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Characterization of the Sterile20 kinase Slik: A regulator of growth in *Drosophila*

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Résumé

La prolifération cellulaire et la croissance tissulaire sont étroitement contrôlées au cours du développement. Chez la *Drosophila melanogaster*, ces processus sont régulés en partie par la kinase stérile-20 Slik (SLK et LOK chez les mammifères) et le suppresseur de tumeur Hippo (Hpo, MST1/2 chez les mammifères) dans les cellules épithéliales. La surexpression de la kinase Slik augmente la taille des tissus chez les mouches adultes. Cependant, les mutants *slik*$^{-/-}$ meurent avant d'avoir terminé leur développement. Lorsqu'elle est surexprimée dans les cellules épithéliales des ailes en voie de développement, cette protéine favorise la prolifération cellulaire. En outre, l'expression de Slik dans une population de cellules conduit à une surprolifération des cellules voisines, même quand elles sont physiquement séparées. Ceci est probablement dû à la sécrétion de facteurs de croissance qui stimulent la prolifération de manière paracrine. En utilisant des méthodes génétiques et transcriptomiques, nous essayons de déterminer les molécules et les mécanismes impliqués. Contrairement à ce qui a été publié, nous avons constaté que Slik ne transmet pas de signal prolifératif en inhibant le suppresseur de tumeur Merlin (Mer, NF2 chez les mammifères), un composant en amont de la voie Hippo. Plutôt, elle favorise la prolifération non-autonome et la croissance des tissus en signalisation par la kinase dRaf (la seule kinase de la famille Raf chez la drosophile). Nous prouvons que dRaf est nécessaire chez les cellules voisines pour conduire la prolifération chez ces cellules. De plus, nous avons utilisé le séquençage du transcriptome pour identifier de nouveaux effecteurs en aval de Slik. Ce qui permettra de mieux comprendre les effets de SLK et LOK chez les humains.

Mots-clés: Slik, Hippo, Merlin, Raf, prolifération, croissance, cellules épithéliales

Abstract

Cell proliferation and tissue growth are tightly controlled during development. In epithelial tissues in *Drosophila melanogaster*, these processes are regulated in part by the Sterile-20 kinase Slik (SLK and LOK in mammals) and the tumor suppressor Hippo (Hpo, MST1/2 in mammals). Slik overexpression leads to an increase in tissue size in flies, whereas, *slik*$^{-/-}$ mutants die before completing development. Overexpressing this protein in the developing wing disc epithelium promotes cell proliferation, consistent with the overgrown wing phenotype in the adults. Moreover, expression of Slik in one population of cells leads to an overproliferation of neighboring cells, even when they are physically separated by a central lumen. This can be explained by secretion of paracrine growth factors, stimulating non-autonomous proliferation that is specific to Slik. We used genetic and transcriptomic assays to define the molecules and mechanism involved in Slik-mediated signaling. Contrary to what has been suggested, we found that Slik does not promote proliferation through the tumor suppressor Merlin (Mer, NF2 in mammals), an upstream component of the Hippo pathway, nor through other components of the Hippo pathway. Rather, Slik promotes non-autonomous proliferation and tissue growth signaling through dRaf (the single Raf family kinase orthologue in Drosophila). We found that dRaf is required in the signal receiving cells to stimulate proliferation. We performed RNA-seq to identify novel downstream effectors of Slik. Characterizing the signaling pathway downstream of Slik in *Drosophila* will shed light on how SLK and LOK function in mammals, and provide insights into their potential involvement during development and in cancer.

Keywords: Slik, Hippo, Merlin, Raf, proliferation, growth, epithelial cell

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Abbreviations

AEL: After egg laying

A/P: Anterior/posterior

Ap: Apterious

aPKC: atypical Protein kinase C

Crb: Crumbs

DAPI: 4',6-diamidino-2-phenylindole

DP: Disc proper

DPP: Decapentaplegic

EdU: 5-ethynyl-2'-deoxyuridine

EGF: Epidermal growth factor

En: Engrailed

ERK: Extracellular signal-regulated kinase

ERM: Ezrin/Radixin/Moesin

Ex: Expanded

FAK: Focal adhesion kinase

Ft: Fat

GCK: Germinal center kinase

GPCR: G-protein coupled receptor

GR: Gustatory receptor

Hpo: Hippo

Hth: Homothorax

JNK: c-Jun N-terminal kinase

KD: Kinase dead

Lgl: Lethal giant larvae

LOK: Lymphocyte oriented kinase

M: Minute

MAPK: Mitogen-activated kinase pathway

Mats: Mob as tumor suppressor

Mer: Merlin

Mthl: Methuselah

NKCC1: Na-K-2Cl

PAK: p21-activated kinase

PM: Peripodial membrane

PP2A: Phosphoprotein phosphatase 2A

RASSF: Ras association family

RNA-seq: RNA sequencing

RTK: Receptor tyrosine kinase

Sav: Salvador

Sd: Scalloped

Ser/Thr: Serine/Threonine

SLK: Ste20 like kinase

SPAK: Stress activated protein kinase

Ste20: Sterile20

Ste20p: Sterile20 protein

STRIPAK: Striatin-interacting phosphatase and kinase

TAO1: Thousand and one amino acid kinase

TNF: Tumor necrosis factor

Wts: Warts

Wg: Wingless

Yki: Yorkie

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1. Introduction

The rates of cell growth, cell division and cell survival during development determine the size of organs and appendages (Conlon and Raff 1999). Tissue growth is a highly dynamic process, where cells compete for growth and survival factors in order to proliferate. Therefore, a net tissue growth means less cell elimination and more proliferation. Though simple, the balance between apoptosis and proliferation needs to be tightly regulated to avoid uncontrolled growth. Moreover, once an organ reaches its "correct" size, mechanisms must be in place to ensure cells stop dividing. How cells know what the "right" size is still a mystery. An even more intriguing phenomenon is regulative development, where stress-induced cell ablation during development stimulates compensatory proliferation to ensure tissues reach their normal adult size (Halder and Johnson 2011). Recently, cell-cell signaling that regulates proliferation as a function of organ size has received much attention with the discovery of Sterile20 (Ste20) kinases and their role in tissue growth. In this thesis, I will highlight important discoveries regarding Ste20 kinases and growth.

1.1 Drosophila as model for studying epithelial tissue growth

Genetics is a powerful tool to study complex biological processes, such as developmental events and behavioural responses. The fruit fly *Drosophila melanogaster* is used as a model to discover the genetic components of development. The variety and sophistication of the genetic methods and tools developed for *Drosophila* are unmatched in any other multicellular organism. In addition, the rapid life cycle, low chromosome number, and small genome size make it an excellent model to study developmental processes. In the following sections, I will discuss the life cycle of *Drosophila* and its use as a model for studying growth.

1.1.1 Drosophila life cycle

Drosophila melanogaster development consists of three stages, embryonic, larval, and pupal, that last about 10 days from fertilization to hatching as an adult fly with a standard diet at 25°C (Figure 1.1) (Ashburner, Golic et al. 2005). After fertilization, eggs are laid and embryogenesis begins. Within 24 hours, a fully formed larva emerges from the egg. The

larvae are specialised for feeding, eating about three to five times their own weight over a period of four days during which they grow drastically (Ashburner, Golic et al. 2005). The larval period is divided into three stages, called instars, separated by molts. The first instar lasts for 24 hours, after which the larva molts twice to enter second instar, and again at 72 hours after egg laying to enter the third instar (Ashburner, Golic et al. 2005). The third instar period lasts two days, allowing the larva to complete growth. It then crawls out of the food and undergoes metamorphosis 6 days after egg laying to become a pupa (Ashburner, Golic et al. 2005). This stage lasts about 5 days, leading to the hatching as an adult fly.

1.1.2 Imaginal discs are model systems to study growth

Inside the growing larvae are a series of tissues, called the imaginal discs, that will form much of the adult body. During the pupal stage, when the majority of larval tissues are degraded, the imaginal discs are remodelled to form the adult body. In particular, imaginal discs are sacs of epithelial cells that give rise to the adult appendages, including eyes, legs and wings. The wing imaginal discs are used as model to study mechanisms controlling tissue growth (i.e. increases in size due to increase in number of cells or increased cell size) during development because of the simplicity of working with them. For example, the single-layered epithelium provides wing imaginal discs with structural simplicity, where expression of genes can be modulated and the consequences can be easily visualized by confocal microscopy. Imaginal discs massively increase in size during the larval stages, where they undergo rapid cell proliferation to increase in size up to 1000-fold, going from 50 cells to 50,000 cells (Johnston and Gallant 2002). This proliferation stage is temporally separated from differentiation, which is largely delayed until the pupal stage, thereby simplifying identification of regulators specifically controlling tissue growth (Neto-Silva, Wells et al. 2009). M

Many of the developmental processes are conserved from flies to humans; therefore discoveries made in *Drosophila* can be extrapolated to humans. For example, epithelial cells composing the imaginal discs are diploid and undergo mitosis like most mammalian cells (Neto-Silva, Wells et al. 2009). The external signals and intrinsic mechanisms regulating disc growth and size during development are conserved in mammals (Wu, Huang et al. 2003). Most cell-cycle and growth regulating genes in *Drosophila* have homologues in mammals. The simplicity of the system and the conservation of genes and processes regulating growth
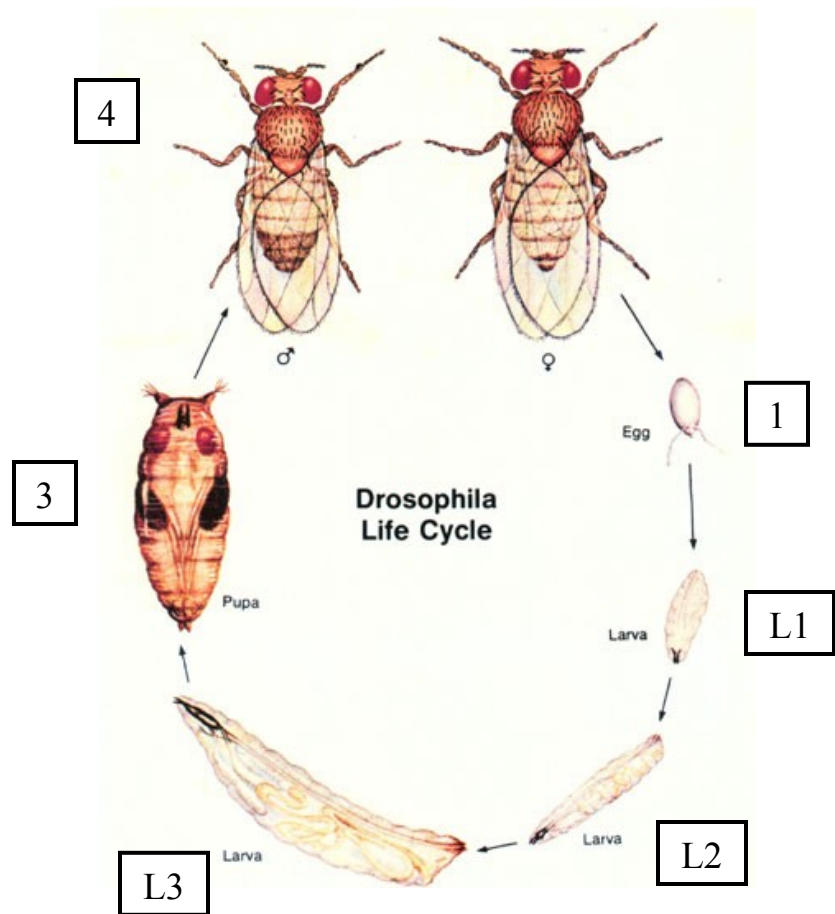
Figure 1.1: Drosophila melanogaster life cycle
The life cycle of *Drosophila melanogaster,* after fertilisation, starts with embryogenesis (1), followed by three larval stages (2): 1st instar (L1), 2nd instar (L2), and 3rd instar (L3). Then, the larva undergoes metamorphosis to form a pupa (3), followed five days later by eclosion of the adult fly (4). (Figure modified from Carolina Biological Supply Company)

during development, therefore make imaginal discs an ideal system to study growth regulators. In the following section, I will discuss the origin of wing imaginal discs and their structure because it is the model system used in this project.

1.1.2.1 Wing imaginal discs

The wing imaginal disc gives rise to the adult wing and thorax. This developing tissue is first formed in the embryo by invagination of the embryonic ectoderm (Cohen, Simcox et al. 1993). Initially, the wing and leg discs share a common primordium formed by a small cluster of cells. Later, through the combinatorial activity of several genes, including Hox genes, *Distal-less* and *vestigial*, these cells separate into distinct imaginal discs (Fuse, Hirose et al. 1996). The wing disc is composed of a cluster of about 50 cells at the end of embryogenesis. During the larval stages, these cells divide rapidly to form the mature wing disc containing about 50 000 cells, forming two discrete layers of epithelial cells called the disc proper (DP) and peripodial membrane (PM) (Figure 1.2). The two monolayers of cells are continuous with their apical membrane facing each other enclosing a lumen. The DP is a highly folded epithelium composed of densely-packed pseudostratified columnar epithelial cells, which will form the wing blade and thorax body wall of the adult fly. The PM is composed of large and flattened squamous epithelial cells.

The process of compartmentalization plays a critical role in proper wing development. A unique feature of the wing disc is the presence of two compartment boundaries: anterior/posterior and dorsal/ventral. Cells committed to either of the compartment do not mix together. The homeobox gene *engrailed (en)* is present only in the posterior compartment of wing discs, where it controls specific developmental programmes that promote posterior identity of these cells. Consequently, cells that do not express *en* are committed to the anterior fate. Another selector gene, gene that confers segment identity, is the LIM-homeobox gene *apterous* (*ap*), which specifies the identity of dorsal compartment cells. Both selector genes regulate expression of several morphogens and patterning factors, including *hedgehog*, *wingless* (*wg*), and *decapentaplegic* (*dpp*) to confer on cells their specific compartmental identities (Neto-Silva, Wells et al. 2009).
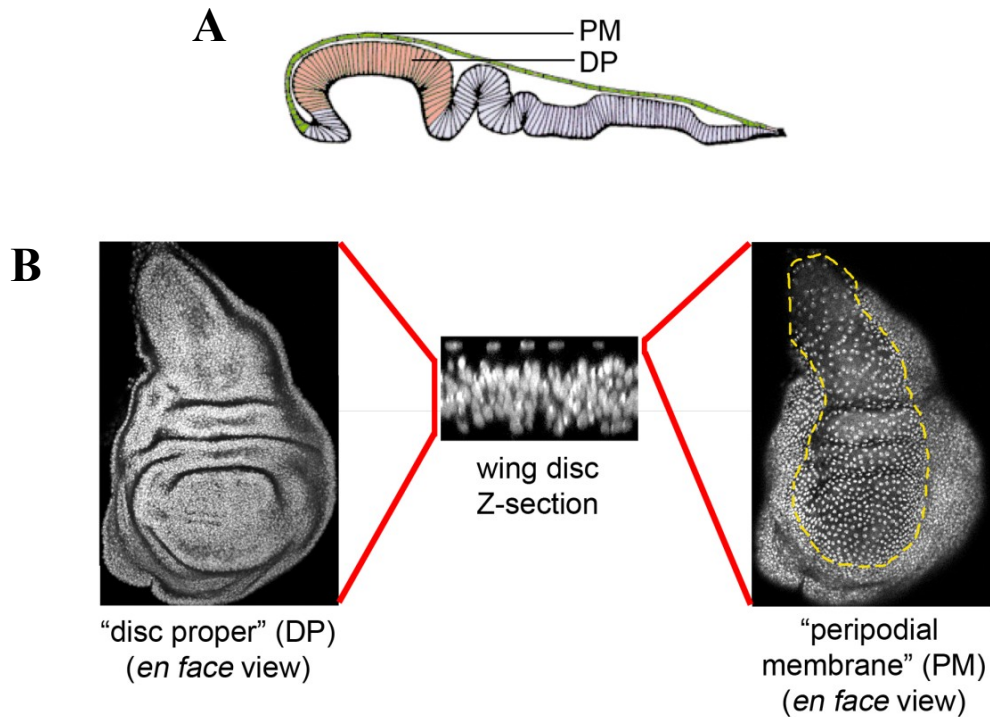
Figure 1.2: The Drosophila wing imaginal disc
(A) Cross-section view of the *Drosophila* wing imaginal disc. The peripodial membrane (PM) and disc proper (DP) form a continuous sac of single layered epithelial cells around a central lumen. DP cells are densely packed pseudostratified columnar cells forming a highly folded epithelium. The red section is called the wing pouch and forms the adult wing after completing development. PM cells (colored in green) are large flattened squamous cells overlying the DP cells. **(B)** DAPI staining of wing imaginal disc imaged by confocal microscopy. The z-section and the *en face* view show clear and distinct differences in the organization of the cells between PM and DP. The DP cell epithelial layer is folded with cells closely packed together. Conversely, PM cells are spaced out and flat.

1.1.3 Studies of imaginal disc growth regulation

Tissue and organ size and shape are tightly controlled during development by mechanisms regulating cell cycle, protein synthesis and apoptosis. These mechanisms coordinate inputs from both extrinsic and intrinsic cues to balance cell proliferation and cell death for a net tissue growth during development (Neto-Silva, Wells et al. 2009). Extrinsic cues are signals arising from the external environment, such as nutrient availability and temperature. Intrinsic cues are inputs from systemic factors, such as hormones and cell-cell interactions. Studies of *Drosophila* imaginal discs, where the high proliferation rate during the larval stage is fueled by nutrient intake, have shed light on the influence of diet on growth. The highly conserved protein kinase dTOR, the *Drosophila* targent of rapamicyn, was identified as the main sensor of nutritional status, which by signaling through the insulin-like signaling pathway, controls the rate of protein biosynthesis by directly regulating the translational machinery and ribosomal biosynthesis (Grewal 2009, Hietakangas and Cohen 2009). Another metabolic regulator is Myc, a member of the conserved family of basic-helix-loop-helix (bHLH) transcription factors. Mammals have three Myc proteins; cMyc, N-Myc and L-Myc. The single *Drosophila* Myc (dMyc) promotes growth by regulating the ribosome biosynthesis (de la Cova and Johnston 2006). Furthermore, dMyc also controls cell cycle progression by acting on Cyclin E, Cyclin D and the retinoblastoma family protein Rb, and apoptosis by inhibiting proapoptotic genes (de la Cova and Johnston 2006). These effects on growth by dMyc are both cell-autonomous and cell-nonautonomous, highlighting the importance of dMyc for growth in *Drosophila.* Patterning factors, such as Wg and Dpp also regulate growth by controlling cell cycle, proliferation and survival during development (Couso, Bate et al. 1993, Zecca, Basler et al. 1995, Johnston and Edgar 1998, Neto-Silva, Wells et al. 2009, Firth, Bhattacharya et al. 2010). For example, *wg* mutant wing discs undergo apoptosis by inducing activity of the pro-apoptotic gene *hid* (Smac/DIABLO and Omi/HtrA2 proteins in mammals)*,* whereas *dpp* mutant cells leave the epithelium and undergo apoptosis through a JNK-mediated pathway (Giraldez and Cohen 2003, Johnston and Sanders 2003, Gibson and Perrimon 2005, Shen and Dahmann 2005). Both Wg and Dpp are shown to regulate cell cycle. Wg induces cells cycle arrest at both G1 and G2 phase of cells at the dorsal/ventral boundary, giving rise to the cells that form the sensory bristles of the adult wing (Johnston and Edgar 1998). In the eye imaginal discs, Dpp induces cell cycle arrest ahead of

the morphogenetic furrow, required for proper cell differentiation (Firth, Bhattacharya et al. 2010).

