing imaginal disc B-B''') Hop knockdown severely decreased levels of JAK/STAT activity (n=35). C-C'') Hipk overexpression leads to increased ectopic JAK/STAT activity (n=24). D-D'') Hop overexpression was lethal at previous conditions used, and therefore larvae were heat shocked 72 hours after egg laying.(n=28) E-E''). Hipk knockdown also reduces levels of JAK/STAT reporter activity in the wing imaginal disc (n=31). F-F'') Hop overexpression with Hipk knockdown resulted in clones that were also killed by apoptosis, however, the clones were able to get much bigger relative to Hop knockdown alone wing disc size (n=27) G-G'')Hop knockdown with Hipk expression lead to decreased STAT within the clone, with Hipk expression severely affecting wing disc size (n=36).

Unfortunately, our epistasis experiments were inconclusive in determining where Hipk acted on the JAK/STAT pathways. Due to the primarily nuclear localization of STAT92E and Hipk, we decided to test if the two proteins could physically directly interact. To do this we used *in situ* proximity ligation assays (PLA for short). PLA is a method used to detect individual proteins and protein-protein interactions in fixed tissue (Soderberg et al., 2006; Wang et al, 2015). In this type of PLA, tissue is fixed and incubated with primary antibodies against the two proteins of interest (or epitope tags contained within them). Next a pair of proximity probes is added. When the proximity probes bind to the specific

primary antibody epitopes a connector oligonucleotide is added that hybridizes to both proximity probes. The ligation of the proximity probes and the connector oligonucleotide leads to the formation of a circular DNA molecule only if the proteins of interest are within 40nm. Once ligated the circular DNA molecule can serve as a template for rolling circle amplification. Ultimately, rolling circle amplification leads to the production of a single-stranded concatemeric copies of the original DNA circle. Because the DNA molecule is still attached via the proximity probe, the amplified DNA copies will be attached to the protein complex of interest. This amplified DNA product can then be visualized by hybridization of fluorescently labelled oligonucleotides that correspond to a sequence on the DNA circle. On a microscope, this hybrization event can be visualized as small, fluorescently labelled dots that stand out from any background noise (Soderberg et al., 2006). As such, an interaction event between two proteins can be visualized with a red fluorescent signal (Figure 18 A,C). In 91% of the wing discs of the genotype *Dpp>Stat92E-Myc, Hipk1M-HA,* there was a PLA signal as seen in Figure 15B (n=56) compared to 0% seen in our negative control genotype *dpp>GFP, Hipk1M-HA* (18B-B''). Surprisingly, increasing magnification showed us that the interaction was primarily taking place outside of the nucleus, as co-stains with DAPI indicated that the interaction between Stat92E and Hipk was occurring outside of the DAPI-stained nuclei (Figure 18 C-C'''' and Figure 19).

To further examine where this PLA event was occurring, we used two newly generated fly lines UAS-MYR-Hipk-HA, which has Hipk localized to the membrane, and UAS-NLS-Hipk-HA which has a nuclear localization sequence. Previously, Myr-Hipk-HA has been shown to localize to the membrane, whereas NLS-Hipk-HA localizes to the nucleus. Interesting, when a PLA was performed with MYR-Hipk-HA and Myc-STAT92E we found a strong signal (Figure 18 E-E''''), consistent with our previous results that the interaction was occurring outside of the nucleus. A negative control of *dpp> UAS-MYR-Hipk-HA, UAS-GFP* was performed to ensure that the interaction was not an artefact, and indeed, no PLA signal was detected in this experiment (Figure 18 D-D''). Performing a PLA between NLS-Hipk and Myc-STAT92E did not result in a positive interaction (Figure 18 F-F''). Together, this data strongly indicate that Hipk's interaction with STAT92E takes place within the cytoplasm, and is associated with the membrane.

