Salt inducible kinases (Siks) are novel regulators of Hipk

by

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Abstract

Homeodomain-interacting protein kinase (Hipk) is an evolutionarily conserved regulator of signaling pathways, controlling growth and patterning, throughout development. Salt-inducible kinases (Siks), that conventionally function as cellular nutrient sensors, were identified in a previous screen in the lab for Hipk modifiers. Overexpression of Hipk caused tissue overgrowth due to neoplasia, that was suppressed when Siks (Sik2 or Sik3) were depleted. On the other hand, synergized tissue overgrowth was observed when Siks and Hipk were co-expressed. We also provide evidence that Siks may regulate Hipk protein levels as well as localization, and that they require their kinase function for modulating Hipk activity. Thus, our work provides a novel mechanism for upstream Hipk regulation, by Siks, also highlighting the potential cytoplasmic roles of Hipk in mediating growth.

Keywords: Hipk, Sik, overgrowth, synergy, delocalization, protein stability

Dedication

To my friends and family, that empowered me in ways that I never knew I needed.

அருவினை யென்ப உளவோ கருவியான்
காலம் அறிந்து செயின்
What is hard for those who act
With proper means and time and tact?

Couplet 483,
Chapter: காலமறிதல் / *Knowing the fitting time*,

Section: பொருட்பால் / *Wealth*

-Thiruvalluvar, 300 BCE

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List of Acronyms

AEL	After egg lay
ACTH	Adreno-corticotropic hormone
Akt	Ak strain transforming / Protein kinase B (PKB)
Arm	Armadillo
AMPK	AMP-activated protein kinase
AMPK-RK	AMP-activate protein kinase – related kinase
bp	Base pair
BSA	Bovine Serum Albumin
CA	Constitutively active
CaMKII	Ca^{2+} / Calmodulin – dependent kinase II
CrebA	cAMP-response element binding protein A
CRISPR	Clustered regularly interspaced palindromic repeats
CRTC	CREB-regulated transcription coactivator
DNA	Deoxyribonucleic acid
dpp	Decapentaplegic
Dvl	Dishevelled
EMT	Epithelial-to-Mesenchymal Transition
HDAC	Histone deacetylase
HDS	High dietary sucrose
Hipk	Homeodomain – interacting protein kinase
Hts	Hu-li tai shao
InR	Insulin Receptor
JAK	Janus Kinase
JNK	Jun Kinase
KD	Kinase dead
lgl	Lethal giant larvae
LKB1	Liver kinase β-1
MEF2C	Myocyte enhancer Factor 2C
MLL	Mixed lineage leukemia
MMP-1	Matrix metalloproteinase-1
NICD	Notch intracellular domain
NSCLC	Non-small cell lung cancer

OGT	O-GlcNAc transferase
PBS	Phosphate-buffered saline
PI3K	Phosphoinositol-3-kinase
PKA	Protein kinase A
PP-1	Protein phosphatase 1
Ras	Rat Sarcoma
RNA	Ribonucleic acid
Sav	Salvador
Scr	Sex combs reduced
Ser	Serine
shRNA	Short hairpin RNA
Sik	Salt-inducible kinase
Slimb	Supernumerary limbs
Src	Sarcoma
STAT	Signal Transducer and Activator of Transcription
SUMO	Small ubiquitin-like modifier
Thr	Threonine
UAS	Upstream Activation Sequence
UBA	Ubiquitin-Associated Domain
Wg	Wingless
WT	Wild type
Wts	Warts
Yki	Yorkie

Chapter 1.

Introduction

1.1. Signaling transduction overview

Every cell in our body and the bodies of other organisms, continually performs a multitude of functions, including respiration, uptake of nutrients, and production of energy to grow, reproduce or survive. Each of these processes is a result of tightly regulated signalling cascades within the cells (Gerhart 1999, Gilbert, 2000). Various stimuli trigger these signals, that elicit a series of protein-protein interactions in the cell. Many of these proteins are enzymes that are activated allosterically upon binding to upstream proteins, and carry out biochemical reactions to transmit the signal to the next player in the sequence (Cooper, 2000). Spatial and temporal regulation of these pathways in tissues ensures proper cell proliferation, differentiation, tissue growth, and body patterning throughout development. Dysregulation at any level of these pathways will disturb homeostasis and cause disease. Understanding the players in these pathways and how they are regulated will help understand the repercussions when they are dysfunctional.

Pathways may be regulated at the genetic, epigenetic, transcriptional, post-transcriptional and post-translational levels (Perrimon, Pitsouli and Shilo, 2012). This thesis will focus on the post-translational level of regulation. Proteins can be modified with carbohydrate groups, methyl groups, acetyl groups, phosphate groups, ubiquityl groups or lipid moieties, that can affect the stability, activity, localization, and function of these proteins. Protein kinases are a class of proteins that effect addition of phosphate groups (PO_4^{3-}) to key amino acid residues (Serines, Threonines or Tyrosines) on other proteins or themselves. Overexpression or malfunction of these kinases have been implicated in many diseases, including cancer and developmental disorders (Reviewed elsewhere in Torkamani et al, 2010; Cicenas et al, 2018). Study of these kinases, their function and dysregulation, is therefore essential to understand the mechanisms of disease onset or progression, and subsequently, the respective treatment strategies. The fruit fly *Drosophila melanogaster* is a suitable, and extensively used model to study the role of kinases in disease and development.

1.2. *Drosophila melanogaster* as a model organism

Drosophila is a widely used model organism to study developmental genetics, owing to the high conservation of genes and proteins between *Drosophila* and humans, complemented by lower genetic complexity in the former (Bier 2005). In addition, the short life cycle, inexpensiveness, ease of storage and maintenance, simple yet highly organized and timed development have made this model highly suited for studying dysregulation in spatially and temporally controlled events easily and quickly. A battery of tools is available for genetic manipulation in *Drosophila* starting from mutant fly strains to tissue specific CRISPR knockout strains for a variety of genes (Venken and Bellen, 2005; Ni et al, 2011; Bier et al, 2018; Ewen-Campen and Perrimon, 2020).

Drosophila has a life cycle of 10 days at 25°C. This begins with the egg stage, where the embryo develops into larvae starting from around 3 days after egg lay (AEL). There are two molting cycles, and the second and third instar larvae can be seen till day 6-7 AEL. After this stage, they form pupae from which adults can emerge at about 10 days AEL. The effects of modulating levels of a gene or protein can be studied at any of the different developmental stages in *Drosophila*. As an added advantage, larvae have epithelial sacs of tissue called imaginal discs that form contain the precursors for the corresponding adult organs.

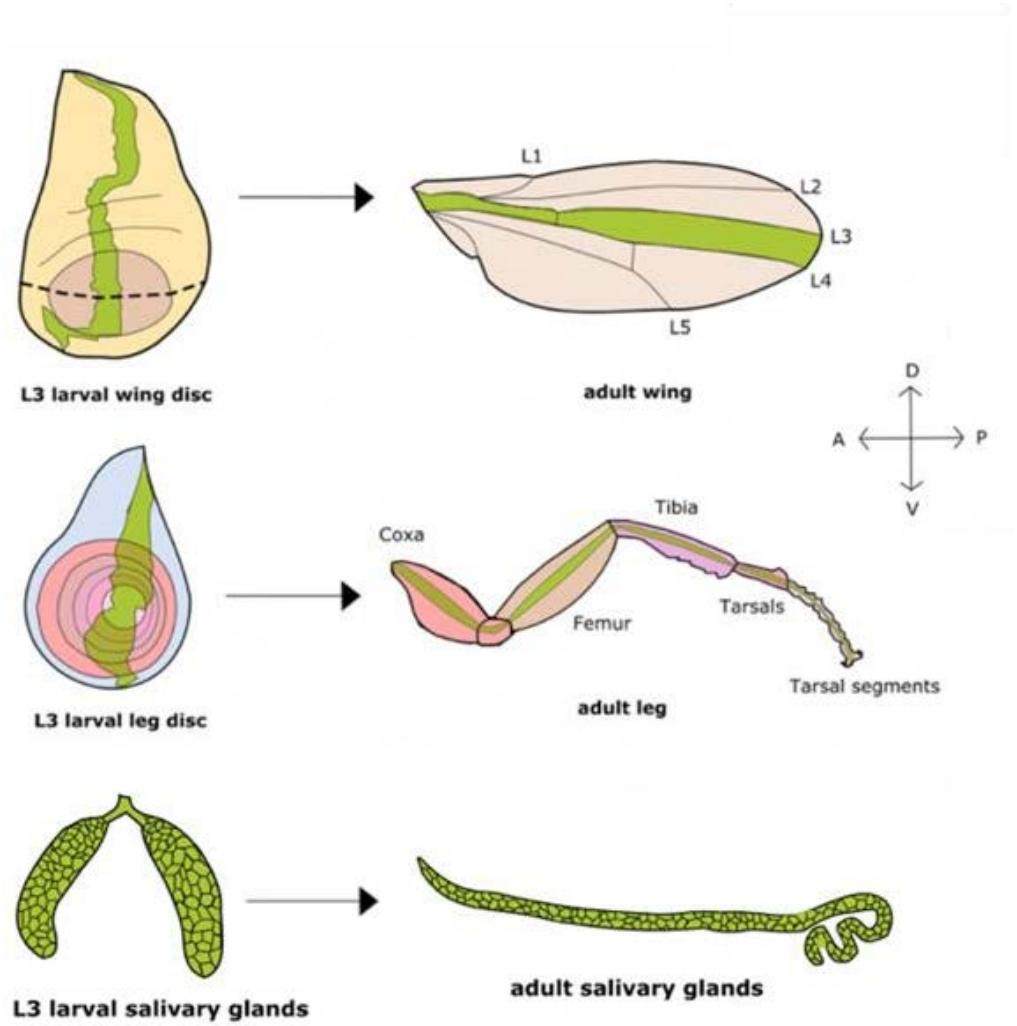


Figure 1 Development of adult organs expressing dpp from corresponding larval imaginal discs

Studies in this project have been performed at both larval (third instar L3 larval) and adult stages of Drosophila. Regions where the gene dpp (decapentaplegic) is expressed are highlighted in light green. Color coded regions indicate the segments of the imaginal discs that give rise to specific regions in the adult leg structures. dpp-Gal4 is the primary driver used in this project.

Throughout development, spatiotemporal regulation by a variety of signaling pathways establishes polarity in all tissues, including imaginal discs (Gilbert, 2000). Polarity is maintained along the anterior-posterior, dorsal-ventral and proximal-distal axes to synchronize and regulate development in all three dimensions. Pathway activators, or morphogens, are spread across tissues in a gradient, to define certain cell

populations and their fates by activating key genetic programs in a concentration-dependent manner (Gilbert, 2000). Cells expressing a unique composition and intensity of specific signaling pathways are therefore destined to form a certain tissue. The Gal4-UAS strategy can be used to alter the expression of a specific gene in a specified tissue or tissue section, at a particular time in development, to observe immediate consequences, or effects later evident in advance developed stages. Fig.1 shows the Gal4 driver primarily used in this study, as well as the regions it is expressed in, both in the larval as well as adult stages.

1.3. The Gal4-UAS system

Originally identified in the yeast *Saccharomyces cerevisiae* (Johnston, 1987; , Giniger et al, 1985) where the transcriptional activator Gal4 binds to the upstream activator sequence (UAS) ahead of the promoters of target genes and drives expression thereof, this system has been adapted to the fruit fly and is a very widely used tool to drive tissue-specific expression of transgenes (Fig. 2). Neither Gal4 nor UAS are genetic sequences naturally present in *Drosophila*. Transgenic flies expressing either Gal4 or UAS alone have been generated and these can be bred to produce offspring bearing both sequences, thereby driving genes in a certain spatiotemporal fashion. Until then, there is no expression of the target gene since either component is absent in the parents. Gal4 sequences have been inserted downstream of various tissue-specific enhancers to ensure spatially restricted expression of Gal4, and subsequently the transgene. Using this powerful tool, the effects of misexpression or knockdown can be studied in a regulated way. (Brand and Perrimon, 1993)

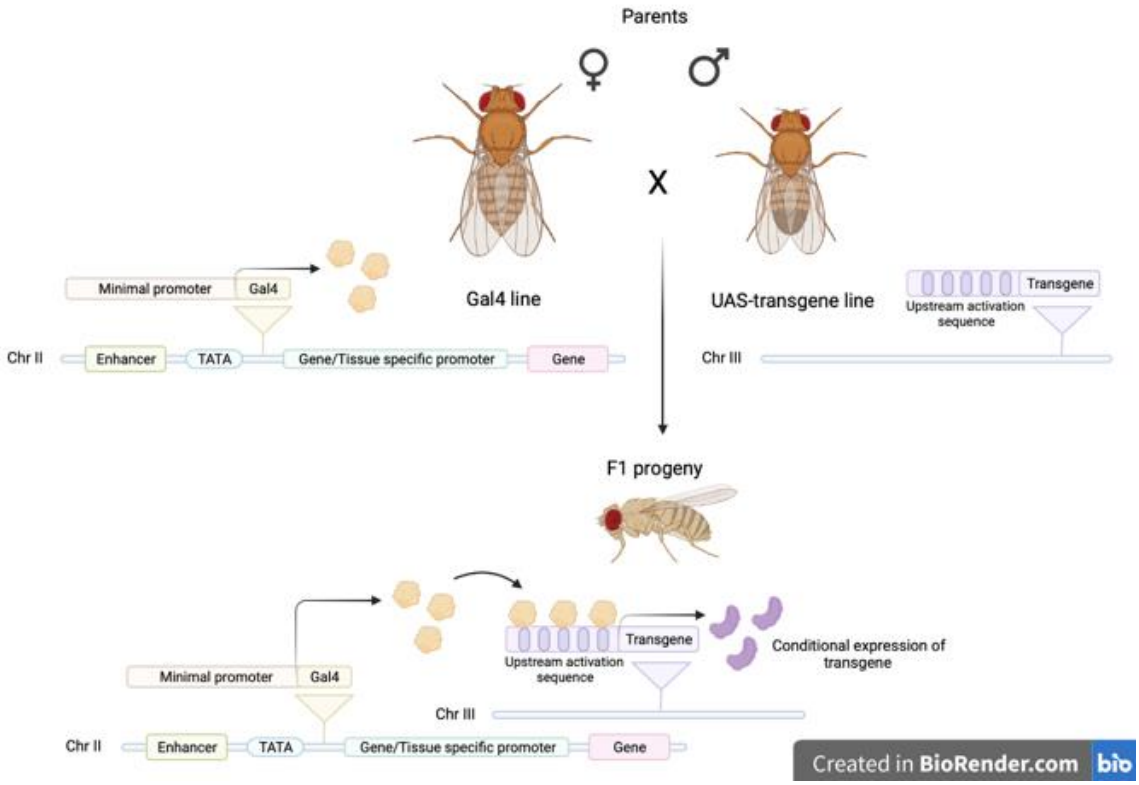


Figure 2 The Gal4-UAS system in Drosophila.

The yeast Gal4-UAS system has been adapted in Drosophila such that the parents express either the Gal4 or UAS transgenes. They are mated to give rise to offspring exhibiting tissue-specific Gal4, and therefore UAS-transgene expression. shRNA constructs can also be introduced downstream of UAS sequences to effect knockdown of target genes endogenously present in Drosophila.

1.4. The Hipk protein kinase family

The homeodomain interacting protein kinase (Hipk) family of protein kinases derive their name from their ability to bind to, and regulate, by phosphorylation of key Ser/Thr residues, those transcription factors that contain homeodomains. This protein domain derives its names from a constituent 180bp long homeobox sequence (McGinnis et al, 1984; Holland, 2001; Holland et al, 2007) that encodes for the DNA-binding homeodomain of the homeodomain transcription factors. These factors both activate and repress expression of various target genes, depending on the context, so as to control

cell fates, tissue patterning and organogenesis during development (Gehring et al, 1994).

Functions of homeodomain proteins are governed by other cofactors that enhance homeodomain protein binding to target DNA sequences. Hipk, or homeodomain interacting protein kinase, was identified initially as one such transcriptional co-repressor, which was found to bind to the NKx-1.2 family of homeoproteins (Kim et al, 1998). Hipks were the first protein kinases to be identified as corepressors for homeodomain transcription factors, and both their kinase and corepressor activities were found to be essential to enhance NKx-1.2 binding to DNA, thereby enhancing the repressor activity of the latter (Kim et al, 1998).

There are 4 paralogs of Hipk in mammals (Hipk1-4), while there is only a single Hipk in *Drosophila*, reiterating the genetic simplicity of the latter. The *Drosophila* Hipk kinase domain is highly conserved and the whole protein sequence is most similar to the widely studied vertebrate HIPK2. Hipk is a primarily nuclear kinase, and is found to strongly localize in nuclear speckles, with minimal cytoplasmic localization (Kim et al, 1998). Subsequent deletion analysis revealed that Hipk contains multiple nuclear localization signals as well as a nuclear speckle retention signal. SUMOylation of Hipk has also been shown to promote and maintain its localization to nuclear speckles (Kim et al, 1998; Huang et al, 2011).