Recent studies suggest that, though all of these factors modulate the rate of tissue growth, local cell interactions are essential for communicating this information. Cells integrate this information and evaluate their growth and survival status relative to their neighbor, and proliferate accordingly. Thus, cells have a greater control over their immediate environment, enhancing tissue plasticity in the context where cells can mount a rapid stress-induced response by changing their growth status by a mechanism called cell competition. This is a process regulating tissue growth and homeostasis during development, where neighboring cells sense metabolic and growth rate differences, and respond relative to their own fitness status (Bryant and Simpson 1984, Johnston and Gallant 2002, de la Cova, Abril et al. 2004). For example, "stronger" cells will proliferate and "weaker" cells will undergo apoptosis. This phenomenon was first discovered in *Drosophila* wing imaginal disc mosaics, where cells heterozygous for *Minute* (*M/+*) mutations in genes that encode important ribosomal proteins get out-competed and eliminated by wildtype cells (Marygold, Coelho et al. 2005). *M/+* cells are at a proliferative disadvantage compared to the other cells. Their elimination stimulates wild type cells to proliferate and fill the vacated space. Local differences in the expression of dMyc also induce cell competition. For example, in genetic mosaic discs, cells mutant for *dmyc* are eliminated by stimulation of the proapoptotic gene *hid* (de la Cova, Abril et al. 2004). The influence of cell-cell interaction induced cell competition on normal growth got more attention with the recent discovery of Ste20 kinases regulating tissue size. Below, I will discuss about Ste20 kinases in general, and more specifically about members of this family in *Drosophila* implicated in regulating tissue growth and organ size.

1.2 Sterile20 kinases

The family of Ste20 kinases share homology to the yeast *Saccharomyces cerevisiae* Ste20 protein (Ste20p). Ste20 kinases have a conserved Ser/Thr kinase domain and a variable non-catalytic domain, allowing interaction with diverse signaling molecules in different physiological environments (Delpire 2009). In mammals, there are 28 members of the Ste20 kinase family divided into two large groups; the germinal center kinase (GCK) and p21-

activates kinase (PAK) families (Figure 1.3) (Sells and Chernoff 1997, Bagrodia and Cerione 1999). This classification is based on the location of the conserved kinase domain – C-terminal for PAKs, and N-terminal for GCKs (Delpire 2009). Furthermore, PAKs contain an N-terminal p21-binding domain (or CRIB domain) and are regulated by the small GTPases Rac1 and Cdc42 (Delpire 2009). The GCK group lack the p21 binding domain. Both PAK and GCK families are further subdivided based on the conservation of the kinase domain into PAK-I, PAK-II, and GCK-I to VIII subfamilies (Delpire 2009).

Many Ste20 kinases are functionally regulated by an auto-inhibitory domain that blocks either their kinase domain or CRIB domain (Creasy, Ambrose et al. 1996, Zhao, Manser et al. 1998, Delpire 2009). Auto- or trans-phosphorylation, which stabilizes the kinase in a proper confirmation for substrate binding, is also essential for their activity (Pike, Rellos et al. 2008). The kinase domain has eleven subdivisions, with the subdomain VIII called the signature sequence serving as the substrate recognition site (Sells and Chernoff 1997). The consensus signature sequence of Ste20 kinases (GTPyWMAPEv) is highly conserved across species, suggesting substrate similarities between yeast and mammals. PAKs and GCKs regulate various intracellular events, including the activation of the mitogen activated protein kinases (MAPK) pathway, growth, cell survival, apoptosis and migration (Sells and Chernoff 1997, Bagrodia and Cerione 1999, Kyriakis 1999, Delpire 2009, Wagner and Sabourin 2009). In the following sections, I will discuss the MAPK pathway and the three *Drosophila* Ste20 kinases that are shown to regulate growth – Hippo, Tao1 (TAO in mammals), and Slik.

1.2.1 Ste20 kinases as activators of MAPK pathways

The yeast Ste20p is a putative mitogen-activated protein kinase kinase kinase kinase (MAP4K) discovered in yeast by using genetic approaches for identifying genes that are targets of the $G_{\beta\gamma}$-protein subunits (Wu, Whiteway et al. 1995). The PAK-like Ste20 kinase phosphorylates the yeast MAP3K Ste11, thereby transmitting the pheromone signal from the G-protein βγ subunits to downstream MAP kinases Fus3 and Kss1 (Wu, Whiteway et al. 1995, Drogen, O'Rourke et al. 2000). Because of the homology to Ste20p, mammalian Ste20 kinases were suggested to be potential MAP4Ks. Indeed, many have since been shown to activate
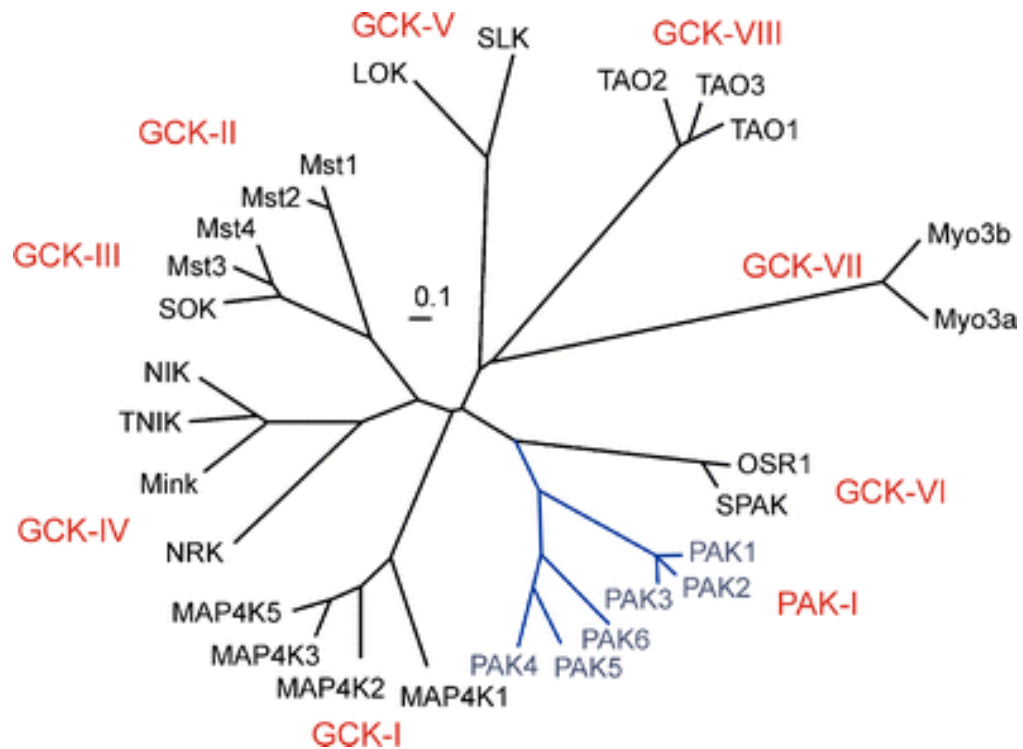
Figure 1.3: Ste20 kinases dendogram
Represented here are 27 mammalian Ste20 kinases. Ste20 kinases are divided into two families – germinal center kinase (GCK) and p21-activated kinase (PAK). These are further divided into subfamilies – PAK-I, PAK-II, and GCK-I-VIII. PAK 4-6 form the family of PAK-II. (Adapted from Delpire 2009)

MAPK pathways either by direct phosphorylation or protein-protein interactions. For example, MAPK1, which is principally expressed in hematopoietic organs, binds and phosphorylates MEKK1 directly to activate the JNK/SPAK signaling pathway (Hu, Qiu et al. 1996, Delpire 2009). MAPK2 is also shown to activate MEKK1, however this function is independent of the catalytic domain (Chadee, Yuasa et al. 2002).

MAPK pathways regulate cellular responses to various extracellular and environmental stimuli, including growth factors, cytokines, neurotransmitters, hormones, cellular stress, and cell adherence, by modulating gene expression through activation of transcription factors (Delpire 2009). MAPK/ERKs (extracellular signal-regulated kinase) are a large group of serine/threonine kinases subdivided into five families: $MAPK^{erk1/2}$, $MAPK^{p38}$, $MAPK^{jnk}$, $MAPK^{erk3/4}$, and $MAPK^{erk5}$. The kinase module of the pathway is composed of three kinases, including MAPK kinase kinase (MAP3K), MAPK kinase (MAP2K), and MAPK (Boulton, Yancopoulos et al. 1990, Boulton, Nye et al. 1991, Mendoza, Er et al. 2011). MAP3Ks are Ser/Thr kinases that are activated either by MAP4Ks phosphorylation, by interaction with small GTP-binding proteins of the Ras or Rho family, or by oligomerization and subcellular localization (Fanger, Gerwins et al. 1997, Mendoza, Er et al. 2011). Activation of MAP3Ks stimulates their Ser/Thr kinase activity leading to the phosphorylation and activation of MAP2K (Siow, Kalmar et al. 1997, Mendoza, Er et al. 2011). MAP2K are termed dual-specificity kinases because they phosphorylate MAPK at a Thr-X-Tyr motif in the activation loop (Gartner, Nasmyth et al. 1992). Once activated, MAPKs then phosphorylate numerous substrates, including transcription factors, other protein kinases, phospholipase, and cytoskeleton-associated proteins, leading to diverse cellular responses.

1.2.2 Ste20 kinases as regulators of growth
One of the conserved functions of the Ste20 family kinases is regulation of the tissue growth. Genetic screens in *Drosophila* led to the identification of three Ste20 kinases playing an important role in regulating growth. Although the mechanisms by which these kinases are regulated are diverse, the common involvement of cell polarity complexes and apical localization suggest an essential role for cell-cell interactions. Recent characterization of these kinases functions and roles will be discussed below.

1.2.2.1 Ste20 kinase Hippo, a regulator of growth in *Drosophila*

The Ste20 Mst1 and 2 kinases are members of the GCK-II family and are homologous to *Drosophila* Hpo. Mst1/2 are redundant in regulating growth via the Mst/Lats/YAP pathway, more commonly known as the Hippo pathway in *Drosophila* (Avruch, Zhou et al. 2011). The pathway is conserved between flies and mammals, and all the components of the pathway share high homology. This network integrates cell-cell contact and cell polarity cues to restrict growth (Reddy and Irvine 2008). Mst1/2 were shown to restrict organ size and suppress tumor/growth by inducing apoptosis both in transformed cells and mice livers (Zhou, Conrad et al. 2009, Lu, Li et al. 2010, Song, Mak et al. 2010).

The Ste20 kinase Hpo is a tumor suppressor regulating growth and tissue size during development (Badouel, Garg et al. 2009). The Hippo pathway is extensively studied in *Drosophila*. Along with Salvador (Sav; Sav1/WW45 in mammals), Warts (Wts; Lats1/2 in mammals), and Mob as tumor suppressor (Mats; Mob1A, Mob1B in mammals), Hpo forms the core of the evolutionarily conserved Hippo pathway (Figure 1.4). This pathway was discovered through genetic screens in *Drosophila,* where it was found to restrict organ growth and tissue size and to promote apoptosis during development by suppressing the activity of the transcription cofactor Yorkie (Yki, YAP/TAZ in mammals) (Kango-Singh, Nolo et al. 2002, Tapon, Harvey et al. 2002, Harvey, Pfleger et al. 2003, Jia, Zhang et al. 2003, Pantalacci, Tapon et al. 2003, Udan, Kango-Singh et al. 2003, Wu, Huang et al. 2003, Reddy and Irvine 2008). Mutations disrupting Hpo, Sav, Mats, or Wts function induce Yki transcriptional activity, leading to overgrowth of *Drosophila* imaginal discs. The mechanism regulating Yki activity is the phosphorylation cascade between the Ser/Thr kinases Hpo and Wts, where active Hpo phosphorylates the WW-domain adaptor protein Sav (Wu, Huang et al. 2003, Wei, Shimizu et al. 2007). Sav then acts as a scaffold protein bringing Wts near Hpo, facilitating Wts and Mats phosphorylation by Hpo (Wei, Shimizu et al. 2007). The Wts/Mats complex in turn phosphorylates Yki, creating a binding site for the 14-3-3 phosphopeptide binding protein, leading to retention of Yki in the cytoplasm and inhibition of its transcriptional activity, which are pro-proliferative and anti-apoptotic (Huang, Wu et al. 2005, Dong, Feldmann et al. 2007, Oh and Irvine 2008, Oh and Irvine 2009, Ren, Wang et al. 2010).

Yorkie forms complexes with various DNA binding transcription factors

Drosophila Yki lacks a DNA binding domain, similar to both mammalian homologues YAP and TAZ. Therefore, Yki forms a complex with other DNA-binding transcription factors, such as Scalloped (Sd), Mothers against DPP (Mad), and a Homothrax-Teashirt (Hth-Tea) complex, to promote tissue growth by stimulating transcription of the *Drosophila inhibitor of apoptosis 1* (*diap1)*, the growth promoter *Myc,* and cell-survival promoter miRNA *bantam (ban)* (Kango-Singh, Nolo et al. 2002, Harvey, Pfleger et al. 2003, Jia, Zhang et al. 2003, Pantalacci, Tapon et al. 2003, Huang, Wu et al. 2005, Nolo, Morrison et al. 2006, Thompson and Cohen 2006, Goulev, Fauny et al. 2008, Lei, Zhang et al. 2008, Peng, Slattery et al. 2009, Neto-Silva, de Beco et al. 2010, Ziosi, Baena-Lopez et al. 2010). Interestingly, Yki association with these DNA-binding transcription factors is tissue specific. For example, though *yki* is essential for growth of all the imaginal discs, *sd* is only required in the wing and *hth* for the eye discs (Campbell, Inamdar et al. 1992, Liu, Grammont et al. 2000, Peng, Zeng et al. 2009). Interestingly, multiple Yki binding partners serve to increase the range of its target genes. For instance, Yki-Sd mediates transcription of *diap1,* whereas *ban* is a target of Yki-Mad and Yki-Hth transcription factors (Peng, Zeng et al. 2009, Oh and Irvine 2011).

Negative regulation of the Ste20 kinase Hpo

Exactly how Hpo is regulated during development is still unclear. Hpo homodimerization and apical membrane localization appear to be important. Homodimerization is thought to be essential to induce transphosphorylation and localization to the membrane, thereby increasing Hpo activity (Glantschnig, Rodan et al. 2002, Lee and Yonehara 2002, Deng, Matsui et al. 2013). Recently, two mechanisms have been identified that negatively impact Hpo activity – dephosphorylation by the phosphoprotein phosphatase 2A (PP2A) and competition for the scaffold protein Sav by Ras association family member (dRASSF; RASSF in mammals) (Polesello, Huelsmann et al. 2006, Ribeiro, Josue et al. 2010). Using genomics and proteomics, the *Drosophila* PP2A portion of the dSTRIPAK (*Drosophila* Striatin-interacting phosphatase and kinase) complex was found to associate with and dephosphorylate Hpo (Ribeiro et al., 2010).
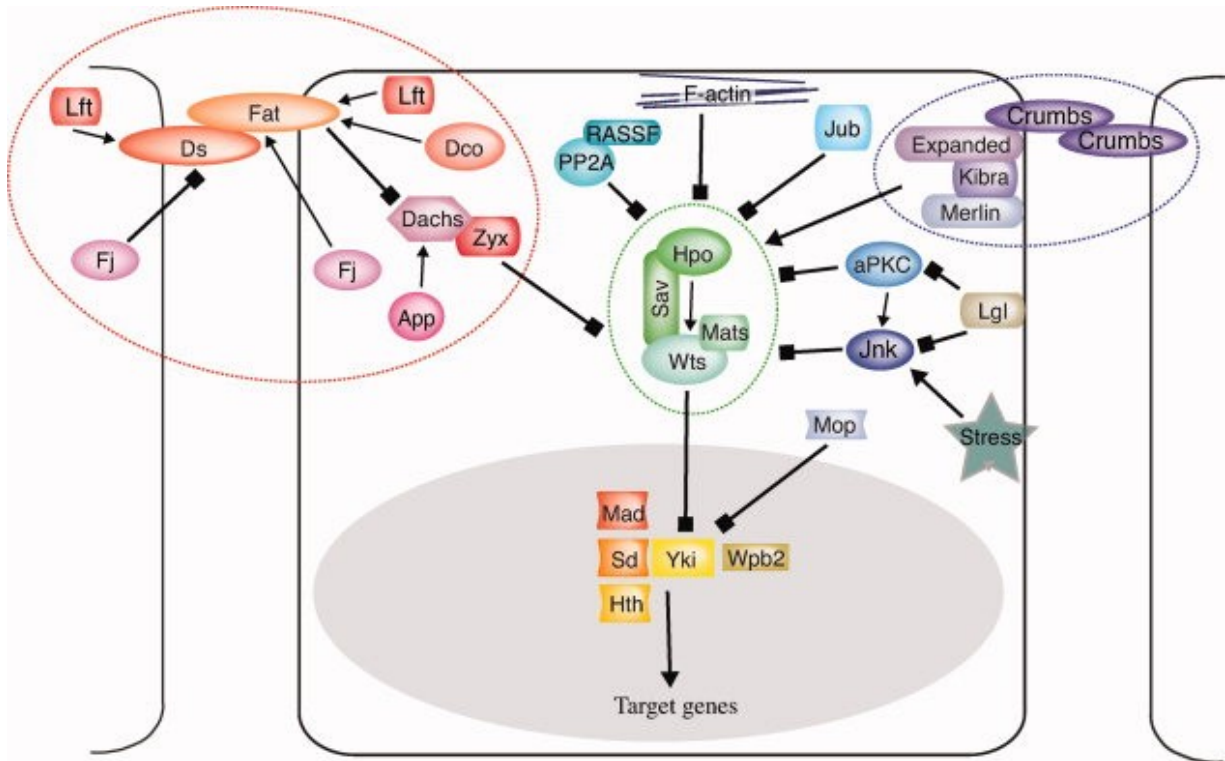
Figure 1.4: Hippo pathway in *Drosophila*
The Hippo tumor suppressor pathway restricts growth and induces apoptosis by negatively regulating the transcription co-activator Yorkie (Yki). The pathway is essentially a phosphorylation cascade mediated by the two Ser/Thr kinases, Hpo and Wts. Active Hpo phosphorylates Sav, which acts as scaffold protein to bring Wts closer to Hpo. Hpo is able then to phosphorylate Wts and Mats, thereby activating Wts kinase activity. Wts in turn phosphorylates Yki, leading to its cytoplasmic retention and inhibition of its transcriptional activity. (Adapted from Staley and Irvine 2012)

The dSTRIPAK complex also forms a complex with dRASSF, facilitating the recruitment of dSTRIPAK to Hpo (Ribeiro, Josue et al. 2010). Both Sav and Hpo have SARAH domains that are required for binding to each others. This interaction is essential for activation of Hpo. On the other hand, the SARAH domain of the *Drosophila* dRASSF binds to Hpo and forms a complex, thereby competing for binding with Sav and restricting Hpo activity (Polesello, Huelsmann et al. 2006).

