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Title of Thesis: Roles of Mind bomb 2, microRNAs and tissue architecture in the regulation of collective cell migration in *Drosophila*

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ABSTRACT

Title of Document:	ROLES OF MIND BOMB 2, MICRO-RNAS AND TISSUE ARCHITECTURE IN THE REGULATION OF COLLECTIVE CELL MIGRATION IN *DROSOPHILA*
	Sunny Trivedi, Ph.D., 2021
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Cell migration is essential in animal development and co-opted during metastasis and inflammatory diseases. Some cells migrate collectively, which requires them to maintain epithelial characteristics, such as stable cell-cell adhesions, while adopting motile characteristics, such as rapid turnover of adhesions and dynamic cytoskeletal structures. How this regulation is unclear but important to study. We examined this issue at the levels of protein regulation, gene regulation, and physical regulation. While investigating *Drosophila* oogenesis, we found that the putative E3 ubiquitin ligase, Mind bomb 2 (Mib2), is required to promote epithelial stability as well as the collective cell migration of border cells. *mib2* mutant follicle cells have drastically reduced E-cadherin-based adhesion complexes and lower levels of actin filaments. Through mass spectroscopy and biochemical analysis, we identified components of Mib2 complexes, which include E-cadherin and α- and β-catenins, as well as actin regulators. We also found new roles for three Mib2 interacting proteins, RhoGAP19D, Supervillin, and Modulo, in border cell migration. We conclude that Mib2 acts to stabilize E-cadherin-based adhesion complexes and promote a robust actin cytoskeletal network, which is important both for epithelial integrity and collective cell

migration. In addition to protein regulators, the Janus Kinase/Signal Transducer and Activator of Transcription (JAK/STAT) pathway is required during border cell migration to modulate gene expression. To get a deeper understanding of JAK/STAT signaling regulation, we examined candidate microRNAs. We focused on microRNAs predicted to regulate or be regulated by STAT signaling components such as Apontic and Slow border cells. Our analysis suggests that *miR-8*, *miR-279, mir-315,* and *let-7* may act within a JAK/STAT signaling feedback loop to influence collective cell migration. In the third type of regulation, prior work suggests that the JAK/STAT activator Unpaired (Upd) asymmetrically signals in egg chambers owing to the tissue architecture. Here, we developed a new tool to assay the distribution of Upd in extracellular regions of egg chambers. This will be used to study how the contours of surrounding cells affect the JAK/STAT activation pattern in follicle cells. In our work, we identified several regulators of cytoskeletal meshwork and JAK/STAT signaling that are crucial in oogenesis and provide avenues for further research. Both collective cell migration and JAK/STAT signaling are involved in autoimmune disorders and metastatic cancer progression, so our results may apply to other contexts.

ROLES OF MIND BOMB 2, MICRO-RNAS AND TISSUE ARCHITECTURE IN THE REGULATION OF COLLECTIVE CELL MIGRATION IN *DROSOPHILA*

By

Sunny Trivedi

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Preface

In this thesis, I describe my research into the molecular regulation of Janus Kinase/Signal Transducer and Activator of Transcription (JAK/STAT) signaling and collective cell migration. In the introduction chapter, I discuss the important roles of JAK/STAT signaling and its relevance to cancer metastasis (Trivedi & Starz-Gaiano, 2018). We describe the molecular components in the highly regulated JAK/STAT signaling pathways and point out how these are crucial in collective cell migration, as well as in other cell types. We also describe the involvement of JAK/STAT signaling in breast cancer, prostate cancer, and blood disorders. We shed some light on the effectors and targets of JAK/STAT signaling in both mammals and *Drosophila*. Our work attempts to encourage the scientific community to use JAK/STAT signaling as a therapeutic target for various disorders. Due to the relative simplicity but impressive genetics available in *Drosophila*, we and others continue using flies to investigate cell migration and its mechanisms (Tolwinski, 2017).

Collective cell migration, including cancer metastasis, requires a very well-coordinated set of mechanisms that can be activated by pathways like JAK/STAT but ultimately depends on changes in cytoskeletal and adhesion proteins (Saadin & Starz-Gaiano, 2016a). The migrating cells must carefully maneuver using a delicate balance of adhesion proteins, polarity proteins, and cytoskeletal proteins, among many other factors. The border cells after specification, rely on regulators such as E-cadherin (E-cad) and actin to detach from the neighboring non-migrating epithelial cells and to move along other cells (Montell et al., 2012), which is similar to what occurs in carcinoma metastasis. This intricate cellular machinery is very dynamic compared to epithelial cells, and more remains to be discovered on how such a difference is regulated.

Related to this cytoskeletal regulation, I dive deeper into characterizing an E3 ligase, Mind Bomb 2 (Mib2), in chapter 2. Although Mib2 was originally proposed to be a JAK/STAT regulator (Müller et al., 2008), we discovered its novel role in cytoskeleton stabilization in egg chambers. Since Mib2 is an E3 ligase, we speculate that Mib2 is monoubiquitinating its target proteins to provide them stability. We show that Mib2 can physically interact with multiple cytoskeletal proteins. Additionally, Mib2 stabilizes β-catenin, E-cad, and actin in epithelial cells. Our studies show *mib2* is required for border cell migration, and the expression of domain mutants suggest that certain regions of Mib2 may have particularly interesting roles in this process. Moreover, our results indicate that *mib2* has a complex regulatory relationship with JAK/STAT signaling in fly ovary.

Given the precise regulation needed to control JAK/STAT signaling, we hypothesized that microRNAs (miRNA) could be involved. In chapter 3, I explain our work on several miRNAs and their involvement in JAK/STAT signaling as well as border cell migration. We show that *miR-8*, *miR-279*, *miR-315*, and *let-7* may associate with JAK/STAT signaling and could be involved in its regulation. Our transcription factor binding site studies reveal that JAK/STAT components, including STAT and two downstream transcription factors, have consensus binding sites near most of these miRNAs. The relevant miRNAs are also implicated in other signaling pathways such as Notch and EGFR, which are a part of circuitous regulation of collective cell migration, so these may function in cross talk (Morante et al., 2013; Saadin & Starz-Gaiano, 2016a; Vallejo et al., 2011). Particularly, the *miR-8* is involved in the border cell migration and should be further focused on for its mechanism.

Chapter 4 summarizes our efforts and highlights the importance of our work. I also suggest several future directions that would expand on all of our results to date and how to best interpret them.

Dedication

I would like to dedicate my thesis work to my family members for their unconditional love, friends for their support, and mentors for their guidance.

Acknowledgments

I am forever grateful to my mom for her above and beyond love for me. It has been a mission of her life that I achieve new heights and grow up to be an upstanding human being. At the end of the day, if nothing is right in my life, I can still look up because I have her. Her words “Sona ne kyathi laage kaant, Sansari mann ma, sona ne laage kyathi kaant - A golden heart can never be rusty with malign thoughts” inspires me to always be kind to others. I am also thankful to have my granny and my aunt for their immense love and care for me. From financial to emotional support, these ladies have always got my back. Last but by no means least, my fiancé has been the rock-solid support behind my success. She gets the highest accolade for her immense love and care, for guiding me through my research blunders, and for sticking with me through thick and thins. Thank you, all ladies, for being four pillars of my life.

Until my Ph.D., I had always been fortunate that I could work with the most amazing mentors. However, during my Ph.D. Dr. Michelle Star-Gaiano blew away all the records. I can say without a doubt that she is an undisputed best mentor ever. She is one of the rare people who has mastered all four; critical scientific thinking, respect for everyone, skillfully managing her busy schedule, and being a super mom. It has been an honor learning from her, and if someday I can be even a fraction of the scientist that she is, I would consider it as my lifetime achievement. May her force be with me forever. A big thanks to my current and past lab members, for sharing some good laughs, discussing nerdy science, and especially not letting me forget my meetings. I am also very grateful to my committee members, Dr. Brewster, Dr. Miller, Dr.

Bieberich, and Dr. Andrew, for showing guiding me and making me a better scientist. I have been fortunate to teach with Dr. Feezer, Dr. Smith, Dr. Wagner, Dr. Fleishmann, and Dr. Eisenmann and learn a great deal of mentoring skills over the years. The biological department staff is the kindest and helpful group of professionals that has great respect in my heart.

I am very thankful to have friends who helped me manage my work-life balance. They made me feel like a home far away from home. The 6 years of my Ph.D. flew by just like that because of our walks, cooking and tea sessions, trips, cricket, and Taarak Mehta Ka Ooltah Chashma sessions.

A Ph.D. may be awarded to an individual but can never be earned by just an individual. Thank you, everyone, for pitching in and being a part of my microenvironment; this success goes to you all.

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Chapter 1: Introduction

Trivedi, S.; Starz-Gaiano, M. *Drosophila* Jak/STAT Signaling: Regulation and Relevance in Human Cancer and Metastasis. *Int. J. Mol. Sci.* **2018**, *19*, 4056. https://doi.org/10.3390/ijms19124056

1.1) Overview

The Janus kinase (JAK) and Signal Transducer and Activator of Transcription (STAT) signaling pathway is crucial in the regulation of immune response, stem cell regulation, and determining cell identities in diverse organisms. In the late 1980s and early 1990s, this signaling cascade was shown to be central to interferon response in humans (reviewed in (Stark and Darnell 2012; O'Shea and Murray 2008)), and its homologs were soon identified in *Drosophila* (Yan et al. 1996; Harrison et al. 1998; H.W. Chen et al. 2002; Brown, Hu, and Hombria 2001; Hou, Melnick, and Perrimon 1996). The demonstration that activating mutations in JAK produced neoplastic growth in flies, particularly in blood cell-like lineages (H. Luo, Hanratty, and Dearolf 1995; H. Luo et al. 1997; Hanratty and Dearolf 1993; Harrison et al. 1995), illustrated the striking similarity between the pathways across the animal kingdom, since soon after, deregulated STAT function was linked to human hematopoietic malignancies, and activating mutations in JAK was linked to leukemia and other myeloproliferative disorders (Lacronique et al. 1997; Peeters et al. 1997; Stark and Darnell 2012; Ward, Touw, and Yoshimura 2000) . Given these parallels, it is no surprise that detailed characterization of the JAK/STAT pathway in *Drosophila* has been very informative about its functional mechanisms in humans.

Here, we broadly compare and contrast the JAK/STAT signaling cascade in mammals and *Drosophila*. We review studies linking key JAK/STAT regulators with human disease, especially blood cell cancer, cancer stem cells, and metastatic cancers derived from breast and prostate. We also describe well-characterized cell types and phenotypes affected by loss and gain of function of JAK/STAT pathway components in *Drosophila* and discuss how flies can be useful for the identification of new pathway regulators. Finally, we explore connections between genes determined to be JAK/STAT regulators in *Drosophila* and their human

homologs that are linked to disease and highlight candidates for further study based on their involvement in both contexts.

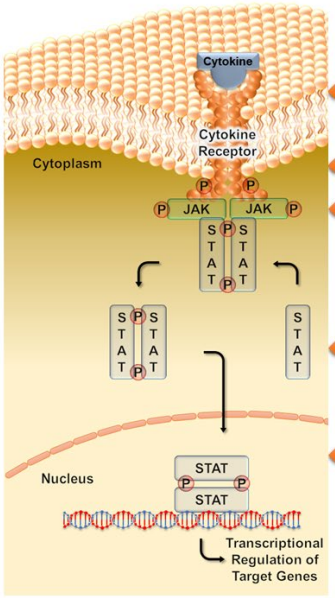
1.2) JAK/STAT signaling overview in flies and humans

Extracellular cues trigger JAK/STAT signaling, which ultimately leads to transcriptional activation of target genes (Figure 1.1). The basic framework for this signaling is the same across species, but the mammalian signaling system includes families of proteins with overlapping roles, while the fly cascade has fewer components and little redundancy. In humans, a set of more than 40 interleukins and cytokines serve as activating cues (reviewed in (Bromberg and Darnell 2000; Schindler and Plumlee 2008; Arbouzova and Zeidler 2006)). In flies, only three proteins hold this function: Unpaired (Upd) 1, Upd 2, and Upd 3 (Harrison et al. 1998; Hombria et al. 2005; L. Wang et al. 2014; Wright et al. 2011). Given the array of activators, mammals have multiple cell-surface receptors that can act singly or multimerize to respond to their diverse set of ligands (Levy and Darnell 2002; Murray 2007). In contrast, only one receptor has been determined in flies, called Domeless (Dome) (H.W. Chen et al. 2002; Brown, Hu, and Hombria 2001; Ghiglione et al. 2002; Silver and Montell 2001). Receptor-ligand binding activates JAK proteins docked to the cytoplasmic portion of the receptor. There are four Janus kinases in humans (JAK1-3 and Tyr2), which bind different receptors. One JAK protein is found in flies, which is most similar to JAK 2. Like most *Drosophila* genes, the gene encoding JAK is named after its loss of function phenotype; due to defective segmentation and skipped segments in the cuticular patterns of late embryos/early larvae, the mutant was named *hopscotch* (*hop*, with similar phenotypes observed for *unpaired* mutants) (Hanratty and Dearolf 1993; Perrimon and Mahowald 1986). Janus kinases have a well-conserved structure, featuring a kinase domain, a similar pseudokinase domain without catalytic activity, and a band 4.1-ezrin-radixin-moesin (FERM) domain that binds to the receptor and contributes to the

regulation of kinase activation upon receptor-ligand binding (Yamaoka et al. 2004). Activated JAK targets a second JAK associated within the same receptor dimer or multimer, and the subsequent phosphorylations create binding sites for cytoplasmic Stat proteins. There exist seven STAT family members in humans (STAT1-4, 5a, 5b, and 6), but only one in flies: Stat92E, which is most similar to STAT5b (Hou, Melnick, and Perrimon 1996; Yan et al. 1996; Sweitzer et al. 1995). Conserved domains in STAT proteins include the coiled-coil, SH2, DNA binding, and transactivation domains (Levy and Darnell 2002). While non-phosphorylated STAT has roles in chromatin regulation (Yang and Stark 2008; Shi et al. 2006; Brown and Zeidler 2008), the best-studied roles for the protein family are those that occur after it is "activated" by phosphorylation. Phosphorylated STAT dimerizes, which promotes its translocation into the nucleus, where it directly binds DNA and recruits transcriptional activators (Levy and Darnell 2002). Thus, the canonical JAK/STAT pathway results in changes in gene expression, including amplifying the expression of its own regulators.