Hipk was found to be essential for normal development, since deletion of Hipk leads to embryonic lethality in *Drosophila* (Lee et al, 2009a). Similarly, mice lacking both HIPK1 and HIPK2 were found to die as embryos (Isono et al, 2006), exhibiting defective neural tube closure and exencephaly, in addition to impaired cell proliferation. Interestingly, mice lacking either HIPK1 or HIPK2 were viable, and grossly normal, suggesting a functional redundancy between the two proteins in mammals.

Interestingly, with time, multiple Hipk protein targets have been identified other than homeodomain transcription factors. Hipk is able to regulate mediators of signalling pathways, and consequently processes like cell proliferation, differentiation, apoptosis, and stress response via these pathway intermediates (addressed in the following section).

1.5. Role of Hipk in signal transduction

As a Ser/Thr kinase, Hipk can act on multiple substrates bearing the protein consensus sequence S/P-R/X-S/T*-P-P/S (Steinmetz et al, 2019). Many of these substrates are signaling intermediates or effectors, thus enabling Hipk to regulate these cascades, and consequently, cell fates, like cell proliferation, cell survival and differentiation.

Hipk can positively regulate the Wingless (Wg/Wnt) pathway via multiple parallel mechanisms (Swarup and Verheyen, 2012; Lee et al, 2009b; Shimizu et al, 2014) (Fig. 3). Hipk can phosphorylate Slimb (supernumerary limbs) in the cytoplasm, and prevent it from triggering proteasomal degradation of Arm (Armadillo), the ortholog of beta-catenin in *Drosophila*. Arm is thus protected from destruction and is free to translocate to the nucleus where it can turn on expression of target genes of the Wg pathway (Swarup and Verheyen, 2012). Hipk is also able to directly phosphorylate Arm in the nucleus and increase its transcriptional activity (Lee et al, 2009b). Hipk2 has also been proposed to stabilize Dishevelled (Dvl) levels in a kinase-independent manner, along with PP-1 (protein phosphatase-1), in zebrafish embryos. Dvl is not degraded in the presence of Hipk-PP1 and can activate the Wg/Wnt pathway components downstream (Shimizu et al, 2014). Hipk can thus regulate cell proliferation, migration, and differentiation via the Wg/Wnt pathway.

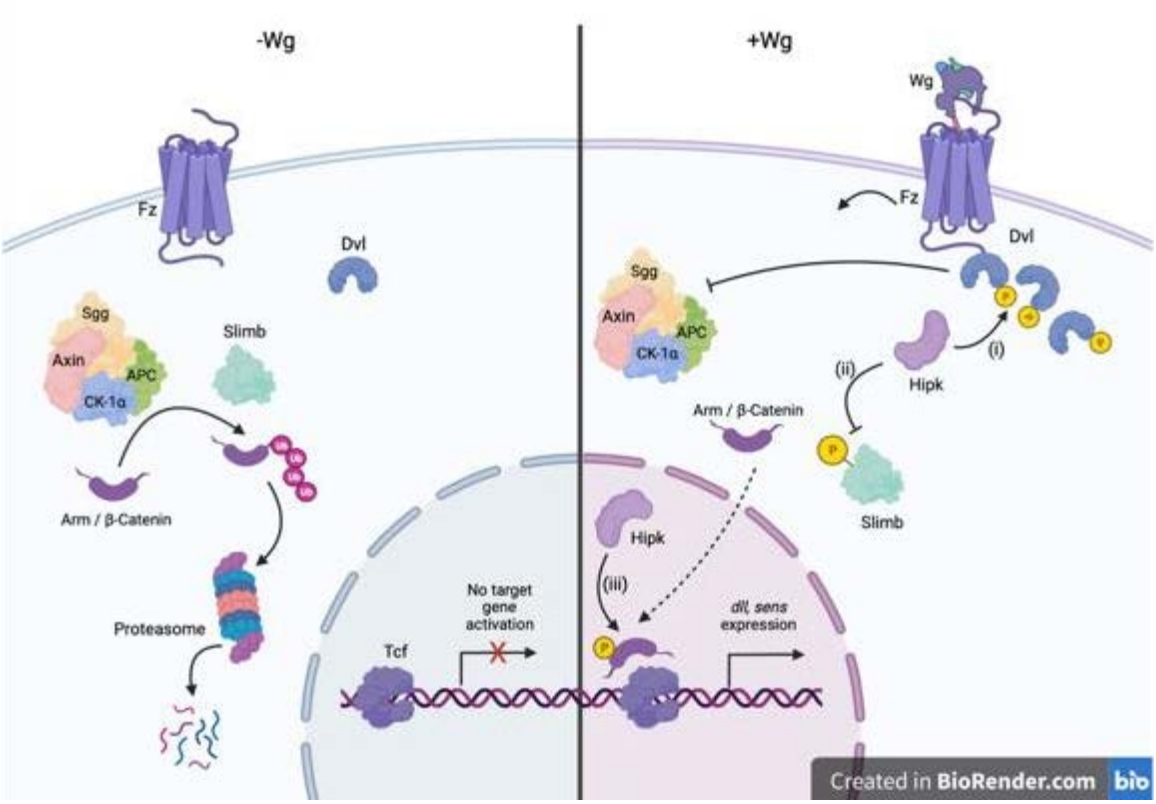


Figure 3 Hipk positively regulates Wg/Wnt pathway via multiple mechanisms:

(i) Phosphorylating and stabilizing Dvl in the cytoplasm, allowing inhibition of the Sgg-Axin-APC-CK-1a complex and subsequent prevention of proteasomal degradation of β-catenin, (ii) Phosphorylating and inhibiting Slimb in the cytoplasm, preventing it from triggering proteasomal degradation of Arm and (iii) Phosphorylating and increasing activity of Arm directly, in the nucleus

Hipk can also positively regulate the Notch pathway in *Drosophila* by phosphorylating the global co-repressor Groucho, preventing it from repressing NICD (Notch intra-cellular domain) (Fig. 4). NICD can consequently turn on expression of target genes of the Notch pathway. Hipk is thus able to regulate cell proliferation, growth and patterning via the Notch pathway. (Lee et al, 2009a)

Hipk can also regulate the Hippo pathway. The pathway consists of a core kinase cassette that phosphorylates and sequesters transcriptional co-activator Yki (Yorkie) in the cytoplasm, when active. Hipk has been reported to phosphorylate Yki and increase its transcriptional activity, therefore increasing target gene expression (Fig. 4). Hipk can

therefore regulate cell proliferation, apoptosis, and tissue size via the Hippo pathway (Chen and Verheyen, 2011; Poon et al, 2011; Steinmetz et al, 2021).

Hipk has also been proven to be essential for JAK-STAT signaling whereby Hipk can directly interact with Stat92E in the cytoplasm and regulate its activity (Fig. 4). Stat92E becomes enriched in the nucleus following Hipk interaction, where it can activate target gene expression (Tettweiler et al, 2019). Hence, Hipk can also regulate cell proliferation, growth, and inflammatory response via this pathway. In the absence of SUMOylation, Hipk was reported to translocate to the cytoplasm where it is able to activate JNK pathway, and consequently apoptosis and cell migration (Huang et al, 2011) (Fig. 4).

Hipk is thus a potent growth regulator, and can control tissue size and patterning by the concerted activation of multiple signaling pathways.

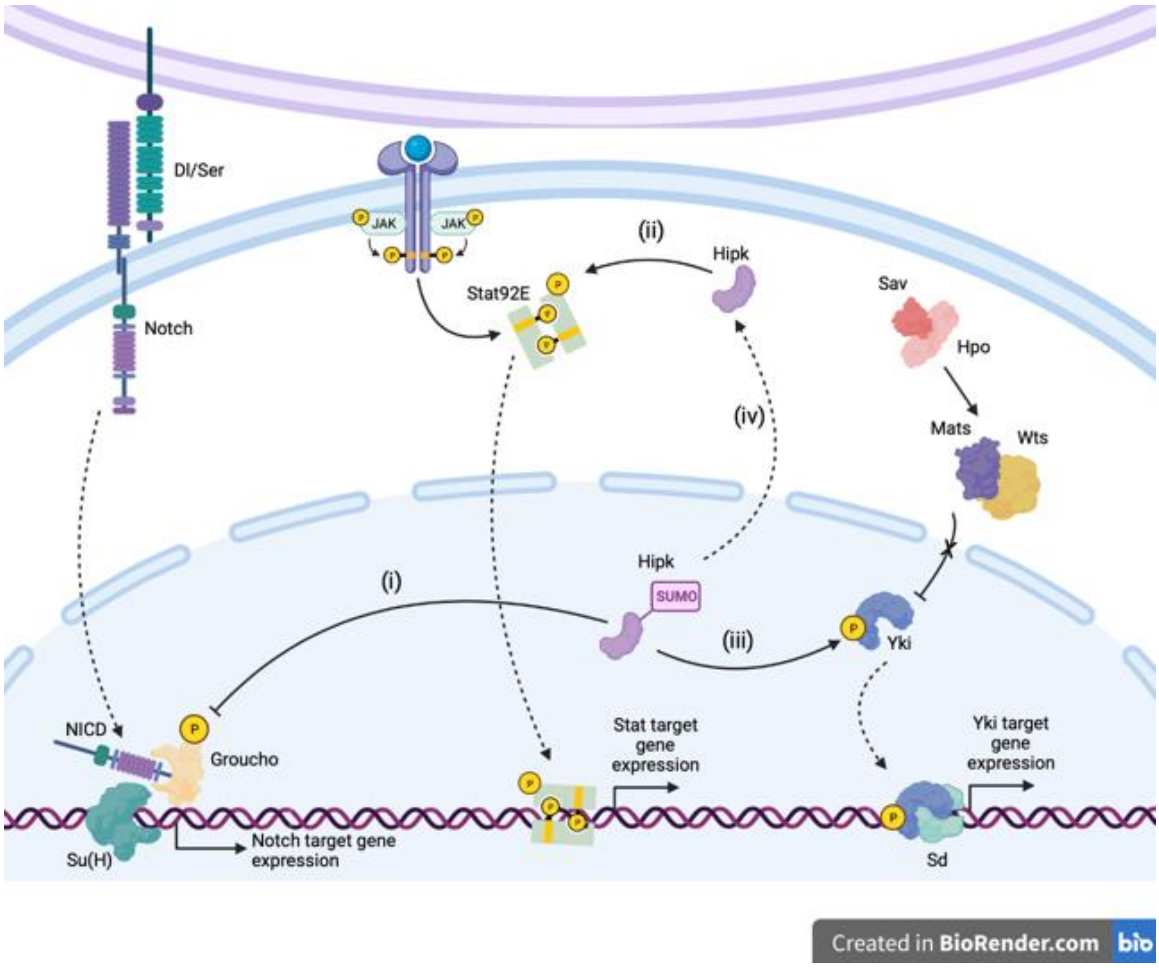


Figure 4 Hipk regulates cell fates via multiple signaling pathways

(i) Hipk can phosphorylate global repressor Groucho in the nucleus, inhibiting its function. NICD is thus free to turn on Notch target gene expression along with Su(H) (suppressor of hairless). (ii) Hipk can phosphorylate Stat92E in the cytoplasm, augmenting its activity and upregulating STAT target gene expression. (iii) Hipk can phosphorylate Yki in the nucleus and promote its activity, thus increasing Yki target gene expression. (iv) Decrease in Hipk SUMOylation can lead to its cytoplasmic translocation (Huang et al, 2011)

1.6. Signal transduction and cancer

Signaling pathways are tightly regulated throughout development to achieve homeostasis and controlled responses in cells. A slight perturbation in these cascades can therefore lead to erroneous growth signals, uncontrolled proliferation, and cancer. An accumulation of genetic aberrations leads to the progression of cancer from benign

overgrowths to invasive malignancies capable of migrating to secondary organs (Hanahan and Weinberg, 2000). Genetic variants of the intermediate components of multiple cell proliferative signaling pathways including Wg, Hippo, Notch, JAK-STAT and JNK (Jun-kinase) have been implicated in many human cancers (Sanchez-Vega et al, 2018; Chen et al, 2012; Vainchenker and Constantinescu, 2013; Wagner and Nebrada, 2009).

Drosophila provides an ideal model system to study the same, because the very signaling pathways that are disturbed in cancers, are responsible for normal tissue growth and patterning during *Drosophila* development. Multiple cancer models have also been established in *Drosophila*, with genetic aberrations in two or more distinct pathway components, that can together cause neoplastic growths showing invasive behaviour. For example, the first *Drosophila* cancer model comprised flies expressing an activated version of the oncogene *ras* (rat sarcoma) coupled with an inactive version of the tumor suppressor gene *scribble,* involved in defining apico-basal polarity in epithelia (Pagliarini and Xu, 2003).

It makes sense to predict that Hipk, being a versatile regulator of multiple signaling pathways, may predispose tissues to cancer, if erroneously expressed. Indeed, elevation in levels of Hipk alone may cause both hyperproliferative phenotypes as well as invasive behaviour. This renders it a potent oncogene, a viable contender for targeted therapeutics and diagnostics, as well as a potential candidate for an overgrowth/tumor model.

1.7. Hipks and cancer

Hipk has been proven to regulate cell proliferation and migration via multiple signaling pathways (Blaquiere et al, 2018), and inhibiting any single pathway could not rescue Hipk-mediated overgrowth. Wong et al, 2019 demonstrated that this could be in part due to the elevation of *dMyc* expression, which is a potent oncogene activated downstream of each of these signaling pathways. Hipk-overexpression has also been shown to cause phenotypes reminiscent of EMT (Epithelial to Mesenchymal transition) like loss of E-Cadherin, elevation in MMP-1 (matrix metalloproteinase-1) levels, cell

migration and secondary tumours (Blaquiere et al, 2018; Tettweiler et al, 2019; Wong et al, 2019). Hipk-overexpressing cells also show elevated levels of glucose uptake and aerobic glycolysis (Wong et al, 2019), that are metabolic changes exhibited by cancer cells (Hanahan and Weinberg, 2011). A positive feedback loop between dMyc and aerobic glycolysis was found to support tumor growth in Hipk overexpressing cells (Wong et al, 2019). Hipk-driven tumours were also demonstrated to have a mitochondrial profile like that implicated in other tumor models in *Drosophila*, with an overall increase in mitochondrial mass due to more abundant mitochondria that are long and highly branched; as well as a rise in mitochondrial activity (Wong et al, 2020). This increase in mitochondrial mass could also be attributed increased dMyc activity in Hipk tumors (Wong et al, 2020). Hipk overexpressing tissues were additionally found to be sensitized to developing higher grade tumors in combination with other established tumor models in *Drosophila* (Blaquiere et al, 2018). These findings support the overexpression of Hipk as a robust tumour model to study both genetic as well as metabolic mechanisms of carcinogenesis in *Drosophila*.

The tumorigenic role of Hipk in *Drosophila* is conserved in higher vertebrates for all 4 mammalian HIPKs have been implicated in a variety of cancers (reviewed in Blaquiere and Verheyen, 2017). Loss of HIPK2 in mice can lead to development of viable, but dwarf adults (Trapasso et al, 2009), and embryonic fibroblasts from these mice may have a decreased proliferative potential. HIPK2 may thus be a proto-oncogene, critical for normal development. However, Wei et al, 2007 report that HIPK2 deficient mice show increased tumors as compared to controls, suggesting a tumor suppressor role for HIPK2. HIPK2 mutants have been implicated in many human cancers, with conflicting roles as both an oncogene (Valente et al, 2015; Tan et al, 2014; Feng et al, 2017; Ricci et al, 2013; Soubeyran et al, 2011; Wei et al, 2007; Li, Arai et al, 2007) and a tumor suppressor (Cheng et al, 2012; Saul and Schmitz, 2013; Deshmukh et al, 2008; Al-Beiti and Lu, 2008). These data suggest that there may be context-specific roles for the Hipk protein family, but they are critical in development and disease. Understanding how Hipk is regulated in multiple contexts is therefore essential to battle a debilitating disease like cancer.

1.8. RNAi screen for Hipk modifiers

The Verheyen lab was interested to identify upstream regulators of Hipk. Jessica Blaquiere and Rubia Chung had carried out a screen for phospho-regulators of Hipk (unpublished data). Hipk was overexpressed using *sca-Gal4* (*scabrous-Gal4*), a notum specific driver. This resulted in severe shortening of the bristles in the notum region of adults, as well as morphological abnormalities of the notum. To identify other kinases that could genetically interact with Hipk, 307 candidate kinases were knocked down by RNA interference using the same driver, while Hipk was overexpressed. If altering the levels of the kinase could modify the phenotype caused due to Hipk overexpression, the two kinases could be putative genetic interactors. Hipk could be negatively regulated by the kinase in question if there was an enhancement of the Hipk-driven phenotype. On the other hand, if there was a suppression of the phenotype, there are multiple possible scenarios - the kinase could act in parallel with Hipk to augment its function, or be an upstream activator of Hipk, or act downstream of Hipk, but be required for it to exert its function. The effects of depleting the kinase by itself, may also influence the phenotypes observed, of course.

Siks, or salt-inducible kinases, emerged as potential Hipk regulators in this screen. *Drosophila* has 2 Siks, and when knocked down using multiple RNAi lines, Sik2 was listed as a putative positive regulator of Hipk, while Sik3 knockdown gave rise to mixed effects, i.e., either suppression or enhancement of the Hipk-mediated shortened bristle phenotype, depending on the RNAi line used (unpublished data). It was clear that Siks may genetically interact with Hipk, since in no scenario did knockdown of Siks leave the Hipk-overexpression phenotype unaltered. This was an interesting finding, for, there had been no previous indication from published literature that Siks could regulate Hipk in a novel function.