Modulation of the Hippo pathway is mainly achieved by a number of upstream regulators, including the Mer-Expanded complex, the transmembrane Cadherin Fat and the actin cytoskeleton. These upstream regulators control the apical membrane localization of the components of the pathway, as discussed below.

Expanded, Merlin, and Kibra complex regulate localization of Hpo
The two cytoplasmic FERM domain proteins Expanded (Ex) and Merlin (Mer), and the WW domain protein Kibra (Kbr) form a complex at the apical membrane of cells and positively regulate Hpo homodimerization (Hamaratoglu, Willecke et al. 2006, Baumgartner, Poernbacher et al. 2010, Genevet, Wehr et al. 2010, Deng, Matsui et al. 2013). A number of transmembrane or membrane-associated proteins act on these proteins to control Hpo pathway activity. For example, Crumbs (Crb), a transmembrane protein involved in epithelial cell polarity containing an intracellular FERM domain, binds to Ex, promoting its localization to the apical membrane. Mis-regulation of Crb leads to its mis-localization, and in turn to the mis-localization of Ex, inducing Yki activity (Chen, Gajewski et al. 2010, Grzeschik, Parsons et al. 2010, Robinson, Huang et al. 2010). In addition to Crb, Lethal giant larvae (Lgl), another cell polarity protein, has tumor suppressor properties, the mechanism of which was unknown until it was linked to the Hippo pathway. The neoplastic tumor suppressor Lgl regulates localization of Hpo and dRASSF, to the apical membrane by antagonistically acting on another polarity complex, the atypical Protein kinase C (aPKC) complex (Grzeschik, Parsons et al. 2010, Menendez, Perez-Garijo et al. 2010).

Regulation of the Hpo pathway by actin cytoskeleton
Cytoskeleton dynamics can also regulate the Hpo pathway, suggesting a tight interplay between cell shape and normal tissue size. The actin capping proteins, Cpα and Cpβ, which

function to regulate actin polymerization, suppress growth by the activity of Yki in flies (Fernandez, Gaspar et al. 2011, Sansores-Garcia, Bossuyt et al. 2011). Similarly, accumulation of F-actin by mutation/downregulation of these actin capping proteins or by decreasing the actin regulatory protein Capulet at the apical membrane stimulates Yki activity in Jnk-dependent and -independent manners (Fernandez et al., 2011; Sansores-Garcia et al., 2011). The *Drosophila* FERM protein Ex also inhibits actin polymerization, thereby reducing F-actin accumulation (McClatchey and Giovannini 2005, Fernandez, Gaspar et al. 2011). Hpo pathway activation in *ex* mutants may thus be related to an increase in F-actin accumulation.

The transmembrane protein Fat regulates localization of Warts, Expanded, and Yorkie

The large transmembrane Cadherin-related protein Fat (Ft), involved in the planar cell polarity pathway, promotes the abundance and apical localization of Wts, Ex, and Yki, thereby positively regulating the Hpo pathway (Bennett and Harvey 2006, Cho, Feng et al. 2006). Dachs, an unconventional myosin acting downstream of Ft, shows differential localization influenced by Ft, where it is apical in Ft mutant cells, and cytoplasmic in Ft overexpressing cells (Mao, Rauskolb et al. 2006). Dachs associates with the *Drosophila* LIM domain protein Zyx, one of several LIM domain proteins implicated in Hpo signaling. Dachs binding stimulates the conformational change of Zyx (Oh and Irvine 2011, Rauskolb, Pan et al. 2011). The LIM domain of Zyx is then available to bind to Wts, leading to the degradation of Wts, thereby providing another means of linking the Ft pathway to the Hpo pathway (Rauskolb, Pan et al. 2011).

Another LIM domain protein that associates with Wts in *Drosophila* is the single Ajuba family protein Jub. It regulates tissue growth and organ size by directly associating with both Wts and Sav, thereby inhibiting the kinase activity of Wts and increasing Yki transcriptional activity (Das Thakur, Feng et al. 2010). Moreover, in *Drosophila,* Jub was shown to link the epidermal growth factor receptor (EGFR) and the Ras-Raf-MAPK pathway to increased Yki activity (Reddy and Irvine 2013). Signaling through EGFR increases the phosphorylation of Jub, and as mentioned above Jub then directly interacts with Wts and Sav and suppresses Wts kinase activity (Reddy & Irvine, 2013). Consequently, active unphosphorylated Yki translocates into the nucleus and transcribes growth promoters and anti-apoptotic genes in EGF-responding cells.

1.2.2.2 Ste20 kinase Tao1, restricts growth via the Hippo pathway

Thousand and one amino acid kinase 1 (TAO1) was first discovered in mammals through amplification by degenerate primers of sequences from rat, that were related to the yeast Ste20p (Hutchison, Berman et al. 1998). Subsequently, two more member of the GCK-VIII subfamily have been cloned – TAO2 and TAO3 (Chen, Hutchison et al. 1999, Tassi, Biesova et al. 1999). These Ser/Thr kinases regulate cell cycle progression, apoptosis, and microtubule dynamics (Hutchison, Berman et al. 1998, Chen, Raman et al. 2003, Mitsopoulos, Zihni et al. 2003, Raman, Earnest et al. 2007, Wu and Wang 2008). Interestingly, both TAO1 and TAO2 have catalytic and non-catalytic activities. For example, cell cycle arrest upon DNA damage mediated by TAO1 and TAO2 requires their kinase activity to induce the p38 MAPK response (Raman, Earnest et al. 2007). However, overexpression of TAO1 has been shown to induce apoptosis in human neuroblastoma cells in a catalytic activity-independent manner (Wu and Wang 2008). The regulatory domain of TAO2 stabilizes microtubules through direct binding, another catalytic-independent function (Mitsopoulos, Zihni et al. 2003, Delpire 2009). The upstream signals regulating TAOs are largely unknown. TAO1 is shown to be phosphorylated *in vitro* by ATM (ataxia telangiectasia mutated), leading to p38 MAPK activation by phosphorylation of MEK3 and MEK6 (Raman, Earnest et al. 2007). TAO3, which in response to stress inhibits the JNK/SAPK (c-jun N-terminal kinase/stress activated protein kinase) MAPK pathway, is negatively modulated by EGF, potentially linking this kinase to growth/survival signaling (Tassi, Biesova et al. 1999, Delpire 2009).

The single *Drosophila* Tao1 was identified by RNAi screening in cultured *Drosophila* cells as a microtubule dynamics regulator (Liu, Rohn et al. 2010). Thereafter, it has been implicated in regulating cell shape, apoptosis and tissue growth (Sato, Hayashi et al. 2007, Liu, Rohn et al. 2010, Boggiano, Vanderzalm et al. 2011).

Depletion of *tao-1* in cultured cells increases microtubule protrusions, suggesting that Tao-1 regulates microtubule dynamic (Sato, Hayashi et al. 2007). Later, Tao-1 was shown to bind microtubules and destabilize plus-end growth, thereby regulating cell form (Liu, Rohn et al. 2010). Tao-1 also regulates apoptosis in germ line cells by inducing the expression of the gene *sickle* (*skl*), thereby potentiating the activity of the protein Hid leading to increase in apoptosis (Sato, Hayashi et al. 2007). Because of its implication in apoptosis and microtubule

stability, Tao-1 became a potential candidate for regulating growth in flies. Tao-1 was later identified as a growth regulator by *in vivo* RNAi screening in *Drosophila,* where its depletion induced overgrowth of the eye (Boggiano, Vanderzalm et al. 2011, Poon, Lin et al. 2011). This led to discovery of Tao-1 as Hpo kinase, where it phosphorylates Hpo at threonine-195 in the activation loop, leading to an increase activity of the tumor suppressor Hippo pathway, thereby restricting tissue growth (Boggiano, Vanderzalm et al. 2011, Poon, Lin et al. 2011).

1.2.2.3 Sterile20 kinase Slik, promote growth during development in *Drosophila*

The Ste20 kinase SLK was discovered in a two hybrid screen for myogenic regulators in mice (Sabourin and Rudnicki 1999). It is a member of the GCK-V subfamily. The other member of that family is the lymphocyte oriented kinase (LOK). SLK and LOK are homologous to the *Drosophila* Ste20 kinase Slik (Hipfner and Cohen 2003). SLK is ubiquitously expressed. During development, it is predominantly enriched in neuronal and muscle tissue (Zhang, Hume et al. 2002, Wagner and Sabourin 2009). On the other hand, LOK is enriched in the lymphoid organs (Kuramochi, Moriguchi et al. 1997). In cultured cells, SLK and LOK have been linked to regulation of various cellular functions. For example, SLK was shown to mediate apoptosis through diverse pathways, including phosphorylating and activating p53, JNK1, and signal-regulating kinase-1 (ASK1) (Sabourin and Rudnicki 1999, Sabourin, Tamai et al. 2000, Hao, Takano et al. 2006, Cybulsky, Takano et al. 2009, Cybulsky, Takano et al. 2010). SLK has also been shown to bind to microtubules and co-localize with cell adhesion signaling molecule, including paxillin and Rac1 at the leading edge of migrating cells, regulating cell adhesion and spreading (Wagner, Storbeck et al. 2008, Quizi, Baron et al. 2013). This is achieved through the phosphorylation of paxillin, the focal adhesion adapter protein, stimulating cell migration through focal adhesion kinase (FAK)/MAPK signaling pathway (Quizi, Baron et al. 2013). Similarly, another study showed that SLK signaling through FAK and Src is necessary for cell migration upon stimulation of the tyrosine kinase receptor HER2/ErbB2/Neu, which is highly upregulated in breast cancer, suggesting a link between cancer cell migration and SLK (Roovers, Wagner et al. 2009). LOK was shown to have an analogous role in lymphocyte, where it regulates lymphocyte adhesion by controlling the distribution of active leukocyte-function-associated antigen 1 (LFA1) by an unknown mechanism (Endo, Toyama-Sorimachi et al. 2000). SLK has been linked to

preserving radial microtubule array through its phosphorylation activity on the p150(Glued) subunit of dynactin, consistent with the fact that SLK associates to microtubules (Zhapparova, Fokin et al. 2013). Considering that SLK is located at the apical compartment of the epithelial cells and that the kinase is implicated in microtubule stabilization, it is logical that SLK would restrict microvilli to the apical side. This is done by regulating the apical localization of the ERM protein Ezrin through phosphorylation (Viswanatha, Ohouo et al. 2012). LOK has also been identified in lymphocytes as an ERM kinase and similarly regulating cytoskeleton rearrangement (Belkina, Liu et al. 2009). SLK cytoskeletal modeling also controls vasodilatation by phosphorylation of RhoA (Guilluy, Rolli-Derkinderen et al. 2008). Another function of SLK that has been identified is its requirement for cycle progression through G_2 (O'Reilly, Wagner et al. 2005). Moreover, SLK also regulates normal muscle and kidney development in mice (Cybulsky, Takano et al. 2004, Storbeck, Daniel et al. 2004, Luhovy, Jaberi et al. 2012, Storbeck, Al-Zahrani et al. 2013).

SLK regulation is achieved mainly through phosphorylation and homodimerization. SLK is phosphorylated at T183 and S189 in the activation segment of its catalytic domain, leading to the kinase domain homodimerization (Delarosa, Guillemette et al. 2011, Luhovy, Jaberi et al. 2012). SLK localization to the microtubule at the leading edge of migrating cells is mediated by c-Src (Wagner and Sabourin 2009) Moreover, LIM domain-binding transcriptional cofactor proteins Ldb1 and Ldb2 directly interact with SLK at the C-terminal AT1-46 homology domain to maintain SLK in an inactive state (Storbeck, Wagner et al. 2009).

Overall, the diverse functions of SLK and LOK that have been reported were mainly identified in cultured cells. The involvement of these kinases in regulation of apoptosis, cell cycle progression and actin cytoskeleton dynamics suggests they may play a greater role in normal and/or pathological tissue growth. Although no clear picture of the physiological functions of SLK and LOK has emerged, recent studies of Drosophila Slik have clearly implicated this kinase in regulating tissue growth and morphogenesis during development.

Slik was discovered in *Drosophila* in a systemic genetic overexpression screen. Overexpression of Slik in posterior cells in the wing discs led to the overgrowth of the

posterior compartment of the wings (Hipfner and Cohen 2003). Two separate functions of Slik have been characterized in flies – it regulates growth and epithelial integrity during development. Interestingly, the effect of Slik on tissue growth is catalytic-activity independent, whereas its function on actin-cytoskeleton dynamics requires its kinase activity. During development, it was found that Slik regulates the rate of cell proliferation and affects cell survival by an unknown mechanism (Hipfner and Cohen 2003). It has been shown that cells mutant for *slik*, are eliminated through apoptosis by activation of the JNK pathway (Hipfner and Cohen 2003). The overgrowth of the adult wings by overexpressing Slik is due to an increase in the rate of cell proliferation in wing imaginal discs (Hipfner and Cohen 2003). Interestingly, this over-proliferation is also detected in cells in the overlying peripodial membrane of the wing discs, where the transgene is not expressed, suggesting a non-autonomous role of Slik (Figure 1.5) (Hipfner and Cohen 2003). This proliferation was suppressed in a *draf* heterozygous background, suggesting that dRaf acts downstream of Slik in the non-autonomous signaling pathway (Hipfner and Cohen 2003). Another potential link between Slik and growth is through Mer (Hughes and Fehon 2006). Slik was shown to regulate Mer phosphorylation, leading to its mis-localisation away from the plasma membrane, thereby presumably inhibiting growth suppression by the tumor suppressor Hippo pathway (Hughes and Fehon 2006).

Genetic analysis indicated that Slik has a separate catalytic-activity dependent function. *Slik* mutant cells are extruded out of the epithelium sheet and most undergo apoptosis, suggesting that Slik's requirement for maintenance of epithelial integrity may explain the increased apoptosis observed in *slik* mutant cells (Hipfner, Keller et al. 2004). During development, Slik regulates cellular architecture by phosphorylating and activating the single ERM family protein Moesin in *Drosophila* (Hipfner, Keller et al. 2004, Carreno, Kouranti et al. 2008). Moesin activation and cortical localization stabilizes F-actin and reduces Rho1 activity, thereby maintaining epithelial integrity (Speck, Hughes et al. 2003). Slik-mediated phosphorylation of Moesin also serves an important function during meiosis, where cells undergo changes in their form. For example, the spatiotemporal regulation of Moesin by Slik during cell division is required for cortical rigidity and cell rounding, important for

spindle orientation and chromosome alignment. (Carreno, Kouranti et al. 2008, Kunda, Pelling et al. 2008).

Little is known about how Slik is regulated. Nonetheless, it was shown that its localization and abundance at the apical region of cells are important for Moesin activation. Regulating these is Sip1, the *Drosophila* orthologue of the ERM binding protein 50 and the Na^+/H^+ exchange regulator NHERF1 (EBP50/NHERF1), which binds both Slik and Moesin, thereby facilitating Moesin phosphorylation (Hughes, Formstecher et al. 2010). Another protein that regulates the function of Slik in the maintenance of the epithelium is Flapwing, the *Drosophila* phosphatase type 1β. It forms a complex with both Mer and Moesin, and co-regulates their dephosphorylation, consequently affecting their localisation and plasma membrane trafficking, and ultimately negatively impacts epithelial integrity (Yang, Primrose et al. 2012).
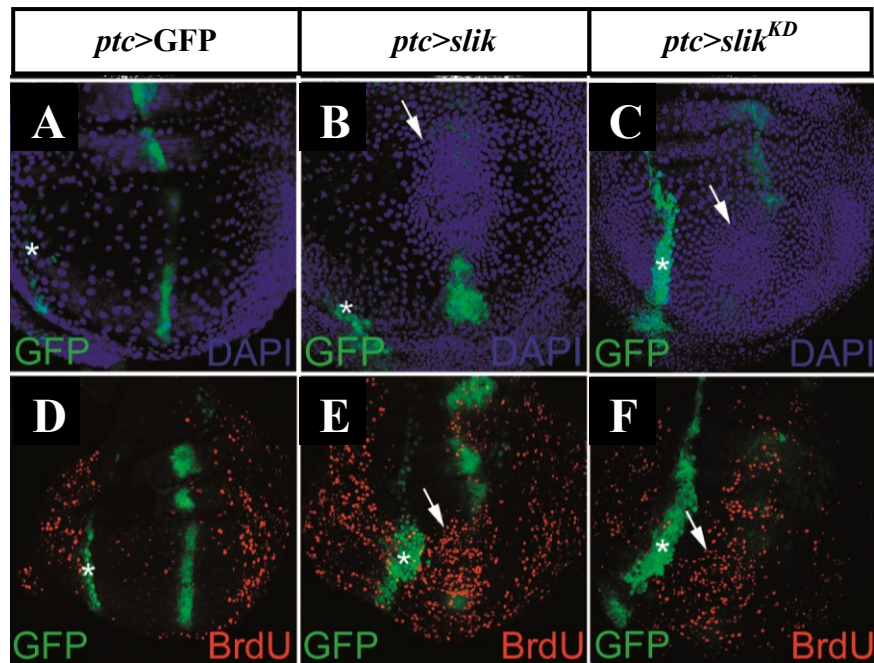
Figure 1.5: Slik nonautonomously stimulates proliferation of PM cells
Confocal images of peripodial membrane (PM) layer of wing imaginal disc stained with DAPI (in blue), labeling nuclei, and BrdU (in red), labeling cells undergoing proliferation. **(A,D)** Wing disc expressing GFP alone in the underlying DP cells, showing flattened, widely spread out DAPI staining and little BrdU labeling. **(B,C,E,F)** Expression of both Slik **(B,E)** and kinase dead Slik (Slik[KD]) **(C,F)** in DP cells drives proliferation of the PM cells, as indicated by the increase in DAPI and BrdU labeling when compared to the wing disc expressing GFP alone. The arrows indicate the high nuclear density **(B,C)** and high BrdU uptake **(E,F)**. (Modified from Hipfner and Cohen 2003)

1.3 Genetic tools

Drosophila offers numerous advantages for genetic studies. For example, it is a genetically simple organism with only four pairs of chromosomes. Fruit flies have numerous external appendages that can be subjected to non-lethal mutation that can serve as markers to follow mutants through generations (Beckingham, Armstrong et al. 2005). In addition to these natural advantages, the creation of balancer chromosomes made *Drosophila* the standout model for genetic studies. Balancer chromosomes have multiple re-arrangements inhibiting homologous recombination. Therefore, they allow mutation of interest to be stably maintained and tracked through many generations (Beckingham, Armstrong et al. 2005). With the discovery of transposable P-elements, transgenes can easily be inserted into fly genome. This led to creation of the GAL4-UAS system, described below, which I used to characterize Slik signaling pathway in the *Drosophila* wing imaginal disc.