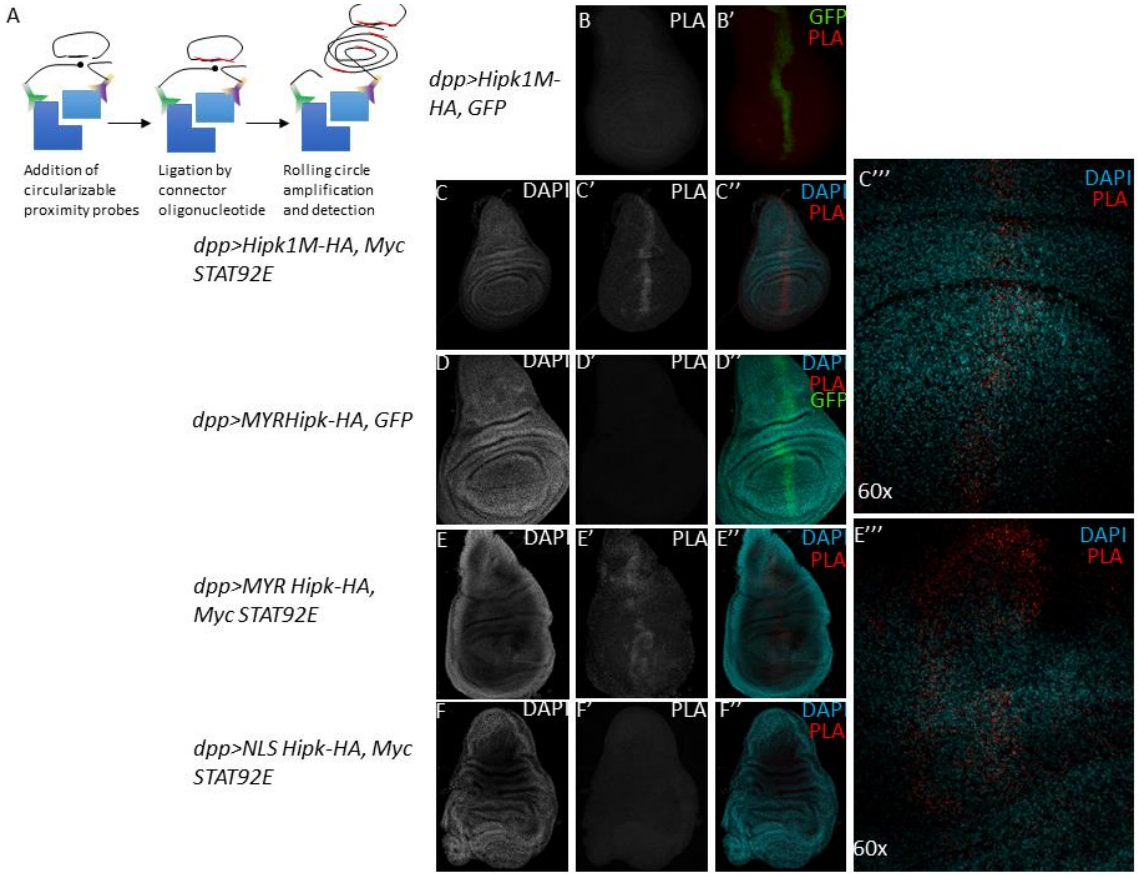


Figure 18 STAT92E and Hipk directly interact in the cytoplasm in fixed tissue.
A) Schematic of PLA: Tissue is fixed prior to addition of proximity probes, which bind to primary antibodies already present. A connecting oligonucleotide is added, and will hybridize to the two probes if they are less than 40nm apart. The ligation forms a circular DNA molecule, which will then amplify. Signal is detected by fluorescently labelled oligonucleotides hybridizing to the multiple copies of the ligating oligonucleotide. B) *dpp>GFP, Hipk1M-HA* negative control gives no PLA signal (n=17). C-C‴) *dpp>STAT92E-Myc, Hipk1M-HA* gives a strong positive PLA signal (n=54). The reaction seems to occur outside of the nucleus (C‴). D-D‴) *dpp>GFP*, *MYR-Hipk1M-HA* does not give a PLA (n=9). E-E‴) *dpp>STAT92E-Myc, MYRHipk1M-HA* gives a strong PLA signal, outside of the nucleus (n=5). F-F‴) *dpp>STAT92E-Myc*, *NLS-Hipk1M-HA* does not give a PLA signal, suggesting nuclear localized Hipk does not interact with STAT92E (n=15).

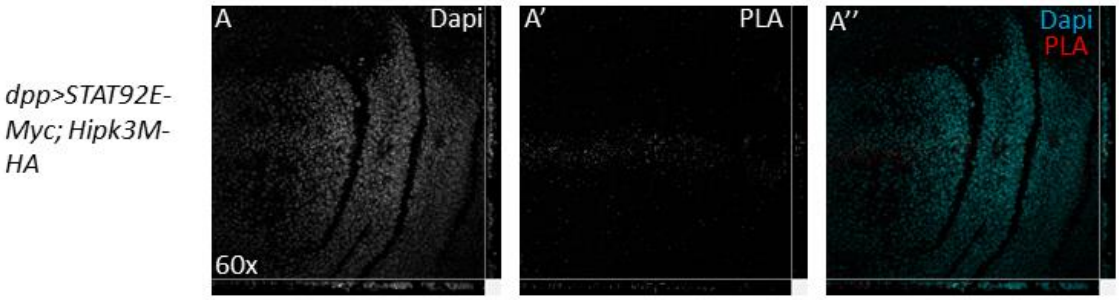


Figure 19 PLA Slices and Controls:
A-A'') Co-expressing STAT92E-Myc with Hipk1M-HA yields a protein-protein interaction (60x magnification).

In addition to the PLA results, we also showed that differentially localized Hipk-HA-tagged proteins affected JAK/STAT reporter activity in wing imaginal discs (Figure 20A). The kinase-dead version of Hipk reduced JAK/STAT reporter activity in clonal tissue, suggesting that Hipk kinase activity is necessary for propagation of the JAK/STAT signal (Figure 20B). Again, we used two newly generated fly lines UAS-MYR-Hipk-HA and UAS-NLS-Hipk-HA to test if JAK/STAT reporter activity is modulated when we express Hipk proteins localized to different areas within the cell. Assessing clones containing NLS we observed slight increases in the JAK/STAT reporter (Figure 20 C-C''). However Hipk localized to the membrane had very strong effects on JAK/STAT reporter activity. This data further suggests that Hipk could interact with STAT92E at the membrane and modulates the output of the JAK/STAT pathway by modulating STAT92E activity at the membrane in some manner (Figure 20 D-D'').