1.9. The Salt-inducible kinase (Sik) family and their functions

Salt-inducible kinases were initially identified and named when their levels were differentially increased in the adrenal glands of rats raised on high Na^+/K^+ salt diets (Wang et al, 1999). Siks act as nutrient sensors in the cell, modulating cellular responses to nutrient conditions. Siks serve to increase nutrient uptake and breakdown

to energy during feeding, and inhibit the same during fasting. Siks belong to the AMPK-related kinase (AMPK-RK) family of Ser/Thr kinases (Manning et al, 2002). All AMPK-RKs have an N-terminal kinase domain bearing a key Thr residue at the activation loop. Upstream kinases activate Siks by phosphorylating this key Thr residue (Licanzo et al, 2004). Downstream of the kinase domain is the UBA (Ubiquitin-associated) domain that when modified post-translationally can allosterically affect activation of Siks (Jaleel et al, 2006). It has also been shown that mutations in this domain can alter SIK localization by mediating their nuclear transport via inhibiting interaction with cytoplasmic sequestering 14-3-3 protein scaffolds (Al-Hakim et al, 2005). The C-terminal regions then possess other important Ser/Thr residues that can be phosphorylated by other upstream kinases like PKA (Protein kinase A), CaMKII (Ca^{2+}-Calmodulin kinase II), etc. to effect regulation of Sik activity or fine-tuning of their functions (Screaton et al, 2004; Berggreen et al, 2012; Henriksson et al, 2012).

Mammals have three paralogs: SIK1-3, while *Drosophila* has two, Sik2 and Sik3, homologous to mammalian SIK2 and SIK3 respectively (Fig. 5). Siks can be activated downstream of Insulin signaling and LKB1 (Liver kinase β-1) via phosphorylation of the Thr residue at the activation loop (Licanzo et al, 2004). Subsequently, Siks can regulate function and localization of transcriptional regulators like CRTCs (CREB-regulated transcriptional coactivators) or HDACs (Histone deacetylases) downstream, to modulate responses to nutrient conditions (Haberland et al, 2009; Altarejos and Montminy, 2011; Screaton et al, 2004; Shaw et al, 2005). Phosphorylation of CRTCs and HDACs leads to their sequestration in cytoplasm (Mihaylova et al, 2011; Koo et al, 2005; Uebi et al, 2010). Once within the nucleus, however, these factors may drive or repress target gene expression in a context-specific manner.

Siks are generally constitutively active in cells via LKB1 mediated phosphorylation, and alterations in cAMP levels serve to regulate Sik function via phosphorylation by PKA, that is activated upon increased cAMP levels in the cell (Licanzo et al, 2004; Berggreen et al, 2012; Henriksson et al, 2012).

In the fed state, Insulin signaling serves to inhibit glucose production by salvaging non-carbohydrate precursors in the cell, i.e., gluconeogenesis, while upon starvation, glucagon signaling stimulates gluconeogenesis to replenish cellular energy stores. Under resting/fed conditions, Siks are activated in the cytoplasm downstream of

the InR-PI3K-Akt (Insulin receptor – phosphatidyl inositol 3 kinase – protein kinase B) cascade. Here, Siks phosphorylate HDACs and CRTCs, sequestering them in the cytoplasm by inducing their association with 14-3-3 proteins (Koo et al, 2005; Uebi et al, 2010). While fasting, however, cAMP levels are higher in cells, activating PKA, that phosphorylates and inhibits Siks in the cytoplasm by promoting association of Siks themselves with 14-3-3 proteins (Sonntag et al, 2018). Transcriptional regulators HDACs and CRTCs are thus free to translocate to the nucleus now to drive the gluconeogenetic gene program.

Siks were found to have redundant functions in mice (Malm et al, 2016) in some contexts while they were found to have opposed functions in others (Sakamaki et al, 2014; Kim et al, 2015). SIK1 and SIK2 have also been shown to undergo auto-phosphorylation on a key Serine residue in the kinase domain (Hashimoto et al, 2008), adding another layer of complexity on the regulation of these kinases.

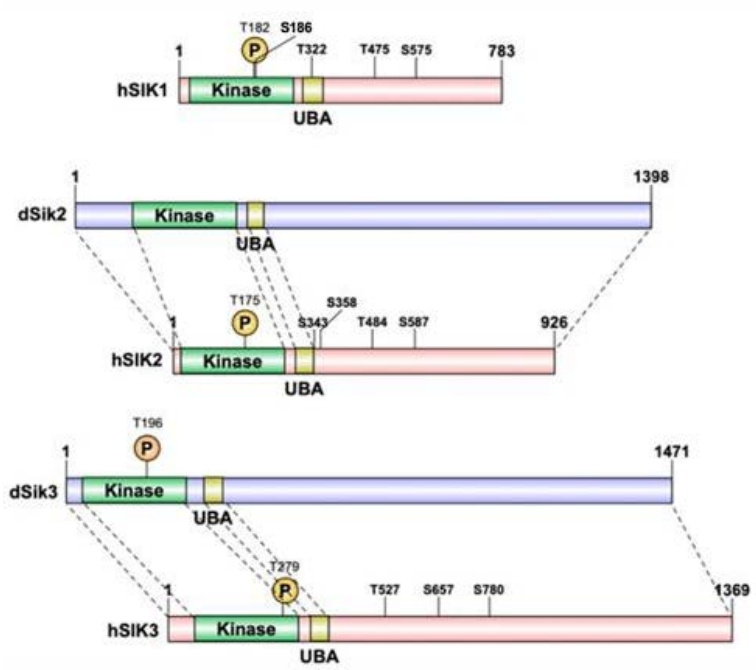


Figure 5 Sequence homology between mammalian (hSIK1-3) and Drosophila (dSik2-3) Siks.

Drosophila dSik2 and dSik3 are most similar to hSIK2 and hSIK3 respectively. Siks belong to the family of AMPK-RKs that have a conserved N-terminal kinase domain with an activation loop marked by a key Threonine residue. Downstream is a UBA domain, followed by C-terminal domain bearing multiple target sites for regulation by upstream kinases.

1.10. Role of Siks in signal transduction

Evidently, Siks are established metabolic regulators that can regulate gene expression as a response to nutritional conditions. Interestingly, other targets have been identified for these multifunctional regulatory kinases.

Siks can negatively regulate the Hippo pathway by directly phosphorylating Sav (Salvador), an upstream scaffolding protein that can negatively regulate activity of Yki, the transcriptional regulator capable of migrating to nucleus to induce target gene expression (Wehr et al, 2012). In *Drosophila,* when Sav is phosphorylated by Siks, its efficiency to scaffold Warts (Wts), the core kinase of the Hippo cascade upstream of Yki, is reduced. Consequently, Wts cannot effectively phosphorylate and inhibit Yki. The latter is thus free to translocate to the nucleus to activate target gene expression, ultimately promoting cell growth.

In a study that followed, larvae raised at high dietary sucrose conditions showed increased activity of Siks in Ras/Src activated tumours via InR-PI3K-Akt signaling (Hirabayashi and Cagan, 2015). Siks could activate Wg signaling via Hippo pathway downregulation in these tumours. In these cells, increased Wg signaling augmented expression of InR, effecting a suppression of insulin resistance, that had developed in the rest of the organism due to high sucrose in the diet. This set a feed-forward mechanism in motion where increased InR expression caused further increase in Insulin signaling, and subsequently Sik activation and so on. The resulting activation of Hippo and Wg pathways could cause tumor growth and progression.

In one study, Sik2 was found to be interact with Lgl (lethal giant larvae), a component of the cell polarity complex, and promote wing growth, while Sik3 modulated the activity of the cell polarity complex so as to restrict tissue growth (Parsons et al, 2017). This study additionally suggested context-specific redundant or opposing roles for the two kinases.

p85α, the regulatory subunit of PI3K, was identified as a putative novel substrate for Sik2. Sik2 can phosphorylate and activate p85α, thereby activating PI3K and the downstream cascade, including Akt and Siks, in a feed forward mechanism to ensure cell survival and metabolism, in ovarian cancer cells (Miranda et al, 2016).

Siks were also shown to be necessary for normal eye development in *Drosophila* (Sahin et al, 2020). However, in an eye tumor model in *Drosophila*, both reduction and activation of Siks gave rise to increased tumor progression. Epistatic interaction was observed between Siks and Notch signaling components, suggesting that Siks may be able to regulate the Notch pathway indirectly, and therefore eye development as well as tumor progression. Notch can act as an oncogene or a tumour suppressor gene depending upon the context (Bolos et al, 2007), thus explaining the dual role of Siks seen in regulating tumour progression in the eye.

1.11. Siks and cancer

It is apparent that Siks can regulate key signaling pathways, proven especially in tumor settings. Misexpressed SIKs have indeed been implicated in multiple human cancers, where the different SIKs act either as oncogenes or tumor suppressors depending upon the context. However, the mechanisms by which Siks can regulate signaling pathways and control tumor progression, in different cancers and contexts, needs to be elucidated to provide effective and targeted therapies in the future.

SIK2 has been reported to promote metastasis and tumour progression in gastric, prostate, colorectal and ovarian cancers (Ahmed et al, 2010; Miranda et al, 2016, Maxfield et al, 2016; Bon et al, 2015). Conversely, the activated cAMP-PKA-SIK axis has been implicated in pancreatic cancers in which SIKs are unable to phosphorylate and inhibit downstream oncogenic targets, thus acting with tumor suppressing functions (Patra et al, 2018). LKB1, a key upstream SIK activator, has been widely implicated as a tumour suppressor in non-small cell lung cancers (NSCLCs) (Matsumoto et al, 2007). SIK1 and SIK3 have been implicated as major downstream effectors of mutant LKB1 in these cancers (Hollstein et al, 2019), thus showing anti-tumorigenic effects.

SIK2 and SIK3 have been reported to have redundant and additional roles in phosphorylating and inhibiting HDAC4, a transcriptional co-repressor of MEF2C (myocyte enhancer factor 2C) (Tarumoto et al, 2018; Brown et al, 2018; Tarumoto et al, 2020). The latter is a transcription factor that is expressed in hematopoietic, muscle and neuronal cell lineages, and usually upregulated by the action of MLL (mixed lineage leukemia protein), the fusion oncoprotein in leukemias. MEF2C can activate target gene

programs promoting cell proliferation and driving leukemias. When HDAC4 is inhibited by Sik-mediated phosphorylation, it can no longer repress MEF2C activity, and leukemias can progress, while genetic or pharmacological inhibition of Siks may stall cancer progression by allowing HDAC4-mediated repression of MEF2C.

Siks thus have both oncogenic and anti-tumorigenic roles that may be attributed to their distinct functions in different tissues, as well as the different signaling networks that are activated to cause tumours in these tissues. Siks may well serve as multi-functional bridges between growing tumours and their response to nutrition, rendering them viable targets for diagnostics and therapeutics, or even lifestyle-alteration treatment strategies, to tackle a debilitating disease like cancer.

1.12. Aims of the thesis

Preliminary results from a previous Hipk modifier screen in the lab identified Sik2 and Sik3 as potential Hipk regulators. Based on these findings, we hypothesized that **Siks could exhibit a novel role in regulating Hipk,** on top of the metabolic functions of Siks.

Hipk overexpression is known to cause tissue overgrowth via the activation of multiple signaling cascades. Using this readout, we aimed to confirm and further understand the nature of the genetic interaction between Siks and Hipk by either knocking down or co-expressing Siks in a Hipk-active background. We expected that altering the levels of Siks would modulate Hipk-driven phenotypes established previously, thus validating the theory that Siks could have a novel function in regulating Hipk. Since Siks are Ser/Thr kinases, we also wished to determine whether they required their kinase function to phosphorylate and regulate Hipk activity. We also aimed to further understand the mechanism by which Siks could regulate Hipk, by performing suitable immunofluorescence or biochemical experiments assaying for possible physical interaction and protein levels, respectively.

Chapter 2.

Materials and Methods

2.1. *Drosophila* fly strains and crosses

Drosophila lines used are listed in Table 1. Wild type flies were w^{1118} (Bloomington)

Table 1 List of Drosophila fly lines

Gal4 driver lines	UAS transgenic lines (from *Drosophila* stock centres)	Kindly donated by the Chung Lab	Kindly donated by the Tapon Lab	Mutant strains
dpp-Gal4,UAS-GFP / TM6B	*yv ; ; UAS-w RNAi* (BL33762)	*UAS-myc-Sik2-WT (III)*	*UAS-Sik2-WT / TM3*	$Sik2^{\Delta 41}$/ $Sik2^{\Delta 41}$ (null) *or Y* (hypomorph) (Kindly donated by the Chung lab)
UAS-GFP ; dpp-Gal4,UAS-HA-hipk^{3M} */ TM6B* (abbreviated as *dpp>Hipk)* (Blaquiere et al., 2018)	*UAS-Sik2 RNAi (III)* (BL55880)	*UAS-myc-Sik2-KD (II)*	*UAS-Sik2 (S1032A) / TM3* (also referred to as *UAS-Sik2-CA)*	$Sik3^{\Delta 48}$*(II)* (Kindly donated by the Montminy lab)
+; Act-Gal4 / SM6A-TM6B	*UAS-Sik3 RNAi (II)* (VDRC 107458)	*UAS-myc-Sik3-WT (II)*	*UAS-Sik3-WT / CyO*	$Sik3^{\Delta 72}$ */ CyO* (Kindly donated by the Montminy lab)
		UAS-myc-Sik3-KD (II)	*UAS-Sik3 (S563A) / CyO* (also referred to as *UAS-Siks-CA)*	

Gal4 drivers were crossed to UAS-transgenes, and larvae or pupae were chosen against the TM6B phenotype. Crosses were carried out at 25°C and 29°C (indicated in experiment descriptions in Chapter 3). *UAS-w RNAi* (BL33762) was used as a titration control throughout the study.

2.2. mRNA extraction, cDNA synthesis and qRT-PCR

dpp-Gal4/TM6B and *+ ; Act-Gal4 / SM6A-TM6B* driver lines were used to drive the UAS transgenes, and 10 non-TM6B larvae were used for total mRNA extraction using the RNeasy Mini Kit (Qiagen 74104). For the mutant strains, 10

larvae from the stocks were used for total mRNA extraction. Following this, 500ng mRNA was used for cDNA synthesis using OneScript Plus Kit (abm G236). qRT-PCR was then performed using the SensiFAST SYBR Kit (Bioline 92005) on the StepOne Real-Time PCR system (Applied Biosystems). See Table 2 below for primers used, with their corresponding 5'→3' sequences.

Table 2 List of qRT-PCR primers

S.No	Target	Forward primer sequence (5' => 3')	Reverse primer sequence (5' => 3')
1	rp49	ATCGGTTACGGATCGAACAA	GACAATCTCCTTGCGCTTCT
2	sik2	CTCGCGTCTTGTCCGACCCAATG	GTATGCCAGCCAAGGAGAGATCTTCG
3	sik3	CGACCAGCAAGATATCCGTG	CTGCGACTCCATCACCTCG
4	hipk	GCACCACAACTGCAACTACG	ACGTGATGATGGTGCGAACTC

All the transgenic and mutant lines used were validated for their expression levels using qRT-PCR (Fig. 6). The UAS-Sik3-RNAi (VDRC 107458) line showed that Sik3 was not effectively knocked down, with both the *dpp-Gal4* and the *Act-Gal4* drivers.

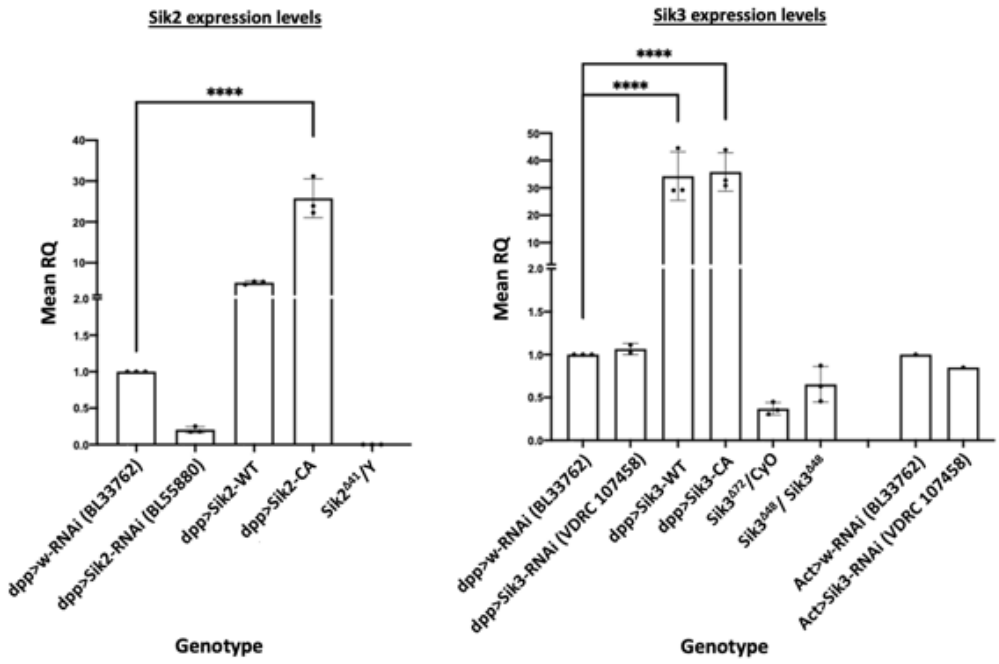


Figure 6 qRT-PCR validation of Drosophila fly lines used in this study.