1.3.1 GAL4-UAS system

The GAL4-UAS system makes it possible to express genes in *Drosophila* in a targeted manner, and has made flies the most tractable metazoan system to study gene functionality implicated in a wide range of processes. GAL4 is a yeast *Saccharomyces cerevisiae* transcriptional activator required for stimulating transcription of genes essential for galactose metabolism. GAL4 can be expressed in *Drosophila* without obvious phenotypic damage (Brand & Perrimon, 1993). This led to the creation of the GAL4-UAS system in *Drosophila,* based on a collection of GAL4-carrying P-elements randomly inserted throughout the genome (Brand and Perrimon 1993, Duffy 2002). Nearby genomic enhancers drive expression of the GAL4 gene in a specific pattern (Figure 1.6A). The GAL4 binds to a cis-acting regulatory sequence called Upstream Activating Sequences (UAS). By placing the UAS upstream of a gene-of-interest and transforming this into flies, it becomes possible to drive GAL4-dependent expression of the gene. When GAL4-expressing flies are mated to flies bearing a UAS-transgene, progeny inheriting both GAL4 and UAS transgenes will express the target gene in a spatial and temporal pattern defined by the enhancer regulating GAL4 expression. For example, when crossed to UAS-GFP transgenic flies, *ap*GAL4 drives GFP expression in the dorsal half of the DP layer of the disc, but not in ventral cells or in the PM, both of which do

not express *ap* (Figure 1.6B). Another driver, *ptc*GAL4 drives expression of GFP specifically in a central stripe of DP cells (Figure 1.6C). A nice feature of these systems is that the part of the discs not expressing the transgenes can serve as an internal control. This facilitates comparisons, for example by immunofluorescence.
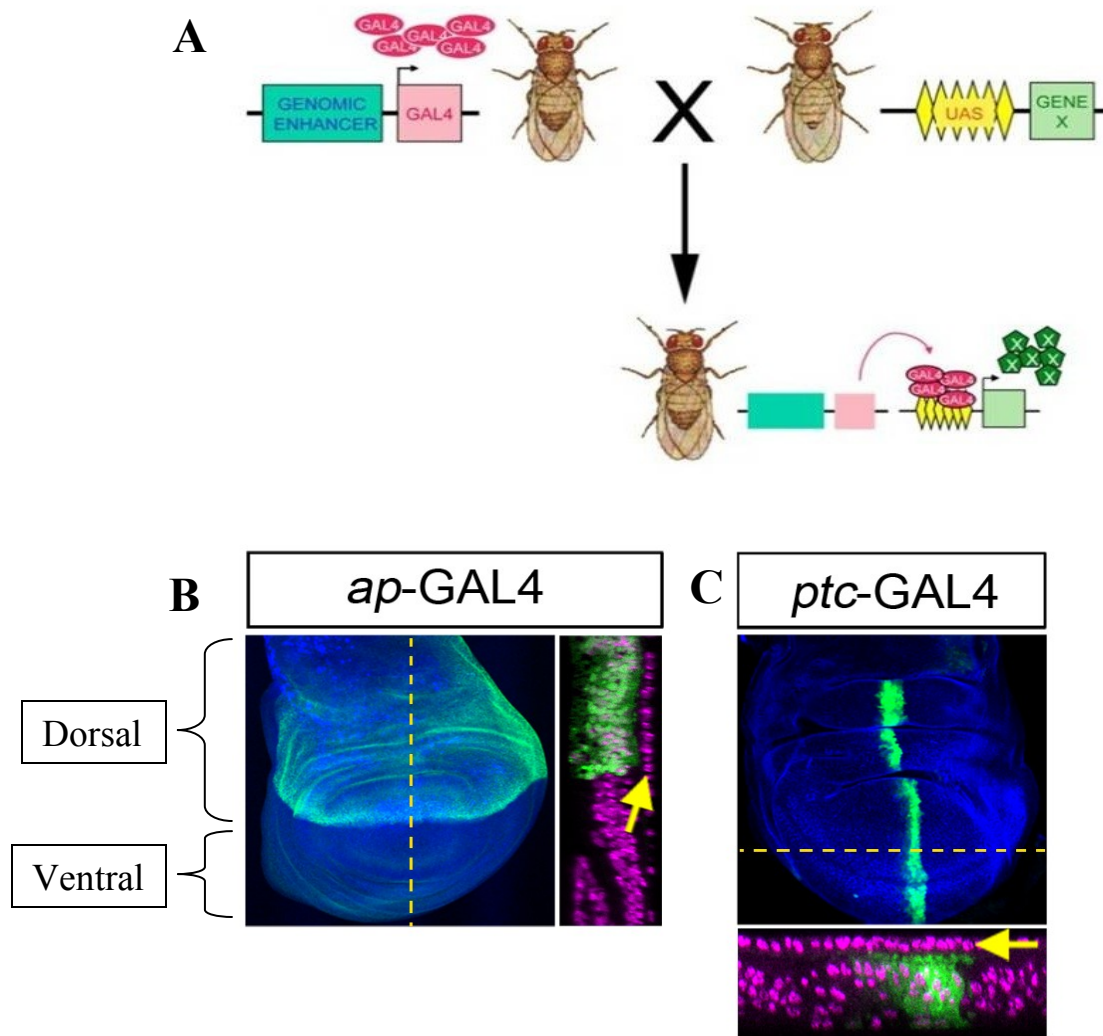
Figure 1.6: The GAL4-UAS system expressing transgenes specifically in DP cells.
(A) Schematic representation of the GAL4-UAS system. GAL4 is expressed under the control of an enhancer. The gene of interest is coupled with UAS sequence and a minimal promoter, which is silent in the absence of GAL4. Once mated, the enhancer will drive expression of GAL4, which then binds to its binding site, UAS, and drives transcription of the target gene in a tissue-specific manner determined by the enhancer. **(B)** The enhancer *ap* is only active in the dorsal compartment of the wing discs. Thus expression of GFP driven by *ap*GAL4 is only detected in the dorsal half of the DP layer of the disc. No expression is detected in PM cells (arrow). **(C)** The enhancer *ptc* drives is active in a central stripe of DP cells. Therefore, *ptc*GAL4 drives expression of GFP only in this central area, and not in PM cells (arrow).

2. Objectives

Understanding mechanisms controlling growth during development is important as it will provide insights into how coordination of both extrinsic and intrinsic cues regulates tissue homeostasis. The discovery of Slik as a regulator of tissue growth rate during development in *Drosophila*, suggests that its mammalian homologue may play a similar role. Therefore, it is of great interest to characterize the Slik signaling pathway, which will allow a better understanding of the role of these kinases in human physiology and disease. Though several components required for Slik to promote epithelial integrity have been identified, the mechanism through which this kinase drives non-autonomous cell proliferation and ultimately tissue growth remains elusive. The main objective of my thesis work was to identify the mechanism of Slik growth regulation using genetic and transcriptomic assays. Specifically, I set out to address three questions:

- **Does Slik signal through the Hippo pathway?** It has been shown that Slik directly phosphorylates Mer, thereby inhibiting Mer activity and driving cell proliferation and tissue growth (Hughes and Fehon 2006). I investigated if there was any genetic interaction between Slik and Mer.

- **What is the relationship between Slik and dRaf?** dRaf has been identified previously as one possible interacting partner of Slik (Hipfner and Cohen 2003). I investigated in which tissue dRaf is required in Slik signal transmission.

- **What are the other effectors of Slik?** Using transcriptomics, I set out to identify potential downstream targets of Slik.

3. Results

3.1 Does Slik signal through the Hippo pathway?

It was shown that Slik phosphorylates Mer, leading to its inactivation and mis-localization (Hughes and Fehon 2006). Loss of Mer membrane polarization inhibits Hpo activity, therefore leading to increased Yki transcriptional activity. Hence, although not tested, it has been suggested that Slik-driven cell proliferation leading to tissue growth acts through the Hippo pathway. Intriguingly, the kinase dead form of Slik, Slik[KD], also drives non-autonomous cell proliferation, suggesting that kinase activity of Slik is dispensable (Hipfner and Cohen 2003). To resolve this apparent contradiction, I set out to test whether Slik acts through the Hippo pathway, via Mer and/or Yki.

3.1.1 Inhibition of Merlin does not drive non-autonomous proliferation of PM cells

Previously, to characterize the role of Slik in *Drosophila* epithelial cells during development, we expressed *slik* using *ptc*GAL4, which is expressed in a stripe of DP cells in the middle of the wing imaginal discs (Figure 1.6). These discs were dissected, labelled with EdU, stained with DAPI, and visualized by confocal microscopy. DAPI stains nuclear DNA and EdU labels cells undergoing DNA synthesis (Figure 3.1). The peripodial layer of control discs expressing GFP using *ptc*GAL4 showed scattered nuclei indicative of broadly spread squamous cells. These PM cells were generally quiescent, reflected by low EdU labeling. In contrast, the PM of discs expressing *slik* showed densely packed nuclei and overproliferation evidenced by an increased number of DAPI positive nuclei and EdU labeling of cells overlying the *ptc*GAL4 stripe in the DP. This suggests that Slik drives proliferation of PM cells in a cell non-autonomous manner. To demonstrate that this effect was due to leaky expression of GAL4 in PM cells, we used another DP cell-specific driver, *ap*GAL4 (Figure 1.6), to express *slik* in developing wings. Again, we saw an increase in DAPI-positive cells and EdU incorporation in the PM in *slik*-expressing discs compared to the control. Interestingly, the observed phenotype is more robust as evidenced by more DAPI-positive nuclei and EdU labeling compared to discs expressing *slik* under the control of *ptc*GAL. *ap*GAL4 appears to be a stronger driver of transcription, perhaps because it drives gene expression in the whole dorsal compartment of the discs. Therefore, the number of DP cells

overexpressing Slik is larger with apGAL4 compared to *ptc*GAL4. It is important to note that the overproliferation of the PM cells is observed mainly in cells overlying the DP cells expressing the Slik transgene. We conclude that *slik* expression in DP cells does indeed drive non-autonomous proliferation of the PM cells.

As mentioned above, it has been suggested that Slik phosphorylates Merlin, thereby inhibiting it, leading to an increase in cell proliferation and tissue growth (Hughes & Fehon, 2006). If so, we reasoned that suppressing Merlin directly, either by expressing a dominant negative form of *mer* (*mer$^{\Delta BB}$*) or depleting it by expressing a long double-stranded RNA (dsRNA) targeting *mer* (referred to as *merRNAi*) should replicate the non-autonomous effects observed when *slik* is overexpressed. To test this hypothesis, I dissected wing imaginal discs from third instar larvae bearing the GAL4 driver alone or expressing *slik*, *mer$^{\Delta BB}$*, or *merRNAi*. As expected, we observed elevated DAPI staining and EdU labelling in the overlaying peripodial cells in discs overexpressing *slik* compared to control (Figure 3.2A). In contrast, the PM of discs expressing the dominant negative form of *mer* looked like the PM of discs expressing *ap*GAL4 alone, where DAPI and EdU labelling showed large quiescent cells (Figure 3.2B). The few cells undergoing proliferation are DP cells located at the edges of the disc. Similarly, when, I depleted *mer* in the wing imaginal discs by expressing *merRNAi* with *ap*GAL4, DAPI staining and EdU labeling of PM cells were normal (Figure 3.2B). We note that both *mer* transgenes drive robust tissue overgrowth in the wing (see below and data not shown). However, depletion of *mer* does not induce non-autonomous proliferation of PM cells. The inability of Mer suppression to phenocopy Slik overexpression suggests that Slik driven non-autonomous proliferation does not result from inhibiting Mer activity.

DP Cells	PM cells	
DAPI/GFP	DAPI	EdU
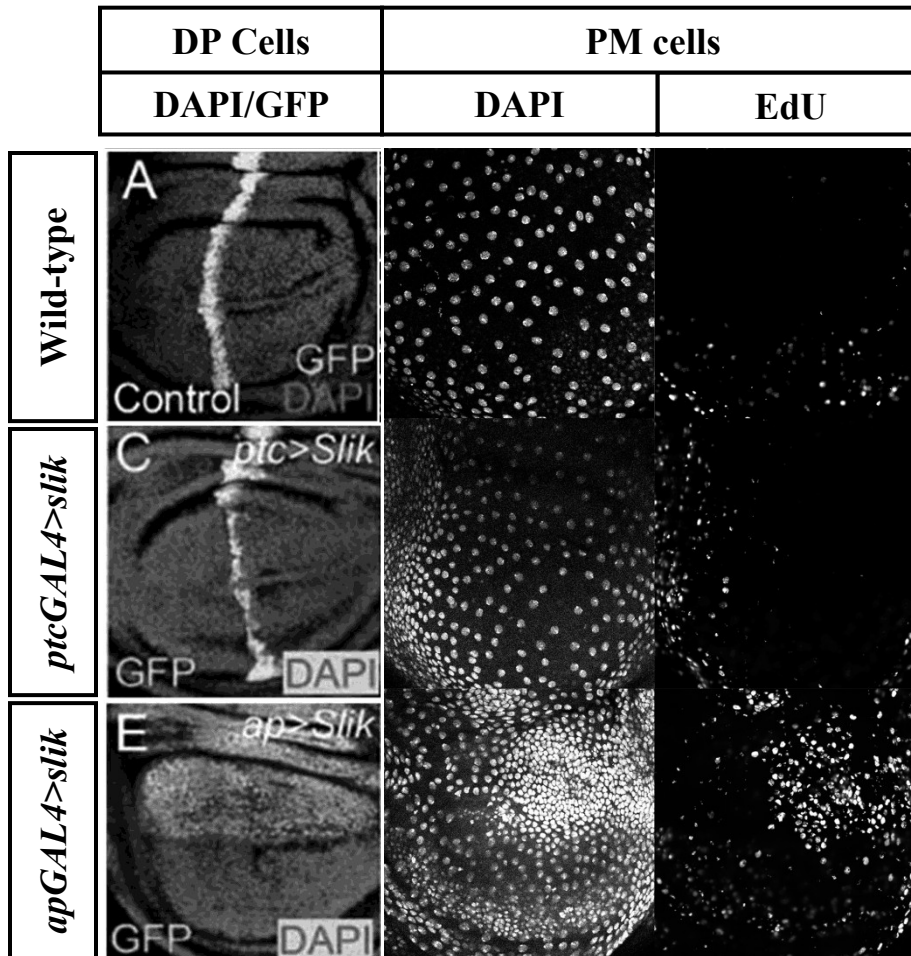
Figure 3.1: Slik drives non-autonomous proliferation of PM cells
Confocal images of wing imaginal discs expressing GFP alone (wild-type) or together with
Slik, using *ptc*GAL4 and *ap*GAL4. Discs were stained with DAPI and GFP, and labeled with
EdU.GFP is expressed in the DP cells. The peripodial layer of wild-type discs show large cells
undergoing little proliferation suggested by low EdU incorporation. Overexpression of Slik
drives proliferation of the PM cells as evidenced by the abnormal cluster of DAPI-positive
cells and the increase in EdU incorporation overlying the Slik-expressing DP cells. Note DP
and PM images are not from the same discs. N= more than 5 discs.
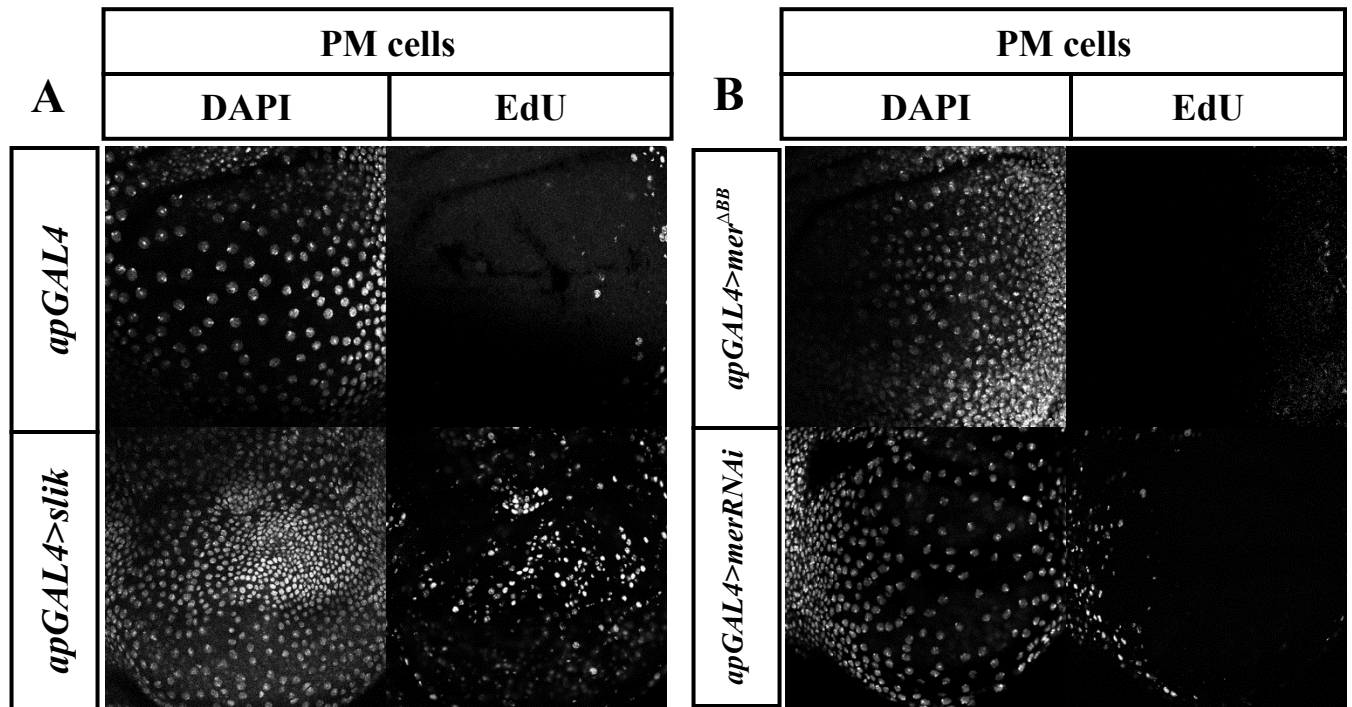
Figure 3.2: Inhibition of Merlin does not stimulate non-autonomous proliferation of PM cells

(A-B) Confocal images of the peripodial membrane of wing imaginal disc expressing *slik*, *mer^{ABB}*, or *merRNAi* (dsRNA targeting *meri*) with *ap*GAL. Discs were stained with DAPI and labeled with EdU. **(A)** PM cells show normal physiology in discs expressing *ap*GAL4 alone. Slik overexpression drives non-autonomous proliferation of PM cells. **(B)** In discs expressing *mer^{ABB}* or *merRNAi*, the peripodial layer is similar to the wild-type disc, where DAPI-positive cells are scattered and the little EdU uptake (upper panel and lower panel respectively).