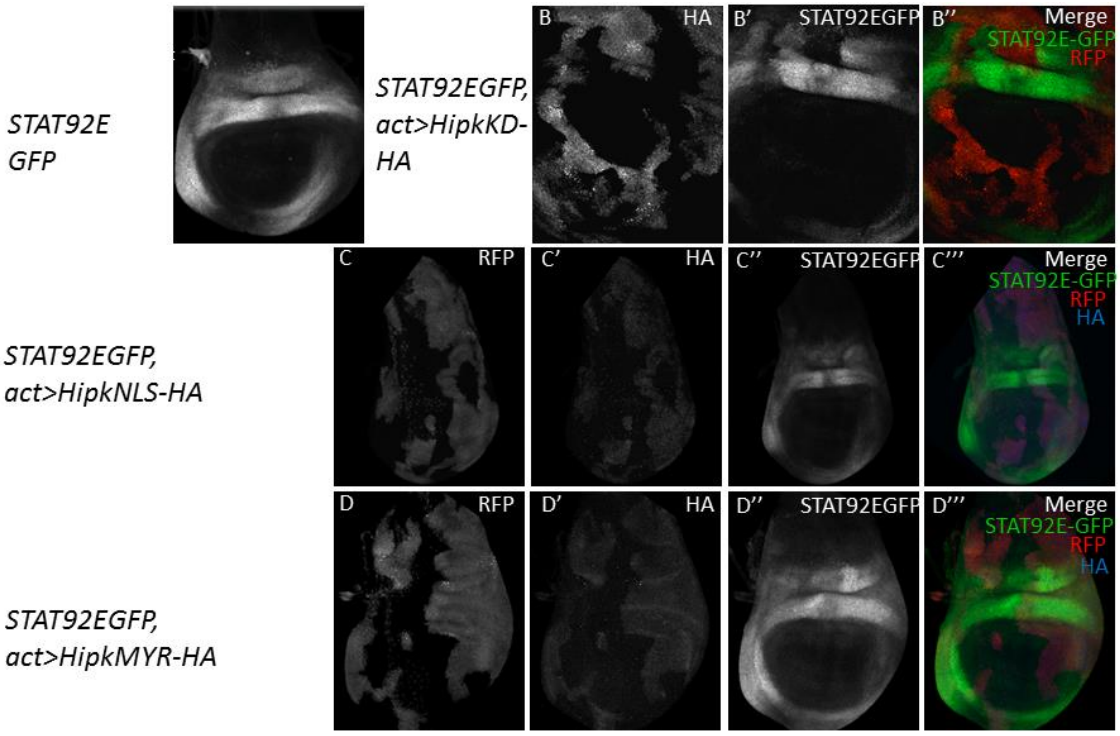


Figure 20 Hipk kinase activity and localization modify Hipk's effect on the JAK/STAT pathway.

A) Wildtype expression of the JAK/STAT reporter 10xSTAT92E-GFP. B) Kinase-Dead Hipk induces severe disruptions in the JAK/STAT reporter (n=14). C) Nuclear localized Hipk induces slight increases in JAK/STAT activity (n=11). D) Myristoylated Hipk induces substantial increases in JAK/STAT activity (n=13).

Chapter 4. Discussion

Evolutionarily conserved signaling networks are composed of components that when mutated, can lead to the formation of tumourous tissue, and these mutations can ultimately give rise to metastatic behaviour in these affected cells. We show here that Drosophila Hipk, an evolutionarily conserved regulator of several signal transduction pathways, is involved in tumourigenesis (Figure 14) (Lee et al 2009a, Lee et al 2009b, Swarup and Verheyen 2012, Chen and Verheyen 2012). Tumours formed by increasing Hipk activity appear similar to melanotic tumours due to over-active JAK/STAT signaling (Arbouzova and Ziedler, 2006). We show Hipk is required for JAK/STAT signaling. Using epistasis experiments we show that Hipk ameliorates activity of the JAK/STAT pathway. Interestingly, when we looked for interactions between the transcriptional effector of the JAK/STAT pathway, STAT92E and Hipk, we found a direct interaction as detected by proximity ligation assay.

4.1. In vivo kinase RNAi screen to identify Hipk regulators

Initially, an in vivo kinase RNAi screen was performed in an attempt to identify phospho-regulators of Hipk activity. From this screen, 58 out of 307 kinases when knocked down, suppressed Hipk activity (Figure 7E). Based on the assumption of the screen, these kinases could possibly be phosphorylating Hipk and regulating its activity. However, it is very unlikely that we identified so many putative phospho-regulators of Hipk activity with this screen, and further experiments would need to be performed in order to conclude that these kinases actually regulate Hipk activity. It is also possible that the kinases we knocked down that enhanced the Hipk over-expression could be interacting with Hipk. In this case, the kinase knockdown could be increasing Hipk activity, as normal levels of the kinase could prevent full Hipk activity. Again, further experiments would need to be performed in order to demonstrate these possible interactions. When 73 kinases were knocked down, one RNAi line would give a suppression of Hipk activity, whereas the other RNAi line would enhance Hipk activity (mixture, Figure 7E). This could have been due to off targets, as RNAi can interact with and downregulate mRNA from other genes. Another possible explanation could be the kinase knockdown affected a different protein, and