As the various STAT proteins in humans can homo- or hetero-dimerize and can be activated by numerous permutations of the ligands, receptors, and JAKs, the combinatorial outcomes are very complex. Thus, the stripped-down pathway that exists in *Drosophila* is important to provide a tractable but still very relevant system for characterization of this signaling cascade and its essential regulators.

JAK-STAT Signaling Core Components	
Drosophila Components	**Human Homologs (Protein Family)**
Unpaired (Upd) 1, 2, 3	Cytokines and Interleukins
Domeless (Dome)	Interleukin-6 receptor (IL receptor family)
Hopscotch (Hop)	Janus Kinase (JAK) 1, 2, 3, Tyk 2
Stat92E	STAT1, 2, 3, 4, 5a, 5b, 6



Drosophila JAK-STAT	Drosophila Regulators	Human Homologs (Protein Family)
Upd Regulators	Jafrac2 Imp	PRDX4 (PRDX1, 2, 3, 4) IGF2BP1 (IGF2BP1, 2, 3)
Dome activation	Et Socs36E	GP130 (IL6 receptor family) Socs5 (Socs1 to 7, CIS)
Hop activation	Socs44A	Socs1 (Socs1 to 7, CIS)
STAT92E activation	Asrij Slbo Socs36E dPias Ptp61f	OCIAD1 (OCIA domain containing proteins) CEBP-D (CEBP-A, B, D, E, G) Socs5 (Socs 1 to 7, CIS) PIAS1 (7 PIAS proteins) PTPN1 (Protein Tyrosine Phosphatases)
STAT92E DNA binding activity	Ken Eya E(bx)	BCL6 (BTB domain containing proteins) Eya2 (Eya1, 2, 3, 4) BPTF (PHD finger domain proteins)
Additional Regulators	BRWD3 Pzg Lig dCsk	PHIP (Bromodomain containing proteins) REST (KZNF transcription factor family) UBAP2 (Ubiquitin associated proteins) CSK (C-Src Kinase family)

Figure 1.1) The *Drosophila* JAK/STAT signaling components and corresponding human homologs and their protein families. Interleukin or cytokine (the Upd family in flies) binds to its receptor (Dome in flies), which activates the associated JAK (Hop in flies) and triggers a chain of events. Activated JAK phosphorylates other JAKs and the receptor, creating a binding site for STAT proteins. Recruited STAT proteins (Stat92E in flies), are then phosphorylated. The activated phospho-STATs dimerize and translocate to the nucleus. The STAT DNA binding domain recognizes promoter and enhancer regions of target genes, resulting in their transcriptional activation. The table in Figure 1.1 lists the core components of the canonical pathway. The table to the right delineates key regulators of the fly JAK/STAT pathway, their respective human homologs, and their protein families.

1.2.1) Requirement of JAK/STAT signaling in development and adulthood

In both flies and mammals, normal early development requires correct JAK/STAT signaling, and pathway misregulation later in life is detrimental. Some components are expressed in a cell-type-specific way. Humans with inborn errors in JAK/STAT genes that are important in blood cell lineages are immunocompromised (Casanova, Holland, and Notarangelo 2012). Additionally, abnormally high JAK and STAT activities in adults have been closely associated with autoimmune disease, cell overproliferation, acquisition of blood cell disorders, cancer progression and poor cancer prognosis (Valentino and Pierre 2006; Boudny and Kovarik 2002; Ward, Touw, and Yoshimura 2000; Dorritie, Redner, and Johnson 2014; Bromberg and Darnell 2000). In light of this, much research is directed at understanding the signaling pathway.

While some null JAK/STAT pathway mutations cause tissue-specific defects, presumably others would not allow human development to term, as evidenced by mouse genetic studies. Mutations in genes encoding positive signaling components result in early lethality in mice or cell-type-specific effects (Akira 1999; Levy and Darnell 2002). For example, mutant *JAK1* and *JAK3* mice have Severe Combined Immunodeficiency (SCID), and *JAK1* mutants also have neurological defects and poor survival past birth; knock out mutations in *JAK2* are embryonic lethal, and mutations in the JAK family member *Tyrosine kinase 2 (TYR2)* result in poor response to pathogens (Yamaoka et al. 2004). Similarly, *Stat1* mutant mice have abnormal immune responses and are more susceptible to infections than wild type (Akira 1999), and show significant neurodegeneration as adults (Campbell 2005). *Stat3* mutant mice die in early embryogenesis and tissue-specific mutations result in changes in the proliferation/apoptosis balance in blood cells, poorer cell motility, and inflammation (Akira 1999; Levy and Darnell 2002). *Stat5a* and *b* have overlapping, required roles in the mammary

gland and ovary development, as well as being important in blood cell proliferation and cytotoxic activity (Akira 1999; Hennighausen and Robinson 2008). Female *Stat5a/b* double knock out mice are sterile.

Mutations that block JAK/STAT signaling in *Drosophila* result in early lethality; however, this can be overcome experimentally using sophisticated genetic tools that allow fly researchers to test mutations in individual cell types or at certain times in development. These types of experiments can be performed by using temperature-sensitive mutations, by tissue-specific expression control through the Gal4/UAS system, or by using clonal mosaic analysis, in which most cells of the organism are heterozygous and small clones of cells are homozygous mutant. (For reviews on methodology and JAK/STAT related tools, see (del Valle Rodriguez, Didiano, and Desplan 2011; Q. Chen, Giedt, et al. 2014) for stat-specific genetic tools). These strategies revealed essential functions for JAK/STAT signaling in sex determination (Jinks et al. 2000; Sefton et al. 2000; Arbouzova, Bach, and Zeidler 2006; Wawersik et al. 2005) as well as in cellular functions in diverse cell types that include fly blood cells (Agaisse and Perrimon 2004; Minakhina and Steward 2006), wing precursors (Ekas et al. 2006; Recasens-Alvarez, Ferreira, and Milan 2017), eye progenitor cells (Zeidler, Perrimon, and Strutt 1999), gut stem cells (Lengyel and Iwaki 2002; Nagy et al. 2016; Buchon et al. 2009), adult testes stem cells (Leatherman and Dinardo 2010; Tulina and Matunis 2001; Kiger et al. 2001) and adult ovary cell types (Beccari, Teixeira, and Rorth 2002; Silver, Geisbrecht, and Montell 2005; Silver and Montell 2001; Ghiglione et al. 2002) (see Table 1.1). (For recent reviews on Drosophila JAK/STAT signaling in specific contexts, see (Amoyel and Bach 2012; Hombria and Brown 2002; Fossett 2013; Bausek 2013)(stem cells), (Amoyel, Anderson, and Bach 2014)(tumors), (Morin-Poulard, Vincent, and Crozatier 2013; Agaisse and Perrimon 2004; Minakhina and Steward 2006)(hematopoiesis and immunity), (Hombria and Sotillos 2013)(morphogenesis), (Silver-Morse and Li 2013)(heterochromatin), (Zoranovic, Grmai, and Bach 2013)(cell-cell

competition), (Keebaugh and Schlenke 2014)(host-parasite interactions)). In many of these contexts, loss of signaling produced abnormal phenotypes, and so too did unusually high levels of signaling. These experiments implicate disruptions in JAK/STAT function to defects in stem cell maintenance, cell survival, proliferative defects, cell fate specification, and cell migration in a variety of tissue types.

1.2.2) JAK/STAT activity regulators

Given the many diverse roles for JAK/STAT signaling, and the fact that either too much or too little signaling can produce abnormal effects, it is not surprising that the pathway is subject to many levels of regulation. Estimates based on *Drosophila* cell culture screens suggest there are on the order of hundreds of regulators (Baeg, Zhou, and Perrimon 2005; Fisher et al. 2012; Muller et al. 2005).

Multiple regulatory proteins were initially discovered in mammalian contexts, then shown to play similar roles in flies. Among the first to be characterized was the family of proteins called Protein Inhibitor of Activated STAT (PIAS). These proteins bind to activated STATs to block DNA binding and transcriptional activity and have roles in the SUMOlyation and downregulation/degradation of signaling components (Rakesh and Agrawal 2005; Starr and Hilton 1999; Wormald and Hilton 2004; Rytinki et al. 2009). There are 7 mammalian PIAS proteins encoded by 4 genes, and each can differentially target STATs or affect other transcription factors. The single PIAS encoded in *Drosophila* has been shown in blood cells and eyes to inhibit STAT activity (Betz et al. 2001). Members of the Suppressor of Cytokine Signaling (SOCS) pathway function as feedback inhibitors of STAT activity in mammals and flies (Levy and Darnell 2002; Zeidler and Bausek 2013). These can bind JAKs or the receptor directly through an Src Homology 2 (SH2) domain and reduce kinase activity, which occurs through recruitment of proteins to promote ubiquitination and degradation (Alexander and

Hilton 2004; Croker, Kiu, and Nicholson 2008). Eight SOCS proteins have been characterized in mammals; several of these have specific regulatory links to certain STATs, as well as roles in downregulating other signaling pathways. Three SOCS proteins are encoded in flies but only two (Socs36E and Socs44A) are known to regulate JAK/Stat signaling (Rawlings et al. 2004; Callus and Mathey-Prevot 2002; Karsten, Hader, and Zeidler 2002) and in some contexts, these also modulate signaling through Epidermal Growth Factor Receptor (EGFR), Ras, and Map Kinases (Amoyel et al. 2016; Almudi et al. 2009). Fly Socs36E has an intrinsically disordered domain and acts to recruit upstream JAK/STAT pathway components to Cullin-dependent degradation (Monahan and Starz-Gaiano 2015), similar to how it acts in human cells. In both flies and mammals, STAT transactivates *Socs* gene expression, creating feedback inhibition of the pathway (Monahan and Starz-Gaiano 2013; Zeidler and Bausek 2013; Callus and Mathey-Prevot 2002; Matsumoto et al. 1997; Matsumoto et al. 1999; Endo et al. 1997). An additional well-characterized class of regulatory proteins are the Protein Tyrosine Phosphatases (PTPs) that directly dephosphorylate JAKs and potentially STATs (Baeg, Zhou, and Perrimon 2005; Aoki and Matsuda 2000; Myers et al. 2001), reversing their activation. This is a very large family in mammals, with cell-type-specific expression, and multiple members have been shown to function in JAK/STAT signaling, especially in a cell-type-specific manner (Mustelin, Vang, and Bottini 2005). So far, one member of this family, Ptp61F, is linked to JAK/STAT signaling in *Drosophila* (Baeg, Zhou, and Perrimon 2005; Muller et al. 2005; Saadin and Starz-Gaiano 2016b). Finally, regulation of receptor internalization via endocytosis is a regulatory mechanism common across species that modulates JAK/STAT signaling (Strous et al. 1996; O.M. Vidal et al. 2010; Devergne, Ghiglione, and Noselli 2007; Kurgonaite et al. 2015; Ren et al. 2015; Radtke et al. 2010).

More recently, flies have been used to identify novel regulators of JAK/STAT signaling. This has been accomplished by identifying mutants with a similar phenotype, by leveraging unbiased genetic enhancement and suppression screening, and by assaying pathway activity in tissue culture cells. Many of the proteins identified in this way have human homologs that may play analogous roles. Given that JAK/STAT activity has important functions in immune response, cell motility, and stem cell maintenance in both humans and flies, genetic analysis in the fly provides a valuable strategy to elucidate critical regulators in these processes. Thus, studying different cellular contexts and using multiple approaches will be useful for determining how all of these components are controlled and contribute to fine-tuning the pathway in normal conditions to prevent pathological states. We will describe JAK/STAT regulation in three contexts: mammalian blood cell differentiation and proliferation, stem cell signaling, and cell motility and metastasis, and will draw parallels with regulation of these processes in flies via JAK/STAT signaling. Finally, we will explore the potential contributions for homologs of *Drosophila* JAK/STAT regulators in metastasis.

1.3) JAK/STAT signaling in blood cell proliferation and cell fate

1.3.1) JAK/STAT signaling and human blood cells

In humans, blood cells are produced from hematopoietic stem cells (HSC), which reside in a stem cell niche in the bone marrow. HSCs divide asymmetrically to renew and make multipotent daughter cells; depending on local factors, these will produce multipotent stem cells restricted either to lymphoid or myeloid lineages. JAK/STAT signaling is important in both this stem cell regulation and differentiation. Stat5 is expressed in HSCs and is necessary for their ability to self-renew (Schepers et al. 2012; Dorritie, Redner, and Johnson 2014), and Stat3 is also active in HSCs but is not strictly required (Y. Kato et al. 2005). Mouse knock out experiments demonstrate that both Stat3 and Stat5 are needed for B-cell development, and Stat5

is necessary for the differentiation of some myeloid cell types (Dorritie, Redner, and Johnson 2014).

Multiple different mutations in the human JAK/STAT signaling pathway or its positive regulators result in defective immune response or proliferative disorders in blood cells (Valentino and Pierre 2006; Dorritie, Redner, and Johnson 2014; Ward, Touw, and Yoshimura 2000; Levy and Darnell 2002). Loss of function mutations have been linked to severe combined immunodeficiency disease (SCID), autoimmune diseases, and inability to fight certain kinds of infections (Casanova, Holland, and Notarangelo 2012; Ward, Touw, and Yoshimura 2000). Conversely, aberrantly high JAK/STAT signaling is well-known for promoting myeloproliferative disorders including, leukemia and lymphoma (Ward, Touw, and Yoshimura 2000; Dorritie, Redner, and Johnson 2014). In these diseases, proliferation and poor differentiation of certain white blood cell types in bone marrow renders the immune system dysfunctional and causes overproliferation and tumors. Tel-JAK2 fusions that constitutively activate JAK are thought to be causative for some cases of chronic myeloid leukemias and acute lymphoblastic leukemia. Other hematopoietic malignancies show activation of JAK1 or JAK3 through different means (Ward, Touw, and Yoshimura 2000; Dorritie, Redner, and Johnson 2014). A rare chronic leukemia called myelofibrosis is most commonly caused by activating mutations in JAK2 (Staerk et al. 2007). This blood cell disorder alters stem cell dynamics in bone marrow and results in scarring or fibrosis of the bone marrow, disrupting the stem cell niche. Similarly, polycythemia vera is a rare neoplastic blood disorder often caused by activating mutations in JAK2 that result in the overproduction of red blood cells (Staerk et al. 2005). Transformation of blood cells by oncogenes often results in aberrant activation of Stat1, 3, or 5, depending on the originating cell type, and this can occur independently from canonical upstream signaling (Ward, Touw, and Yoshimura 2000; Dorritie, Redner, and

Johnson 2014). This activation can promote proliferation, differentiation defects, and stem cell-like character or neoplasia.