3.1.2 Inhibiting Merlin does not enhance Slik overexpression effects

Another method to test whether Slik signals through Mer is to investigate for genetic interactions. Genetic interaction is observed when alterations in two genes produce a phenotype that is different than alteration of either gene individually (St Johnston 2002). Therefore, we scrutinized whether inhibiting Mer would affect the Slik-driven non-autonomous proliferation of PM cells. In wing imaginal discs co-expressing *slik* and *merRNAi* with *ap*GAL4, we observed an increase in DAPI-positive nuclei and EdU incorporation compared to peripodial layer of *ap*GAL4 alone (Figure 3.3, upper panels). However, the extent of non-autonomous proliferation was not noticeably enhanced compared to expressing *slik* alone. Similarly, we investigated if reducing *mer* gene dosage would affect Slik driven cell proliferation. In *mer*$^{+/-}$, we observed a similar increase in nuclei and proliferation in the PM upon *slik* overexpression as in a wild-type background (Figure 3.3, lower panels). We conclude that inhibition of Mer fails to enhance the Slik-driven non-autonomous proliferation of the PM cells, consistent with Slik acting through a different effector.

3.1.3 Slik does not regulate transcription of the Yorkie target gene expanded

Although Mer not involved, we tested whether Hpo pathway could still be implicated. To achieve this, I looked for genetic interaction between Slik and Yki. The Hippo pathway restricts growth by negatively regulating Yki. Thus, if Slik inhibits the Hippo pathway, we expect it to promote Yki activity to drive cell proliferation and tissue growth. As readout of tissue growth, we measured the size of the *Drosophila* adult wings, which are sensitive to growth effects. *ptc*GAL4 drives genes expression between the third (L3) and fourth (L4) wing veins. The area enclosed between veins L3 and L4 as a ratio of the whole wing area gives a quantifiable value that is highly reproducible (Hipfner and Cohen 2003). The dominant negative forms of *mer*, *hpo* and *ft* drive tissue growth when expressed under a GAL4 driver and are used as control in this assay (LaJeunesse, McCartney et al. 1998, Wu, Huang et al. 2003, Matakatsu and Blair 2012). Expression of the dominant negative forms of each with *ptc*GAL4 increased the area bounded by veins L3 and L4 by about 20% (Figure 3.4A). This overgrowth was reduced by a small but highly significant amount when the transgenes were expressed in a *yki* heterozygous background (p<0.001), consistent with the function of Yki
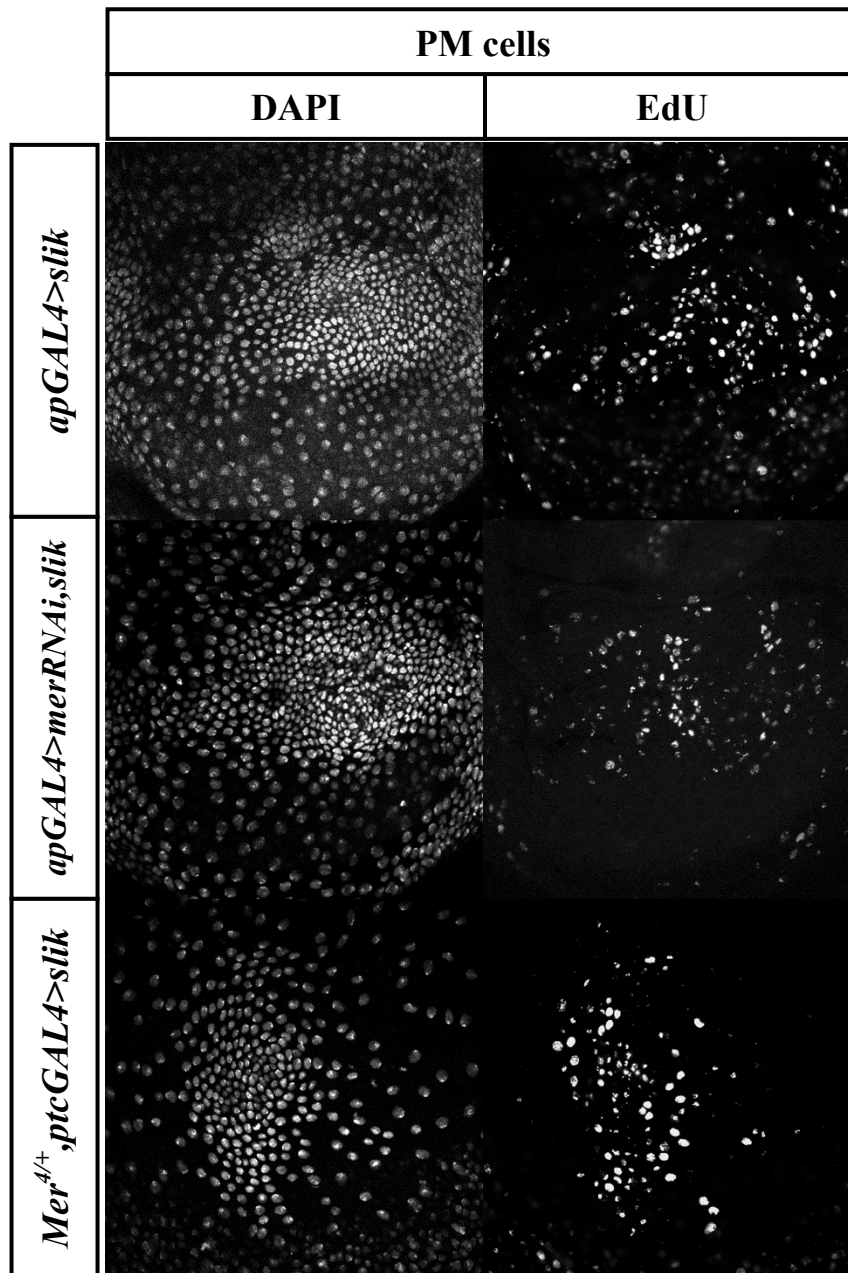
Figure 3.3: Slik driven non-autonomous proliferation of PM cells is Merlin independent
Confocal images of the PM of wing imaginal discs expressing *slik* alone, coexpressing *slik* and *merRNAi* with *ap*GAL4, or expressing *slik* with *ptc*GAL4 in a *mer* heterozygous background. Discs were stained with DAPI and labeled with EdU. Depletion of *mer* in *slik* overexpressing cells or overexpression of s*lik* in a mer$^{+/-}$ background did not enhance the amount of the non-autonomous proliferation of PM cells. N= at least 4 discs for each genotype.

downstream of Mer, Hpo and Ft. Overexpression of *slik* with *ptc*GAL does not lead to a strong enhancement of growth in the adult wing because of a compensating increase in apoptosis (Hipfner & Cohen, 2003). However, co-expression of *slik* and *p35,* the viral caspase inhibitor that blocks apoptosis, led to an increase in the area bounded by veins L3 and L4 of about 5% (Figure 3.4B). Interestingly, co-expressing *slik* and *p35* in a *yki* heterozygous background produced the same extent of overgrowth, suggesting that Slik-driven tissue growth is unaffected by *yki* gene dosage. As with *mer*, the lack of genetic interaction between *slik* and *yki* is consistent with Slik not acting through the Hippo pathway.

The lack of involvement of Yki was further substantiated when I examined Yki target gene expression, an alternative approach to investigate the relationship between Slik and the Hippo pathway I investigated if Slik regulated the expression of *ex*, which is the best characterized transcriptional target of Yki. Though *ex* is a target of Yki, it is also a tumor suppressor in *Drosophila*. This FERM domain protein acts through a negative feedback loop, activating the Hippo pathway and thus inhibiting Yki activity, providing another layer of regulation (Hamaratoglu, Willecke et al. 2006). The expression of *ex* increases when the activity of the Hippo pathway is reduced (Hamaratoglu, Willecke et al. 2006). The ex^{LacZ} enhancer trap, a P-element transposon inserted upstream of the *ex* gene and downstream of its enhancer, provides a convenient readout for Hippo pathway activity (Genevet, Wehr et al. 2010). Discs expressing GFP alone, *slik* or dsRNA transgenes targeting three components of the Hippo pathway (referred to as *merRNAi*, *hpoRNAi*, and *ftRNAi*) under the control of *ap*GAL4 were dissected, and stained with antibodies labeling GFP, Slik, Mer, Hpo, or Ft, respectively (Figure 3.4A-E, first row). We observed decrease in Mer, Hpo and Ft staining in the dorsal half of discs expressing the respective dsRNA transgenes, suggesting that the transgenes were functional (Figure 3.4A-E, first row). Further, we also stained the discs with anti beta-galactosidase antibody to visualize the *ex* enhancer activity (Figure 3.4A-E, second row). In control GFP-expressing discs, ex^{LacZ} was broadly expressed, with two characteristic stripes of upregulation spanning the middle of the discs. As expected, when depleting components of the Hippo pathway, we observed an increase in beta-galactosidase staining in the dorsal, *ap*-expressing half of the disc. On the other hand, discs overexpressing *slik* had a similar level of LacZ staining in dorsal and ventral cells, suggesting that Slik does not regulate
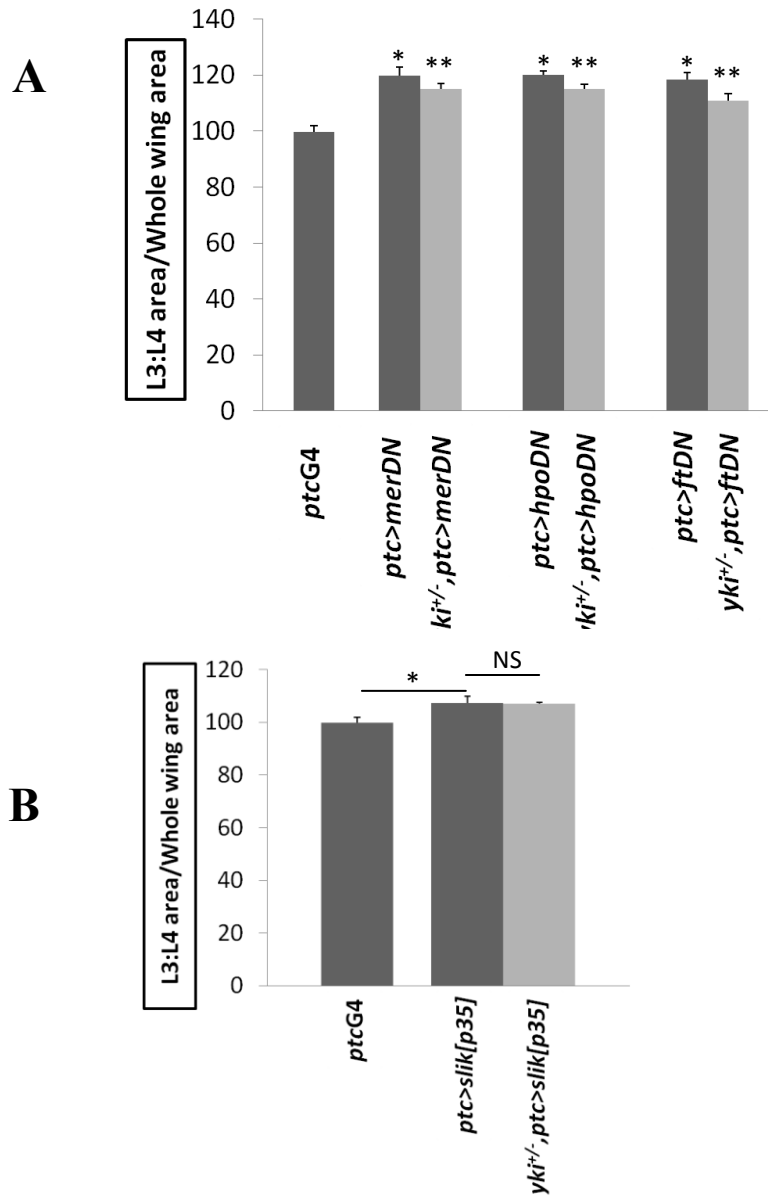
Figure 3.4: Slik driven tissue growth is Yki independent
(A-B) Quantification of growth by measuring the ratio of the area between veins L3 and L4 as a function of total wing area. **(A)** Inhibition of the Hippo pathway by expressing the dominant negative form of *mer, hpo,* and, *ft* with *ptc*GAL4 causes overgrowth of the adult wing, which is sensitive to *yki* dosage. **(B)** Expression of *slik +p35* using *ptc*GAL4 also leads to overgrowth, but is independent of y*ki* gene dosage. Data represents the average from 20 wings. (Two-tailed *T*-test, *: *T*-test versus GFP-expressing control, *p<0.001*. **: *T*-test versus control (y*ki*$^{+/+}$) background, *p<0.001*)

expression of *ex*. To confirm this, we performed quantitative real-time PCR (QPCR) on cDNA synthesised from total RNA extracted from discs expressing *slik,* and *hpo^{K71R}* (a dominant negative form of *hpo*) with *ap*GAL4, and from *ap*GAL4 alone discs as control. Expression of *slik* was upregulated by around 5-fold in *slik* overexpressing discs when compared to the control (Figure 3.4F). In discs expressing the dominant negative form of *hpo,* expression of *slik* was similar to wild-type. As expected, expression of *ex* was statistically significantly increased by around 1.8-fold in discs in which Hippo pathway was inhibited (p<0.001). On the other hand, *slik* overexpressing discs did not show differential expression of *ex*, consistent with the previous results. We conclude that Slik does not regulate expression of the Yki target gene *ex*. Taken together, our analysis of the connections between Slik, Mer, and Yki suggest that it is unlikely that Slik drives growth and cell proliferation by inhibiting the Hippo pathway.
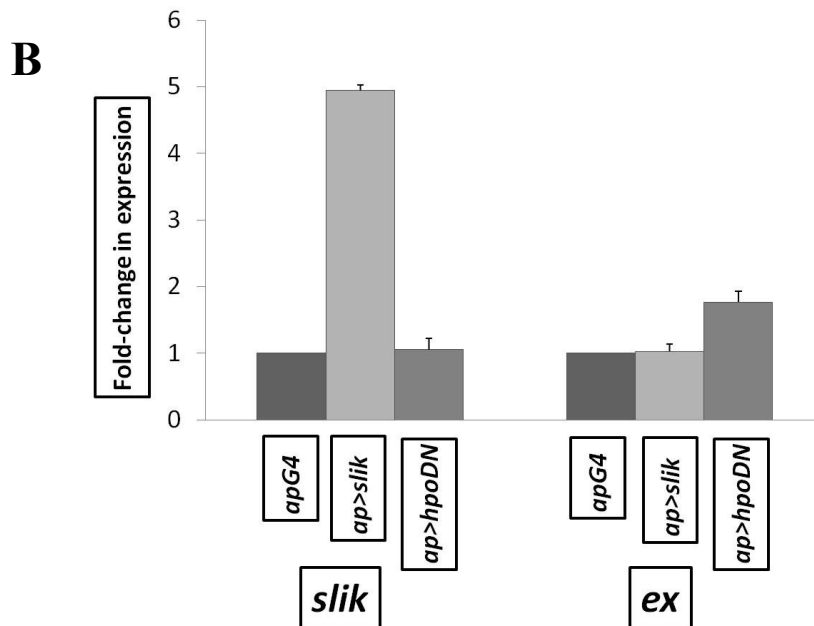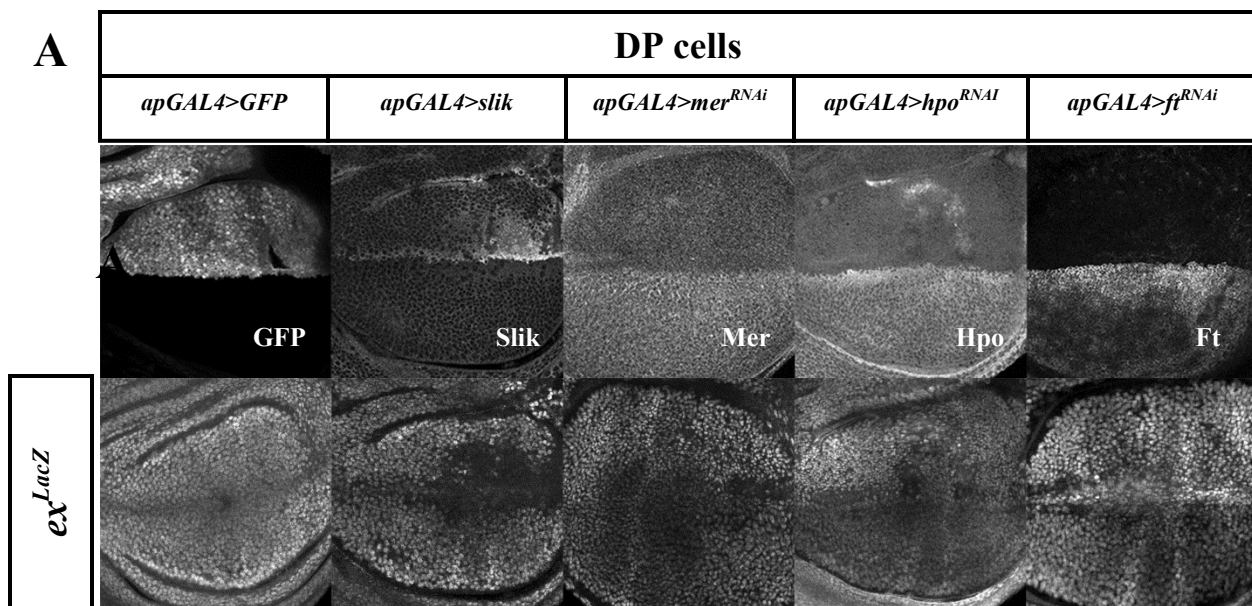
Figure 3.5: Slik does not regulate Yorkie target genes
(A) Confocal images of the disc proper of the wing imaginal discs expressing GFP, *slik, merRNAi, hpoRNAi,* or *ftRNAi* under the control of *ap*GAL4. The upper panels show staining with antibodies against GFP, Slik, Mer, Hpo, and Ft respectively. Expression of dsRNA targeting *mer, hpo,* and *ft* eliminates Mer, Hpo and Ft staining suggest that the RNAi transgenes are functional. The lower panels are the same discs labeled with anti-beta-galactosidase antibody. Inhibiting the Hippo pathway increases β-gal staining in the ventral compartment. Slik overexpressing discs have an uniform β-gal staining. **(B)** Quantitative-PCR analysis of expression of *slik* and *ex* in discs expressing *ap*GAL4 alone or driving expression of *slik* and *hpo*^{K71R}. However, Slik overexpressing cells does not transcriptionally change *ex* expression.