subsequent unknown interactions could be responsible for the enhancement or repression of Hipk activity. These unknown interactions could also be responsible for some of the kinases that when knocked down, lead to suppressed Hipk activity. To confirm that the kinase we knocked down is specifically modulating Hipk activity, we could re-introduce a transgene for the kinase we knocked down. If this re-introduced kinase transgene gave a phenotype that resembled Hipk over-expression, we could propose that the kinase we knocked down (and re-introduced in this experiment) is acting directly on Hipk protein itself. This screen makes the assumption that Hipk is phosphorylated to become active, but it is possible that Hipk phosphorylation can actually decrease Hipk activity. Phosphorylation of Hipk on a residue outside of the activation loop could result in Hipk activity suppression. As such, some of the kinases we knocked down that enhanced Hipk activity could be kinases that are normally required to phosphorylate and inhibit Hipk activity. Finally, we presume the kinases we knock down are upstream of Hipk, and are regulating Hipk through phosphorylation. However, it is possible that these kinases are downstream of Hipk, and could possibly be regulated by Hipk-induced phosphorylation. Collectively, it is important to mention that the initial kinase screen only gave us *possible* phospho-regulators of Hipk activity, and further experiments are necessary to confirm these kinases as phospho-regulators of Hipk.

4.2. Hipk-Hop or Hipk-STAT92E Interaction?

In the second Chapter we discuss a role for Hopscotch as a putative phospho-regulator of Hipk. As we attempted to characterize how Hipk modified the JAK/STAT pathway it appeared that Hipk could not act on the JAK/STAT pathway in the absence of Hop, and data suggesting that Hipk is required for the JAK/STAT drove us to look at other components of the pathway. Interestingly, we observed a direct interaction between Hipk and STAT92E. This however, results in a discrepancy: How can Hipk be regulated by Hop when we see strong evidence that Hipk actually regulates STAT?

My PLA results showed that Hipk needed to be localized to the membrane in order to interact with STAT92E. Other components of the JAK/STAT pathway, namely Dome and Hop, are located at the cell membrane. This is where STAT92E gets phosphorylated by Hop on a tyrosine residue for full activation. I previously mentioned that vertebrate

Hipk2 needs to be phosphorylated on Y354 for full activation, substrate affinity and localization (Saul et al 2012). This Y354 phosphorylation site is conserved in Drosophila. One possibility is that Hipk's likely interaction with STAT92E primed Hipk to be phosphorylated by Hop on Y354. If Hop was knocked down, less STAT92E would associate with the complex at the membrane, as Hop would not be able to form SH2 docking sites that are required for STAT92E to bind. Because we show Hipk interacts with STAT92 directly, then less Hipk would be associated with this Dome/Hop/STAT92E complex at the membrane, and Hipk's activity would be decreased because it would not be able to be phosphorylated on its activation loop residue by Hop. This model would be consistent with results from Chapter 2, as we saw decreased Hipk activity when Hop was knocked down in multiple contexts. This would also be consistent with results from Chapter 3 as Hipk directly interacts with STAT92E at the membrane. Instances of proteins priming sites for subsequent reactions is seen in the Wnt pathway. With no Wg ligand is present, CKI phosphorylates β-catenin at S45. This phosphorylation event is necessary to initiate a GSK-3β dependent phosphorylation events that result in the degradation of β-catenin (Amit et al, 2002). Further experiments such as kinase assays to test if Hop phosphorylates Hipk at this residue could indicate that the Hop-Hipk phosphorylation event could occur (see Figure 21).