Since activating mutations lead to disease, several drugs are currently used therapeutically to suppress JAK/STAT signaling in patients. AG490 was the first JAK inhibitor characterized, and it has been used in the treatment of acute lymphoblastic leukemia, and other drug analogs have been shown to be effective in blocking acute myeloid leukemia progression/survival in cell culture (Dorritie, Redner, and Johnson 2014; Ward, Touw, and Yoshimura 2000; Faderl et al. 2005; Meydan et al. 1996). A JAK1/2 inhibitor, ruxolitinib, is the only therapeutic agent for the treatment of myelofibrosis in the US, but this drug is expensive. Tofacitinib (Xeljanz), a pan-JAK inhibitor, modulates immune response via JAK1/3 and STAT1 (Ghoreschi et al. 2011), and is approved in the US for treatment of rheumatoid arthritis. The JAK inhibitor ADZ1480 has been found to be effective in blocking growth and survival of cell lines derived from multiple different solid tumors/carcinomas (Dorritie, Redner, and Johnson 2014). Clinical trials are underway. While these drugs are promising, many have significant side effects, indicating that better drugs are needed.

1.3.2) *Drosophila* hemocytes as a model for blood disorders

Drosophila hemocytes show many similarities to human white blood cells and play critical roles in the immune response. The fly innate immune system acts through the production of antimicrobial peptides and the phagocytosis of pathogens. *Drosophila* has an open circulatory system and do not require oxygenated red blood cells. However, three types of blood cells differentiate from hemocytes: plasmatocytes, which phagocytose bacteria and are most similar to human macrophages, crystal cells, which are responsible for melanization of pathogens, and lamellocytes, which encapsulate large foreign invaders like wasp eggs (Lanot et al. 2001; Morin-Poulard, Vincent, and Crozatier 2013; Keebaugh and Schlenke 2014). The

main hematopoietic stem cells reside in the larval lymph gland. The posterior signaling center of the lymph gland produces Unpaired, which induces JAK/STAT signaling in hemocyte progenitors, maintaining stem-like character and preventing them from differentiating too soon (Agaisse and Perrimon 2004; Krzemien et al. 2007).

Like in mammals, JAK/STAT interacts with multiple signaling pathways in the hematopoietic compartment to control hemocyte differentiation and proliferation. Interactors include the combined homolog of Platelet-derived growth factor/Vascular-Endothelial growth factor, PVF2 (Munier et al. 2002; Bruckner et al. 2004), the Hippo pathway transcription factor, Yorkie (Anderson et al. 2017), the homolog of early B-cell factor, Collier (Krzemien et al. 2007), the GATA factors U-shaped, which promotes prohemocytes, and Pannier, which promotes differentiation (Sorrentino, Tokusumi, and Schulz 2007; H. Gao, Wu, and Fossett 2009; Minakhina, Tan, and Steward 2011). The JAK/STAT signaling regulator Asrij is expressed in blood cells and other cell types, suggesting it is important in differentiation as well (Inamdar 2003), and it regulates endocytic turnover of signaling components (Khadilkar et al. 2014). Interestingly, many of the transcriptional targets of STAT are conserved between fly hemocyte-derived tumors and HeLa cells (Bina et al. 2010), again supporting the idea that the signaling pathway is similar. Recent work indicates that quiescent hemocyte stem cells reside in adult flies, in addition to larval lymph glands, and are activated in response to infection (Ghosh et al. 2015), but the effects of JAK/STAT signaling on this have not yet been revealed. However, since JAK/STAT signaling is activated upon immune challenge in *Drosophila*, the pathway is necessary for effective immune response in larva and adults (Morin-Poulard, Vincent, and Crozatier 2013; Zeidler, Bach, and Perrimon 2000; Agaisse et al. 2003; Minakhina and Steward 2006; Agaisse and Perrimon 2004).

A dominant, activating mutation in *Drosophila* JAK, Hop^{Tum}, has provided an invaluable tool in the study of JAK/STAT signaling in flies (Hanratty and Dearolf 1993). This point mutation creates a single glycine to glutamic acid change in the pseudokinase domain, which renders the catalytic domain constitutively active (H. Luo, Hanratty, and Dearolf 1995; H. Luo et al. 1997; Hanratty and Dearolf 1993; Harrison et al. 1995). Mutants die in larval stages with dark, neoplastic tumors of a melanotic blood cell subtype, reminiscent of human leukemia (Dearolf 1998). A different point mutation, Hop^{T42}, creates an activating amino acid substitution in the kinase-like domain, which results in phenotypes similar to those due to Hop^{Tum} expression (H. Luo et al. 1997). When this conserved amino acid is substituted in mouse JAK2, the mammalian protein likewise is constitutively activated. Interestingly, expression of a causative fusion protein in acute myeloid leukemia, Runx1/AML1-ETO also caused expansion of hemocyte precursors (Sinenko et al. 2010), supporting the idea that *Drosophila* hemocytes provide a reasonable model for leukemia mechanisms.

A number of drug screens for JAK/STAT inhibitors have been performed using a cultured cell line derived from *Drosophila* embryonic hemocyte (B.H. Kim et al. 2010; B.H. Kim et al. 2008; Thomas et al. 2015b; Thomas et al. 2015a). Notably, drugs suppressing JAK activity in humans also work against the fly counterpart, indicating functional and structural conservation across species. For example, ruxolitinib inhibits JAK/STAT signaling in *Drosophila* cell culture (Thomas et al. 2015a). Notably, these studies showed that methotrexate specifically inhibits JAK/STAT signaling as well as the commonly-used ruxolitinib (Thomas et al. 2015b). Transferring this finding to cultured Hodgkin lymphoma cells, it was shown that methotrexate could suppress signaling even in the presence of the V617F activating mutation in JAK. Additionally, a novel compound MS1040 was identified a specific JAK inhibitor in fly cells and mammals (Eggert et al. 2004), which could have clinical relevance. In another interesting line of work, approved chemotherapeutic agents for humans were administered to

flies bearing a genetically-induced intestinal stem cell tumor (Markstein et al. 2014). Although some drugs could reduce the tumors, one class additionally caused overproliferation of normal stem cells. This effect was mediated via JAK/STAT activation and may reveal clues to the biology of tumor recurrence in humans. Since combination therapy is a strong direction for future treatments of myeloproliferative disorders and cancers, more work to identify specific and effective drugs is needed, and JAKs are a good target. These studies illustrate the utility of drug screening in *Drosophila*.

1.4) JAK/STAT regulation of stem cell character

1.4.1) JAK/STAT signaling, carcinomas, and cancer stem cells

Besides its involvement in blood cell cancers, JAK/STAT signaling activation is common in carcinomas. Activated Stat3 and Stat5 have both been linked to many types of carcinomas, as they promote tumorigenesis and cancer stem cells, and this has been extensively reviewed (Levy and Darnell 2002; Levy and Gilliland 2000; Bromberg and Darnell 2000; Calo et al. 2003; H. Yu et al. 2014; Islam et al. 2015). Cancer stem cells are rare, but can proliferate and are believed to contribute to resistance to treatment and cancer recurrence. STAT inhibition slows cancer cell growth and may change stem-like character; therefore, drugs to block the pathway, like ADZ1480, are heavily being investigated. One model posits that the cancer recurrence common with Stat-positive cancers is due to the ability of Stat to provide signals that induce niche-like properties, maintaining cancer stem cells non-autonomously (H. Yu et al. 2014). As with hematopoietic stem cells, cancer stem cells are subject to many regulatory factors that impact the decisions to self-renew or differentiate. Since these stem cells are rare and usually hard to identify, it is helpful to examine stem cell regulation in other contexts. Research in *Drosophila* has provided strong insight into regulation of stem cells and the niches that support them.

1.4.2) *Drosophila* testes stem cells

The *Drosophila* testis is an outstanding model for examining stem cell regulation, as it is well-characterized, clearly organized, and genetically accessible. Adult testes have two stem cell populations, the germline cells and the somatic cyst stem cells (Bausek 2013; Issigonis and Matunis 2011). Distal hub cells and cyst stem cells both act as a stem cell niche for the germline stem cells to maintain pluripotency. As cells divide and move away from the niche, they begin to differentiate. Thus, the testis provides a well-suited context to study how different signals influence the balance of stem cell numbers and act in the stem cell niche. As in blood cells, gain or loss of JAK/STAT signaling in the testes results in dramatic phenotypes, disrupting stem cell regulation and proliferative control. In this case, low STAT activity leads to a loss of somatic stem cells, which in turn also causes germ line stem cell loss due to differentiation (Leatherman and Dinardo 2010; Tulina and Matunis 2001; Kiger et al. 2001; Singh et al. 2010; Issigonis et al. 2009). Conversely, somatic overactivation of the pathway leads to excessive cyst stem cells, which expands the stem cell niche and leads to production of higher numbers of germline stem cells. In the fly testes somatic stem cells, the transcription factor Zinc finger homeodomain 1 (Zfh1) is the key effector of JAK/STAT signaling, and itself is necessary and sufficient to promote stem cell fate (Leatherman and Dinardo 2008, 2010). The JAK/STAT negative regulators Socs36E and Ptp61F are important in this tissue as well (Singh et al. 2010; Issigonis et al. 2009; Amoyel et al. 2016; Issigonis and Matunis 2012). Higher Ptp61F leads to more differentiated cells(Issigonis and Matunis 2012). Loss of *Socs36E* leads to excessive signaling and overproliferation of stem cells and changes in stem cell adherence to the niche (Issigonis et al. 2009), which enables mutant cells to outcompete wild type neighbors (Amoyel et al. 2016). While these phenotypes are due in part to changes in JAK/STAT signaling, these regulators also have other targets. For example, Socs36E downregulates Map Kinase signaling in the testes, in addition to JAK/STAT activity (Amoyel et al. 2016).

Key transcription factors and epigenetic regulators have been found to impact the output of JAK/STAT activity in testes and provide feedback on signaling to prevent it from getting too high or too low. The B-cell lymphoma Bcl6 homolog, Ken and Barbie (Ken), is needed in the somatic stem cells to repress the JAK/STAT inhibitor Ptp61F; thus, Ken indirectly maintains high enough levels of JAK/STAT activity for stem cell self-renewal (Issigonis and Matunis 2012). Ken opposes STAT activity by repressing some STAT target genes. The positive STAT regulator nucleosome-remodeling factor (NURF) acts to promote JAK/STAT-mediated stem cell maintenance and prevents differentiation (Cherry and Matunis 2010). Conversely, Enhancer of polycomb, a part of a histone acetyltransferase complex (L. Feng, Shi, and Chen 2017; L. Feng, Shi, et al. 2018), and Ubiquitously transcribed tetratricopeptide repeat gene on the X chromosome (dUTX), a histone demethylase (Tarayrah et al. 2013), both increase *Socs36E* expression and downregulate STAT activity. These function to counteract JAK/STAT signaling and promote somatic cell differentiation instead of stem cell maintenance. Similarly, the transcription factor Apontic suppresses JAK/STAT signaling to limit the number of somatic stem cells and thus limit the size of the stem cell niche, also by promoting expression of *Socs36E* and probably a STAT-directed microRNA (Monahan and Starz-Gaiano 2016; Terry et al. 2006). These results implicate chromatin regulation in STAT target gene expression, and illustrate the tight regulation required on the pathway to make the amount of signaling optimal.

1.5) JAK/STAT signaling promotes cell motility and metastasis

JAK/STAT signaling activation has been linked to more metastatic cancers for a number of tumor types (Table 1.1). A number of lines of evidence support this idea, from higher levels of signal detected in human metastases to xenograph and cell culture assays, which show that carcinoma cells are more invasive or motile in response to higher JAK/STAT activity,

especially that of Stat3 and Stat5 (Valentino and Pierre 2006; Dorritie, Redner, and Johnson 2014; Ward, Touw, and Yoshimura 2000; Levy and Darnell 2002). To draw comparisons to the fly model, we will focus on two well-studied cases, breast and prostate cancers below. We focus our discussion to recent results linking JAK/STAT signaling to the acquisition of cell motility. It is worth noting that metastatic disease can also be promoted by immune system activation, which can be JAK/STAT dependent, but we will concentrate on cell-autonomous means of promoting cell motility. We go on to describe how JAK/STAT signaling and its regulation is critical in the proper determination of a motile cell type in *Drosophila*- the border cells – and we outline how border cell behaviors capture aspects of metastatic cell migration.

1.5.1) JAK/STAT signaling in breast cancer metastasis

About 80% of diagnosed breast cancers are invasive, which threatens advancement to metastatic disease (Y. Feng, Spezia, et al. 2018). Metastatic cancer is much more likely to be lethal than carcinoma *in situ*. JAK/STAT signaling is a key regulator of cell migration and proliferation in this context (Valentino and Pierre 2006; Dorritie, Redner, and Johnson 2014; Ward, Touw, and Yoshimura 2000; Levy and Darnell 2002). In particular, JAK2-STAT5b signaling is often overactivated in tumor cell proliferation and metastatic spread of breast cancer. An analysis of multiple breast cancer cell lines showed that STAT5b is often constitutively phosphorylated at Y699 and activated (Yamashita et al. 2003). Invasion into Matrigel is commonly used to assess a cell type's metastatic potential. STAT5b silencing significantly inhibited invasion of a metastatic breast cancer cell line (T47D), compared to controls. These data suggest there is significant participation of JAK2-STAT5b in promoting metastasis. Further studies show that combinational drug therapy targeting JAK2-STAT5b signaling inhibited breast cancer metastasis (Sp et al. 2015). Drug application downregulated STAT5b nuclear localization, binding activity, and downstream target gene expression.