3.2 What is the relationship between Slik and dRaf?

Many Ste20 kinases activate the MAPK pathway by acting on the upstream regulator MAP3K. Previous analyses identified genetic interactions between Slik and two MAPK pathways in *Drosophila*, those mediated by JNK and ERK. Most importantly, Slik showed strong genetic interaction with the MAP3K dRaf, an upstream component of the ERK pathway (Hipfner & Cohen, 2003). Removing a single copy of *draf* was sufficient to inhibit Slik driven non-autonomous proliferation of the PM cells and overgrowth of adult wings (Hipfner & Cohen, 2003). No genetic interaction was detected between Rolled (*Drosophila* ERK) and Slik, raising the possibility that dRaf does not signal through the canonical ERK pathway to transduce the Slik growth signal (Hipfner & Cohen, 2003). As a first step toward further characterizing the signaling pathway activated downstream of Slik, we investigated the relationship between Slik and dRaf in more detail.

3.2.1 Slik activates dRaf in the overlying PM cells

We proposed two possible models for Slik-mediated signaling (Figure 3.6A). In the first model, Slik activates dRaf in the DP cells, leading to the production and/or release of a nonautonomously-acting factor that drives growth. This factor then binds to its receptor on the plasma membrane of the PM cells and activates signaling to promote their proliferation. The second model proposes that Slik and dRaf act in different sets of cells, i.e. that Slik activates the production or release of the nonautonomously-acting factor independently of dRaf. This factor then binds to its receptor on PM cells, stimulating dRaf activity in PM cells leading to their proliferation. We performed genetic assays to investigate the validity of these models. If model 1 is correct, we predicted two things – first, that depleting *draf* should suppress Slik-driven non-autonomous proliferation of PM cells; and second, that expression of constitutively active dRaf should drive non-autonomous proliferation. To test the first prediction, we made use of a dsRNA transgene targeting dRaf (referred to as *drafRNAi*). In control experiments, we showed that expression of *drafRNAi* downregulated dRaf protein levels when coexpressed with a myc-tagged dRaf transgene, and also blocked the phenotypic effects of dRaf overexpression (Fig 3.6 B), indicating that the *drafRNAi* is functional. We then coexpressed *slik* together with *drafRNAi* using *ap*GAL4. Interestingly, depletion of *draf* did not suppress the non-autonomous proliferation of PM cells driven by Slik as reflected by the dense nuclei

staining and increased BrdU uptake in the peripodial layer (Figure 3.6C, 1st panels). This observation suggests that dRaf is not required in DP cells for Slik driven non-autonomous proliferation of PM cells. To further confirm this, we expressed a constitutively active form of dRaf (dRaf*) with *ap*GAL4, and tested if it was sufficient to drive non-autonomous proliferation of PM cells (Figure 3.6C, 2nd panels). Consistent with the previous result, we observed little BrdU incorporation and wide spread nuclei in the PM layer, a phenotype similar to wild type discs. This indicates that activating dRaf in DP cells is not sufficient to drive non-autonomous proliferation of PM cells. Together, these results favour Model 2, in which Slik stimulates release of a nonautonomously-acting factor that binds to its receptor on PM cells and leads to increased dRaf activity in PM cells, driving their proliferation.
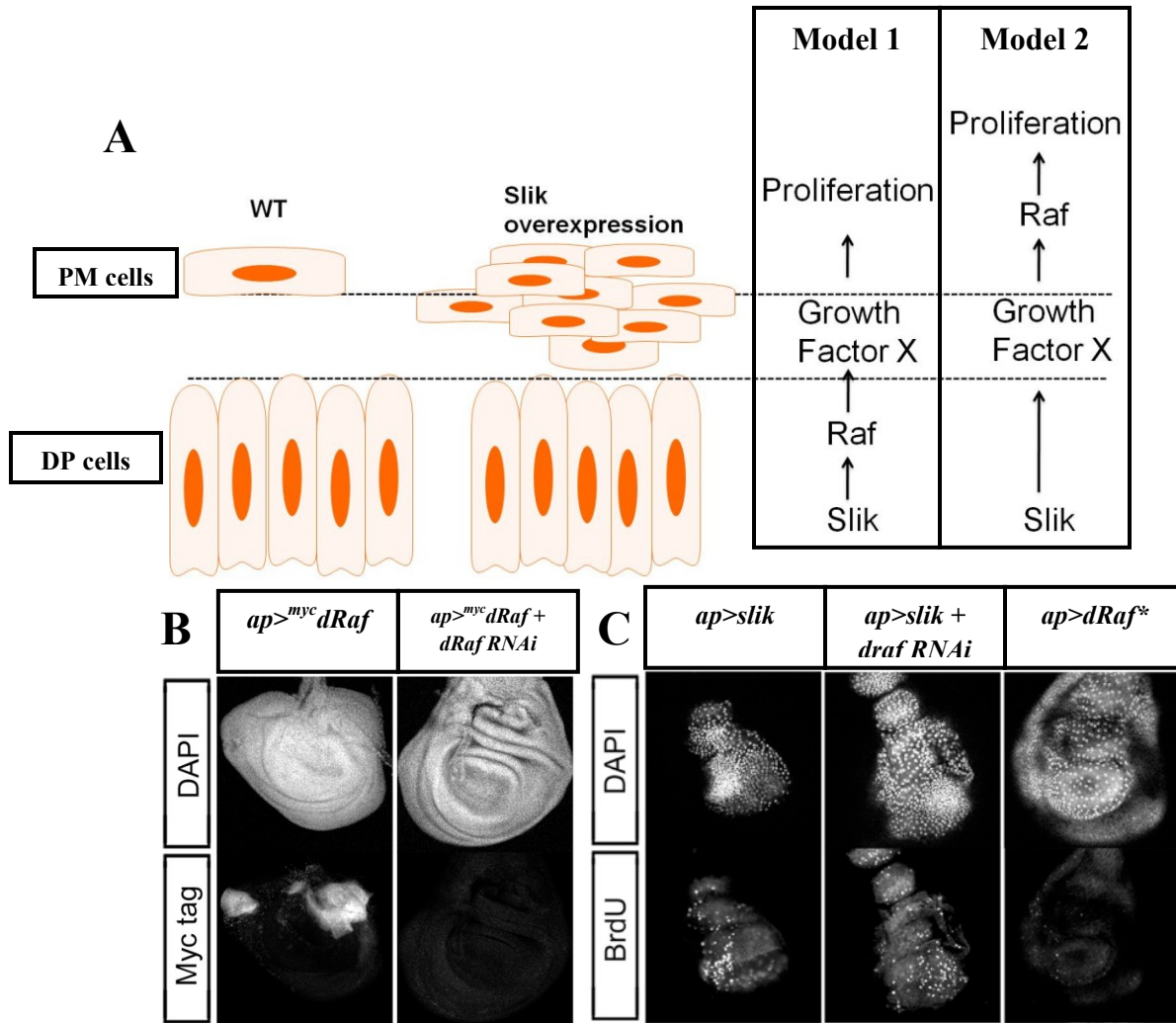
Figure 3.6: Slik activates dRaf in the signal receiving cells

(A) Two proposed models for the Slik-mediated signaling regulating growth during development. In model 1, Slik activates dRaf in DP cells stimulating secretion of a growth factor, leading to PM cell proliferation. In model 2, Slik stimulates release of a growth factor that activates dRaf in PM cells, then leading to PM cell proliferation **(B)** Confocal images of disc proper stained with DAPI and anti-Myc. Expression of Myc epitope-tagged dRaf causes loss of most of the dorsal tissues (1st panel). Co-expression of dsRNA that targets dRaf restores normal tissue and loses myc staining, suggesting that *drafRNAi* is functional (2nd panel) **(C)** Confocal images of the peripodial layer of the wing imaginal discs expression *slik* alone or together with *drafRNAi,* or expressing the constitutively active *draf* alone with *ap*GAL4. Depletion of *draf* does not affect Slik-driven non-autonomous proliferation of PM cells suggested by the densely packed DAPI-positive nuclei and increased BrdU uptake. Constitutive active Draf in DP cells does not promote PM cell proliferation, demonstrating that activation of dRaf is not sufficient to drive non-autonomous proliferation.

3.3 Discovery of downstream targets of the Ste20 kinase Slik

In order to understand the mechanism by which Slik regulates growth during development, we wanted to identify the factor that activates proliferation of PM cells. Since, Slik signals through dRaf, it seemed likely that the factor in question would act on receptor tyrosine kinases (RTK). However, RTK ligands are not the only proteins able to influence RTK signaling. Many proteases and glypicans also modulate extracellular signalling by regulating the spread and polarization of the extracellular signals (Fico, Maina et al. 2011). Glypicans can potentiate the action of receptor-ligand binding by capturing secreted factors and increasing their concentration or availability near to the receptor (Nybakken and Perrimon 2002). It can also stabilize ligand-receptor complexes, or allow formation of multivalent complexes between the receptor and the ligand, increasing the efficiency of receptor activation (Nybakken and Perrimon 2002). Proteases on the other hand are shown to convert glypicans as secreted signal antagonists. For example, in the *Drosophila* wing discs, the alpha/beta hydrolase Notum was shown to cleave the glypican dally-like when bound to Wg, shedding it off the cell surface, making it unavailable for signaling (Giraldez, Copley et al. 2002, Kreuger, Perez et al. 2004). To pinpoint the nonautonomously-acting factor that transduces Slik signaling, we used both a candidate-based approach and an unbiased approach.

3.3.1 Candidate based approach identifies Pvf3 as a potential downstream effector of Slik

We assembled a list of 21 genes whose products were previously documented to regulate growth or patterning in discs by acting non-autonomously (Table 3.1). From this list, we looked for expression in wing discs by semi-quantitative real-time (RT)-PCR. Of these, we found only *pvf3* to be reproducibly upregulated in *slik* overexpressing discs in more than one RNA preparation, suggesting that *slik* may regulate expression of *pvf3* (data not shown). To confirm this, we performed quantitative RT-PCR analysis of gene expression. Consistent with the previous analysis, *pvf3* was upregulated by about 2.5-fold in discs in which Slik was overexpressed using *ap*GAL4 (Figure 3.7). This effect was specifically dues to the overexpression of *slik*, as *pvf3* expression was unaffected by Hpo[K71R] expression (Figure 3.7).

To see if increased *pvf3* expression is responsible for Slik-driven non-autonomous cell proliferation, we depleted it by expressing a dsRNA targeting *pvf3* (referred as *pvf3RNAi*) in

Slik overexpressing DP cells. Surprisingly, Slik non-autonomous proliferation was not suppressed by depleting *pfv3* as evidenced by the presence of dense DAPI-positive nuclei and increased EdU incorporation in PM cells (Figure 3.7B, upper panel). There are three *pvf*s in *Drosophila, pvf1*, *pvf2* and *pvf3*. It has been shown that *pvf2* and *pvf3* have redundant function in the regulation of hemocyte migration, proliferation and size in fly cell culture (Munier, Doucet et al. 2002, Bjorklund, Taipale et al. 2006, Harris, Schnittke et al. 2007). We therefore reasoned that *pvf2* may compensate for *pvf3* depletion. Therefore, we expressed dsRNA targeting both *pvf2* and *pvf3* in cells overexpressing Slik. Again, this did not suppress Slik-driven non-autonomous proliferation (Figure 3.7B, lower panel). These results are only preliminary, as we have not confirmed the functionality of *pvf2* and *pvf3* dsRNA transgenes. However, the data suggest that *pvf3* might not be required for non-autonomous proliferation of PM cells stimulated by Slik. However, further analysis is needed to confirm this.

Genes	Family
Argos	EGF
Branchless	FGF
Dally	Glypican
Delta	EGF
Dpp	TGF-β
Dlp	Glypican
Eiger	TNF
Hedgehog	SHH
Gurken	EGF
Keren	EGF
Pyramus	FGF
Pvf1	PDGF and VDGF-like factor
Pvf2	PDGF and VDGF-like factor
Pvf3	PDGF and VDGF-like factor
Spitz	EGF
Thisbe	FGF
Upd	Morphogen
Upd2	Morphogen
Upd3	Morphogen
Vein	EGF
Wingless	Wnt

Table 3.1: List of non-autonomously acting factors in discs
The table contains the list of all factors that act non-autonomously in *Drosophila* developing tissue. We used semi-QPCR to identify factors in this table that are differentially expressed in *slik* overexpressing discs, which led to the discovery of Pvf3 as potential mediator of Slik signaling.
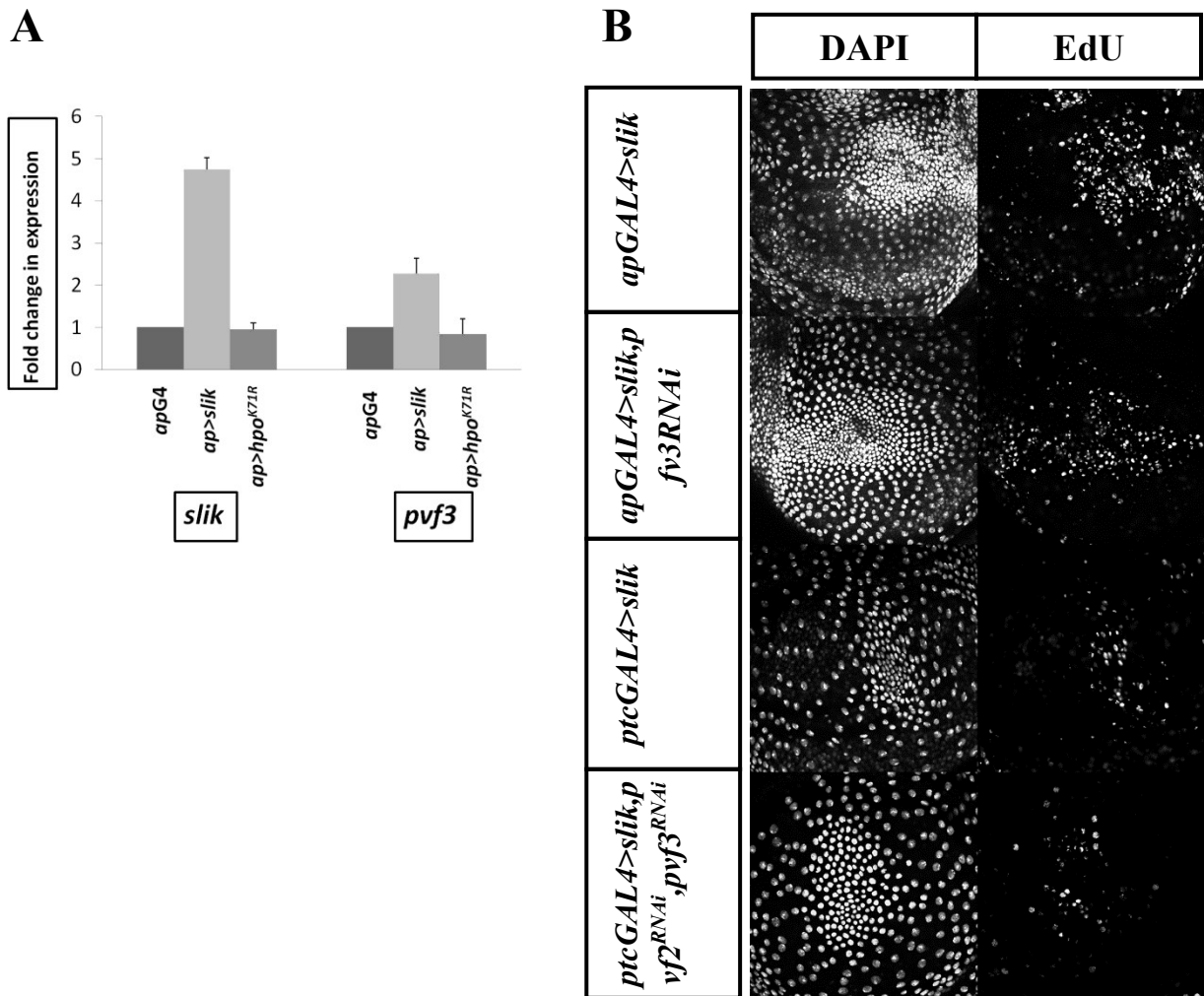
Figure 3.7: *pvf3* is upregulated in *slik* overexpressed discs
(A) Quantitative real-time PCR analysis of expression of *slik* and *pvf3* in discs of the genotypes ptcGAL4/+, *ptc*GAL4/UAS-*slik,* and *ptc*GAL4/UAS-*hpo*[K71R]. Discs overexpressing Slik specifically upregulates *pvf3* by about 2.5-folds. **(B)** Confocal images of the peripodial layer of the wing imaginal discs expressing either *slik* together with *pvf3RNAi* under the control of *ap*GAL4 or *slik* together with *pvf2-pvf3RNAi* under the control of *ptc*GAL4. Depletion of either *pvf3* or *pvf2* and *pvf3* does not suppress the Slik-driven non-autonomous proliferation of the PM cells.

3.3.2 Whole transcriptome sequencing to identify possible downstream targets of Slik

The differential expression of *pvf3* in cells overexpressing *slik* suggests that Slik might stimulate a broader transcriptional change in cells. To identify the downstream targets and to understand the general effects of Slik overexpression, we performed a transcriptomic analysis by RNA-sequencing (RNA-seq). We compared gene expression profiles of wing imaginal discs isolated from larvae expressing *ap*GAL4 alone, which acted as wild-type control, and larvae expressing *slik* with *ap*GAL4. We prepared duplicate samples for each genotype, consisting of about 60 discs each and tested between two RNA extraction methods – RNeasy from Qiagen and TRIzol from Invitrogen. TRIzol extraction gave the best output with the highest quality RNA, assessed using an Agilent Bioanalyzer. We obtained two tight peaks corresponding to the 23S and 18S ribosomal RNA (rRNA) for each of the samples, suggesting excellent RNA integrity (Figure 3.8A). The RNA samples were then submitted to the IRCM Molecular Biology Core Facility to generate cDNA libraries, followed by whole-transcriptome sequencing at Genome Quebec using an Illumina sequencer.