Expression levels of Sik2 and Sik3, relative to endogenous control RP49, were determined using the primers listed in Table 1. Samples were assayed in triplicate, and the experiment was repeated two times

2.3. Imaginal disc dissection, antibody staining and quantification

L3 larval imaginal discs or salivary glands were dissected in phosphate-buffered saline (PBS) and fixed in 4% paraformaldehyde (PFA) for 15 min at room temperature. After fixation, samples were washed with PBS with 0.1% Triton X-100 (PBST). After blocking with 5% BSA (Bovine serum albumin) in PBST for 1 hour at room temperature, samples were incubated with primary antibodies overnight at 4°C. The primary antibodies used include rabbit anti-Hipk (1:200) (Blaquiere et al., 2018), rabbit anti-dMyc (d1-717) (1:500, Santa Cruz sc-28207), mouse anti-Arm (1:200, DSHB N2 7A1) and mouse anti-myc-tag (1:200, Millipore 4A6 05-724). After washing with PBST, samples were incubated with Cy3- and/or Alexa Fluor 647-conjugated secondary antibodies (1:500, Jackson ImmunoResearch Laboratories, Inc.), DAPI (4, 6-Diamidino-2-Phenylindole, Dihydrochloride) (final concentration: 0.2 µg per mL, Invitrogen D1306) for 1 hour at room temperature. Samples were mounted in 70% Glycerol/PBS after wash. Images were taken on a Zeiss LSM880 with airyscan confocal microscope and processed using Zen Blue software or Image J.

For quantifying delocalization, an image analysis program was set up on Zen Blue to identify DAPI/nuclei and set up cellular boundaries, otherwise known as zones of influence around the nuclei. The threshold signal intensity is optimized for all channels. Cell diameters and resolution between adjacent cells are optimized, and average Hipk intensity is quantified within each cell (nuclear + cytoplasmic signal) as well as each nucleus. These values were then plotted using GraphPad Prism as stacked bars. (Maximum-intensity projection/extended depth-of-focus images were used for image analysis).

2.4. Pupal dissection, pharate leg mounting and quantification

Pupae were dissected in ethanol and mounted in Aquatex (EM Science). Pharates were imaged in ethanol using the Leica MZ6 modular stereomicroscope and images were processed using Image J. Legs of all pharates from a genotype were mounted and incubated at 60°C for 15 minutes and imaged under the Zeiss Axioplan 2 microscope using Optika Imaging software. Imaged legs of were scored for leg

malformation as follows. Malformed legs were those that had lost all tissue architecture in the tarsals while partially rescued or non-malformed legs showed distinct tarsal segments and occasionally even the tibia. The scoring was plotted using Fiji or ImageJ software for quantification.

2.5. Protein lysate preparation and Western blotting

10 larval heads and salivary glands were dissected into, and lysed with, 1× Cell Lysis Buffer (Cell Signaling Technology), supplemented with 1× Protease Inhibitors (Roche), 1 mM phenylmethylsulfonyl fluoride (PMSF) and 1 mM sodium fluoride (NaF). The tissues were mechanically homogenized and sonicated for 3× 5 secs. Lysates obtained after centrifugation for 10 min were stored in 1× Laemlli buffer at -20°C. Protein lysates were resolved on 8% SDS/PAGE gels (at 90V for 120 mins) and then transferred to nitrocellulose membranes (at 20V for 75 mins). Membranes were blocked with 5% skimmed milk before primary and secondary antibody incubation. Images were acquired by a FujiFilm LAS-4000 Chemiluminescent Scanner. The primary antibodies used include loading control: mouse anti-β-Tubulin (1:1000, abm G098), mouse anti-myc-tag (1:200 Millipore 4A6 05-724). HA-tagged Hipk was also detected using HRP-conjugated anti-HA-tag (6E2) antibodies (1:2000, Cell Signaling Technology 2999). Membranes incubated with primary antibodies are washed and stripped in β-mercaptoethanol – SDS buffer for 30 min at 56°C before blocking and probing with the next primary antibody.

Chapter 3.

Results

3.1. Loss of Siks can suppress Hipk-overexpression phenotypes

A previous RNAi screen in the lab (performed by Jessica Blaquiere and Rubia Chung) identified Sik2 and Sik3 as possible regulators of Hipk. Earlier graduate student in the lab, Dr. Kenneth Kin-Lam Wong, followed up on this data, and obtained some preliminary results (Wong, 2020). I then decided to take over and continue working on this study after he graduated from the lab. Overexpression of Hipk using the *dpp-Gal4* driver causes overgrowth in tissues expressing *dpp* like the legs (Fig. 7B, F). This is in line with previous findings in the lab reporting tumorigenic effects of Hipk (Blaquiere and Verheyen, 2016; Blaquiere et al, 2018; Tettweiler et al, 2019; Wong et al, 2019; Chen and Verheyen, 2012; Blaquiere et al, 2014). As our lab has shown before, these malformations are manifestations of neoplastic growth caused by Hipk expression. This phenotypic readout was used to follow up on the preliminary data from the screen. Hipk was overexpressed when Siks were depleted either by RNA interference or using loss-of-function mutants. These mutants were generated by imprecise excision of P-elements in the exons of Sik2 (Choi et al, 2012) or Sik3 (Wang et al, 2011). Overexpressing Hipk yields neoplastic leg malformations in pharate adults at 29°C (Fig. 7B, F, J). When levels of Siks are lowered in this background, there is a partial suppression of the overgrowth in pharate legs, suggesting that Siks must be required for Hipk to mediate such tumorigenic effects (Fig. 7C-E, G-J). Additionally, when both Siks were knocked down (Fig. 7E), the suppression of overgrowth was stronger than in case of the individual knockdowns. This supports the theory that Siks may have redundant, as well as additive effects in Hipk regulation.

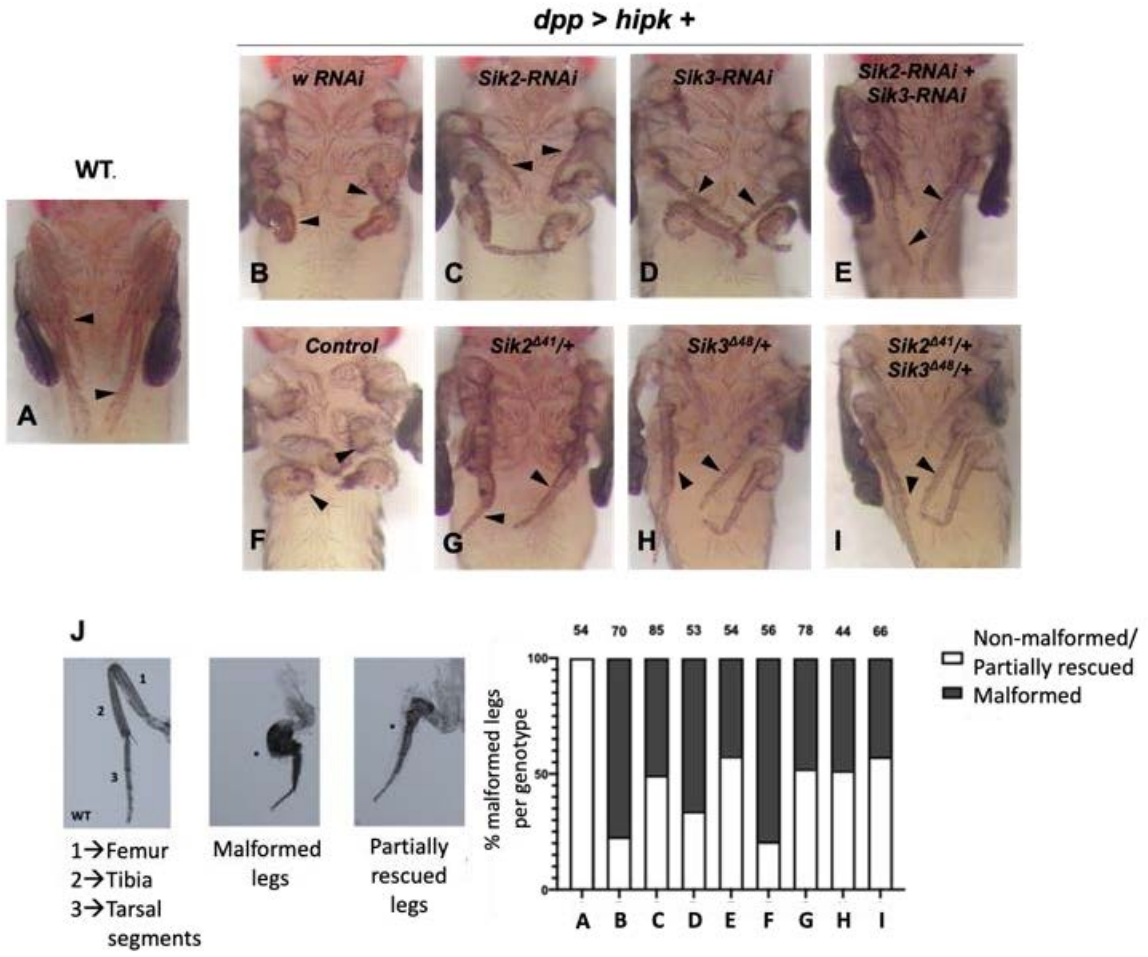


Figure 7 Depleting Siks can partially rescue Hipk-mediated tissue overgrowth at 29℃

At 29℃, Hipk overexpressing pharates develop malformed legs (Comparing B, F to A). When Siks are knocked down (C-E) or Hipk is overexpressed in heterozygous Sik2$^{\Delta 41}$or Sik3$^{\Delta 48}$ mutants (F-I), there is a segmental rescue of Hipk-induced neoplastic leg malformations. When both Siks are knocked down (E) or when Hipk is overexpressed in pharates that are mutant for one copy of Sik2 and Sik3 (I), the suppression of Hipk-induced overgrowth is stronger than when loss of only one Sik is depleted (C, D, G, H). This suggested that Siks not only have redundant but additive functions in regulating Hipk. The rescue is quantified in J (biological replicates = 3, N>=10 each time)

3.2. Siks synergize with Hipk and cause tissue overgrowth

We were interested to see what the effects were when Sik levels are increased in the presence of Hipk. We co-expressed WT (Wild-type) or CA (constitutively active)

forms of Siks with Hipk using the *dpp-Gal4* driver at 29°C. To generate constitutively active forms of Siks, Serine residues targeted for phosphorylation by PKA (Protein kinase A) to inhibit Siks, are substituted with Alanines instead (Wehr et al, 2012). As a result, PKA cannot phosphorylate Siks and inhibit their activity, rendering the latter constitutively active. Hipk overexpressing discs show a broader GFP stripe due to Hipk-induced cell proliferation, as well as distorted tissue architecture (Comparing Fig. 8E, E' to 8A, A'). Overexpression of WT/CA Siks by themselves using the *dpp-Gal4* driver could not cause tissue growth (Fig. 8B, B'-E, E'). When Siks and Hipk were co-expressed, however, a broader GFP stripe could be observed in *dpp>Hipk+Sik-WT* wing discs (Fig. 8G, G'-H, H') as compared to the controls. In all discs of *dpp>Hipk+Sik-CA* larvae more massive tissue overgrowth is observed, with the GFP region expanding to almost the entire disc, accompanied by a complete loss of tissue architecture (Fig. 8I, I'-J, J'). This suggests that Siks synergize with Hipk and can cause tissue overgrowth. Presumed wing discs are shown in Fig. 8I, I'-J, J', since this phenotype is too severe to definitively identify the specific discs. Furthermore, these larvae do not survive beyond early pupal stages.

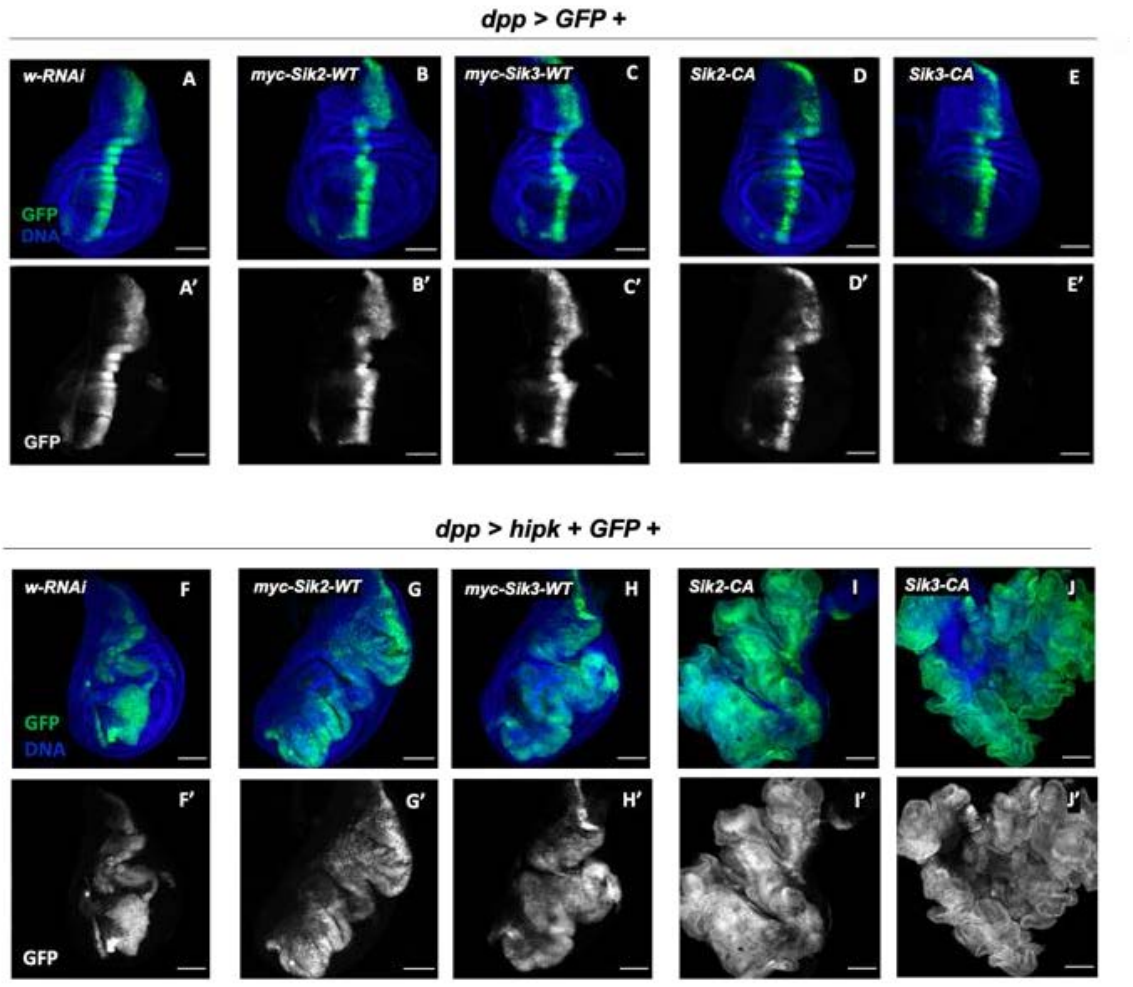


Figure 8 Siks synergize with Hipk to cause tissue overgrowth.

At 29°C, wing discs overexpressing Hipk or WT/CA-Siks or both in the dpp domain were observed after staining with DAPI. Hipk overexpressing wing discs show a widening of the domain and distorted tissue architecture (Compare F, F' to A, A'). GFP is also expressed in this domain along with the transgenes, allowing tracking of their expression. Overexpression of Siks alone, either their wild type (B, B'-C, C') or their constitutively active (D, D'-E, E') forms cannot induce tissue overgrowth. However, when co-expressed with Hipk (G, G'-J, J'), WT/CA-Siks can synergize with Hipk and cause dramatic tissue overgrowth. Presumed wing discs are shown in I, I'-J, J'. Scale bar = 100μm. This experiment was repeated thrice with N>=10 each time.

The expression of UAS-driven transgenes by Gal4 can be modulated by using different growth temperatures. Gal4 is temperature sensitive, and its activity is greater at higher temperatures (Duffy, 2002). When I examined the effect of growing these crosses at 25°C, *dpp>Hipk+Sik-WT/CA* larvae could survive until the pharate stage, and growth

synergy is evident in both larval and pupal stages, as shown in Figs. 9 and 10 respectively. Since the transgenes are expressed at lower levels, the severity of the *dpp>hipk* phenotype is reduced and appears to be below a threshold and appears almost wildtype, but the enhancement by Siks is still apparent. In the larval stage, Sik-Hipk co-expressing wing discs show a slight widening of the GFP stripe, with a mild distortion of tissue architecture (Fig. 9G, G'-J, J') as compared to the control discs overexpressing either Hipk alone (Fig. 9F, F') or Siks by themselves (Fig. 9B, B'-E, E').