Downregulation of JAK2-STAT3 by overexpression of WW domain-containing oxidoreductase (Wwox), which inhibits JAK2 phosphorylation, attenuates cell migration *in vitro* and suppresses metastasis *in vivo* (R. Chang et al. 2018). A particularly hard-to-treat subtype of breast cancer is Triple Negative Breast Cancer (TNBC), which lacks human epidermal growth factor receptor 2 (HER2) and hormone receptors for estrogen and progesterone (Aysola et al. 2013). JAK2 is often amplified in TNBC cell lines and specific inhibition of JAK2-STAT5 signaling with the drug ruxolitinib reduces proliferation of cells in culture, as well as tumor growth *in vivo* (Balko et al. 2016), suggesting this is a good avenue for developing treatment regimes. Upstream of JAK2 and Stat3, IL6 is also linked to breast cancer metastasis: IL6 expression is higher at the invasive leading edge of human primary breast cancer cells (Q. Chang et al. 2013). Overexpression of IL6 signaling induced metastasis and tumor growth *in vivo* mouse models, while its downregulation suppressed growth and reduced metastatic progression. Thus, the core components of canonical JAK/STAT signaling seem to be integral in mediating cell proliferation and metastatic disease.

Layered over the core signaling, conserved regulators of JAK/STAT signaling also impact human breast cancer metastasis, although not always as expected. Given the key role of JAK2, it is clear that regulators mediating its degradation have a critical function in preventing overactivation. SOCS protein family members are known for their negative regulation of JAK/STAT signaling (Croker, Kiu, and Nicholson 2008; Krebs and Hilton 2000). Socs1 is overexpressed in TNBC tissues and cell lines (Qian, Lv, and Li 2018). Socs1 is significantly associated with distant metastasis and its downregulation suppresses the proliferation of TNBC. Overexpression of Socs1 protein correlates with lymph node metastasis, large tumor size and advanced clinical stage in TNBC patients, but it is not likely to be targeting JAK/STAT in this case. In contrast, SOCS3 promotes ubiquitination of JAK2 to reduce its expression and regulate cytokine signaling, while TrkC (tropomyosin-related kinase C) prevents the SOCS3 mediated

ubiquitination of JAK2 (M.S. Kim et al. 2016). TrkC first binds and interacts with the c-Src/JAK2 complex to increase JAK2 and STAT3 levels, which induces Twist 1 and 2 expression. Carcinomas that become metastatic are thought to undergo an epithelial-to-mesenchymal-transition (EMT), in which they lose apical basal polarity, reduce E-cadherin-based adhesions, and adopt the characteristics of migratory, loosely connected mesenchymal cells. Twist 1 and 2 are known to be EMT-promoting transcription factors that may elevate metastatic potential.

Additional conserved regulators are relevant to breast cancer, although direct modulation of JAK/STAT activity has not been defined in all cases. PTP1B acts as an antiproliferative agent in this context. PTP1B acts a negative regulator of both STAT5 and JAK2 activation in invasive breast cancer cell lines (Johnson et al. 2010). Breast cancer patients with distant metastases show high levels of PTP1B protein (Liao et al. 2017). Another important JAK/STAT activity regulator is the transcriptional repressor BCL6. BCL6 appears to be important to promote mesenchymal properties of breast cancer cells (J.-M. Yu et al. 2015). E-cadherin is often downregulated during cell invasion to allow detachment from the tumor, and BCL6 serves a transcriptional repressor of *E-cadherin* in breast cancer cells. In breast metastatic lesions, reduction of activated nuclear STAT5a levels correlated to increase in BCL-6 cellular expression (Tran et al. 2010). Lastly, PIAS has not yet been shown to be a direct JAK/STAT signaling regulator in human breast cancer metastasis, but it is involved in the disease. Inhibition of the ligase activity of PIAS1 increased metastases to bone in mice after injection of human breast cancer cells (Dadakhujaev et al. 2014). Further investigation of these regulators of JAK/STAT signaling and of the pathway itself may shed more light on the intricate molecular mechanisms of breast cancer metastasis.

1.5.2) JAK/STAT signaling in prostate cancer metastasis

As with other carcinomas, JAK/STAT core components IL-6, JAK2, and STAT5, and other key regulators of this pathway are implicated in promotion of prostate tumor growth and metastasis, and there is no highly effective treatment to cure metastatic disease. Activated STAT5a/b indicates a poor prognosis (Hoang et al. 2015). Abnormal STAT5 activation is detected in 61% of distant prostate cancer metastasis, including 81% of those in lymph nodes in human clinical samples (Gu et al. 2010). Moreover, two human prostate cancer cell lines with metastatic potential (DU145 and PC-3) displayed a three-fold increase in cell migration in wound healing assays upon STAT5 activation. Furthermore, when DU145 cells expressing activated STAT5 were injected into mice, it resulted in eleven times more lung metastases compared to control injections without activated STAT. This indicates that STAT activation can drive metastasis. However, this mechanism might be dependent on Src kinases instead of JAK. STAT5 has been shown to interact with androgen receptor via its DNA binding domain and protect it against proteosomal degradation, which can induce tumor growth in prostate cancer cells and may not require JAK (Hoang et al. 2015). IL-6 receptor has also been shown to promote prostate cancer metastasis. Soluble IL-6 binds to gp130, which activates it, but inhibition of this reduced cell migration of DU145 cells in scratch assays and, conversely, increased soluble IL-6R expression in DU145 cells reduced their adhesion by 25% (Santer et al. 2010). IL-6 also downregulated the misshapen tumor suppressor in prostate cancer cell lines.

Further evidence for activated JAK2-STAT5a/b signaling leading to metastasis comes from the findings that this pathway regulates EMT markers (Talati et al. 2015). The activated signaling induces mesenchymal markers including the transcription factor Twist and stem cell factor BMI1, a polycomb group repressor component, and represses epithelial markers including E-cadherin in human prostate cell lines, xenograft mouse models, and patient derived

explant cultures. Interestingly, reduction of Twist suppressed the activated STAT pro-migration effects. STAT5 activation also prompted significant reduction of E-cadherin expression in xenographs using a cell line that normally has high levels of this adhesion molecule. The *in vivo* inoculation of DU145 prostate cancer cells with JAK2-STAT5a/b activated through expression of prolactin increased tumor metastasis by 69% in mice. Additionally, inhibition of JAK2 with the drug AZD1480 blocked JAK2-STAT5a/b signaling and suppressed prostate tumor growth in both cell culture and mouse models (Gu et al. 2013). Additional human JAK and STAT homologs with partial similarity to Hop and Stat92E, also have implications in prostate cancer (daSilva et al. 2013), which are not discussed here. Thus, clearly the IL6-JAK2-STAT5 axis plays a regulatory role in prostate cancer and metastasis.

Disruptions of key, conserved regulators of JAK/STAT signaling are also implicated in human prostate cancer. The SOCS family appears to be involved. SOCS1 causes significant reductions in wound closure and invasive behavior when it is stably expressed in prostate cancer cell lines (Villalobos-Hernandez et al. 2016). In mouse models having metastatic tumors, none of the SOCS1 expressing mice had macro metastasis as compared to the controls, suggesting a role for SOCS1 in metastasis suppression. SOCS1 expression was significantly reduced in patients with metastatic prostate cancer, likely due to overexpression of a regulatory microRNA. SOCS1 likely acts on the MET/Hepatocyte Growth Factor receptor tyrosine kinase. These data make SOCS1 a strong candidate to be investigated as a JAK/STAT regulator in this context. GP130, a receptor subunit of IL-6, increases the invasiveness of prostate cancer cells and reduces E-cadherin levels *in vitro* (Shariat et al. 2011). Patients with aggressive prostate cancer had elevated levels of soluble GP130. Thus, GP130 might exert upregulation of JAK/STAT signaling to contribute to prostate cancer metastasis and proliferation.

Other typical JAK/STAT regulators may function differently than expected in prostate cancer. Although PTP1B inhibits STAT activity, other PTPs seem to promote tumorigenesis or metastasis. PTP1B is overexpressed at the protein level in clinical samples of prostate tumors (Lessard and Labb 2012). The copy number of the gene encoding PTP1B was also increased by more than 20% in metastatic tumor samples. PTP1B silencing did not highly affect cell proliferation; however, it drastically impaired the migration and invasive properties of tumor cells in culture. These results suggest PTP1B functions independently from conventional JAK/STAT inhibition in prostate cancer metastasis. PIAS1 another protein expected to reduce JAK/STAT activity had elevated protein expression in primary tumors as well as in metastatic lesions upon sample analysis of patients with prostate cancer (Puhr et al. 2016). PIAS1 expression increased further after chemotherapy in resistant cells. Short term inhibition of PIAS1 resulted in reduced cell proliferation while long-term inhibition triggered apoptosis *in vitro*. Thus, like PTP1B, PIAS1 seems to have roles independent from suppressing proliferative JAK/STAT signaling. Although confirmation is needed to determine these mechanisms, it is interesting that multiple JAK/STAT signaling regulators are implicated in prostate cancer metastasis, even if they work through different modes of action.

1.5.3) JAK/STAT promotes border cell migration in the ovary

The first evidence for JAK/STAT signaling promoting cell motility came from *Drosophila* border cells, a subset of somatic follicular cells in the adult ovary (Silver and Montell 2001; Ghiglione et al. 2002; Beccari, Teixeira, and Rorth 2002). Egg chambers in the ovary, which will each give rise to one egg, develop as a set of germline cells surrounded by a somatic monolayer epithelium of follicle cells. (For a general fly oogenesis patterning review see (Duhart, Parsons, and Raftery 2017; Denef and Schupbach 2003)). At each pole of the developing egg chamber, polar cells form, which secrete Upd and Upd 3 (Harrison et al. 1998;

L. Wang et al. 2014; Van de Bor et al. 2011). STAT signaling is critical in this population early for regulating apoptosis and permitting survival of two polar cells (Borensztejn et al. 2013). Secreted Upd acts as a morphogen to pattern the follicular epithelium (Xi, McGregor, and Harrison 2003). In response to Upd, about 12 nearby post-mitotic cells activate STAT signaling and approximately six maintain high levels of STAT activation and become the motile border cells (Starz-Gaiano et al. 2008; Ghiglione et al. 2002; Silver and Montell 2001; Van de Bor et al. 2011). At a key time in egg development, these cells become motile, invade into the adjacent germline tissue, then migrate to the oocyte as a group surrounding the non-motile polar cells, where they are together required for patterning and egg structure. This chemotactic migration integrates signals from multiple signaling pathways, including EGF and PDGF/VEGF(Saadin and Starz-Gaiano 2016a). In addition to JAK/STAT's requirement in the acquisition of border cell motility, the cell cluster's continued migration relies on STAT activity (Silver, Geisbrecht, and Montell 2005). A key effector of this migratory fate decision is the transcription factor Slow Border Cells (Slbo, a homolog of human CEBPD). Interestingly, not only is STAT activation necessary for cell migration, but it is sufficient among epithelial cells in the egg chamber to induce motility (Silver and Montell 2001; Beccari, Teixeira, and Rorth 2002). Ectopic or continuous overactivation of the pathway results in too many cells becoming migratory. Thus, the ovary provides an ideal context for identification of JAK/STAT pathway regulators of cell motility, and the border cells are an outstanding model system for investigating genes that promote migration, which include many that are activated in metastasis (Naora and Montell 2005; Yoshida et al. 2004; Stuelten, Parent, and Montell 2018).

Border cell migration has been likened to an epithelial-to-mesenchymal transition (EMT), however, border cells move as a collective that retains some epithelial character. In particular, apical-basal polarity components are required (Pinheiro and Montell 2004; H. Wang et al. 2018; Sotillos et al. 2013). Additionally, while downregulation of E-cadherin is a classic

marker of EMT, in border cells it must be maintained. In fact, E-cadherin is an important downstream target activated by STAT and Slbo. Toward the center of the cluster, E-cadherin is highly concentrated and required there to maintain cluster integrity (Niewiadomska, Godt, and Tepass 1999; Cai et al. 2014), and probably is needed to maintain association between the Upd-secreting polar cells and the motile border cells to maintain STAT activity. At the outer edges of the cells, E-cadherin is needed for traction to move over germline cells, but its protein level is lower, presumably due to cell adhesions being constantly remodeled. Some current studies suggest carcinomas may also metastasize as mixed cell clusters similar to border cells (Stuelten, Parent, and Montell 2018; Friedl and Gilmour 2009; Haeger et al. 2015; Hegerfeldt et al. 2002; Khalil et al. 2017).

Since high levels of JAK/STAT signaling result in additional motile border cells, loss of function mutations in negative regulators yields this phenotype. Mutations in *Socs36E*, aSocs5 homolog, or Protein tyrosine phosphatase *Ptp61F*, a PTPN1 homolog, both disrupt border cell migration by allowing too many cells to become motile, due to excessive JAK/STAT signaling (Monahan and Starz-Gaiano 2013; Silver, Geisbrecht, and Montell 2005; Saadin and Starz-Gaiano 2016b). Interestingly, *Socs36E* transcription is activated in response to STAT activity, functioning as an autoregulatory break on the signaling system (Monahan and Starz-Gaiano 2013). Ptp61F is thought to reduce JAK/STAT signaling by dephosphorylation of the receptor or JAK or both, which is also the case in for the mammalian PTPs(Baeg, Zhou, and Perrimon 2005; Saadin and Starz-Gaiano 2016b).

A number of additional JAK/STAT regulators involved in border cell migration have been identified and characterized through unbiased genetic screens. In particular, numerous transcriptional regulators influence pathway output. The Bcl6 homolog Ken functions to promote sufficiently high JAK/STAT activity levels by transcriptionally suppressing a *stat-*

targeted microRNA, mir-279 (Yoon, Meinhardt, and Montell 2011). While a homologous microRNA has not been identified in humans, other microRNA regulators exist that target signaling components (H. Yu et al. 2014). An additional transcription factor in this regulatory network in border cells is the JAK/STAT feedback inhibitor Apontic, although the closest human homolog for this protein, Fibrinogen Silencer Binding Protein, is not very well studied (Starz-Gaiano et al. 2008). Apontic is activated by the Eyes Absent (EYA) transcriptional factor and promotes expression of *mir-279* and *Socs36E* to keep JAK/STAT signaling from getting too high (Starz-Gaiano et al. 2009; Monahan and Starz-Gaiano 2013). Recent studies have shown that a component of a chromatin remodeling complex, encoded by *Brahma*, genetically interacts with *Stat92E*, indicating that epigenetic regulation plays a key role in modulating the transcriptional output of the pathway (Saadin and Starz-Gaiano 2016b), as is the case in *Drosophila* testes stem cells (L. Feng, Shi, and Chen 2017; Cherry and Matunis 2010; Tarayrah et al. 2013; Maimon, Popliker, and Gilboa 2014).