We expected to observe complex transcriptional changes, including both cell-autonomous and non-autonomous responses due to Slik activity. Slik induces cell proliferation and apoptosis, which increases bioenergetic and biosynthetic demand (Schieke, McCoy et al. 2008). Thus, we predicted that some of the cell-nonautonomous effects of Slik might lead to expression level changes of genes whose product are involved in processes like cell cycle regulation, apoptosis, metabolism, and translation. Though the cell non-autonomous transcriptional changes are informative, we were more interested in cell-autonomous changes. These could include genes whose product encode for proteins capable of transmitting signals to neighbouring cells – such as transmembrane proteins or secreted factors including growth and patterning factors, secreted glycoproteins, glypicans and proteases. These differentially expressed genes might be potential mediators of Slik growth signaling.

The sequencing mapped 14000 genes to the reference genome. When we compared the expression profiles between the replicates, we obtained a correlation coefficient of 1 for both samples (Figure 3.8B). A correlation coefficient of 0.99 was observed when comparing the expression profiles between Slik and wild-type samples. This suggests that the results from the two biological replicates were highly reproducible, and that there were not dramatic

differences in expression profiles between control and Slik samples. Statistical analysis by DESeq and edgeR packages identified 140 and 498 statistically significantly differentially expressed genes respectively ($p<0.05$). genes identified by DESeq were also in included in the edgeR analysis. Therefore, we preferred using the DESeq list for downstream application because it is more stringent at filtering outliers. To validate the differential expression results, we tested a number of candidates by Q-PCR analysis. We were able replicate similar fold-changes in expression level of genes that were highly upregulated (for example *tektin-C* and *CG42330*), genes that were slightly differentially expressed (for example *mthl4* and *pp24-2*), and genes that were highly downregulated (for example *CG18110* and *ppk20*) (Figure 3.8C). As expected, target genes directly regulated by Hpo/Yki, including *ex, eiger,* and *dco* (Oh, Slattery et al. 2013) did not show up as differentially expressed in the screen, confirming our previous experiments. Surprisingly, *pvf3* did not appear to be strongly differentially expressed in the transcriptome sequencing (although it was about 1.2-fold higher in Slik-overexpressing discs). We also failed to observe changes in expression of any other obvious growth factors.

To investigate the functions of the 140 genes, we used gene ontology analysis to cluster genes based on their functional characteristics using DAVID bioinformatics resources (Huang da, Sherman et al. 2009). We obtained five annotation clusters which showed significant enrichment and false discovery rates lower than 10% (Table 3.2). As expected, we observed genes significantly enriched in functional annotation clusters such as electron transport, oxidative phosphorylation, respiratory chain, and mitochondria inner membrane. These genes were highly upregulated in the *slik* overexpressing cells. Cell proliferation, DNA replication and protein translation are energy intensive processes that require increase in ATP production, underlying the importance of cellular metabolism for growth (Almeida, Bolanos et al. 2010). Mitochondria are the major source of cellular energy production. It has been shown recently the presence of active APC/C-Cdh1, which ubiquitinates the glycolysis-promoting enzyme 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase, isoforms 3 (PFKFB3) leading to its degradation, inhibits cultured cell proliferation under serum stimulation (Almeida, Bolanos et al. 2010). Another evidence of the importance of cellular metabolism is the increase in mitochondrial membrane potential and respiration observed during cell cycle progression through G_1 phase (Schieke, McCoy et al. 2008). Growth and cell proliferation is

accompanied by an increase in glycolysis, suggesting that the mitochondria adapt to the required energy demands to tightly coordinate cell proliferation and cell cycle progression, a phenomenon termed the Warburg effect (Warburg 1956, Almeida, Bolanos et al. 2010). Therefore, upregulation of genes implicated in respiration in Slik overexpressed cells might be a direct effect of Slik on their gene expression, or more likely, it could be simply due to the increased demand in ATP production due to cell proliferation.

Intriguingly, we observed an enrichment of genes clustered under the term G-protein coupled receptor (GPCR) signaling pathway. GPCRs are interesting because they are membrane-bound and in some cases are implicated in development (Fredriksson, Lagerstrom et al. 2003, Nordstrom, Lagerstrom et al. 2009). Some Ste20 kinases have been identified to signal downstream of GPCRs. For example, in yeast, the Ste20p transduces the pheromone signals activated by the GPCR Ste2/3 (Sprague, Cullen et al. 2004). Therefore, it wouldn't be surprising if Slik is also implicated in GPCR signaling, although they might not be expected as downstream transcriptional target. In our RNAseq data, we observed two families of genes that are enriched in this cluster – the Methuselah (Mthl) family and the Gustatory Receptors (GR). It has been shown that mutations in the Mthl family of proteins extends longevity and stress resistance in *Drosophila* (Ja, Carvalho et al. 2009). During embryonic development, four *mthl* family genes are expressed in the wing discs – *mthl3, 4, 6,* and *8* (Patel, Hallal et al. 2012). In our transcription profile study, all four of them were differentially expressed. We observed massive downregulation of *mthl6* and *mthl8*, whereas *mthl4* and *mthl3* were upregulated by 2-fold and 1.6-fold respectively. Expressions of *mthls* are not restricted to developing wings, and can be detected in embryos during gastrulation, development of the gut, heart, lymph glands and later in the larval central nervous system (Patel, Hallal et al. 2012). This expression pattern is comparable to the orthologue of Mthl in beetles, suggesting a greater role for Mthl in organ morphogenesis during development (Song, Ranjan et al. 2002, Patel, Hallal et al. 2012). However, their functions in these tissues have not been defined. GRs are tastants that detect nutrient rich food and avoid toxicity (Clyne, Warr et al. 2000). The GR family contains 68 receptors in *Drosophila* which are expressed in many cell types, extending their role beyond being the detection of tastants and pheromones (Park and Kwon 2011). In our transcription profile, we find three that were differentially regulated *Gr61a*, *Gr64a*, and

Gr64b. Gr64a and Gr64b were highly upregulated in *slik* overexpressing cells. Slik might regulate expression of GPCRs such as *mthl* and/or *Gr* to drive growth in *Drosophila.* However, further analyses are needed to assess their potential as downstream effectors of the Slik signaling pathway. We also observe enrichment of genes with terms like ion channel, sodium channel, and sodium channel activity. Ion channels have been implicated in regulating many biological processes, including those involved in maintaining tissue homeostasis such as proliferation, differentiation and apoptosis (Razik and Cidlowski 2002, Lang, Foller et al. 2005, Schonherr 2005). For example, it has been shown that growth factors can trigger the entry of Ca^{2+} in proliferating cells, and subsequent Ca^{2+} oscillation to activate numerous cellular events, such as depolarization of actin filaments (Lang, Ritter et al. 2000, Lang, Foller et al. 2005). Consequently, the actin depolarization leads to the activation of the Na^+/H^+ exchanger and/or the Na^+, K^+, $2Cl^-$ cotransporter (NKCC) leading to increase in cell volume, which is required for cell division (Lang, Busch et al. 1998, Lang, Foller et al. 2005). Interestingly, two Ste20 kinases, SPAK and OSR1, are well-known to phosphorylate and activate NKCC1 (Piechotta, Lu et al. 2002, Piechotta, Garbarini et al. 2003). This membrane transport protein is known to regulate cell volume, cell proliferation and survival, epithelial transport, neuronal excitability, and blood pressure (Russell 2000). In our screen, we observed various sodium and calcium channels that were differentially expressed in Slik overexpressing discs. Mainly, the pickpocket (ppk) genes, which encode for Degenerin/Epithelial Sodium Channel (DEG/ENaC) subunits, showed transcriptional changes. The DEG/ENaC is found to be essential for mechanical nociception in sensory cells of *Drosophila* larvae (Zhong, Hwang et al. 2010). Slik might regulate these ion channels to induce influx of ions which leads to increase in cell size, necessary for cell proliferation (Lang, Foller et al. 2005). We intend to pursue with genetic analysis of the 140 candidate genes to identify possible downstream effectors of Slik signaling.
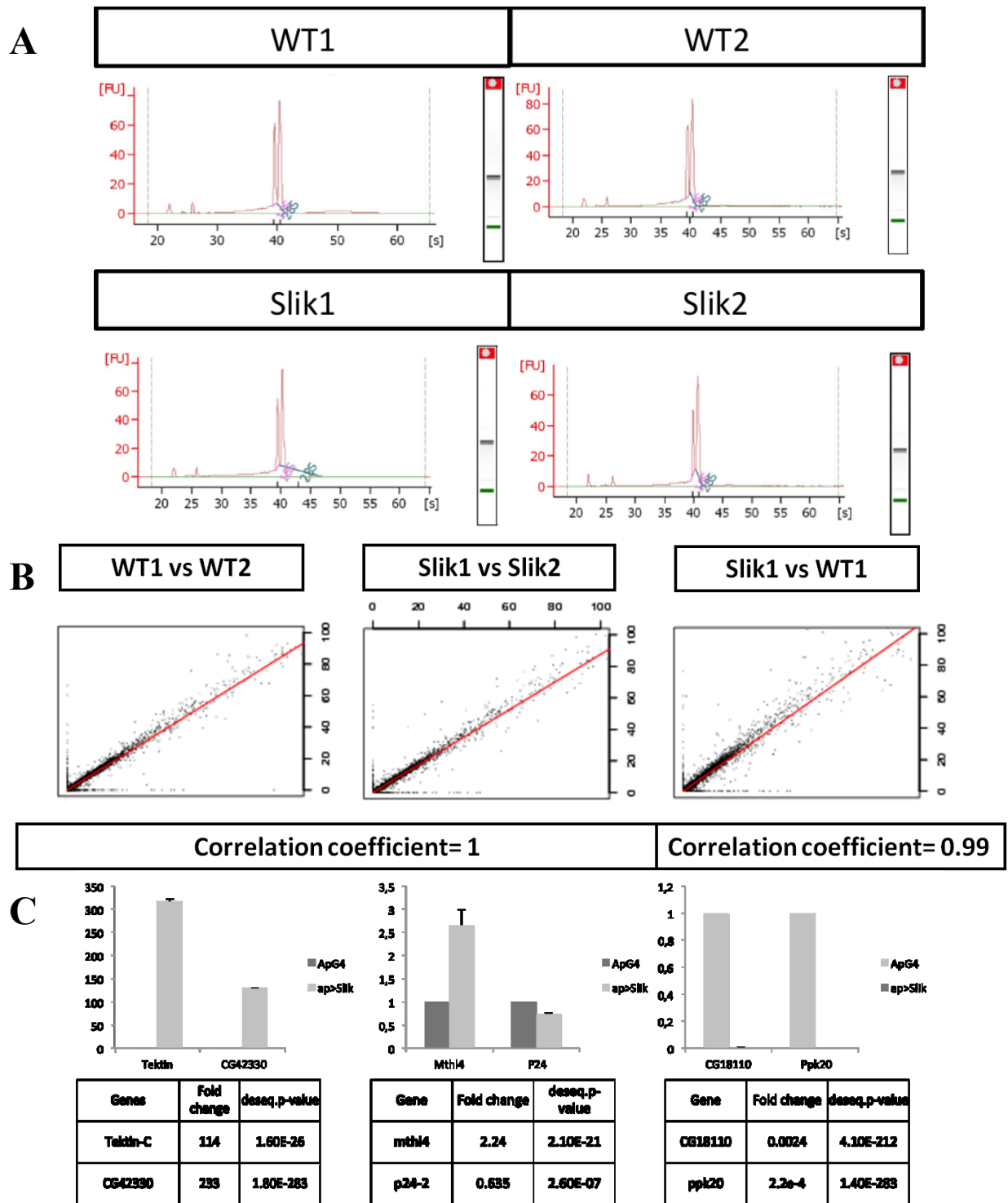
Figure 3.8 Whole transcriptome sequencing to identify potential Slik downstream targets
(A) TRIzol extracted total RNA analysed by Agilent Bioanalyzer. Peaks correspond to 23S and 16S rRNAs. **(B)** Scatterplot of gene expression levels in pairs of sample as indicated. **(C)** Validation of selected differentially expressed genes by QPCR.

Annotation cluster 1		Number of genes identified	Total number of genes	Enrichment score: 6.78	
Categories	Terms			*p* value	FDR (%)
SP_PIR_KEYWORDS	Membrane	42	132	1.71E-13	2.01E-10
SP_PIR_KEYWORDS	Transmembrane	33	132	7.64E-09	8.99E-06
SP_PIR_KEYWORDS	Transport	21	132	4.02E-08	4.73E-05
Annotation cluster 2				Enrichment score: 3.36	
SP_PIR_KEYWORDS	oxidative phosphorylation	8	132	1.48E-11	1.74E-08
SP_PIR_KEYWORDS	membrane-associated complex	7	132	4.18E-10	4.91E-07
SP_PIR_KEYWORDS	respiratory chain	7	132	1.06E-08	1.25E-05
SP_PIR_KEYWORDS	mitochondrion inner membrane	8	132	8.33E-07	9.80E-04
SP_PIR_KEYWORDS	electron transport	6	132	6.55E-06	0.00770996
Annotation cluster 3				Enrichment score: 2.69	
SP_PIR_KEYWORDS	oxidative phosphorylation	8	132	1.48E-11	1.74E-08
SP_PIR_KEYWORDS	membrane-associated complex	7	132	4.18E-10	4.91E-07
SP_PIR_KEYWORDS	respiratory chain	7	132	1.06E-08	1.25E-05
SP_PIR_KEYWORDS	mitochondrion inner membrane	8	132	8.33E-07	9.80E-04
SP_PIR_KEYWORDS	electron transport	6	132	6.55E-06	0.00770996
SP_PIR_KEYWORDS	electron transfer	4	132	3.41E-05	0.04017226
Annotation cluster 4				Enrichment score: 2.6	
INTERPRO	Methuselah, N-terminal	5	114	4.45E-06	0.00590709
SP_PIR_KEYWORDS	glycoprotein	16	132	3.23E-05	0.03800599
UP_SEQ_FEATURE	transmembrane region	22	47	3.85E-05	0.04997583
SP_PIR_KEYWORDS	g-protein coupled receptor	10	132	3.51E-04	0.41173537
SP_PIR_KEYWORDS	transducer	10	132	6.06E-04	0.71100966
GOTERM_BP_FAT	G-PCR protein signaling pathway	12	90	7.22E-04	1.05993368
Annotation cluster 5				Enrichment score: 2.5	
SP_PIR_KEYWORDS	ion transport	10	132	9.36E-05	0.11013571
SP_PIR_KEYWORDS	Sodium transport	6	132	1.17E-04	0.137927
GOTERM_BP_FAT	sodium ion transport	7	90	1.95E-04	0.28796476
GOTERM_BP_FAT	monovalent inorganic cation transport	9	90	7.35E-04	1.07866603
GOTERM_MF_FAT	sodium channel activity			8.07E-04	1.02741942

Table 3.2: Enrichment of functional annotation clusters
Significantly enriched annotation clusters identified by DAVID are represented in this table
(*p*<0.01, FDR<10%).

4. Discussion and Conclusion

The Ste20 kinase Slik regulates fundamental biological events such as cell proliferation, cell survival, and maintenance of epithelial integrity in *Drosophila*. In general, the mechanisms regulating these processes ensure for normal development and are conserved from flies to human. The two homologues of Slik in mammals, SLK and LOK, have been shown to be involved in many processes, including cell apoptosis, migration and adhesion. Whereas the characterization SLK and LOK function has been hampered by a lack of models to study their functions in a physiological environment (SLK) or mild phenotypes likely due to functional redundancy (LOK), *Drosophila* has proven to be a great model system for studying the function of this kinase family. Considering the high degree of conservation of pathways mediating developmental processes, identification of downstream effectors of Slik could potentially lead to discovery of SLK and LOK targets and a better understanding of their functions.

4.1 Slik does not signal through the Hippo tumor suppressor pathway

It has been suggested that Slik signals through Mer to drive cell proliferation and tissue growth during development. Specifically, it has been shown that Slik directly phosphorylates Mer, inhibiting its tumor suppressor activity and promoting membrane localization (Hughes and Fehon 2006). Mer is related to the member of the ERM family of protein, containing a similar N-terminal FERM domain (Golovnina, Blinov et al. 2005). It is possible that Slik phosphorylates Mer considering that Slik is a known ERM kinase. However, in this thesis, I provided evidence suggesting that it is unlikely that Slik drives growth through Mer regulation in *Drosophila*. Using genetic assays, we showed that Slik drives cell proliferation and tissue growth independently of Merlin, and by extension the Hippo pathway. I demonstrated that blocking the function of Mer is not sufficient to stimulate non-autonomous proliferation, which would be expected if Slik drove growth by inhibiting Mer. Furthermore, we did not detect genetic interactions between *slik* and *mer*, suggesting that Slik and Mer may not be involved in the same pathway. If Slik were to signal through Mer, I expect Slik to regulate the Hippo pathway, since Mer is an upstream regulator of that tumour suppressor pathway. To investigate this possibility and to further confirm our Slik and Mer genetic assays, we showed

that there is no genetic interaction between Slik and Yki, the transcription co-factor targeted by the Hippo patwhay. Slik-driven overgrowth of adult wings was insensitive to *yki* gene dosage, in contrast to the overgrowth stimulated by blocking functions of Mer, Hpo and Ft. We also demonstrated by using an enhancer trap that Slik does not enhance transcription of *ex,* a target gene of Yki. To validate our genetic results, we performed QPCR analysis of gene expression and observed no transcriptional changes of the *ex* gene in cells overexpressing Slik, nor did we see changes in Yki target genes in our RNAseq analysis. Taken all together, we conclude that Slik does not act through Mer or the Hippo pathway to promote cell proliferation and tissue growth.

In wild-type cells, both Mer and Slik are localized at the apical side of the cell (Kissil, Johnson et al. 2002, Hipfner and Cohen 2003). However, in Slik mutant clones, it has been shown that Mer loses its apical localization and accumulates at the basolateral compartment of the cell, suggesting that Slik regulates Mer localization (Hughes and Fehon 2006). It is known that Slik promotes epithelial integrity by phosphorylating and activating the ERM protein Moesin, which connects the actin cytoskeleton to the plasma membrane. This linkage is essential for proper cell shape and the appropriate organization of apical membrane structures. It has been reported that epithelial cells null for Slik loses their apical-basal polarity and are extruded from the epithelial sheet (Hipfner and Cohen 2003). Therefore, the accumulation of Mer in the basolateral compartment seen in the Slik mutant cells might be due to the loss of the epithelial integrity rather than the direct effect of Slik on Mer. The genetic interaction between Slik and Mer reported by Hughes et al. is also unconvincing. They reduced *slik* gene dosage in cells expressing the truncated from of *Mer* that lacks the Slik phospho sites and observed a slightly enhanced reduction of the wing size compared to expressing Mer alone. Therefore, it's hard to interpret the results. Further, they did not provide data about the wing size of *slik* heterozygous flies. A proper experiment would be to overexpress *slik* in *mer* heterozygous background and look for enhancement of growth compared to overexpression of *slik* alone.