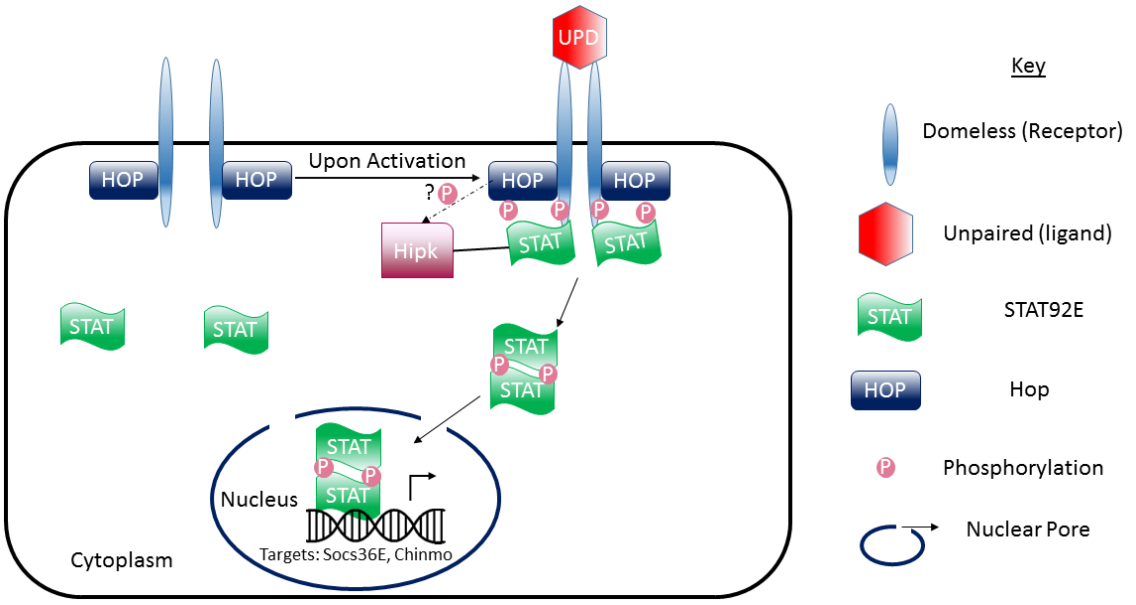


Figure 21 Possible Model for a Hipk-Hop-STAT92E Interaction
In this model, Hipk's interaction with STAT92E at the membrane brings Hipk in close contact with the Hop/Dome/STAT92E complex at the membrane. This membranous localization could allow for Hop-mediation tyrosine phosphorylation of Hipk on Y354, a core activation loop residue.

4.3. Hipk and Cancer

As described in the introduction elevated Hipk levels are seen in cervical cancers, apiloctyic astrynomas, kidney fibrosis, idiopathic pulmonary fibrosis, colorectal cancer cells and is involved in several other proliferative diseases (Al-beiti and Lu 2008, Cheng et al., 2012; Deshmukh et al 2008; D'orazi et al., 2006; Fan et al, 2011; Saul and Schmitz, 2013, Yu et al, 2009). However, several papers show decreased expression of vertebrate Hipks is correlated with several types of cancers including thyroid and breast carcinomas, papillary thyroid carcinomas, and follicular thyroid carcinomas (Pierantoni et al 2002, Lavra et al, 2011). Hipk2 deficiency has also been linked to chromosomal instability via cytokinesis failure, increasing tumourigenicity in mouse embryonic fibroblasts (Valente et al, 2015). Colon cancer tumours with increased expression of Hipk had a better outcome than tumours with low expression (Soubeyran et al 2011). Hipk2 over-expression leads to stabilization of p53 proteins, a known tumour suppressor (Wang et al, 2001). Furthermore, Hipk has also been known to promote apoptosis by phosphorylating the tumour suppressor p53 at serine-46 under ultraviolet radiation conditions (D'orazi et al, 2002). This phosphorylation even turns on p53-induced target genes, which promotes apoptosis.

However, all our Drosophila Hipk phenotypes demonstrate that Hipk elevation results in over-proliferation and tumour formation, consistent with several studies that see elevated expression levels when monitoring Hipk2 levels in cancer, but inconsistent with several other studies.

So how do we explain these discrepancies between Hipk acting as a tumour suppressor or an oncogene? A simple explanation is the idea that Hipk plays different roles in these cancers simply depending on the context. As many cancers have several mutated signaling components, Hipk levels in various cancers could be dependent on the other known/unknown proteins that are mis-expressed in that type of cancer. Another possible explanation is the idea of temporal changes of Hipk activity in various cancers. We, with others, suggest that Hipk expressivity in cancers varies depending on the stage of the tumour, the type of cancer, and Hipk mutation levels (D'Orazi et al, 2006, Albeiti and Lou 2006). D'Orazi et al suggest that Hipk2 expression in human colorectal cancers tended to correlate inversely with the staging of the tumours. On the other hand, Hipk2 levels were significantly lower in stage I tumours compared to stage II/III tumours (Albeiti and Lou, 2006). As such, it is possible that at least initially, elevating Hipk levels aids in overgrowth and proliferation of malfunctioning cells. At later stages, low Hipk levels could be detrimental to tumour suppression, as Hipk is unable to phosphorylate p53 to induce apoptosis, thus aiding in the well-being and growth of tumourous tissues. Because of the discrepancies between when and how Hipk expressivity is modulated in the described cancers, it could be fruitful to analyze Hipk expression, mutations, duplications and/or deletions in tumours at multiple stages in human patients. This could give us an idea of whether or not to attempt to amplify Hipk levels, or try to inhibit Hipk activity, possible therapeutic designs we could use to try and slow progression of the tumour. Another area of focus could be cancers where JAK/STAT signaling is severely affected, such as Hodgkin's lymphoma, which has increased levels of Stat 5, 6 and a JAK2 mutation (Crochet et al., 2006). This could also be assessed in cell culture, as we could obtain Hodgkin lymphoma cell lines, and test whether Hipk2 knockdown or inhibition through some small-molecule modulated division capability or survivability of these cancerous cells.

4.4. Development of Hipk-Activity Inhibitors

Hipk regulation is very tightly controlled. Hipk2 levels in unstressed levels are kept low through ubiquitination mediated by proteins such as Siah-1 and WSB-1 (Winter et al, 2008, Choi et al, 2008). Hipk2 activity is also regulated through cis-autophosphorylation and trans-autophosphorylation of its activation loop (Saul et al, 2013; Siepi et al, 2013). Saul et al. identify other phosphorylation sites that were not subject to phosphorylation by Hipk protein itself, and suggest that Hipk could be regulated at these other residues through a separate trans-phosphorylation event.

Here, we attempted to identify trans-phosphorylation regulators of Hipk function. Unfortunately, our data is insufficient to fully identify our putative kinase phospho-regulator, Hopscotch, as directly regulating Hipk function. Furthermore, our data suggest that Hipk acts on STAT92E rather than Hop.