In border cells, JAK/STAT regulation is also controlled by cellular trafficking mechanisms. Exocytic regulation of Upd release from polar cells requires SNARE components and NSF and alpha-Snap, which reset the SNARE complexes to permit vesicle trafficking (Saadin and Starz-Gaiano *in press*). Endocytic control regulates turnover of activated receptors, which can downregulate signaling (Devergne, Ghiglione, and Noselli 2007; Silver, Geisbrecht, and Montell 2005; O.M. Vidal et al. 2010) Additional regulatory components for JAK/STAT signaling at the molecular and cell biological level are currently under investigation.

1.6) Drosophila-JAK/STAT regulators implicated in human metastatic diseases

The power of *Drosophila* genetics lies in the ability to identify new components of a process in an unbiased way. This strategy has been fruitful in determining many components of JAK/STAT signaling in the cell types discussed above as well as in other contexts that are

beyond the scope of this review. As is clear, JAK/STAT signaling is functionally and mechanistically conserved between flies and humans, and many regulators are shared between these pathways. Still more regulatory players have been characterized to act on *Drosophila* JAK/STAT signaling but are not yet connected to this pathway in humans. In the following section, we discuss the human homologs of positive and negative *Drosophila* JAK/STAT regulators in cancer progression and metastasis (see Table 1.1). In many cases, the mechanisms by which these regulators cause/are involved in/result in cancer are unknown. We propose that their known interactions with JAK/STAT signaling in *Drosophila* lay the groundwork for exploring their potential involvement in different types of cells and understand their mechanisms in cancers.

1.6.1) Positive *Drosophila* JAK/STAT Regulators in Metastasis

Beside core components, multiple positive regulators of the *Drosophila* JAK/STAT signaling pathway have human homologs that are implicated in cancer progression and metastasis (Table 1.1). The ovarian protein OCIAD1 is overexpressed in metastatic cancer tissue compared to primary ovarian tumor tissues (Sengupta et al. 2008). Overexpression of OCIAD1 in the presence of lysophosphatidic acid induced cell adhesion to collagen and laminin in human ovarian cancer cell lines. Thus, OCIAD1 is a key positive regulator of ovarian cancer metastasis and its *Drosophila* homolog is a positive regulator of JAK/STAT signaling in hematopoiesis (Kulkarni et al. 2011). OCIAD2 is often associated with OCIAD1, and it also promotes STAT3 activation and cell migration in human cell culture (Sinha, Bheemsetty, and Inamdar 2018). This supports the hypothesis that OCIAD1 may upregulate metastatic potential via JAK/STAT regulation in human ovarian cancer.

Elevated levels of the Pleckstrin homology domain-interacting protein (PHIP) is predictive of distant metastasis and reduced survival in human melanoma (de Semir et al.

2012). Silencing PHIP in an ovarian cancer cell line reduced invasion into Matrigel by more than one-third and reduced metastatic potential by about half in a mouse model. Given that PHIP is a known positive regulator of JAK/STAT in flies, JAK/STAT involvement in PHIP-dependent metastasis is worth further investigation.

Silencing of the IGF2BP1 gene, which encodes an mRNA binding factor, caused reduced cell proliferation, migration and invasiveness of a cervical cancer cell line and HeLa cells in wound healing and trans-well migration assays (Y. Su et al. 2016). Conversely, overexpression of IGF2BP1 in fibroblasts elevated their invasiveness in Matrigel (T. Kato et al. 2007). In patients, high expression of this protein was associated with lung cancer progression. In vitro assays showed that IGF2BP1 downregulation by a microRNA reduced migration and invasion of an osteosarcoma cell line (Qu et al. 2016). Moreover, miR-150 suppressed tumor growth in an osteosarcoma xenograft mouse model via repression of IGF2BP1 (Y. Luo et al. 2015). In both hepatocellular carcinoma and glioblastoma, downregulating IGF2BP1 reduces tumor proliferation and invasion potential in their respective cell lines (Zhou et al. 2014). Thus, IGF2BP1 behaves as a pro-metastatic agent, consistent with a predicted role in promoting JAK/STAT activity, like its homolog acts in fly testes (Toledano et al. 2012).

PRDX4 is linked to brain cancer progression. Molecularly, it appears to be a thioredoxin peroxidase. The PRDX4 gene and protein are both upregulated in human glioblastoma multiforme (GBM) and mouse models of this disease (T.H. Kim and Song 2012). *In vitro* suppression of PRDX4 caused a reduction of GBM stem cell-like proliferation and prolonged survival in orthotopic transplantation to mouse. The PRDX4 homolog in flies, Jafrac2, promotes hemocyte overgrowth by upregulating JAK/STAT activity (Radyuk et al.

2013). Further investigation is required to determine if PRDX4 has a role in metastasis through JAK/STAT signaling.

The transcription factor CEBPD acts as a pro-metastatic regulator in urothelial and lung carcinomas. CEBPD overexpression increased urothelial cell migration and showed an increase in invasion potential *in vitro* (Y.-H. Wang et al. 2015). Inhibition of MMP2 significantly blocked CEBPD-induced migration and invasion properties. Additionally, lung tumor metastasis was significantly lower when tumor cells were injected CEBPD null mouse compared to a control. However, this suggests an indirect effect of CEBPD in metastasis. Overexpression of CEBPD in lymphatic cells increased cell migration *in vitro*, and conversely its repression inhibited it. In flies, the CEBPD homolog, Slbo, is a key transcriptional target of activated STAT in border cells (Silver and Montell 2001); thus, we propose CEBPD may be activated and function analogously to promote migration during tumor metastasis.

1.6.2) Negative *Drosophila* JAK/STAT Regulators in Metastasis

As is clear, abnormal activation of JAK/STAT can result in tumorigenesis and metastasis. Thus, negative regulators of the pathway could have roles in disease progression as well, if they are lost or blocked. As many negative regulators have been characterized in *Drosophila*, these are worthy of attention. The human proteins BPTF, UBAP2, REST and EYA2 have *Drosophila* counterparts that act to repress JAK/STAT signaling in various cell types (Table 1.1). While changes in each of these factors are associated with human cancer progression and metastasis, a direct connection to JAK/STAT signaling is less clear.

The nucleosome-remodeling factor (NURF) BPTF has been shown to be present in various cancers with different metastatic potentials. Examination of patient tumor samples indicates that BPTF is a suppressor of lung cancer metastasis to brain (Grinberg-Rashi et al. 2009). In hepatocellular carcinoma patients, BPTF was associated with low E-cadherin levels,

high tumor numbers, and more vascular invasion (Xiao, Liu, Fang, et al. 2015). Colorectal cancer patients who had higher BPTF expression tended to have poor survival (Xiao, Liu, Lu, et al. 2015), which suggests BPTF promotes cancer progression. In a cultured melanoma cell line, BPTF suppressed proliferative capacity and significantly reduced metastases upon intravenous injection in nude mice (Dar et al. 2015). However, in a different melanoma line, increased BPTF expression induced cell proliferation (Dar et al. 2016). Consistent with the latter, knockdown of BPTF in lung adenocarcinoma cell lines inhibited cell proliferation and lung cancer growth in *in vivo* mouse models (Dai et al. 2015). Given the repressive function of its *Drosophila* homolog (E-Bx) in JAK/STAT signaling, we hypothesize that BPTF could be involved in human cancer metastasis in part by suppressing JAK/STAT signaling. However, BPTF can be associated with pro- or anti-metastatic roles, which may mean changes in its expression are passive or indirect, and that it may act in human cancer pathogenesis via multiple mechanisms, or in cell-type-specific ways.

UBAP2 contains a ubiquitin-associated domain, so it is presumed to function in protein turnover. *UBAP2* is overexpressed at the gene level in samples collected from castration-resistant prostate cancer patients (Latonen et al. 2016). Additionally, UBAP2 is significantly overexpressed in advanced prostate cancer and is even higher in metastatic prostate cancer. In prostate cancer cell lines, reduction of UBAP2 copy number significantly reduced cell growth. On the other hand, UBAP2 was underexpressed in hepatocellular carcinoma (HCC) patient samples (D.-S. Bai et al. 2016). In HCC cell lines, knockdown of UBAP2 enhanced invasion, proliferation, and tumor growth *in vivo*. The *Drosophila* homolog mutates to embryonic lethality but has only been shown to affect JAK/STAT signaling in eye progenitor cell growth (Baumgartner, Stocker, and Hafen 2013), suggesting that it participates in cell-type-specific regulation. Thus, it seems that UBAP2 might have tissue dependent role in cancer metastasis and whether it acts via regulating JAK/STAT signaling in this context is worth further study.

Reduction of another putative JAK/STAT regulator, the transcription factor REST, increases LIN28A expression and tumor growth both in cell culture and *in vivo* models (Gunsalus et al. 2012). The tumor samples that showed low REST expression also showed local invasion. REST is also a proposed clinical marker for advanced prostate cancer (H. Liang et al. 2014). However, in spite of data suggestive of REST having an anti-proliferative role, there has not yet been demonstration of JAK/STAT signaling involvement in this function.

Lastly, the transcription factor EYA2 is upregulated in lung adenocarcinoma patient samples, and upon its knockdown in cell culture, tumor growth and invasion potential was suppressed (T. Gao et al. 2015). EGFR has been shown to activate EYA2 resulting in breast cancer growth, EMT, invasion, and lung metastasis *in vitro* and *in vivo* (Y. Liang et al. 2017). However, a majority of pancreatic adenocarcinoma patients showed loss of EYA2 in tumor cells (Vincent et al. 2014). Knockdown of EYA2 in pancreatic cancer cell lines increased cell proliferation, and stable EYA2 expression reduced metastasis in mouse xenographs. Further investigation on EYA2's mode of action and possible involvement of JAK/STAT signaling in cancer metastasis is an interesting area yet to be understood.

In summary, most of the human homologs of *Drosophila* positive JAK/STAT regulators are shown to be promoters of human cancer progression and/or metastasis. Understanding their mode of action in cancer progression possibly via JAK/STAT signaling could provide us a new direction towards therapeutic interventions.

***Drosophila* JAK/STAT component**	***Drosophila* tissue**	**Human homolog**	**Metastatic cancer type**
Signal Transducer and Activator of Transcriptio n92E	Brain (Copf et al. 2011) Embryo (Hou, Melnick, and Perrimon 1996; Yan et al. 1996)	Signal Transducer and Activator of Transcription 5b	Brain Cancer (Cao et al. 2011) Breast cancer (Sp et al. 2015; Yamashita et al. 2003)

(Stat92E)	Eyes (Ekas et al. 2006; Zeidler, Perrimon, and Strutt 1999) Hindgut (Johansen, Iwaki, and Lengyel 2003; Li et al. 2003) Lymph Glands (H. Gao, Wu, and Fossett 2009) Ovaries (Silver and Montell 2001; Beccari, Teixeira, and Rorth 2002; Xi, McGregor, and Harrison 2003; Borensztejn et al. 2013; Baksa et al. 2002) Primordial Germ Cells (Sheng et al. 2009; Li, Xia, and Li 2003; Brown, Zeidler, and Hombria 2006) Testes (Tulina and Matunis 2001; Kiger et al. 2001) Trachea (Li et al. 2003) Wing Disc (Recasens-Alvarez, Ferreira, and Milan 2017)	(STAT5b)	Colorectal Cancer (Wolf et al. 2012; H. Xiong et al. 2009; Klupp et al. 2015) Melanoma (Wellbrock et al. 2005) Pancreatic Cancer (Moser et al. 2012) Prostate Cancer (Gu et al. 2010; Hoang et al. 2015)
Hopscotch (Hop)	Brain (Copf et al. 2011) Embryo (Hou, Melnick, and Perrimon 1996; Perrimon and Mahowald 1986) Eyes (H. Luo et al. 1999; Zeidler, Perrimon, and Strutt 1999) Haltere Disc (Recasens-Alvarez, Ferreira, and Milan 2017) Hemocytes (Hanratty and Dearolf 1993) Hindgut (Li et al. 2003) Leg Disc (Recasens-Alvarez, Ferreira, and Milan 2017) Lymph Glands (Sorrentino, Tokusumi, and Schulz 2007) Somatic Muscle (Y.H. Liu et al. 2009) Ovaries (Xi, McGregor, and Harrison 2003; Beccari, Teixeira, and Rorth 2002; McGregor, Xi, and Harrison 2002; Silver and	Janus Kinase 2 (JAK2)	Breast Cancer (Balko et al. 2016; R. Chang et al. 2018; M.S. Kim et al. 2016) Bone Cancer (Yun et al. 2017) Cervical Cancer (C.L. Luo et al. 2016) Colorectal Cancer (X. Liu et al. 2015) Melanoma (Shin et al. 2017) Pancreatic Cancer (Das et al. 2015) Prostate Cancer (Talati et al. 2015; Gu et al. 2013)