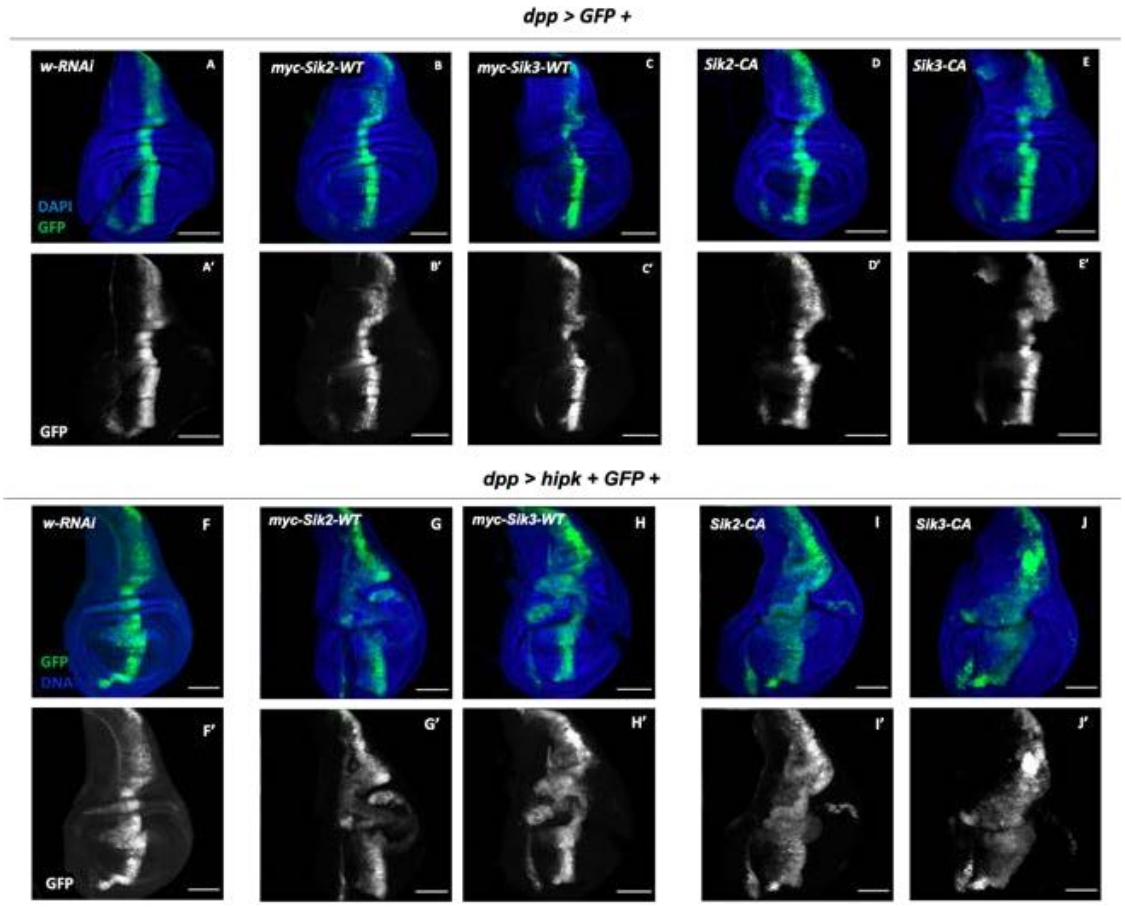


Figure 9 Siks synergize with Hipk to cause tissue overgrowth.

At 25℃, wing discs overexpressing Hipk or WT/CA-Siks or both from the dpp-Gal4 driver were observed after staining with DAPI. Wing discs overexpressing either Siks alone (B, B'-E, E') or Hipk alone (F, F') do not show an apparent tissue overgrowth. Wing discs co-expressing Siks and Hipk (G, G'-J, J') show tissue overgrowth characterized by increase in number of GFP positive cells expressing both proteins, and a distorted tissue architecture, suggesting an evident synergy between the kinases. Scale bar = 100μm. This experiment was repeated thrice with N>=10 each time.

In the pupal stage, pharates show evidently malformed legs only when Siks and Hipk are expressed together (Figs. 10G, G'-J, J'), which is not observed when either of the proteins alone is overexpressed (Fig 10A-F, F'). The experiment in Fig. 10 was performed by former graduate student in the lab, Dr. Kenneth Kin-Lam Wong, and the *UAS-Sik-CA* genotypes are referred to as *UAS-Sik-SA* by him, highlighting the Ser→Ala mutations in the transgenes, that renders the Siks constitutively active.

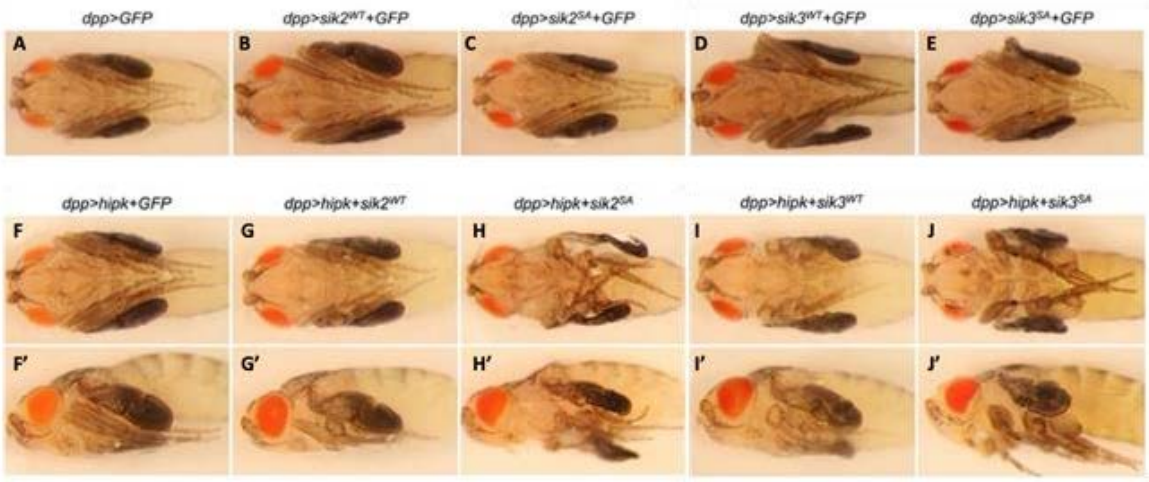


Figure 10 Siks synergize with Hipk to cause tissue overgrowth.

At 25°C, pharate adults overexpressing Hipk, or WT/SA(CA)-Siks, or both, using the dpp-Gal4 driver, were observed. Neither pharates overexpressing WT/CA Siks (B-E) nor Hipk (F, F') develop malformed legs, and phenocopied WT pharates (A). Pharates co-expressing Hipk and Siks however (G, G' – J, J') show neoplastic leg malformations. Pharates images in C, E, J and J' are males while the other adults are females. This experiment was performed by former graduate student in the lab, Dr. Kenneth Kin-Lam Wong (Wong, 2020).

3.3. Siks can modulate Hipk activity

Elevating Hipk levels can activate multiple signaling pathways to cause increased cell proliferation and tissue overgrowth. This can be ascertained by staining the larval imaginal discs with antibodies against readouts of these signaling pathways. An increase in Hipk activity can cause a localized increase in *dMyc* expression via many or all of these cell-proliferative pathways (Wong et al, 2019). More intense Arm staining can also be observed in cells overexpressing Hipk, as the latter can stabilize Arm and further

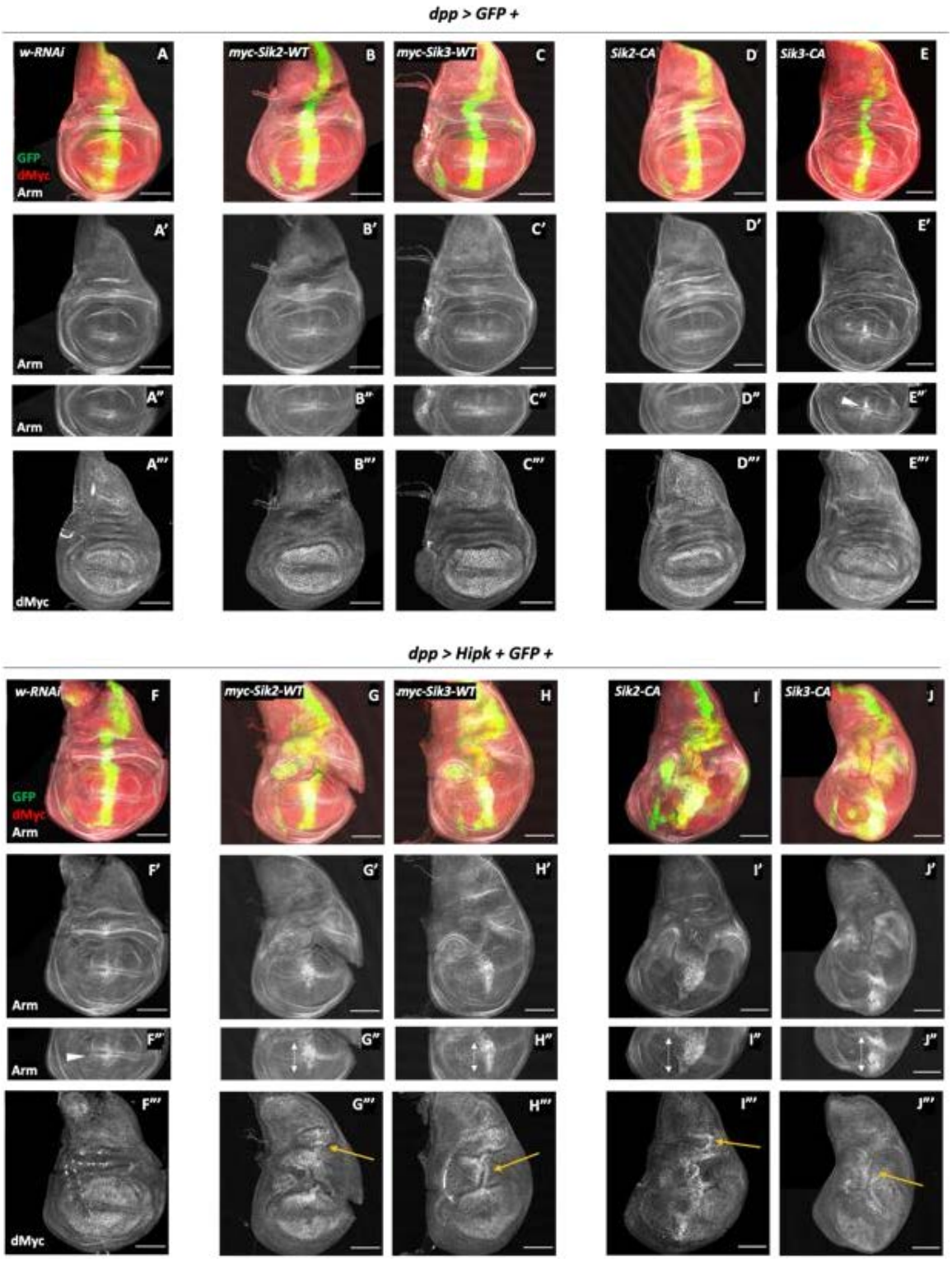
augment its activity in the Wg pathway (Swarup and Verheyen, 2012; Lee et al, 2009b; Shimizu et al, 2014).

To determine whether Siks can increase Hipk activity, at 25°C, we stained wing discs overexpressing Hipk with antibodies against dMyc and Arm, and co-expressed WT/CA Siks in this background. If Siks modulated Hipk activity, we would expect to see a difference in the levels of the readouts. Indeed, the Arm immunofluorescence signal at the intersection of the D/V and A/P boundaries extended dorsoventrally on either side when Siks were co-expressed with Hipk (Fig. 11 G', G"-J', J": white double-headed arrows). This extension was not observed in discs overexpressing Hipk alone (Fig. 11F', F"), but a localized increase in Arm signal intensity due to increased Hipk activity (indicated by single white arrowhead), as compared to *dpp>GFP+w-RNAi* controls was apparent. The Arm signal intensity was markedly increased in Sik-Hipk co-expressing discs as compared to controls. This is quantified in Fig. 11K. The ratio of Arm fluorescence intensities within the dpp domain (region 1 of wing disc in Fig. 11K), at the intersection of the D/V-A/P boundaries and outside the dpp domain, but at the D/V boundary, (where a light streak of the Arm stain can be seen – region 2 of wing disc in Fig. 11K), is quantified, and compared across genotypes. Co-expression of the constitutively active Siks and Hipk led to higher increase in Arm staining than did co-expression of WT-Siks with Hipk. This suggested that Hipk activity can increase with increase in Sik function.

The number of dMyc staining cells was similarly increased in Sik-Hipk co-expressing wing discs in the dpp domain (yellow arrows in Fig. 11G"'-J"'). However, such an increase was not observed in the dpp domains of control discs overexpressing either Siks alone (Fig. 11B"'-E"') or Hipk alone (Fig. 11F"') under these conditions. This data is quantified in Fig. 11L. Ratio of dMyc intensity within the dpp domain in the wing pouch (region 1 in wing disc of Fig. 11L) to its intensity elsewhere in the wing pouch (region 2 in wing disc of Fig. 11L) was measured and plotted. In this case again, CA-Sik-Hipk co-expressing discs show increased dMyc signal in transgene expressing cells as compared to the WT-Sik-Hipk co-expressing discs. These results imply that activity of Hipk increases in a Sik-dependent fashion.

This experiment was performed at 25°C, since Sik-Hipk co-expressing wing discs dramatically overgrown and lost all tissue architecture at 29°C. The GFP stripe in that

situation spanned most of the disc area, and fluorescence intensities of Arm or dMyc in these Hipk expressing cells could not be compared to the internal control regions of the disc, expressing neither Hipk nor GFP.


dpp > GFP +
w-RNAi
A
GFP
dMyc
Arm
myc-Sik2-WT
B
myc-Sik3-WT
C
Sik2-CA
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E
A'
B'
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Arm
A''
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dMyc
dpp > Hipk + GFP +
w-RNAi
F
myc-Sik2-WT
G
myc-Sik3-WT
H
Sik2-CA
I
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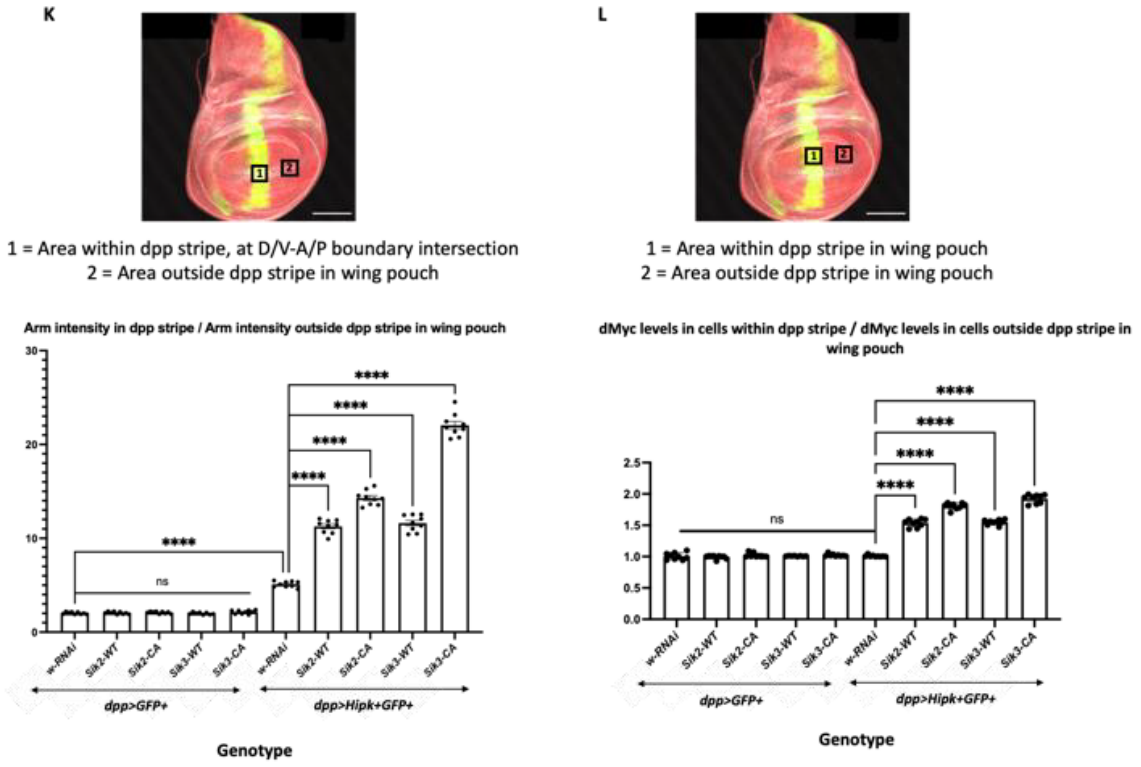


Figure 11 Siks can modulate Hipk activity.