This does not suggest that we should not attempt to identify kinase regulators, as well as other compounds that could modify Hipk activity. Indeed, recent work describes the synthesis of a selective inhibitor of Hipk2 (Cozza et al, 2014). The researchers developed a compound called TBID, which was found to reduce Hipk2 activity in vitro and in vivo. Other scientists discuss the use of microRNAs to regulate Hipk activity. Conte and Pierantoni (2015) review how microRNAs regulate Hipks expression. Furthermore, in collaboration with Dr. Roger Linnington, our lab may screen through novel small-molecule compounds from bacteria from the North-West coastline of North America for those that could modify Hipk activity in cancerous cell lines. All in all, a number of ways to affect Hipk activity already exist, or are being newly identified, but identifying scenarios where modifying Hipk levels is a useful therapeutic approach is necessary, and will differ on individual patients and the specific mutations in their tumours.

4.5. Hipk-STAT92E Interaction Mechanism

We provide evidence that Hipk modulates JAK/STAT target gene expression. We further show that Hipk likely interacts with STAT92E, and present data that the Hipk kinase domain is required for its effect on the JAK/STAT pathway. We also provide preliminary

evidence that this is occurring at the cell membrane, where the Hop/Domeless/STAT92E receptor complex lies**.** As such, it is possible to suggest that Hipk directly phosphorylates STAT92E, but further experiments such as a kinase assay would be required to verify this model. However, before going forward, there is one major issue that needs to be addressed about the possible Hipk-induced phosphorylation reaction of STAT that is: which domain of STAT92E responds to Hipk-mediated phosphorylation?

All STAT proteins must be phosphorylated on a conserved tyrosine residue on the C-terminal side of the protein for full activation. Without this phosphorylation event, STATs are unable to form dimers via their Src homology 2 (SH2) domains, and induce transcription. Hipk was initially identified as a serine-threonine kinase, and as such, phosphorylates only on serine or threonine residues. However, recently vertebrate Hipk2 was shown to autophosphorylate on a tyrosine residue (van der Laden, 2015), suggesting that Hipk proteins can, in fact, phosphorylate tyrosine residues. There is no evidence yet that Hipk can phosphorylate a substrate on a tyrosine. Hop knockdown induces disruptions in levels of JAK/STAT activity. Over-expressing Hipk in a Hop knockdown background is unable to rescue the disruptions in JAK/STAT reporter activity, and could possible indicate that Hipk is unable to phosphorylate this tyrosine residue. However, further experiments would need to be performed to conclude this idea. We do not believe that Hipk is phosphorylating the Y700 residue required to activate Stat, as simultaneous *hop* knockdown and Hipk overexpression led to a similar decrease in JAK/STAT reporter activity as *hop* knockdown alone, suggesting that Hipk overexpression could not compensate for loss of Hopscotch (Figure 16D), which would normally phosphorylate STAT92E on this tyrosine residue for its activation. Furthermore, Hipk is generally seen as a regulator, and not necessarily as a component, of most signal transduction pathways and for these reasons, it seemed unlikely that Hipk is phosphorylating STAT92E on this essential tyrosine residue. As such, I tried to bioinformatically identify another region within STAT92E that could undergo phosphorylation by Hipk and serve as a sight of modulation of Stat activity.

Vertebrate STATs 1a, 3, 4 and 5 have a conserved motif in their C terminal domain called PMSP. Phosphorylation of a serine residue in that motif (S727) is required for maximal activation of transcription by both STAT1 and STAT3 (Wen et al, 1995). As Hipk

is a serine/threonine kinase, we tried to identify this domain in Drosophila STAT92E, but unfortunately this domain does not appear to be conserved. However, we were able to identify several serine/threonine residues close to the C-terminal end of Drosophila STAT92E, which could represent possible phosphorylation sites where Hipk could be acting on STAT92E (Figure 22). Admittedly, the data presented here is insufficient to suggest Drosophila Hipk acts on these residues of STAT92E and further experiments are required to determine this possible phosphorylation event. However, we recently discovered evidence that vertebrate Hipk2 can phosphorylate STAT3 in cos7 mammalian cells (Matsuo et al, 2001). Experiments to determine if Hipk and STAT92E interact include co-immunoprecipitation assays between Hipk and STATs which would be useful to confirm the PLA experiments presented in this thesis. Furthermore, cloning truncated versions of Hipk and/or STAT92E and perform PLA/co-immunoprecipitation experiments could be useful to determine which domains are necessary for this potent Hipk/STAT92E interaction. Finally, it would be extremely interesting to determine if this interaction is conserved through vertebrates, and using luciferase assays we hope to discover if Hipk2 modulates JAK/STAT activity in HeLa cell lines.