	Montell 2001; Ghiglione et al. 2002) Trachea (Li et al. 2003) Testes (Tulina and Matunis 2001) Wing Disc (Recasens-Alvarez, Ferreira, and Milan 2017)		
Domeless (Dome)	Brain (Copf et al. 2011) Embryo (H.W. Chen et al. 2002; Brown, Hu, and Hombria 2001) Eye (Tsai and Sun 2004) Hindgut (Johansen, Iwaki, and Lengyel 2003) Lymph Gland (Sinenko et al. 2010; Khadilkar et al. 2014) Ovaries (Ghiglione et al. 2002; Medioni and Noselli 2005; Xi, McGregor, and Harrison 2003) Trachea (Brown, Hu, and Hombria 2001) Wing (Recasens-Alvarez, Ferreira, and Milan 2017)	Interleukin Receptor 6 (IL-6)	Breast Cancer (Q. Chang et al. 2013) Bone Cancer (Tu et al. 2012) Colorectal Cancer (Schneider et al. 2000; Zeng et al. 2017; X. Zhang et al. 2018) Hepatocellular Carcinoma (Pu et al. 2018) Ovarian Cancer (X. Zhang et al. 2018; Zou, Zhang, and Xu 2016) Pancreatic Cancer (Grunwald et al. 2016) Prostate Cancer (Santer et al. 2010)
Eye transformer (Et)	Lymph Gland (Makki et al. 2010)	Glycoprotein 130 (GP130)	Melanoma (Lacreusette et al. 2006) Prostate Cancer (Shariat et al. 2011)
Ken and Barbie (Ken)	Embryo (Kuhnlein, Chen, and Schuh 1998) Eyes (Lukacsovich et al. 2003) Genitalia (Kuhnlein, Chen, and Schuh 1998; Lukacsovich et al. 2003)	B Cell CLL/Lymphoma 6 (BCL6)	Breast Cancer (Tran et al. 2010)

	Ovaries (Yoon, Meinhardt, and Montell 2011) Testes (Issigonis and Matunis 2012)		
Protein Tyrosine Phosphatase (Ptp61f)	Eyes (Betz et al. 2001) Ovaries (Saadin and Starz-Gaiano 2016b) Testes (Issigonis and Matunis 2012)	Protein Tyrosine Phosphatase (PTP1B)	Breast Cancer (Johnson et al. 2010; Liao et al. 2017) Colorectal Cancer (Q. Chen, Li, et al. 2014) Esophageal Cancer (X.-M. Wang et al. 2013) Lung Cancer (Julien and Dub 2007) Melanoma (J. Liu et al. 2018) Ovarian Cancer (Fan et al. 2013) Prostate Cancer (Lessard and Labb 2012)
Protein Inhibitor of Activated STAT (Pias) (Su(var) 2-10)	Eyes (Betz et al. 2001) Hemocytes (Betz et al. 2001) Ovaries (Ghiglione et al. 2002)	Protein Inhibitor of Activated STAT (PIAS1)	Breast Cancer (Dadakhujaev et al. 2014) Gastric Cancer (P. Chen et al. 2012) Prostate Cancer (Puhr et al. 2016)
Suppressor of Cytokine Signaling (Socs44A)	Wings (Rawlings et al. 2004)	Suppressor of Cytokine Signaling 1 (SOCS1)	Breast Cancer (Qian, Lv, and Li 2018) Colorectal Cancer (David et al. 2014) Hepatocellular Carcinoma (Gui et al. 2017) Melanoma (Scutti et al. 2011) Prostate Cancer (Villalobos-Hernandez et al.

			2016)(Villalobos-Hernandez et al. 2016)
Suppressor of Cytokine Signaling (Socs36E)	Notum (Callus and Mathey-Prevot 2002) Ovaries (Monahan and Starz-Gaiano 2013; Silver, Geisbrecht, and Montell 2005) Testes (Amoyel et al. 2016; Issigonis et al. 2009; Singh et al. 2010) Wing Disc (Karsten, Hader, and Zeidler 2002; Callus and Mathey-Prevot 2002; Rawlings et al. 2004)	Suppressor of Cytokine Signaling 5 (SOCS5)	Colorectal Cancer (M. Su et al. 2018) Liver Cancer (Sanchez-Mejias et al. 2018)
Enhancer of Bithorax (E(Bx))	Testes (Kwon et al. 2009)	Bromodomain PHD Finger Transcription Factor (BPTF)	Brain Cancer (Grinberg-Rashi et al. 2009) Colorectal Cancer (Xiao, Liu, Lu, et al. 2015) Hepatocellular Carcinoma (Xiao, Liu, Fang, et al. 2015) Lung cancer (Dai et al. 2015) Melanoma (Dar et al. 2016; Dar et al. 2015)
Lingerer (Lig)	Eyes (Baumgartner, Stocker, and Hafen 2013) Imaginal Discs (Baumgartner, Stocker, and Hafen 2013) Ovaries (Costa et al. 2013)	Ubiquitin Associated Protein 2 (UBAP2)	Hepatocellular Carcinoma (D.-S. Bai et al. 2016) Prostate Cancer (Latonen et al. 2016)
Putzig (Pzg)	Heart (Cammarato et al. 2011) Wing Disc (Kugler et al. 2011)	RE1 Silencing Transcription Factor (REST)	Breast Cancer (Gunsalus et al. 2012)

			Prostate Cancer (H. Liang et al. 2014)
Eya	Eyes (Bonini, Leiserson, and Benzer 1993; Rayapureddi et al. 2003; W. Xiong, Dabbouseh, and Rebay 2009) Salivary Gland (Vining et al. 2005) Somatic Muscle (Y.H. Liu et al. 2009) Ovaries (J. Bai and Montell 2002; Medioni and Noselli 2005; Starz-Gaiano et al. 2009) Testes (Fabrizio, Boyle, and DiNardo 2003)	Eyes Absent 2 (EYA2)	Brain Cancer (Wen et al. 2017) Breast Cancer (Farabaugh et al. 2012; Krueger et al. 2014)
Asrij (Arj)	Head (Aradska et al. 2015) Hemocyte (Khadilkar et al. 2014; Inamdar 2003) Lymph Gland (Kulkarni et al. 2011) Trachea (Inamdar 2003)	Ovarian Cancer Immunoreactive Antigen Domain Containing 1 (OCIAD1)	Ovarian Cancer (Sengupta et al. 2008)
Bromodomain and WD Repeat Containing Protein 3 (BRWD3)	Eyes (W.Y. Chen et al. 2015) Heart (Cammarato et al. 2011) Midgut (Ihry and Bashirullah 2014) Salivary Gland (Ihry and Bashirullah 2014) Testes (Wasbrough et al. 2010)	Pleckstrin Homology Domain Interacting Protein (PHIP)	Melanoma (Bezrookove 2014; de Semir et al. 2012)
IGF-II-mRNA-Binding Protein (Imp)	Head (Aradska et al. 2015) Ovaries (Munro et al. 2006) Testes (Fabrizio, Boyle, and DiNardo 2003; Toledano et al. 2012)	Insulin-like Growth Factor II Binding Protein 1 (IGF2BP1)	Bone Cancer (Qu et al. 2016) Cervical Cancer (Y. Su et al. 2016) Hepatocellular Carcinoma (Jiang et al. 2017; Yuan, Meng, and Wang 2017; J. Zhang et al. 2015)

			Lung Cancer (T. Kato et al. 2007)
Thioredoxin peroxidase 2 (Jafrac2)	Head (Aradska et al. 2015) Heart (Cammarato et al. 2011) Hemolymph (Radyuk et al. 2013)	Peroxiredoxin 4 (PRDX4)	Brain Cancer (T.H. Kim and Song 2012) Lung Cancer (Hwang et al. 2015)
Slbo Border Cells (Slbo)	Ovaries (Montell, Rorth, and Spradling 1992)	CCAAT Enhancer Binding Protein Delta (CEBPD)	Lung Cancer (Min et al. 2011) Nasopharyngeal Carcinoma (Hsiao et al. 2013) Urothelial Cancer (Y.-H. Wang et al. 2015)
C-Terminal Src Kinase (dCSK)	Eyes (M. Vidal, Larson, and Cagan 2006) Imaginal Discs (Gerlach, Eichenlaub, and Herranz 2018) Ovaries (O'Reilly et al. 2006)	C-Terminal Src Kinase (CSK)	Colon Cancer (Rengifo-Cam et al. 2004; Nakagawa et al. 2000)

Table 1.1) Roles for *Drosophila* JAK/STAT signaling components and their human homologs in development and cancer metastasis. The left side of the table shows canonical *Drosophila* JAK/STAT components and tissues in which they are required or highly expressed. The right side of the table lists the closest human homologs and the types of metastatic tumors in which they are involved.

1.7) Outlook

The portrait of JAK/STAT signaling in human cancer metastasis is far from complete and is obviously very complex. The description we provide here fortifies the idea that further explanation of JAK/STAT regulation in *Drosophila* is warranted and useful for uncovering new genes with roles in human disease. Interestingly, new levels of regulation are becoming apparent in multiple *Drosophila* cell types. These suggest that future studies are needed in particular to evaluate vesicular trafficking regulation and epigenetic control of JAK/STAT

activity and target gene expression. Additional drug screening in *Drosophila* is also likely to be very informative. Further studies of the candidates described here, and new ones will likely shed insights on regulation of cancer metastasis by JAK/STAT signaling or additional linked pathways.

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Chapter 2: Mind bomb 2 stabilizes E-cadherin-based cell adhesion and the actin cytoskeleton to promote epithelial organization and cell migration in *Drosophila*

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2.1) Introduction

Collective cell migration is a dynamic phenomenon required in animals during embryonic development and adulthood. The unchecked regulation of this normally well-orchestrated cell movement can result in pathological disorders such as metastasis, poor wound healing, and birth defects (Friedl et al., 2012; Friedl & Gilmour, 2009; Mehlen & Puisieux, 2006; Theveneau & Mayor, 2011). Fundamental aspects of cell migration are known to be governed by cytokine and growth factor pathways that promote directed cytoskeletal and adhesive changes. Less is known, however, about how these are coordinated in groups of migrating cells since these can often retain epithelial character (Seetharaman & Etienne-Manneville, 2020). Thus, further investigation of the molecular signals governing collective cell migration is essential to gain insights into key regulators and to identify therapeutic targets when aberrant migration occurs.

Drosophila egg development serves as an excellent system to study epithelial organization and collective cell migration owing to tractable genetic tools, well-understood signaling mechanisms, and conserved, but relatively low genomic complexity (Chen et al., 2014; Hudson & Cooley, 2014; L. A. Manning et al., 2015; Prasad et al., 2007). The fly ovary contains ovariole chains made up of a series of developing egg chambers from stage 1 to 14 (King, 1970). Egg chambers consist of 15 nurse cells and an oocyte at the posterior end, surrounded by somatic, follicular epithelial cells (Figure 1.1A). At mid-oogenesis, high levels of Janus Kinase/Signal Transducer and Activator of Transcription (JAK/STAT) signaling in a small set of anterior follicle cells induce them to become the motile border cells (Beccari et al., 2002; Ghiglione et al., 2002; Silver & Montell, 2001). Along with two polar cells, the border cells detach from the epithelium and the group collectively migrates between nurse cells towards the oocyte. This process requires continuous STAT signaling and dynamic regulation

of cytoskeletal and adhesion proteins including actin, myosin, E-cadherin, and β-catenin (Montell et al., 2012; Silver et al., 2005). Interestingly, this regulation must be controlled inter- and sub-cellularly to enable cluster cohesion while promoting movement, and how this occurs is not entirely clear.

Previous RNAi based genetic screens showed that Mind bomb 2 (Mib2) can negatively regulate Stat activity (Müller et al., 2008) and may impact border cell migration (Saadin & Starz-Gaiano, 2016b), making it a candidate regulator of collective cell movement. Mib2 is a putative E3 ubiquitin ligase evolutionarily conserved between *Drosophila,* vertebrates and mammals, and it can bind select target proteins to drive their ubiquitination *in vitro* (Koo et al., 2005). Mib2 has five distinct conserved domains that are likely involved in protein-binding, including MIB-specific domains and RING domains (Domsch et al., 2017; Koo et al., 2005). *In vitro* ubiquitination assays with frog and mouse Mib2 suggest that the RING domains possess E3 ligase activity and, like Mind bomb, promote Notch ligand activation (Koo et al., 2005; Takeuchi et al., 2003). Mouse Mib2 physically interacts with α-Actin in skeletal muscles (Takeuchi et al., 2003). The human ortholog, Skeletrophin, targets the intracellular region of the Notch regulator Jagged 2 and is overexpressed in multiple myeloma cells (Takeuchi et al., 2005). In the late stages of fly embryogenesis, *mib2* mutants undergo major muscle deterioration independent of Notch signaling, suggesting a yet unidentified mechanism to regulate muscle integrity (Carrasco-Rando & Ruiz-Gómez, 2008; Nguyen et al., 2007). While fly Mib2 has been shown to bind directly to the non-muscle myosin component Zipper (Zip) (Carrasco-Rando & Ruiz-Gómez, 2008), its direct involvement in ubiquitination has not been shown, and a role in cell migration has not been identified. Thus, the exact mode of action for fly Mib2 remains uncharted territory.

Our current study indicates that Mib2 regulates cell migration and epithelial cell structure in *Drosophila* egg chambers by directly impacting adhesion complexes and actin organization. We find that Mib2 is highly expressed in follicle cells and is required for efficient border cell migration. Follicle cells mutant for *mib2* show a dramatic reduction in E-cadherin based adhesion components and cortical actin, and exhibit shortened epithelial cell heights. These *mib2* mutant phenotypes are distinct from Notch-related defects. We identified multiple cytoskeletal and adhesion proteins that physically and genetically interact with Mib2, including E-cadherin and regulators of actin organization. Our data suggest that Mib2 has a pleiotropic effect in the dynamic regulation of certain cytoskeletal components during *Drosophila* oogenesis. Thus, we hypothesize that the key role of Mib2 in this context is to bind to and promote the stability of E-cadherin-based adhesion complexes and promote certain types of actin filament organization.

2.2) Materials and Methods

2.2.1) Fly stocks and husbandry

All fly lines and crosses were kept at 25ºC. For fattening, the flies were fed dry yeast overnight at 29ºC for optimal UAS-GAL4 expression and high yield of stage 9-10 egg chambers. The next day the female flies were dissected and ovarioles were stained to observe stage 10 egg chambers for migration index and protein expression. In our study we defined defective migration if the cluster is lagging by 1 cluster length in stage 9 or 10. For protein expression studies we performed intensity analysis using DAPI as our reference. See extended materials for genotypes.

We used the flp-FRT system (reference) to generate negatively marked mutant clones in follicle cells.

2.2.2) Mosaic Clones:

To induce negatively marked mutant clones we crossed *hsFLP* flies (BDSC 6) with the *Ubi-GFP* flies (BDSC 5629) followed by crossing their F1 males with $mi2^{1}$*,FRT40A/CyO* virgins. The F2 flies were given hea shock at 37ºC twice a day for three days. Flies were kept at 25ºC for two days to get maximum number of stage 10 mutant egg chambers. The flies were incubated at 29ºC overnight with yeast. The female flies with straight wings were dissected to extract and immunostain the ovaries.