At 25°C, when compared to controls (A-A'"), Hipk overexpressing discs show a localized increase in Arm intensity at the intersection of the dorsoventral and anterioposterior boundaries (F', F", quantified in K), possibly due to the stabilization of Arm by Hipk (Fig. 3) Under these conditions, discs overexpressing Hipk were unable to show an apparent increase in dMyc levels in the hinge region or wing pouch (F'", quantified in L). Overexpression of Siks (B-B'" to E-E'") alone did not lead to an apparent increase in Arm or dMyc levels (quantified in K, L). Sik-Hipk co-expressing discs (G-G'" to J-J'") however, showed a strong increase in Arm signals (spread dorsoventrally along the A/P boundary, indicated by white double headed arrows in G"-J"), as well as dMyc levels (yellow arrows in G'"-J'"), suggesting Siks can augment Hipk activity. K shows the quantification of Arm intensity in the dpp stripe at the D/V-A/P boundaries (region 1) relative to the region outside the dpp stripe, but along the D/V boundary (region 2) where there is a slight intense streak of Arm as compared to away from the boundary in the wing pouch. L shows the quantification of dMyc signal intensity in the dpp stripe in the dorsal region of the wing pouch (region 1) relative to its intensity elsewhere in the wing pouch (region 2). CA-Sik co-expression with Hipk led to stronger increase in Arm and dMyc intensities in transgene expressing cells, as compared to WT-Sik and Hipk coexpressing discs, suggesting an increase in Hipk activity with Sik activity. Scale bar = 100μm. This experiment was performed twice and N>=10 for all genotype each time.

3.4. Kinase activity of Siks is required to modulate Hipk overexpression phenotypes

Evidence obtained so far suggested that Siks could be putative novel regulators of Hipk. We now wanted to understand the mechanism by which Siks could regulate Hipk. Since Siks were identified in the initial phospho-regulator screen for Hipk, and Siks are Serine-Threonine kinases, we tested whether the kinase function of Siks had a role in Hipk regulation. Kinase dead (KD) constructs of Siks (generated by substituting key lysine residues in the kinase domain with methionines, Choi et al, 2011; Choi et al, 2015), were co-expressed with Hipk at 29°C. Unlike the WT Siks (Fig. 12B-B', D-D'), KD-Siks could not lead to synergized overgrowth in wing discs (Fig. 12C-C', E-E') when co-expressed with Hipk.

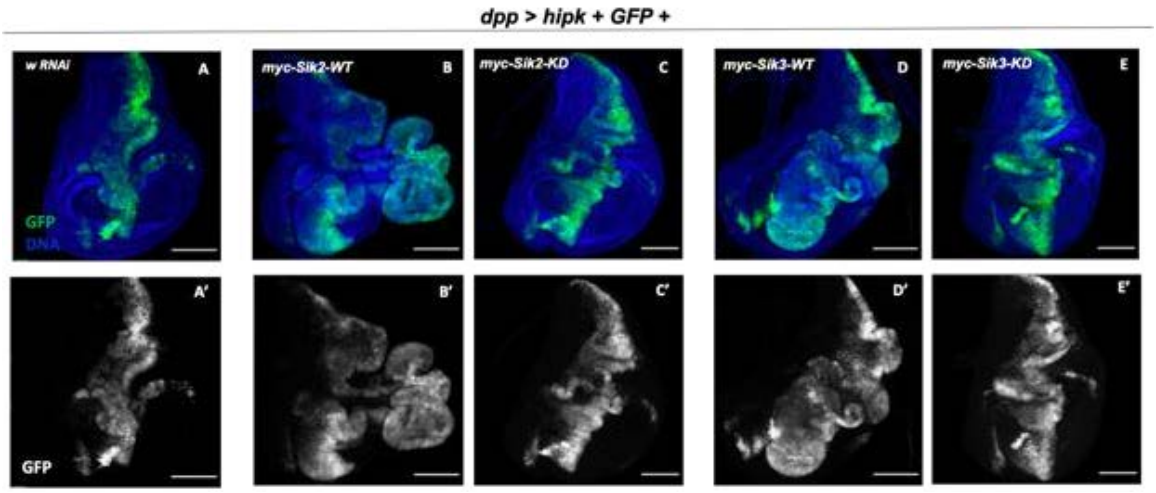


Figure 12 Siks need their kinase activity for modulating Hipk-induced phenotypes.

Hipk-mediated tissue overgrowth is evident in wing discs overexpressing Hipk at 29°C (A, A'). This phenotype is exacerbated upon co-expression with WT-Siks (B, B'; D, D'), suggesting growth synergy. However, when KD-Siks are co-expressed with Hipk, such a synergized tissue overgrowth could not be observed. Scale bar = 100μm. This experiment was repeated three times and N>=20 wing discs in all genotypes each time.

3.5. Co-expression of Sik2 or Sik3 and Hipk can cause developmental delay

In addition to causing synergized overgrowth at 29°C, when WT-Siks are co-expressed with Hipk, there is a delay in the developmental timing – with an extended larval phase (Fig. 13B-B'", D-D"') that lasts from Day 6AEL – Day12AEL followed by pupariation starting day 12AEL, unlike controls that pupariate from Day 7AEL (Fig. 13A-A'"). The larvae continue to grow during this extended developmental stage, with massively overgrown imaginal discs, increased hemolymph and decreased fat body. This is a common phenotype observed in *Drosophila* tumor models (Bilder, 2004; Menut et al, 2007; Doggett et al, 2015; Turkel et al, 2013). Although they can pupariate, these organisms die in the early pupal stage itself, owing presumably to the severe overgrowths. When KD-Siks are co-expressed with Hipk, however, there is no delay in the development (Fig. 13C-C"', E-E"'), and the timing is akin to the controls. The images shown in Fig. 13 are all from 1 day egg-lays, ensuring that the crosses are comparable in terms of their developmental timings. Pharates from both *dpp>Hipk+w-RNAi and dpp>Hipk+Sik-KD* develop neoplastic leg malformations at 29°C (Fig. 13 A"', C"', E"').

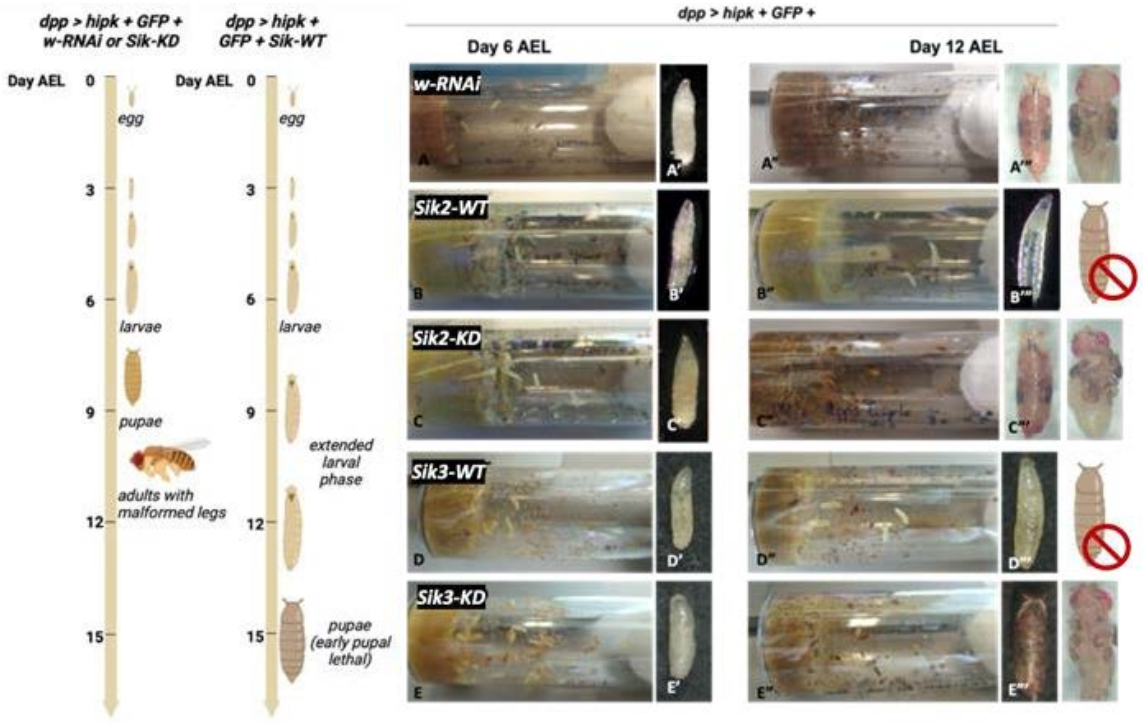


Figure 13 Co-expression of WT-Siks and Hipk causes a developmental delay

At 29°C, Hipk overexpressing larvae pupariate at around day 7AEL, and pharate adults with malformed legs develop at this temperature (as seen previously in Fig. 7) (A-A'''). When WT-Siks are co-expressed with Hipk, developmental delay, characterized by an extended larval phase till day 12AEL is observed (B-B''', D-D'''). Larvae continue to grow, with evident organomegaly, increased hemolymph and fat body wasting. The co-expression of KD-Siks with Hipk does not cause such a marked delay in development (C-C''', E-E'''), suggesting the importance of Sik kinase function in Hipk regulation. Pharate adults in A''', C''' and E''' show neoplastic leg malformations at this temperature, while those co-expressing WT-Siks and Hipk are usually lethal in early pupal stages. This experiment represents three biological replicates.

3.6. Co-expression of Siks and Hipk can cause cytoplasmic enrichment of the two proteins

We were interested to see whether Siks can co-localize, or physically interact with Hipk, suggesting a mechanism for their genetic interaction – i.e., direct phosphorylation, and regulation. To test whether Siks and Hipk could co-localize, and where these proteins could potentially interact, L3 larval salivary glands from *dpp>Hipk+myc-Sik-WT/CA* larvae were dissected and stained for myc-tagged Siks and

Hipk. Salivary glands were chosen because of the large size of the cells that enable clear distinction between nuclei and cytoplasm, and because *dpp-Gal4* is expressed in those cells.

When WT-Siks are co-expressed with Hipk at 29°C, the salivary glands continue to grow through the extended larval phase (Figs. 13B-B"', D-D"'). At day 6AEL, the size of the salivary glands (Figs. B, C) is similar to those of *dpp>Hipk+w-RNAi* larvae (Fig. 14A). However, at day 12AEL, *dpp>Hipk+Sik-WT* glands (Figs. 14F, G) are larger than *dpp>Hipk+w-RNAi* glands (Fig. 14A). However, when the KD-Siks are co-expressed with Hipk, the salivary glands are not enlarged (Figs. 14C, E). The larvae in this case do not exhibit an extended growth phase, and salivary glands cannot be observed at day 12AEL for these organisms, like in case of those co-expressing WT-Siks and Hipk. The enlargement of salivary glands being observed only in case of WT-Sik co-expression with Hipk reiterated the finding in the wing discs that the kinase function of Siks must be necessary in regulating Hipk (Fig. 12).

Interestingly, however, upon co-expressing WT-Siks with Hipk, a primarily nuclear protein, Hipk gets delocalized to the cytoplasm (Figs. 14B', D', F', G'). This delocalization seems to begin around day 6AEL (Figs. 14B', D') and is complete by day 12AEL (Figs. 14F', G'). This does not occur when KD-Siks are co-expressed with Hipk (Figs. 14C', E').

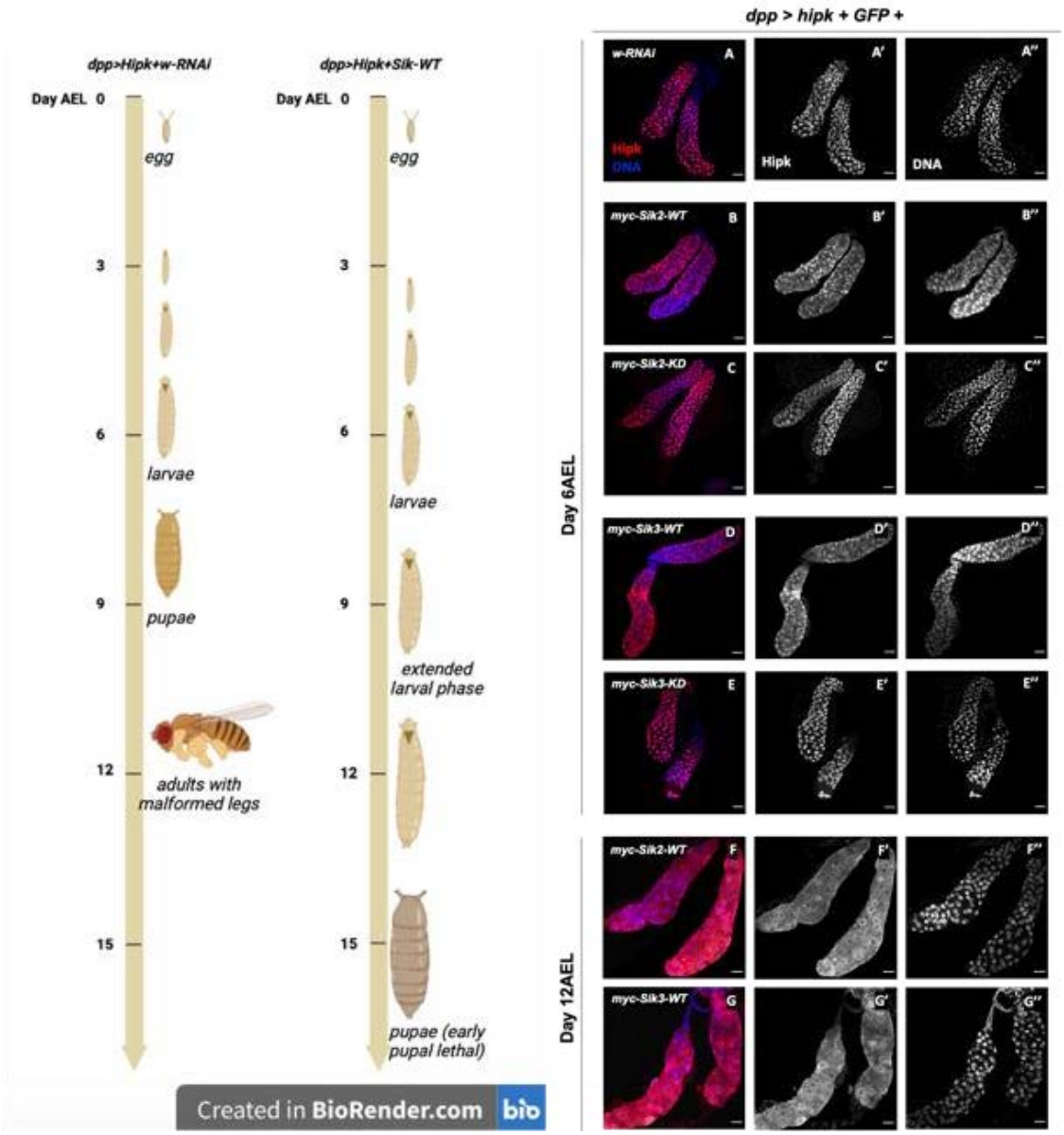
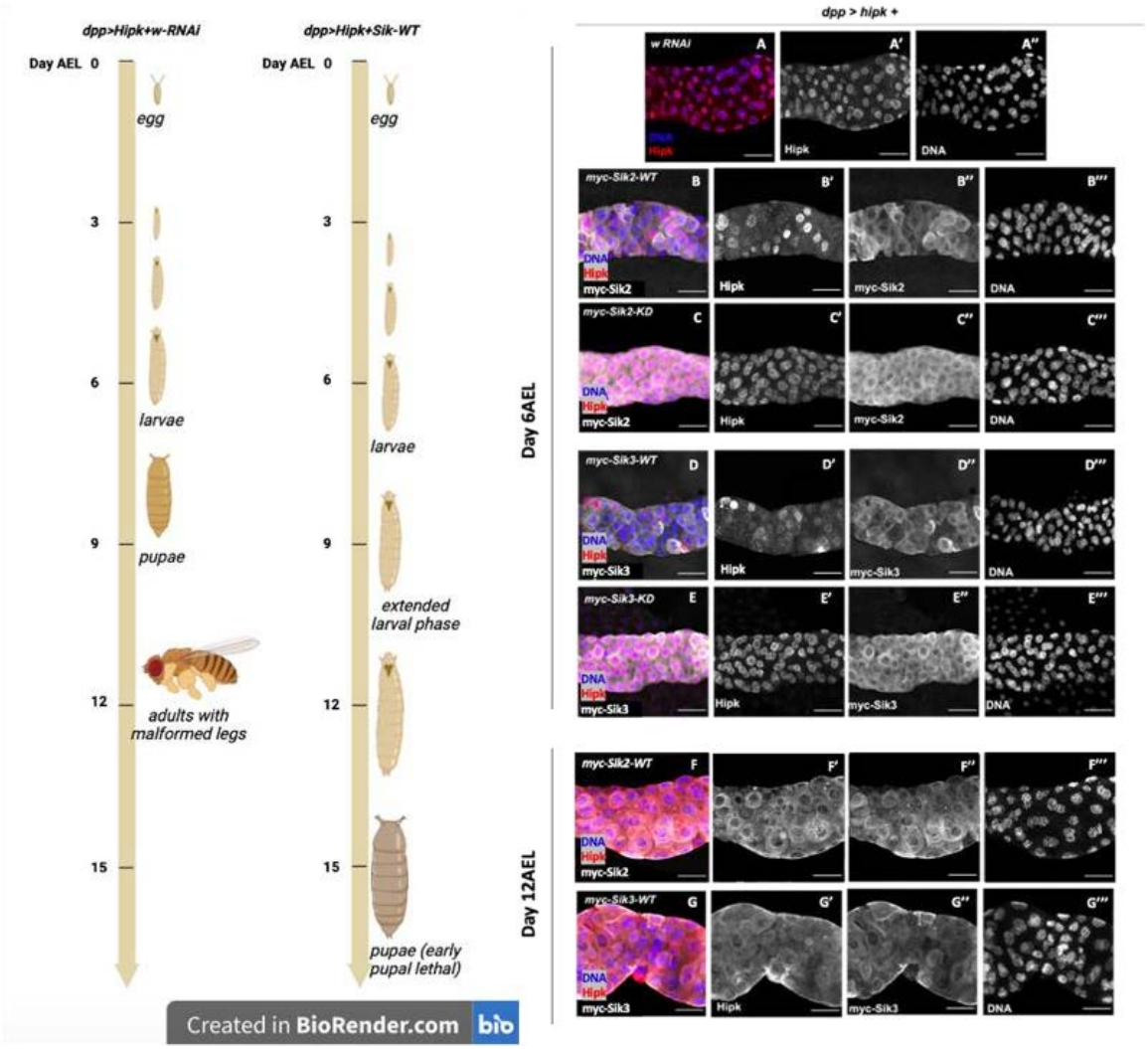


Figure 14 Co-expressing Siks and Hipk can cause organomegaly and Hipk delocalization.