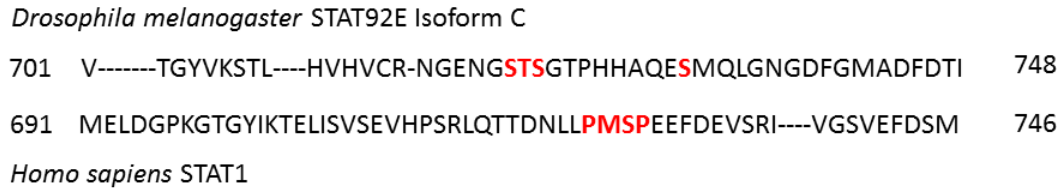


Figure 22 Serine residues required for vertebrate STAT activity are not conserved in Drosophila

Comparison of C-terminal amino acid sequences between Drosophila STAT92E and human STAT1. The PMSP motif that is required for maximal STAT activity in vertebrates is not conserved in Drosophila, howevere, there are several serine/threonine residues in Drosophila STAT92E that could be phosphorylated.

4.6. Conclusion:

Signal transduction pathways are crucial for co-ordinated development and growth of multicellular organisms. Dysregulation and mutations of components in these pathways can often lead to tumourigenesis. Evolutionarily conserved Homeodomain-Interacting-Protein-Kinase (Hipk) is a strong growth regulator of many signal transduction pathways, and elevated levels of *hipk* lead to tumour-like masses. While many known regulators of

Hipk exist, we attempted to identify novel phospho-regulators of Hipk activity. Here we present evidence that Hopscotch, a core tyrosine kinase of the JAK/STAT cascade, is a putative phospho-regulator of Hipk activity in multiple contexts. We show that modulation of Hipk expression levels modifies JAK/STAT activity in both wildtype and tumourous tissues. Finally, we show that Hipk interacts with the JAK/STAT transcriptional effector STAT92E at the membrane.

Taking all this data into account, I suggest that Hipk's interaction with STAT92E primes Hipk for Hop-mediated phosphorylation of Hipk on its Y354 activation loop residue. Several experiments would need to be performed to conclude this hypothesis. In the future, it would be fruitful to determine which domains of Hipk and STAT92E are necessary for their interaction at the cell membrane.

Our work provides a role for Drosophila Hipk in tumourigenesis and regulation of the JAK/STAT cascade. Compiling evidence for assessing levels of Hipk family proteins in cancer suggests that inhibition or activation of Hipk family members could be a useful therapeutic tool to combat diseases such as cancer. However, the underlying mechanisms for Hipk functions in normal tissues is necessary, and as we move forward, we hope to better utilize our knowledge of Hipk functions to combat disease.

Chapter 5. Methods

5.1. Fly lines and Crosses

Flies were kept and crossed at 25°C unless otherwise stated. Wildtype flies were *w*^{1118} (Bloomington). Fly lines used include *UAS-HA-Hipk1M, UAS-HA-Hipk3M, hipk*^{4} (Lee et al, 2009), *dpp-Gal4* (Staeling-Hampton *et al*, 1994), *w; UAS-Stat92E-Myc/CyO-Wg-LacZ* (gift from Sotillos et al, 2013). *UAS-Mekk1RNAi* (VDRC #110339 and #25529), *UAS-HopRNAi* (VDRC #102830, #v40037), *Cdk8RNAi* (VDRC #45370 and #107187), UAS-*HipkRNAi* (VDRC #108252), *eyegone-Gal4 (*Jang et al, 2003)*, patched-Gal4*, *scabrous-Gal4*, *UAS-LacZ (on II), UAS-LacZ (on III), UAS-GFP (on II, #BL5430), UAS-GFP (on III, #BL5431), FRT79 (#BL1997)* (Bloomington Drosophila Stock Centre), *Hop-WT-3xHA (FlyORF #FO01803), 10x Stat92E-GFP* (Bach et al, 2003), *UAS-HA-Hipk1M-MYR, UAS-HA-Hipk1M-NLS*.

To generate *hipk*^{4} clones, *hsflp122;GFP, FRT79/TM6B* females were crossed to *hipk*^{4}*FRT79/TM6B* males. Flies were transferred to a new vial after 24 hours, and the vial with offspring was placed in a 38 °C water bath for 105 minutes 48 hours after egg laying.

To generate Actin flip-out clones for UAS constructs in the epistasis experiment the *Hsflp122;Stat92EGFP/(CyO);Act>CD2>UAS-Gal4 RFP/TM6B* virgin females were crossed to the UAS lines described previously, and placed in a 38°C water bath for 14 minutes 48 hours after egg laying. Any crosses using Hop-WT-HA were placed in a 38°C water bath for 20 minutes 72 hours after egg laying. After heat shock, all fly larvae were placed at 29°C.