2.2.3) Immunohistochemistry and microscopy

Ovarioles were extracted from fattened flies into dissection media (1x Schneider's *Drosophila* medium by Thermofisher Scientific (21720-001), 10% FBS, 0.6% Pen/Strep) and fixed with 4% paraformaldehyde (0.1M KPO4 buffer) for 10 mins at RT. Fixed ovarioles were rinsed and washed 3 times with NP40 buffer (0.05 M Tris HCl, pH 7.4, 0.15 M NaCl, 0.5% Nonidet P-40 (Igepal CA-630, Sigma-Aldrich), 1 mg/ml BSA) (McDonald et al. 2006). For staining, ovarioles were incubated with respective primary antibody in NP40 buffer overnight at 4ºC. See extended materials for antibodies and concentrations. Ovarioles were rinsed and washed 3 times with NP40 buffer then incubated with Alexa Fluor secondary antibodies in NP40 buffer overnight at 4ºC followed by DAPI staining for 10 mins. Ovarioles were rinsed and washed 3 times with NP40 buffer and incubated then mounted in 70% Glycerol. Samples were observed using either the Zeiss LSM 900 confocal with Airyscan 2 or Carl Zeiss AxioImager Z1 and captured using AxioVision acquisition system. Post image processing was performed using ImageJ and Adobe Illustrator.

Primary Antibodies:		
mouse anti-Armadillo (Arm)	1:40 (N2 7A1)	DSHB
mouse anti-E cadherin (Ecad)	1:25 (8C2)	DSHB
Rabbit anti-GFP	1:500 (A-1112)	DSHB
Guineapig anti-Mindbomb2 (Mib2)	1:150 (gift)	(Carrasco-Rando & Ruiz-Gómez, 2008)
mouse anti-Discs large (Dlg)	1:100 (4F3)	DSHB
Anti-Qua antibody	1:50 (6B9)	DSHB
Anti-catenin alpha	1:50 (DCAT1)	DSHB
Anti-Hts	1:50 (1B1)	DSHB
Anti-spectrin-alpha	1:50 (3A9)	DSHB
Mouse anti-FLAG	1:1000 (F1804)	Sigma Millipore
Secondary antibodies:		
Alexa Fluor 488 (Goat anti Rabbit)	1:400 (A-11008)	TFS
Alexa Fluor 488 (Goat anti Rat)	1:400 (A-11006)	TFS
Alexa Fluor 568 (Donkey anti Mouse)	1:400 (A-11037)	TFS
Alexa Fluor 488 (Goat anti Gp)	1:400 (A-11073)	TFS
Stains:		
DAPI	1:1000 (D1306)	TFS
Phalloidin	1:500 (A12380)	TFS

Table 2.1) List of antibodies used in this study

2.2.4) Quantitative real-time PCR analysis

The HSP70-GAL4 virgin female flies were crossed with respective UAS RNAi lines at 25ºC. The F1 flies were heat-shocked at 37ºC for 3 times a day for 3 days. Additionally, for other experiments, the C306-GAL4 virgin female flies were crossed with respective UAS RNAi or overexpression lines at 25ºC. The mCherry RNAi was used as control for all qRT-PCR experiments. All F1 progeny were fattened at 29ºC overnight and ~20 pair of ovaries were dissected. The total RNA was extracted using the Qiagen RNeasy mini kit with DNase I digestion. Using BioRad iScript, ~1µg/µl cDNA was synthesized from total RNA. qRT-PCR reactions (3 biological and 3 technical replicates for each sample) were set up with 1µg/µl cDNA, 2ul primer mix (10µM), and 10 µl iTaq Universal SYBR Green Supermix (BioRad) in a 20 ml reaction. Primers were designed according to the fly primer bank (Hu et al., 2013) (http://www.flyrnai.org/flyprimerbank).

2.2.5) Immunoprecipitation, western blot, and mass spectrometry

Fattened flies were dissected on ice in IP buffer (PBS pH7.6, Halt protease and phosphatase inhibitor (78442, Thermofisher), 1mM PMSF, 1mM DTT). For each sample ~200 pair of ovaries were dissected and snap frozen in liquid nitrogen and stored at -80ºC. Frozen ovaries were homogenized using 400 µl lysis buffer (50mM tris-HCL pH 7.5, 100mM NaCl, 0.2% Np40, 5% glycerol, 1.5mM MgCl2, 1mM EDTA, 1mM EGTA, added fresh: 1mM DTT, Halt protease and phosphatase inhibitor (78442, Thermofisher), 1mM PMSF, 0.05mM Mg132) and incubated on ice for 20 mins. Samples were centrifuged at 16,000 RPM at 4ºC for 15 mins. Supernatant was incubated with pre-washed magnetic A/G beads (88803, Thermofisher) and respective primary antibodies at 4ºC overnight. The beads were washed 3 times with lysis buffer on ice. For mass spectrometry analysis, we immunoprecipitated proteins using FLAG M2 antibody (Millipore Sigma, F1804) from CantonS flies, FLAG tagged ImpL2expressing flies, and FLAG-GFP tagged Mib2 expressing flies. The immunoprecipitated protein complexes bound onto the beads were digested using trypsin digestion kit (89895, Thermofisher). The samples were treated with a detergent kit (88305, Thermofisher) to remove any salt from the samples. The purified samples were then submitted to the MCAC core facility at UMBC for separation on the Bruker NanoElute HPLC, followed by dual Trapped Ion Mobility Spectrometry (TIMS) on the Bruker timsTOF pro-mass spectrometer to identify peptide abundance. Raw data was analyzed with PEAKs Studio to identify peptide abundance. Among the extensive list of proteins that bound to Mib2, we excluded the proteins that had peptide count lower than 5, ribosomal, chromatin related, uncharacterized proteins, and additional isoforms. We included proteins that were exclusively in the Mib2 IP or that had higher peptide counts in Mib2 IP compared to the controls. The relative abundance is achieved by the following formula, standardize [Peptide count, average (peptide count of sample and both controls), standard deviation (peptide count of sample and both controls)]. The experiment

was repeated twice, and the trends reported are representative of both experiments. Additionally, for Western blot analysis, the immunoprecipitated beads were boiled at 100ºC for 15 mins with 4X SDS loading dye. Samples were loaded onto 4-20% polyacrylamide pre-cast gels by BioRad for SDS gel electrophoresis. Next, proteins were transferred from the gel onto the PVDF membrane by Western blot overnight at 4ºC. The membranes were blocked with 3% BSA in TBST and incubated with primary antibody solutions made in TBST overnight at 4ºC. Membranes were washed three times in TBST for 5 mins and incubated with HRP conjugated secondary antibodies for 2 hrs at RT. Membranes were once again washed in TBST three times for 5 mins and developed using ECL developing solution (Biorad). Blots were imaged using a Biorad Chemidoc XRS+ imaging station, and further processed, cropped, and oriented in Adobe Illustrator.

2.3) Results

2.3.1) Mib2 is expressed throughout oogenesis and cytoplasmically enriched in follicle cells

To understand a potential role of Mib2 in collective cell migration, we first examined its protein expression in egg chambers. Immunofluorescence staining using an antibody directed against Mib2 (M Carrasco-Rando et al., 2008) revealed its presence throughout oogenesis (Supp. Figure 2.5) and at stage 8, it is especially prominent within the cytoplasm of follicular epithelial cells (Figure 2.1B-B’’’) and the oocyte. At stage 9 when the border cell cluster has been specified and detaches from the anterior epithelium, Mib2 expression is maintained at high levels in both polar cells and border cells (Figure 2.1C-E’’’) and is enriched at the oocyte periphery. During the completion of the migration phase, stage 10, the border cell cluster retains Mib2 expression albeit at slightly reduced levels. The expression profile was confirmed using flies expressing a genetically tagged Mib2 (Sarov et al., 2016) (Supp. Figure

2.6). Based on this expression pattern, we hypothesized that Mib2 functions in border cell migration and the maintenance of the follicular epithelium in ovaries.

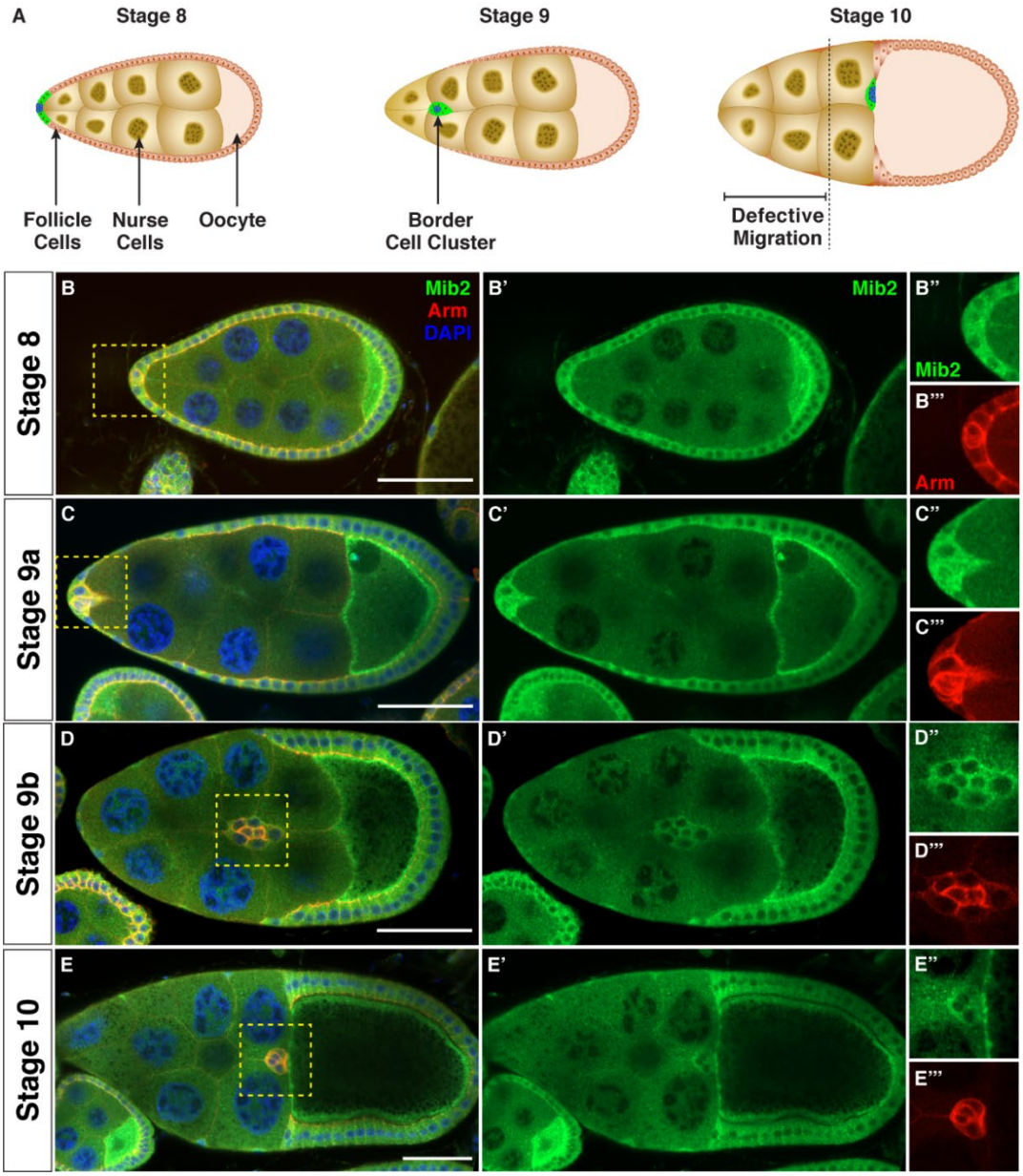


Figure 2.1) Mib2 expression is enriched in the follicular epithelium of developing egg chambers. A) Schematic representation of *Drosophila* egg chambers of stages 8, 9, and 10, which is when border cells transition from epithelial cells to motile cells and migrate to the oocyte. Green cells represent border cells, and blue cells represent polar cells. B-E)

Immunofluorescence staining shows that Mib2 is expressed in a spatially restricted pattern at different stages of oogenesis as indicated. Mib2 protein (green) is highly localized at the cortex and in the cytoplasm of follicle cells including border cells (boxed) and is also detected cytoplasmically in the oocyte early and later at the oocyte cortex. β-catenin (Arm) expression is shown in red, and nuclei are stained with DAPI in blue. The insets focus on fated or migrating border cell clusters. Scale bar is 50μm.

2.3.2) *mib2* is required for border cell migration

To test if Mib2 regulates border cell migration, we examined *mib2* mutants and RNAi knockdowns specifically in the anterior follicle cells. As $mib2^{1}$ null mutants are homozygous lethal (Nguyen et al., 2007), we characterized the effect of null mutations in heterozygosis. We stained egg chambers with antibodies directed against β-catenin (encoded by *armadillo* (*arm*) in flies) and E-cadherin (E-cad) (encoded by *shotgun* (*shg*)), which detects the border cell clusters. In controls, the border cells migrated in alignment with the flattened domain of outer follicle cells at stage 9 and arrived at the anterior of the oocyte by stage 10, having completed migration (Figure 2.2A, F). In contrast, 29% of $mib2^{1}$/+ heterozygous mutant egg chambers showed incomplete border cell migration in stage 10 egg chambers (Figure 2.2 C, F). Separately, we used a border cell cluster-specific Gal4 (Brand & Perrimon, 1993) to express *mib2* RNAi under UAS control (Dietzl et al., 2007; Manseau et al., 1997). In these cases, we observed delayed border cell migration in 35-40% of egg chambers at stage 9, and incomplete migration in 25–30% of stage 10 egg chambers (Figure 2.2B, F). qPCR analysis confirmed that Gal4-mediated *mib2* RNAi resulted in significantly less detectable *mib2* mRNA than in controls (Supp. Figure 2.6). Overexpression of *mib2* did not have any effect on migration alone (Figure 2.2D), but fully rescued the migration defect in $mib2^{1}$/+ heterozygotes (Figure 2.2E, F). These results demonstrate that *mib2* is required for normal border cell migration.