Interestingly, though blocking Mer activity does not induce proliferation of PM cells, it does stimulate overgrowth of the adult wings. This put forward the idea that Slik and inhibition of the Hippo pathway drive growth by alternative pathways. Many pathways

contribute to regulate growth during development. Slik might signal through such pathways. For example, Ste20 kinases, such as NCK and GCK are known to activate MAPK pathways through the interleukin and TNF receptor effector proteins TRAF2 and TRAF6 (Baud, Liu et al. 1999, Chadee, Yuasa et al. 2002). In *Drosophila*, the TNF Eiger has been shown to induce apoptosis through JNK signaling (Ma, Yang et al. 2013). It is unlikely that Eiger would act downstream of Slik pathway because we failed to identify Eiger in the RNA-seq results. Various mechanisms integrate both intrinsic and extrinsic cues in order to respond to the growth requirement during development. One such input is nutrient availability, which is sensed by the insulin/PI3K pathway (Grewal 2009). However, it has been shown that the increase in tissue size driven by Slik overexpression is due to increase in cell proliferation, and not due to increase in cell size, in contrast to the PI3K/insulin pathway (Stocker and Hafen 2000, Hipfner and Cohen 2003). Therefore, Slik probably does not signal through the PI3K/insulin pathway. Pathways such as EGFR/ERK, Dpp, Wg, and Notch also regulate growth by providing survival cues and inducing cell proliferation (Johnston and Edgar 1998, Milan, Perez et al. 2002, Firth, Bhattacharya et al. 2010). However, Wg and Notch signaling regulate cell cycle progression – Wg accelerates the G_1/S transition and Notch accelerates the G_2/M transition (Giraldez and Cohen 2003). Slik appears to uniformly accelerate the different cell cycle phases (Hipfner and Cohen 2003), suggesting that these pathways might not contribute to Slik-driven cell proliferation.

4.2 Slik signals through dRaf in the signal receiving PM cells

We followed up on our previous report that demonstrated a genetic interaction between *slik* and *draf* (Hipfner and Cohen 2003). In animals heterozygous for *draf* mutations, in which all cells in the wing discs have reduced *draf* gene dosage, Slik-driven non-autonomous proliferation is strongly suppressed (Hipfner and Cohen 2003). This could be because – A) Slik activates dRaf in DP cells leading to the secretion/production of a growth factor that stimulates PM cell proliferation, or B) Slik activity in DP cells triggers secretion/production of a growth factor, which activates dRaf in PM cells, stimulating their proliferation. We provided two pieces of indirect genetic evidence that dRaf activity was required in the cells receiving the non-autonomous signal, located in the PM of the wing imaginal discs. First, we demonstrated that depleting *draf* in DP cells does not inhibit non-autonomous proliferation

driven by Slik. Second, consistent with the previous result, we showed that expressing a constitutively active form of *draf* in DP cells was not sufficient to drive proliferation of PM cells. Therefore, our results support a model where Slik activates the release of a growth factor from DP cells that stimulates dRaf in the overlying PM cells, leading to cell proliferation. A direct way to test the requirement of dRaf in PM cells would be to either – co-express dsRNA targeting *draf* in PM cells alone overlaying the *slik* expressing DP cells, or to express the constitutively active *draf* in the PM cells. According to our model, we would expect the depletion of *draf* from PM cells to completely suppress the Slik overexpression phenotype, similar to the *draf*$^{+/-}$ experiment. On the other hand, expression of *draf** in PM cells should stimulate PM cells proliferation. For the moment, it isn't technically possible because there are no GAL4 drivers able to specifically express transgenes in PM cells.

The Raf family of proteins are Ser/Thr kinases regulating a multitude of processes including apoptosis, cell cycle progression, differentiation, and proliferation (Roskoski 2010). In mammals, there are three Rafs – A-RAF, B-RAF, and C-RAF. Theses kinases participate in MAPK cascade of RAS-RAF-MEK-ERK, acting as MAP3K's (Zebisch and Troppmair 2006). RTKs mediate signaling via the small GTPase Ras. When Ras is bound to GTP, it is in an active state and is able to recruit Raf to the membrane (Schlessinger 2000). Raf can be then activated by different mechanisms – by phosphorylation and dephosphorylation of key residues, and by protein-protein and protein-lipid interactions (Dhillon and Kolch 2002). According to our genetic results, the most likely model for Slik activity is that it stimulates production or secretion of an RTK ligand, which bind to its receptor in the plasma membrane of PM cells, leading to the activation of dRaf. Although there is some genetic evidence that Slik mediated signaling might not involve the dRaf canonical pathway (Hipfner and Cohen 2003), how dRaf transduces Slik signaling to induce PM cell proliferation remains unresolved.

4.3 Pvf3, a potential downstream target of Slik

We performed a candidate and a non-candidate based approach to identify the signal that induces non-autonomous proliferation downstream of Slik. Of 24 nonautonomously-acting factors in the wing discs that we tested by semi-QPCR, we found only *pvf3* was upregulated. This upregulation was confirmed by QPCR. However, dsRNA-mediated

depletion of *pvf3* didn't suppress Slik-induced proliferation. Since, Pvf2 and Pvf3 possibly have redundant function in flies – we tested the effect of depleting both on Slik-driven non-autonomous PM cell proliferation, and found that it was not affected. This suggests that Pvf3 might not be required for Slik growth signaling.

Interestingly, *pvf3* encodes a PDGF and VDGF-like growth factor in flies and activates the RTK Pvr (Duchek, Somogyi et al. 2001). In cultured flies, Pvf2 and Pvf3 have redundant function in establishing an autocrine growth signaling loop through the Pvr/Ras/Raf/MEK/ERK pathway (Sims, Duchek et al. 2009). Since, we have identified dRaf as downstream target of Slik, Pvf3 was an attractive candidate for relaying Slik-mediated growth signaling to the PM cells. The lack of an effect of *pvf2RNAi* and *pvf3RNAi in vivo* in reducing Slik-driven non-autonomous proliferation may be due to the inefficiency of the *pvf* dsRNA transgenes. One way to do so is to perform a QPCR analysis of gene expression in discs expressing the *pvf2/pvf3* dsRNA transgene. We would expect lower *pvf2/pvf3* expression level in these discs compared to wild-type if the transgenes are functional. Nonetheless, further analysis is needed to conclude whether Pvf3 interacts with Slik. For example, we could test if overexpression of *pvf3* can induce non-autonomous cell proliferation in the wing disc, as our model would predict. We could also investigate whether Slik signaling is dependent on Pvr, which would be expected if Pvf3 interacts with Slik. The results from these experiments will allow an effective conclusion whether Pvf3 is functionally involved in Slik signaling pathway.

4.4 RNA-seq identifies possible mediators of Slik signaling pathways

To discover potential downstream target of Slik signaling, we performed RNA-seq analysis, which identified 140 statistically significant differentially expressed genes. Pvf3 did not show up in the transcriptome sequencing. This could be due to low level of expression of *pvf3* coupled with insufficient depth of the sequencing (although our analysis included 80 million reads from each sample). Consistent with our genetic analysis, genes encoding components of the Hippo pathway or its target genes also did not show transcriptional changes. This supports our conclusion that Slik does not signal through the Hippo pathway via Mer.

As expected, we observed complex cell-autonomous and cell-nonautonomous transcriptional changes as a result of Slik overexpression. One possible example of the non-autonomous effect of Slik is the upregulation of mitochondrial genes. During cell division, there is evidence of increased demand in ATP synthesis and membrane biogenesis (Almeida, Bolanos et al. 2010). Presumably, the Slik-driven cell overproliferation stimulates genes involved in cellular respiration in a cell non-autonomous manner. One way to confirm this would be to perform a transcription profiling of wing discs expressing *slik* in a *draf* heterozygous background. Because removing a functional copy of *draf* gene suppresses the non-autonomous cell proliferation, the transcriptional response would solely be due to the cell-autonomous effect of Slik overexpression. This would enable the identification of direct Slik downstream targets. Nevertheless, the RNA-seq identified many genes that could be differentially expressed due to Slik cell autonomous effects. As mentioned above, we observed enrichment of two families of GPCRs – the Gustatory Receptors and Methuselah. GPCRs are membrane bound receptors that transduce signals when bound to extracellular ligands. There is a connection between Ste20 kinases and GPCRs, as Ste20p acts downstream of the GPCRs Ste2 and Ste3. However, it's unclear why Mthl and GR would be downstream targets of Slik. The most likely explanation would be that Slik activity acts as a positive feedback loop that upregulates transcription of *mthl* and *Gr*. The functional regulations of these receptors are still unclear. Recent studies have indentified two ligands for Mthl, Stunted A and B (Cvejic, Zhu et al. 2004). When genes encoding these ligands were mutated, flies have an enhanced life span and are resistant to oxidative stress (Cvejic, Zhu et al. 2004). The transcriptional changes of *mthl* genes observed in the analysis suggest a greater role for these genes in cell proliferation and growth. Interestingly, the known ligands of Mthls were not identified as differentially expressed genes, suggesting that Mthl receptors might have other ligands yet to be discovered. RNA sequencing also identified altered expression of many genes encoding for ion channels. These are relevant candidates because Ste20 kinases, such as SPAK and OSR1 are shown to regulate ion transporter NKCC1 (Piechotta, Lu et al. 2002). Upregulation of these transporters suggest that Slik may regulate secretion of several factors rather than a single specific growth factor that induces cell proliferation.

To isolate candidates that are functionally important for Slik-driven growth response, one could perform a genetic screen of the 140 candidate genes identified by RNA-seq. The Vienna Drosophila RNAi Center has readily available fly stocks bearing dsRNA transgenes targeting 90% of all genes, including most of the 140 candidate genes. As a preliminary screen, we could express these RNAi transgenes alone and together with *UAS-slik,UAS-p35* using *ptc*GAL4. This will allow quick visualization of enhancement and suppression of the growth of that part of the adult wing bounded by the veins L3 and L4. We would be interested in isolating genes which, when depleted using dsRNA transgenes, either enhance or suppress the Slik overexpression phenotype, suggesting a genetic interaction between that gene and Slik. Genes whose depletion enhances the Slik overexpression phenotype might be genes that are negatively regulated by Slik. Therefore, inhibiting them increases Slik-driven tissue growth. On the other hand, these could also include genes activated as a feedback response to limit Slik activity. Since Slik stimulates cell proliferation, one can assume that apoptotic and tumor suppressor genes are upregulated to resist the effects of Slik expression. Therefore, depleting these genes would naturally enhance Slik-driven cell proliferation and tissue growth. On the other hand, genes whose depletion suppresses the Slik overexpression phenotype might be direct effectors of Slik-mediated signaling or genes whose product is involved in protein translation, oxidative metabolisms and membrane biosynthesis. As explained above, processes such as respiration and protein synthesis are required for cell division. Therefore, components regulating these pathways may be activated indirectly due to the effect of Slik of cell proliferation. To confirm our findings, we could test if co-expression of these RNAi transgenes with Slik blocks or enhances Slik-mediated non-autonomous proliferation of the PM cells. Results from these experiments would allow us to better characterize the Slik signaling pathway, and establish a list of potential downstream effectors.

5. Materials and methods

5.1 Genetics

5.1.1 Genetic mutants used in this study

mer^4: nonsense mutation, Gln170 to stop, (LaJeunesse, McCartney et al. 1998)

yki^{B5}: null mutant, (Huang, Wu et al. 2005)

5.1.2 Transgenes

$EPSlik/cyo^{KrGFP}$: P-element insertion, (Hipfner and Cohen 2003)

UAS-draf*: gain of function, (Brand and Perrimon 1994).

$UAS\text{-}ft^{\Delta ICD}/TM6$: antimorph, amino acid deletion at position 4620-5147 from the intracellular domain of Fat (Matakatsu and Blair 2012).

$UAS\text{-}hpo^{K71R}/TM6$: antimorph, kinase dead mutant (Wu, Huang et al. 2003).

$UAS\text{-}mer^{ABB}/TM6$: antimorph, deletion of the seven amino acids (^{170}YQMTPEM^{177}) (LaJeunesse, McCartney et al. 1998)

5.1.3 RNAi transgenics files used in the result section

ftRNAi: from VDRC, Transformant ID: 9396

hpoRNAi: from VDRC, Transformant ID: 7823

merRNAi: from VDRC, Transformant ID: 1484

rafRNAi: from VDRC, Transformant ID: 9831

5.1.4 Adult wing size measurements

Adult flies were preserved in PBS (140 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 8.1 mM NaH_2PO_4) containing 10% EtOH and 30% glycerol. Adult wings were dissected and mounted in Canada Balsam mounting medium on a glass slide covered with cover slip for 2 days incubation. A Leica DM4000 upright fluorescence microscopy was used to capture images. ImageJ was used to process the images and calculate the ratio of area between veins L3-L4 to the whole wing. This ratio was then plotted in Microsoft Office Excel.

5.1.5 Antibody staining of wing imaginal discs

Antibodies used

Antibodies used were as follows: Guinea pig anti-Slik (Hipfner and Cohen 2003) at 1:250; Guinea pig anti Ex at 1:2500 (Laughton); Rat anti-Ft at 1:1000 (Helen McNeill); Guinea pig anti-Hpo at 1:400 (George Halder); Guinea pig anti-Mer at 1:2500 (Rick Fehon); Rabbit anti-βGal at 1:200 (Invitrogen); Mouse anti-GFP at 1:200 (Torrey Pines).

Antibody staining protocol

Wandering *Drosophila* 3rd instar larvae were collected in PBS. These larvae were cut in half, and the anterior halves were inverted using dissection forceps. Carefully, without damaging the discs, the fat body, gut and salivary glands were removed. The remaining anterior half of the body, which contains the wing discs, was transferred in an Eppendorf containing PBS on ice. These discs were then fixed in 4% PFA in 0.2% PBT (PBS + 0.2% Tween) for 20 minutes at room temperature. After fixation, the larvae were washed three times for 5 minutes with 0.2% PBT, and blocked for 1hr with BBT (0.2% PBT + 0.3% BSA). Afterwards the larvae were incubated overnight with primary antibodies in PBT at 4°C. Next, these wing discs were incubated in secondary antibodies for two hours at room temperature in dark, after which they were washed two times with 0.2% PBT. Incubation with DAPI was done for 10min at room temperature in the dark. This was followed by two washes for 5 min. Larval bodies were stored in mounting medium (90gm of glycerol, 2.11gm n-propyl Gallate and 10mL PBS) before being mounted on glass slides and covered with cover slips.

Confocal microscopy

All confocal images were captured with LSM 700 from Zeiss and processed using the Zen software package.

5.1.6 EdU labeling

Wandering larvae were collected in Schneider's medium (Sigma). These larvae were dissected as described above. Incubation with 1000ul of Scheider's medium and 1ul of EdU (from 10mM stock) was done for 1hr at room temperature on a nutator. After this incubation,

2 washes with PBS were carried out. Larvae were then fixed with 4% PFA at room temperature for 20 min followed by 3 washes with 0.3% PBT for 10min. The larval bodies were then washed twice with 3% BSA in PBS at room temperature for 5min, before being incubated with the Invitrogen detection cocktail (as described in the manufacturer's protocol) for 30min at room temperature. After, these were washed with 3% BSA in PBS at room temperature for 5min. Incubation with DAPI (1:2000 in 0.3% PBT) was carried out for 20min, before being mounted in glycerol containing 0.5% n-propyl gallate on a glass slide and visualized by confocal microscopy.

5.2 Molecular biology

5.2.1 QPCR

Total RNA was extracted from third-instar wing imaginal discs using TRIzol reagent (Invitrogen) by following the manufacturer's instructions. cDNA was then generated using oligo (dT) (NEB) and SuperScript III(Invitrogen) by following the manufacturer's protocol. Oligos for qPCR were designed using Primer3 website. To differentiate between genomic DNA and coding regions, the forward and reverse pairs were separated by exon-intron-exon boundary. QPCR was then performed in 384-well plates using 1ul of cDNA (6.25ng), 0.25ul of each primers (10uM stock), 2.5ul of SYBR Green (Life Technology), and H_2O to 5ul in ViiA 7 (Applied Biosystems).

The QPCR system was programmed as follows:

- 50ºC for 2 minutes hold
- 95ºC for 10 minutes hold
- 35 cycles of:
    - 95ºC, 15 seconds
    - 58ºC, 30 seconds

QPCR Oligos designed using Primer3:

Slik_F	AGAAGTCCTTAGTCAAAGATCC
Slik_R	CCAGATTTCTGTTGATGAAAGC
Mer_F	TGCTCCTCGTTGACACGACTCTTT

Mer_R	AGAAGAGCATGGAGCACCTGCAAA
Ft_F	ATATAGATTCGGGCAACAATGG
Ft_R	TGCTATCGATTCCAAACTCTG
CG42330_F	AGCTGTACGAAACCGATCC
CG42330_R	AGTTGGTGTATTTGCGCAG
Ex_F	CCGTATGTAAACAGACGGC
Ex_R	AATTGGCTTAGAGGGTATTGG
Pvf3_F	CCACAACGTTGAGCTGCACTTCTT
Pvf3_R	CGTGTGGTTCACAAAGGTTCGCTT
Mthl4_F	GATCTACACTGGCGATATGAC
Mthl4_R	ATGTAAATCGCCGAAAGGAC
Ppk20_F	TCTAATTGCCAATCTGGGTG
Ppk20_R	AGGTAGTAGATCAACTCGAAGG
CG18110_F	GTACTTCCAGGAGGAGACC
CG18110_R	CCTCCATAGGAAACCATTAAGTC
P24-2_F	TTTCCAAGCGATTTGCCAAGCGAG
P24-2_R	AGAACAATTTGGATCCGCGCAACG

5.2.2 RNA-seq

About 60 wing imaginal discs from 3rd instar larvae were dissected for each genotype. We isolated total RNA from these discs using TRIzol and the quality was assessed using an Agilent Bioanalyzer. The RNA samples were then submitted to IRCM molecular biology core facility to capture mRNA using poly(T)-coated magnetic beads, followed by reverse transcription to generate cDNA library and whole-transcriptome sequencing at Genome Quebec using an Illumina sequencer. With the help of the bioinformatic facility in IRCM, the obtained Ensembl annotation from the Illumina iGenomes was aligned to the *Drosophila melanogaster* reference genome (dm3) with TopHat. Almost 14 000 genes were mapped, and subsequent analysis was done using DESeq and edgeR packages.

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