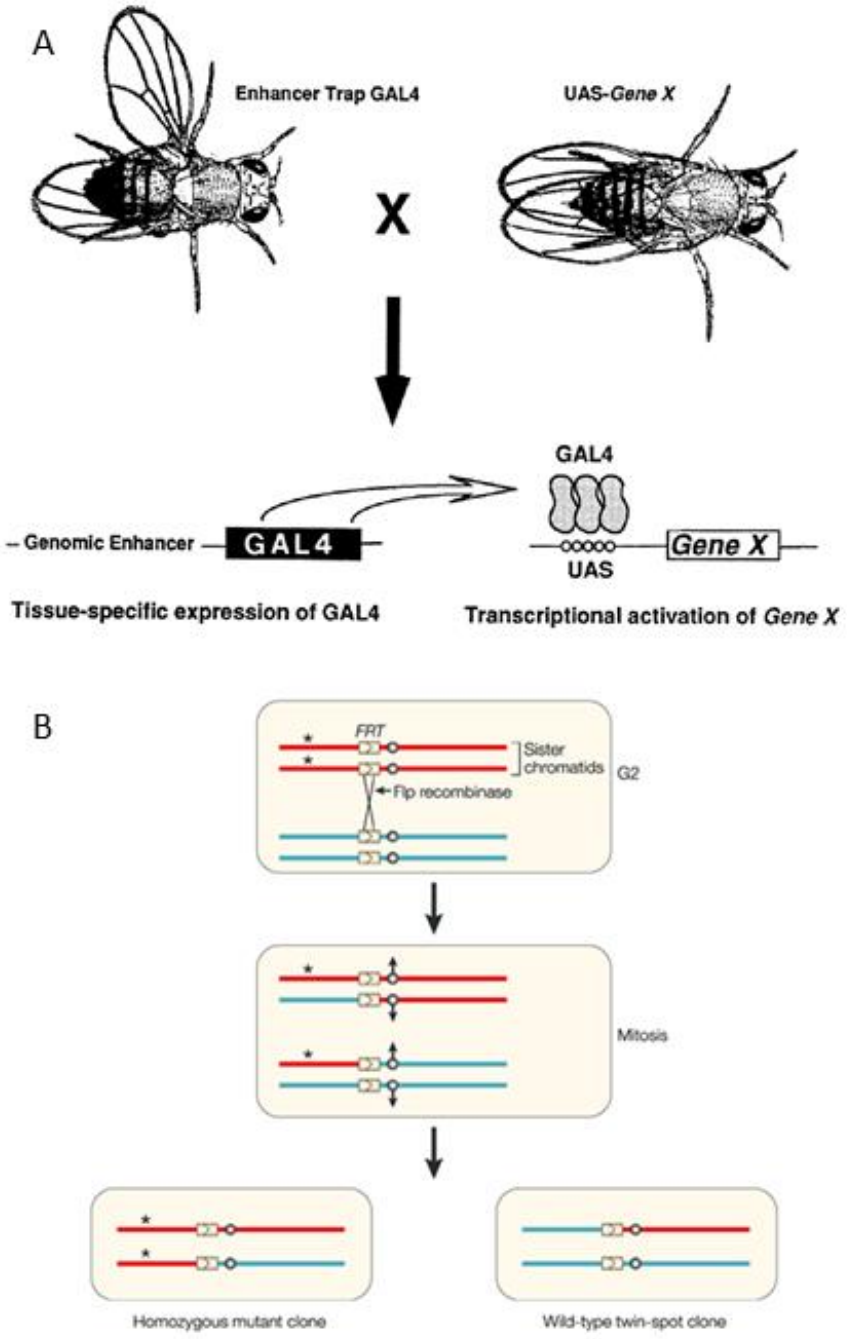


Figure 23 ***Drosophila melanogaster*** **as a genetic tool.**
A) Schematic of UAS/GAL4 system: Tissue specific GAL4 binds to UAS to mediate transcription of the gene of interest (Taken from Brand and Perrimon, 1993) B) Schematic of mitotic clone induction:Flippase mediates recombination at FRT sites, allowing for formation of a homozygous mutant clone (Taken from Johnston et al, 2002)

5.2. Antibody Staining

All stainings and PLA were done using donkey serum blocking reagent (Jackson Immunoresearch). Primary antibodies include anti-mouse Armadillo 1:50 (DSHB #N27A1), anti-mouse β-galactose 1:2000 (Cell Signaling #Z378A), anti-Chicken β-galactose-1:1000 (Abcam ab9361), anti-rabbit HA 1/2000 (Cell Signaling #3724s), anti-mouse Myc 1/200 (ABM #G019), anti-goat CyclinE 1/100 (Santa Cruz sc-15903) and anti-mouse Wingless 1/50 (DSHB 4D4). Secondary antibodies used include anti-chicken FITC (#703-095-155), anti-goat Cy3 (705-165-003), anti-mouse 647 (715-605-151), anti-mouse Cy3 (715-165-151), anti-rabbit 647 (711-605-152) (all from Jackson Immunoresearch), and all at 1:300 dilution.

5.3. Proximity Ligation Assay

All reagents supplied by Duolink-In Situ including: PLA proximity probe Mouse Minus-#A40109, PLA proximity probe Rabbit Plus- #A40106, Detection Reagents- Box #A40411 (separate components include, Ligase-#A34802, Polymerase- #A34803, Ligation 5x stock- #A40109, Amplification Stock Red- #A34703). Primary antibodies were used at the concentrations listed above at 4°C over a period of two nights, and discs were washed 7 times before the PLA experiment was performed. All other elements of the PLA followed the standard protocol provided described by Wang et al (2015).

5.4. Imaging

Whole larval and notum images were taken at 40x magnification using a Canon EOS Rebel T1i mounted to a Leica dissecting microscope. Adult legs were mounted in Aquatex Mounting Media and imaged with Axioplan 2 Microscope. Wing and eye imaginal discs were mounted in 70% glycerol in PBS, or in Dulbecco's Mounting Media with DAPI, and imaged upon the Nikon A1-r Confocal Microscope. All images were processed in Photoshop and figures were made in Powerpoint and exported as tif files.

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