At 29°C, Hipk is a primarily nuclear protein (A'), evident from salivary glands stained with antibodies against Hipk. In the presence of WT-Siks, organomegaly is evident in salivary glands of giant larvae at day 12AEL (F, G). Additionally, in cells of these glands, Hipk begins to migrate to the cytoplasm at day 6AEL, (B', D') and is almost entirely cytoplasmic by day 12 AEL (F', G'). In the presence of kinase dead Siks however, Hipk remains nuclear in salivary glands of larvae dissected at day 6AEL (C-C"', E-E"'). These larvae do not have an extended larval phase until day 12 AEL. Scale bar = 100μm. This experiment was repeated at least thrice and N>=20 salivary glands for all genotypes in each case. Panel on the left depicts this developmental delay in Sik-Hipk co-expressing larvae at 29°C as compared to Hipk-overexpressing larvae.

To better visualize this delocalization, Fig. 15 shows these salivary glands at 20x magnification, and stained with antibodies against myc-Sik in addition to Hipk, to determine the localization of Siks and Hipk, and whether they can also possibly co-localize. Since an antibody against endogenous Siks was not available, one against the myc tag was used, to detect myc-tagged exogenous Siks. The tissues are compared at day 6AEL (Fig. 15A-A''' to E-E'''), as well as day 12AEL, for larvae expressing both WT-Siks and Hipk (Fig. 15F-F''' to G-G'''). At day 6AEL, it is apparent that Hipk possibly starts migrating out of the nucleus (Fig. 15B', D') when WT-Siks are co-expressed with Hipk, and this migration seems to be complete by day 12AEL (Fig. 15F', G'). No such delocalization is observed when KD-Siks are co-expressed with Hipk (Fig. 15C', E'), and these glands develop in a similar manner to the *dpp>Hipk+w-RNAi* ones. The degree of delocalization (fraction of cytoplasmic Hipk in total Hipk levels) is quantified in Fig. 15H.


dpp>Hipk+w-RNAi
dpp>Hipk+Sik-WT
Day AEL 0
egg
3
larvae
6
pupae
9
adults with malformed legs
12
15
extended larval phase
pupae (early pupal lethal)
Created in BioRender.com
dpp > hipk +
w RNAi
Hipk
DNA
myc-Sik2-WT
myc-Sik2-KD
myc-Sik3-WT
myc-Sik3-KD
Day 6AEL
Day 12AEL

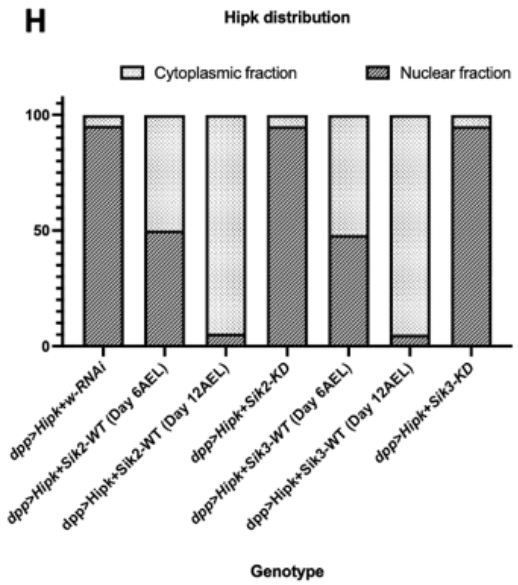

H
Hipk distribution
Cytoplasmic fraction
Nuclear fraction
100
50
0
dpp>Hipk+w-RNAi
dpp>Hipk+Sik2-WT (Day 6AEL)
dpp>Hipk+Sik2-WT (Day 12AEL)
dpp>Hipk+Sik2-KD
dpp>Hipk+Sik3-WT (Day 6AEL)
dpp>Hipk+Sik3-WT (Day 12AEL)
dpp>Hipk+Sik3-KD
Genotype

Figure 15 Siks can alter Hipk localization

Panel on the left shows developmental delay in Sik-Hipk co-expressing larvae at 29°C, and 20x magnified salivary glands are shown on the right panels at the indicated time points after egg lay. When co-expressed with WT-Siks but not KD-Siks, Hipk begins to show nucleocytoplasmic delocalization at day 6AEL (B-B"', C-C"', D-D"', E-E"'), which seems to be complete by day 12AEL (F-F"', G-G"'). Siks are majorly cytoplasmic in all these cases (B"-G")The distribution of Hipk in the nucleus and the cytoplasm is represented in H (N=10). Nuclear Hipk begins to migrate to the cytoplasm when WT-Siks are co-expressed with Hipk (around 50% by day 6AEL and completely by day 12 AEL). No such delocalization can be observed when KD-Siks are co-expressed with Hipk, and these larvae pupariate after day 6AEL. Scale bar = 100μm. This experiment was repeated at least thrice and N>=20 salivary glands of all genotypes in each case.

Enhanced delocalization at the cellular level is shown in 63x magnified salivary glands Fig. 16. Organomegaly of the *dpp>Hipk+Sik-WT* salivary glands is apparent at this level as well, with bigger nuclei and cells (Fig 16. B-B"', D-D"') as compared to controls (Fig. 16A-A"'). This delocalization is not apparent in *dpp>Hipk+Sik3-KD* cells (Fig. 16C-C"', E-E"'). Cells in Fig. 16 B-B"', D-D"' belong to salivary glands of larvae dissected at day 12AEL to highlight fully translocated Hipk in the cytoplasm. Controls and *dpp>Hipk+Sik3-KD* salivary glands were from day6AEL, after which the larvae pupariate, following the usual developmental timeline.

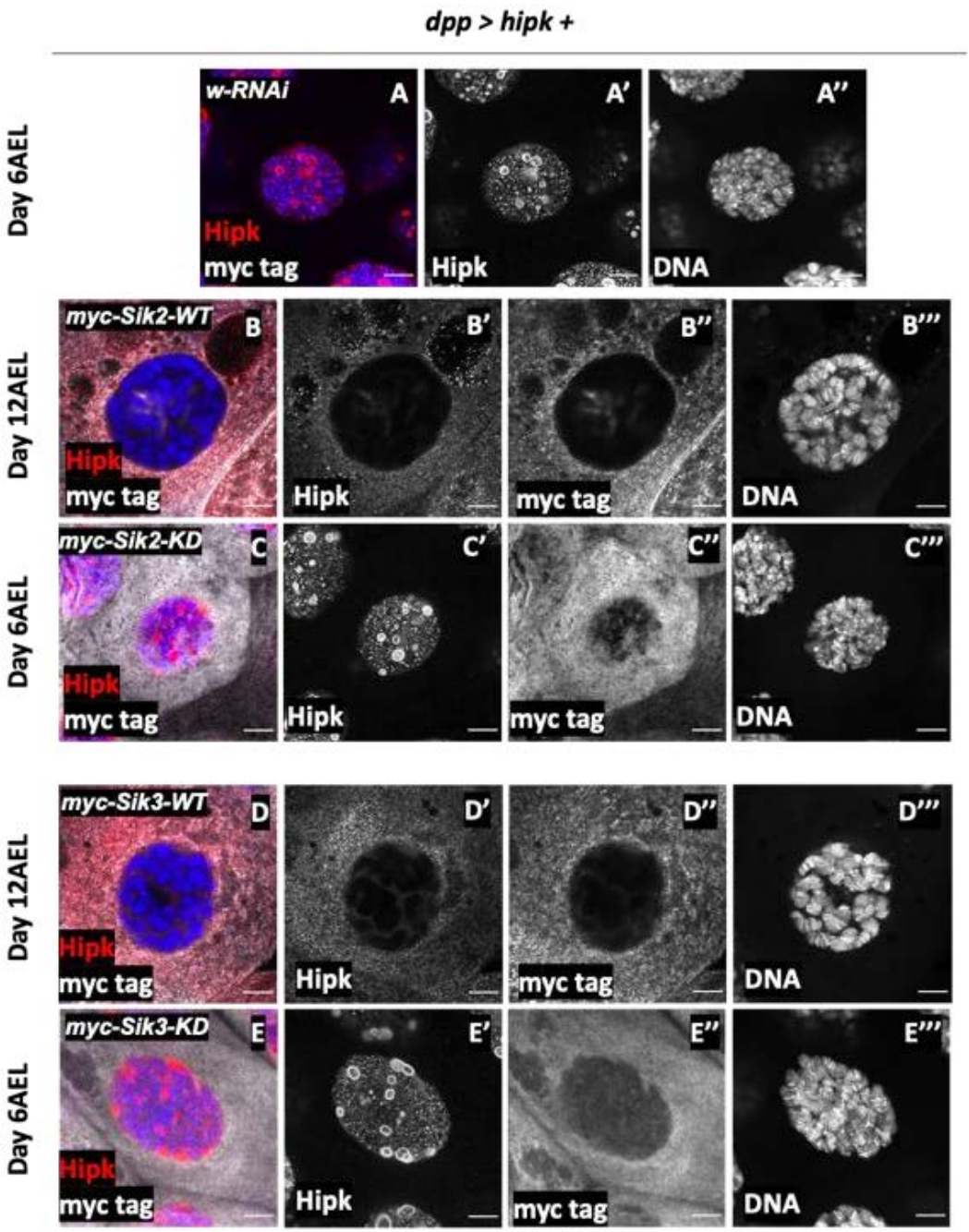


Figure 16 Siks can alter Hipk localization

63x magnified L3 larval salivary gland epithelia at 29℃ overexpressing Hipk show nuclear localization of the latter (A-A'). In the presence of WT-Siks, however, complete delocalization of Hipk to the cytoplasm can be observed by day 12AEL (B-B', D-D'). Hipk however remains nuclear in the presence of KD-Siks (C-C', E-E'). Siks are found to majorly localize to the cytoplasm in all these cases (B"-E"). Scale bar = 10μm. This experiment was repeated at least three times and N>=10 salivary gland cells of all genotypes in each case.

Exogenous Siks are usually found to be uniformly distributed throughout the nuclei, cytoplasm and even cell membranes (Fig. 17). However, strikingly, in the presence of Hipk, Siks also become enriched in the cytoplasm (Figs. 15B", C", D", E"). This suggested that Hipk could alter the localization of Siks as well, potentially altering their functions in turn.

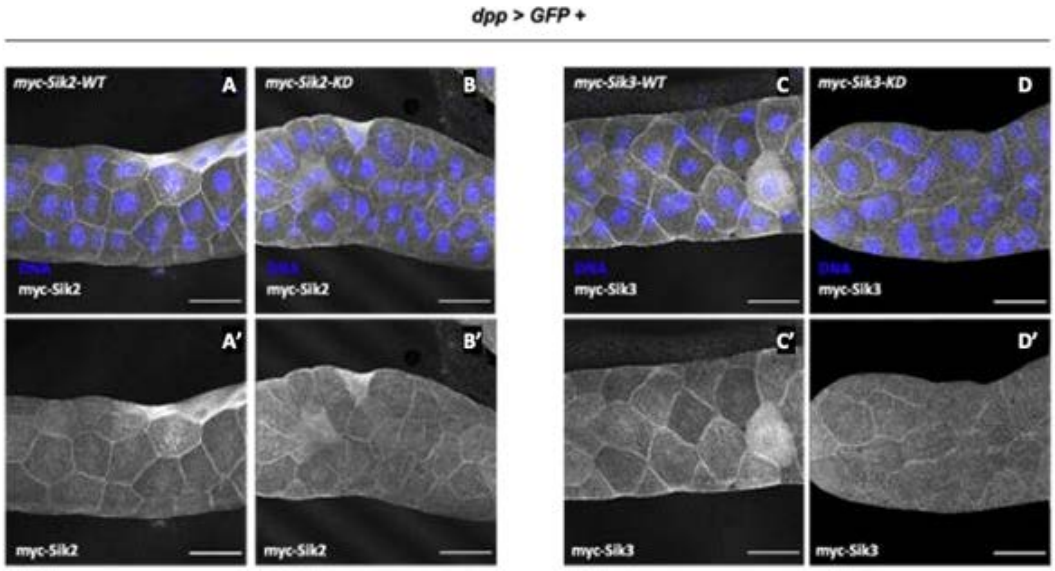


Figure 17 Siks are uniformly distributed throughout the cell

Exogenous WT/KD-Siks are found to be distributed uniformly throughout cells of the salivary gland at 29℃. Siks are found to localize equally to nuclei, cytoplasm and even cell membranes. Scale bar = 100μm. This experiment was repeated thrice with N>=10 glands in each case.

3.7. Siks can possibly stabilize Hipk protein

In addition to studying effect of Siks on Hipk activity, we wanted to understand the effect of altering levels of Siks on Hipk protein levels. This could further explain the increase in Hipk activity in the presence of Siks as well. O-GlcNAc transferase (OGT), an enzyme that transfers the sugar O-GlcNAc onto Ser/Thr/Tyr residues on target proteins had been previously identified in the lab as an upstream regulator of Hipk (Wong et al, 2020). It was found to synergize with Hipk to cause overexpression, not

unlike Siks, and was also necessary for stable expression of Hipk protein. We wanted to investigate whether a similar mechanism was at play between Siks and Hipk. Protein lysates were collected from L3 larvae expressing Hipk in combination with co-expression or knockdown of Siks (Fig. 18). We observed that when Siks were depleted, levels of Hipk were lower; and when constitutively active Siks were co-expressed, Hipk levels were greater, than in *dpp>Hipk+w-RNAi* lanes. Therefore, it is possible that Siks can not only stabilize exogenous Hipk protein levels, but may be required for the stability of the same. This experiment was extended to determine levels of Hipk when expressed with WT and KD Siks. However, the results of this experiment were not consistent, preventing us from making any conclusions just yet.

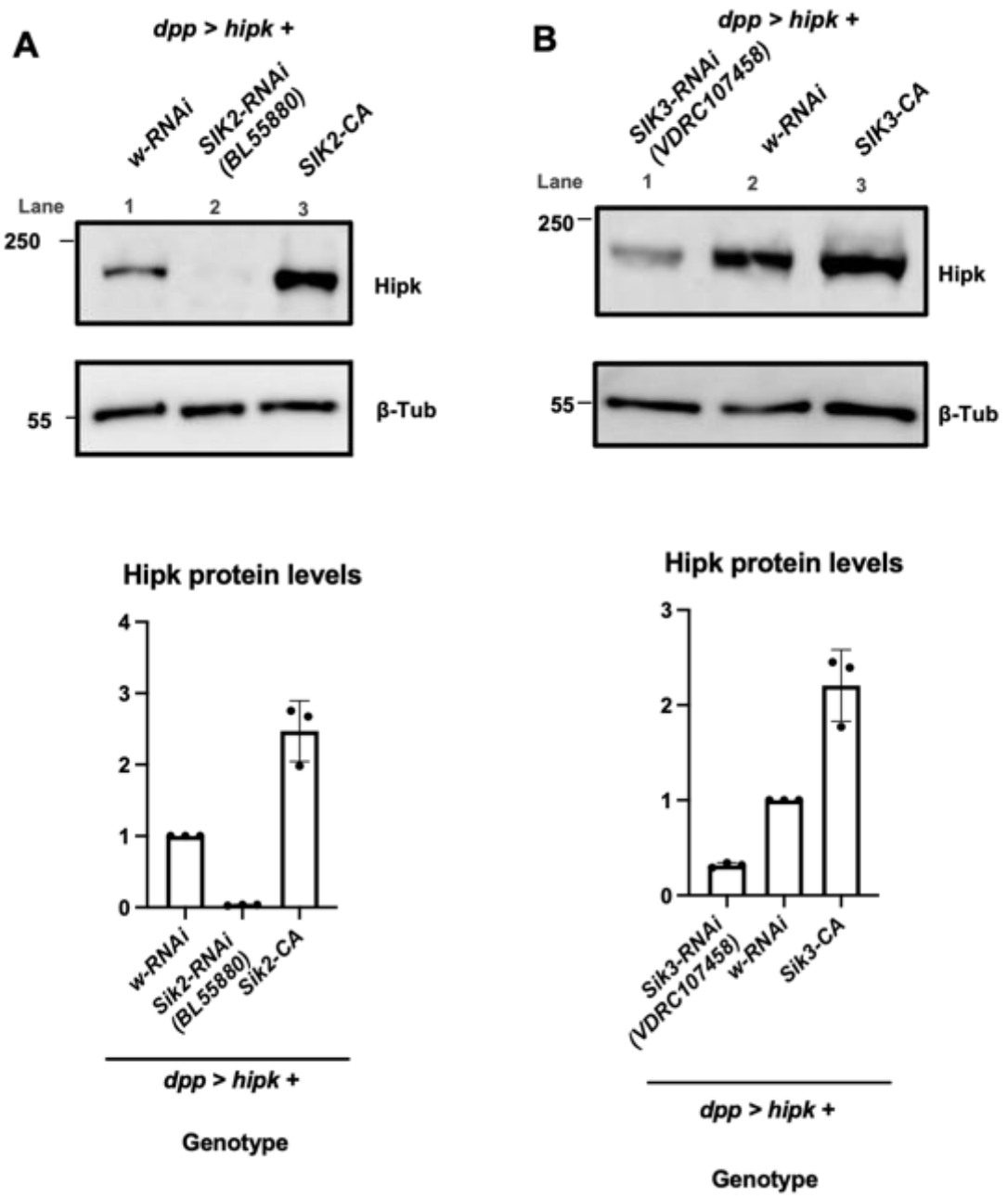


Figure 18 Siks can possibly stabilize Hipk.

At 25℃, depletion of Sik2 (A, lane 2) or Sik3 (B, lane 1) can decrease Hipk protein levels relative to dpp>Hipk+w-RNAi (A, lane 1; B, lane 2). Conversely, co-expression of CA-Sik2 (A, lane 3) or CA-Sik3 (B, lane 3) led to an increase in Hipk protein levels, and the levels of Hipk protein for genotypes in each blot is quantified in the corresponding plots below for three biological replicates. Siks can thus stabilize Hipk protein.

Chapter 4.

Discussion

4.1. Sik2 and Sik3 are novel regulators of Hipk

Siks are established metabolic regulators that have been conventionally known to regulate lipid and glycogen stores in response to feeding or fasting in mammals (Licanzo et al, 2004). Siks have also previously been proven to regulate signaling pathways like Hippo and Notch via phosphorylation of key pathway intermediates (Wehr et al, 2012; Sahin et al, 2020). Here we prove that Siks have a novel function in regulating Hipk, possibly by phosphorylation. Depletion of Siks via RNA interference can partially suppress Hipk-driven overgrowth phenotypes (Fig. 6) while elevating Sik and Hipk levels simultaneously can cause synergized overgrowth in tissues (Figs. 8, 9). Kinase-dead forms of Siks were unable to cause such a growth synergy under the same conditions, indicating that Siks can directly or indirectly phosphorylate Hipk to regulate its activity (Fig. 11). Expression of Siks can also lead to an increase in Hipk activity (Fig. 10) as evident from the increase in readouts of pathways promoted by Hipk, namely dMyc and Arm. Since Siks have been implicated in multiple human cancers (Ahmed et al, 2010; Miranda et al, 2016; Bon et al, 2015), but their mechanisms of carcinogenesis still under study, it may be possible that Siks can promote Hipk activity and drive overgrowth, leading to tumor progression. Siks can thus prove to be a novel therapeutic target in cancers.

The co-expression of Siks and Hipk at 29°C also led to an extended larval phase (Fig. 12), which is a characteristic trait of many tumour models in *Drosophila* (Bilder, 2004; Menut et al, 2007; Doggett et al, 2015; Turkel et al, 2013). The larvae continue to grow, exhibiting phenotypes of organ wasting or cachexia, with increased hemolymph, decreased fat body and dramatically overgrown imaginal discs. Cachexia refers to the involuntary loss of muscle and adipose tissues frequently associated with cancers. Hirabayashi and Cagan in 2015 demonstrated that overexpression of Sik2 in *ras^{V12}/scribble* tumours led to similar peripheral organ wasting, and systemic insulin resistance. Two other groups at the same time (Figueroa-Claravega and Bilder, 2015; Kwon et al, 2015) showed that this systemic insulin resistance was evaded by tumors by

a localized increase in insulin signaling, thus enabling tumours to grow. Tumor-secreted ImpL2 (Insulin antagonist) was found to be necessary and sufficient to cause systemic organ wasting. It is therefore possible that similar mechanisms may be at play in Hipk-tumours when Sik2 or Sik3 is co-expressed, leading to tumor tissue overgrowth with simultaneous systemic cachexia. Moreover, since Siks transmit insulin signals downstream, it is possible that Sik-expressing Hipk tumors cause localized increase in insulin signaling in a similar manner, evading insulin resistance, and allowing tumour growth.

4.2. Siks can regulate Hipk protein stability and localization

Depletion of Siks led to decreased Hipk protein levels, while on the other hand, co-expression of Siks led to higher Hipk protein levels (Fig. 16). It was thus evident that Siks are able to somehow regulate Hipk protein stability, possibly by phosphorylation of key residues on the latter. Additionally, the expression of Siks also led to translocation of Hipk from the nucleus to the cytoplasm (Figs. 13-16). This suggested that Siks may also regulate Hipk activity by altering their localization. Huang et al, 2011 showed that a decrease in SUMOylation of Hipk can result in its delocalization to the cytoplasm. Thus, it may be possible that Siks regulate the SUMOylation pathway negatively, so as to cause cytoplasmic enrichment of Hipk, indirectly. Hipk has been shown to regulate multiple pathways like JAK-STAT and Wg by phosphorylating key pathway intermediates that are found in the cytoplasm (Swarup and Verheyen, 2012; Lee et al, 2009b; Shimizu et al, 2014; Tettweiler et al, 2019). These hint toward a cytoplasmic role for Hipk that is yet understudied. Our results thus also underscore such a novel role for Hipk to drive tumours by modulating pathway components in the cytoplasm.

Takemori et al. reported in 2002 that Siks are distributed throughout the nuclei and cytoplasm in the resting state, in line with our findings that exogenous Siks seem to have such a uniform distribution throughout cells in the resting state (Fig. 17). However, in the presence of ACTH, the Adreno-corticotropic hormone, Siks from the nuclei move to the cytoplasm via PKA-mediated phosphorylation at the C-terminal S577 residue. In the presence of Hipk, we found that both WT and KD Siks became enriched in the cytoplasm. It will thus be interesting to investigate whether Hipk overexpression can activate PKA and delocalize nuclear Siks to the cytoplasm. Wang et al. (2020)

demonstrated that Hipk overexpression can lead to a decrease in PKA mediated phosphorylation of Hts, another PKA target associated with the synapse, as well as Hts localization, either directly or indirectly. It is thus possible that Hipk can affect Sik localization directly, or indirectly via PKA regulation.

4.3. Lingering Questions and Future Directions

Kewei Yu, currently a PhD candidate in the Verheyen lab, is following up on the data presented in this thesis to further understand the effect of Siks on development by themselves, as well as their mechanism of interaction with Hipk.

Siks are known to regulate metabolic responses in cells according to their nutrient status (Licanzo et al, 2004; Haberland et al, 2009; Altarejos and Montminy, 2011; Screaton et al, 2004; Shaw et al, 2005). It would therefore be informative to understand whether regulation of Siks by Hipk can be influenced by diet. Former graduate student in the lab, Dr. Kenneth Kin-Lam Wong, obtained some preliminary results that are shown in Fig. 19. When raised on a high-sucrose diet, Hipk overexpressing flies develop moderate neoplastic leg malformations at 25°C (Fig. 19A). When Siks are depleted in this background, there is a partial suppression of this Hipk-mediated overgrowth (Figs. 19B-D). This rescue is quantified in Figs. 19E-F. In E, adults of each genotype are scored for how many of their legs (0-6) are malformed, while in F, each of these legs are scored based on the severity of their malformations on a scale of absence of malformation to severely malformed legs. It is possible therefore, that Siks can regulate Hipk activity in response to dietary modulation in a novel mechanism. Hipk activity has also been previously shown to be augmented by the function of OGT (O-GlcNAc transferase) under high dietary sucrose conditions (Wong et al, 2020). OGT transfers the O-GlcNAc sugar residues to target Serines, Threonines or Tyrosine residues on target proteins. Since Siks function downstream of the insulin pathway (Haberland et al, 2009; Altarejos and Montminy, 2011; Screaton et al, 2004; Shaw et al, 2005), it is possible that Siks transmit the signal downstream to Hipk, thus explaining an increase in Hipk activity under high dietary sucrose conditions. Sik can therefore act as another upstream Hipk regulator like OGT, possibly explaining the effect of high dietary sugars on cancer progression, proving a link between metabolic regulation and cell proliferation.

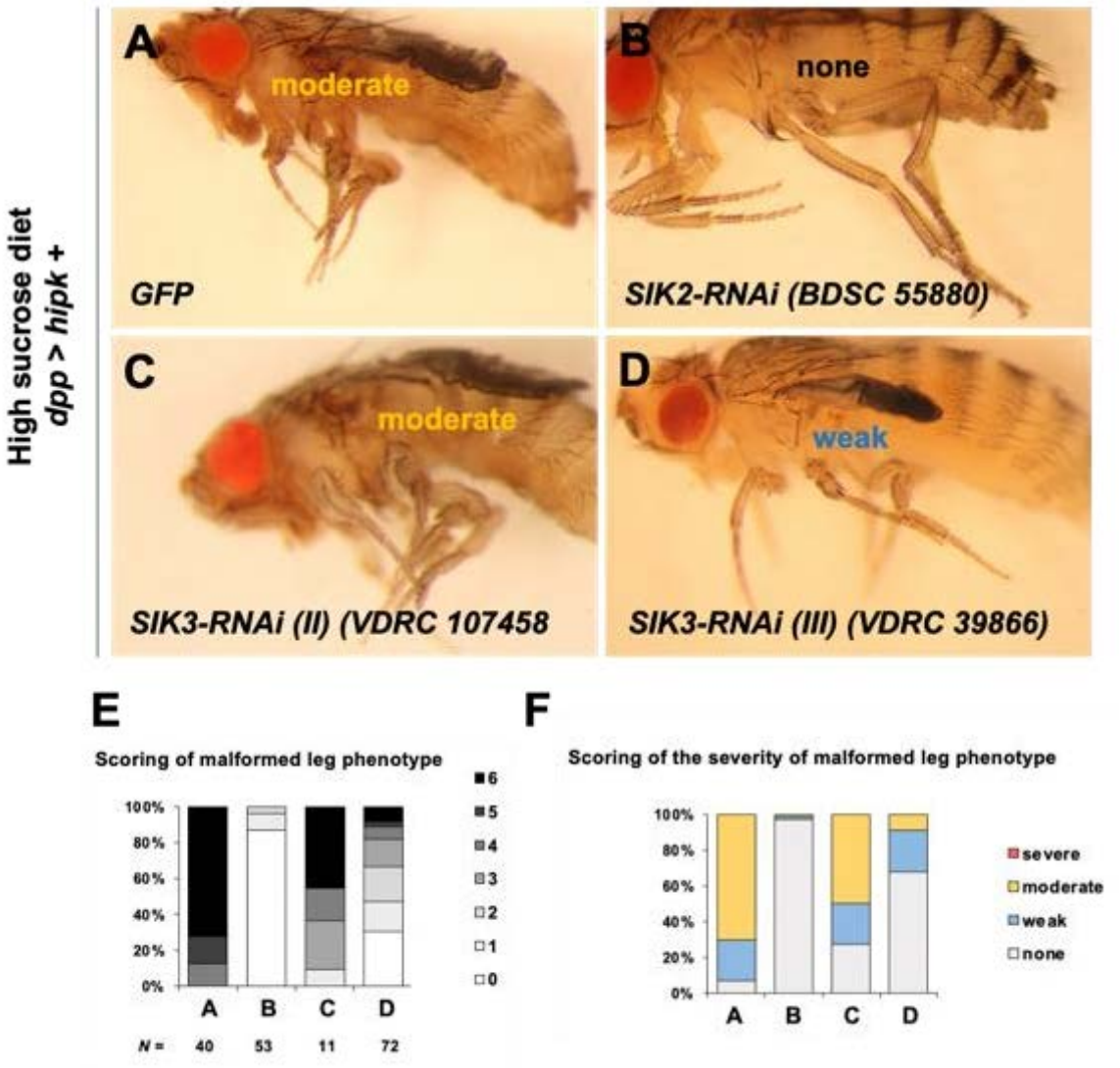


Figure 19 Depletion of Siks can partially rescue Hipk-driven leg malformation under HSD

At 25℃, flies overexpressing Hipk develop moderate neoplastic leg malformations when raised on a high-sucrose diet (A). When Siks are knocked down in this background (B-D) there is a partial suppression of Hipk-driven malformations, quantified in E and F. In E, each adult of a genotype is scored for how many legs are malformed, while in F, the severity of the malformed legs is scored from none (absence of malformation) to severely malformed legs. This experiment was performed by former graduate student in the lab, Dr. Kenneth Kin-Lam Wong (Wong, 2020)

Former graduate student in the lab, Dr. Kenneth Kin-Lam Wong had also demonstrated that Sik2 can physically interact with Hipk, or immunoprecipitate with Hipk

in a complex, using lysates collected from larvae overexpressing Sik and Hipk. It will be important to see whether Sik3 is able to similarly interact with Hipk as well, since Sik2 and Sik3 both seem to regulate Hipk in a similar fashion. This investigation will also lend useful information as to whether there are other intermediate components downstream of Siks that transmit signals and regulate Hipk, or whether Siks' regulation of Hipk is direct.

There are certain limitations to studying protein expression and localization in larval salivary glands. These tissues, in addition to being ideal for studying possible physical interaction / co-localization of two proteins, are also the largest secretory organs in Drosophila. Mature salivary glands secrete a copious amount of glue proteins that help the larvae adhere to in preparation for pupariation (Andrew, Henderson and Seshaiah, 2000). The secretory pathway is upregulated downstream of CrebA (cAMP-response element binding protein A), and in turn, *CrebA* expression is under the control of homeotic genes like *Scr* (sex combs reduced) (Andrew et al, 1994) as well as, CRTCs may be able to modulate CrebA target gene expression. Hipk regulates homeodomain gene expression while Siks regulate CRTC activity (Kim et al, 1998; Haberland et al, 2009; Altarejos and Montminy, 2011; Screaton et al, 2004; Shaw et al, 2005). Salivary glands continue to grow and secrete throughout the larval phase, and may be influenced by altered levels of expression of either Hipk or Siks by themselves. Taken together, these factors suggest that immunofluorescence experiment revealing Sik and Hipk localization, must be validated in another tissue system, for example, the larval fat body, or ovarian follicle cells.

It also remains to be clarified whether cytoplasmic enrichment of Hipk is due to prevention of Hipk nuclear import in the first place by Siks when they are overexpressed, or, due to Sik-mediated translocation of nuclear Hipk outside to the cytoplasm. This step in the mechanism can reveal the preferred target site for Sik on Hipk, as well as the effects of phosphorylation by Sik on this residue(s). Determining growth synergy when Siks are co-expressed with nuclear-targeted as well as cytoplasm-targeted Hipk can provide clues as to which mechanism is more likely – Siks preventing nuclear import of Hipk, or Siks recruiting nuclear Hipk to the cytoplasm.

Considering the results obtained in this study, as well as pertinent literature previously published, it is possible that Siks get enriched in the cytoplasm in the

presence of Hipk, and recruit Hipk from the nucleus, to regulate cellular pathways together in the cytoplasm, and drive tumor overgrowth. Siks can also regulate these pathways in parallel, or via their interaction with Hipk. It will therefore be promising to dissect the effect of cytoplasmic Hipk on various signaling pathways by overexpressing cytoplasmic restricted Hipk.

4.4. Conclusion

Hipk is a central regulator of gene expression governing growth and development. Its role in development is reinforced since erroneously expressed mammalian HIPKs are implicated in multiple human cancers. Identification of upstream regulators of Hipk such as Siks can therefore prove important in combatting cancers where such Hipk variants are upregulated. Incidence of cancers is multiplied in obese individuals, underscoring the metabolic implications in tumorigenesis. Siks are emerging multi-functional kinases that are able to modulate responses to nutrient conditions, as well as independently regulate cell proliferation and tissue growth. In tumours therefore, Siks may possibly act as bridges between nutrient regulation and tissue growth, to prove as an effective diagnostic and therapeutic target. Chemical inhibition of Siks with components like HG-9-91-01 (Clark et al, 2012) also thus appear to be potential therapeutic strategies in combating Sik-upregulated cancers.

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