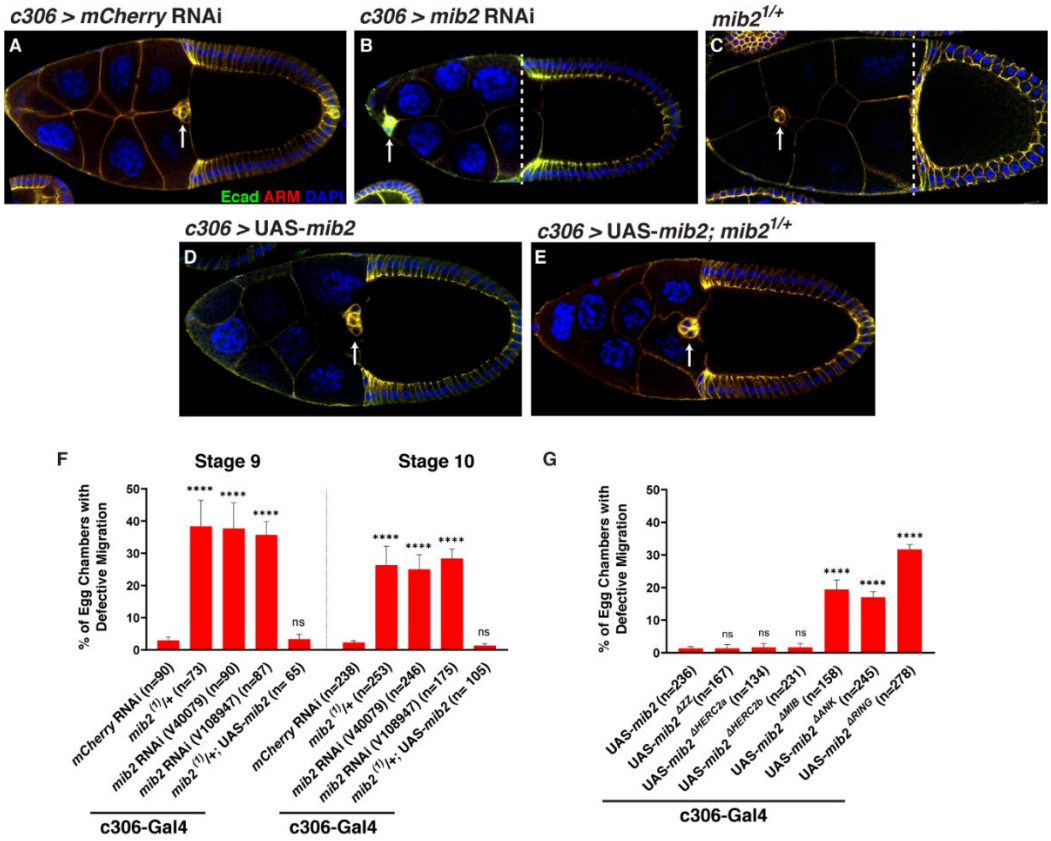


Figure 2.2) Mib2 regulates border cell migration. A-E) Egg chambers immunofluorescently stained for Arm protein, shown in red, and E-cad, shown in green; nuclei are shown by DAPI staining in blue. A) The anterior follicle cell-specific Gal4 line, c306, promotes expression of *mCherry* RNAi (control), resulting in normal migration of the border cell cluster. B) Expression of *mib2* RNAi in anterior follicle cells results in defective border cell migration, with cells remaining close to their anterior starting point (arrow). C) An egg chamber from a heterozygous null mutant, *mib2*^{1}/+, shows defective border cell migration, with cells having moved only about 25% of the normal migration distance (arrow). D) Overexpression of *mib2* in anterior follicle cells with UAS-*mib2* does not disrupt border cell migration or egg chamber morphology. E) Overexpression of *mib2* in the *mib2*^{1}/+ mutant background shows normal cell

migration indicating rescue of the defect shown in (C). F) Quantification of the penetrance of defective border cell migration at stage 9 and 10 in the indicated genotypes. G) Quantification of the penetrance of defective border cell migration due to overexpression of different Mib2 domain deletion lines in anterior follicle cells. Statistical analysis was performed using a one-way ANOVA test, **** represents $p \leq 0.0001$. n= number of egg chambers analyzed.

In addition to overexpressing the full-length protein in anterior follicle cells, we overexpressed versions of Mib2 that had deletions in different domains (Domsch et al., 2017) within a wild-type background. Mib2 has eight conserved domains; two HERC2, a ZZ zinc finger domain, two Mib domains, eight ankyrin repeats, and two RING domains. Like full-length overexpression, expression of Mib2 with a ZZ deletion or HERC deletion did not disrupt border cell migration. However, Mib2 overexpression with deletions in the MIB, ANK or RING domains resulted in significant migration defects (Figure 2.2G and Supp. Figure 2.7). This supports the idea that Mib2 interacts with different partners to exert its function in cell migration and suggests that disruption of certain domains results in dominant-negative effects.

2.3.3) Mib2 is required for adhesion and cytoskeletal complex maintenance and epithelial organization

Next, we used mosaic clonal analysis to investigate the loss of *mib2* function in egg chambers. We initially aimed to observe mutant border cells; however, despite examining over one thousand clones in follicle cells, we identified no mutant border cell clusters. This suggests that *mib2* is required in initial border cell specification and/or survival. Nevertheless, mutant clones in the follicular epithelium turned out to be very informative about the molecular function of Mib2. In particular, we found that loss of *mib2* drastically affects the expression of proteins known to be important in border cell migration.

Cadherin-based adhesion complexes, including E- and N-cadherin and the adaptors α- and β-catenin that link them to the actomyosin network, all have required roles in follicle cell organization and structural integrity (Cai et al., 2014; Niewiadomska et al., 1999a; Pacquelet & Rørth, 2005; Peifer M, 1993; Sarpal et al., 2012; Tanentzapf et al., 2000). Additionally, these complexes must be dynamically regulated during cell migration. Compared to neighboring wild-type cells, *mib2* mutant follicle cells showed a dramatic reduction in adhesion complexes and actin. Specifically, we observed severely reduced levels of the homophilic adhesion molecule E-cad in mutant follicle cells (Figure 2.3 A-A''). Interestingly non-cell-autonomous effects were also observed, where a wild-type cell in contact with a mutant cell showed a partial reduction of E-cad at the juxtaposed surface (Figure 2.3 C-C''). In several instances when the mutant clone was large (more than 20 cells), we observed that the *mib2* mutant follicle cells were disorganized or led to bending in the epithelium (Figure 2.3 B-B''). Notably, such phenotypes are similar to what has been observed in *shg* mutant clones in follicle cells (Niewiadomska et al., 1999a; Pacquelet & Rørth, 2005), however, the *mib2* mutant clonal phenotypes appear to be less severe. For instance, we never observed a mispositioned oocyte, which is characteristic of E-cadherin loss (Niewiadomska et al., 1999b), and the follicle cell layer was less disorganized in *mib2* mutants, suggesting some adhesion may be preserved.

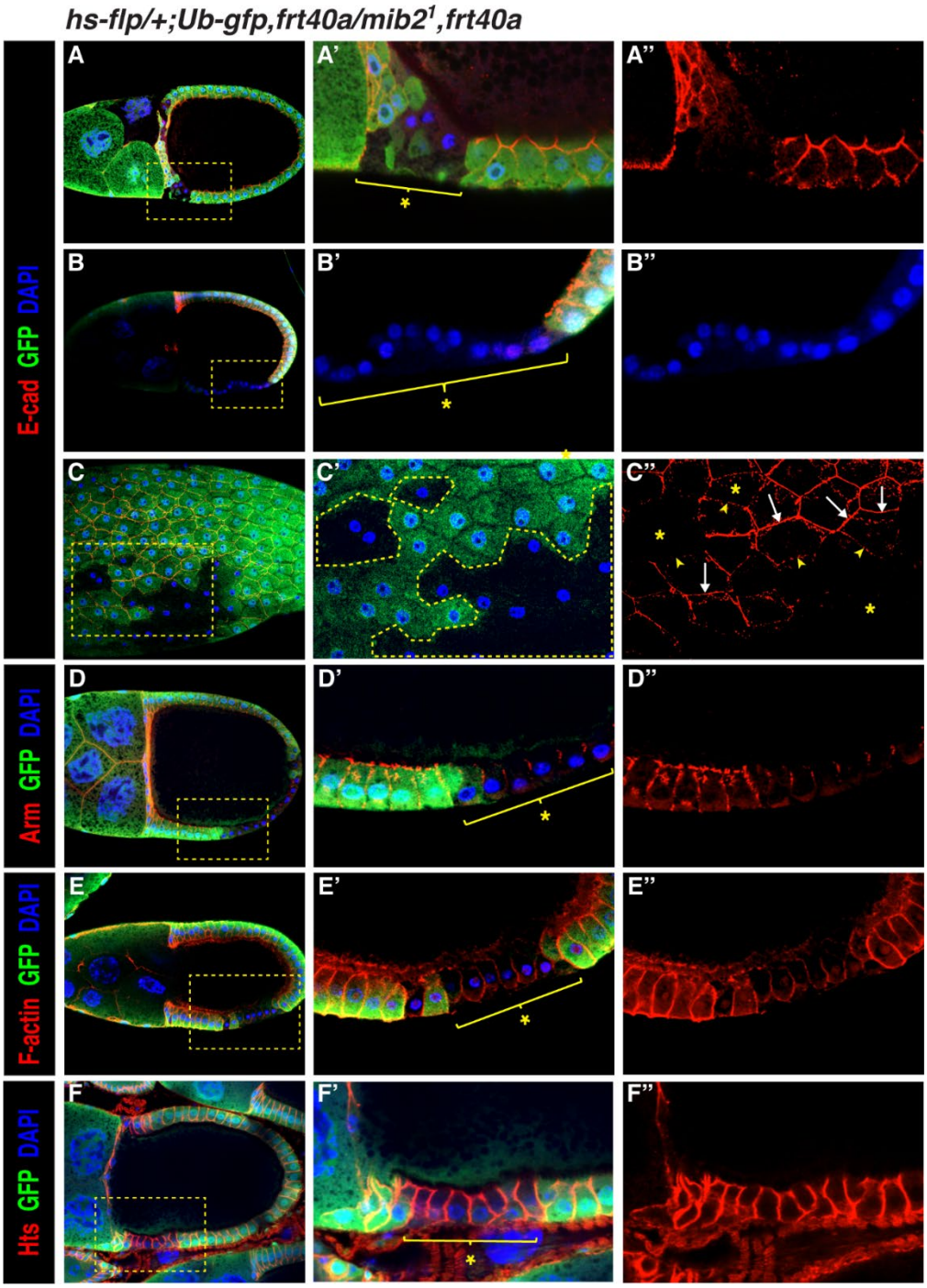


Figure 2.3) ***mib2*** **is required for stable and correctly localized adhesion complexes and actin filament expression.** A-E) Negatively marked *mib2*^{1} mutant clones in the follicular epithelium of stage egg chambers lack GFP and are marked by bracket and an asterisk in A-D and outlined in E. Wild-type cells are marked in green and nuclei are shown in blue. A-A'')

mib2 mutant follicle cells have undetectable E-cad expression (shown in red) at the cell surface. B-B'') An egg chamber at stage 10 with a large $mib2^{1}$ mutant clone (more than 20 cells) shows a disrupted, uneven epithelial layer. C-C'') Patches of $mib2^{1}$ mutant cell clones in a stage 13 egg chamber show greatly reduced E-cad expression, and neighboring wild type cells are also affected. The asterisks mark mutant cells, arrows point to wild-type/wild-type cell boundaries, and arrowheads point to wild type-mutant cell boundaries. D-D'') Mutant follicle cells show a dramatic reduction of Arm expression (shown in red) at apicolateral and lateral surface of follicle cells in a stage 10 egg chamber. E-E'') F-actin, detected by phalloidin staining (shown in red), is reduced at the cell surface and the expression also appears to be lower in the cytoplasm in mutant cells at stage 10. F-F'') Hts (shown in red) remains unchanged in mutant follicle cells and shows the columnar epithelial organization of follicle cells.

We next examined the effects of loss of *mib2* on other adhesion complex components and the cytoskeleton. The adaptor protein β-catenin (Arm) associates with cadherins and α-catenin as part of a complex that links all of them to the actin cytoskeleton. In *mib2* mutant cells, we saw a near-complete loss of apical β-catenin while lateral expression was generally lower and restricted to a much smaller region (Figure 2.3D-D''). Generally, the lateral surface of epithelial cells seemed to be reduced, and cells were often shorter in the apical-basal axis. Additionally, cortical F-actin was markedly reduced in mutant follicle cells (Figure 2.3E-E'') and the cytoplasmic level also appeared to be lower. This phenotype is distinct from *E-cad* (*shg*), *α-cat*, or *arm* mutant follicle cells where F-actin is unchanged or upregulated, although these mutant cells are often shorter and less organized (Pacquelet & Rørth, 2005; Sarpal et al., 2012; Tanentzapf et al., 2000). Among egg chambers with distinct mutant clones, the reductions in Arm, F-actin, and E-cad expression/localization are 100% penetrant ($n > 200$ each). However, other cytoskeletal proteins, such as the adducin-like protein, Hu li tai shao (Hts) (Yue & Spradling, 1992), appeared normal in *mib2* mutant clones (Figure 2.3). Notably,

we did not observe any phenotypes associated with the disruption in Notch signaling (for example, egg chamber fusions or changes in stalk/polar cell fates) (Duhart et al., 2017), indicating *mib2* is unlikely acting in this pathway in follicle cells. Since both *mib2* and *shg* (Niewiadomska et al., 1999b) are required for border cell migration, we performed a simultaneous knockdown of both by their respective RNAi in anterior follicle cells. The F1 generation fly development could not proceed past pupal stages confering the lethality, possibly owing to the cumulative effect of double knockdown and their genetic interaction. These data indicate that Mib2 is crucial in the maintenance and/or correct subcellular localization of adhesion complexes and certain cytoskeletal components.

2.3.4) Mib2 associates with adhesion and cytoskeletal components

To understand Mib2's role in epithelial cell regulation better, we sought to identify the proteins to which it binds. We immunoprecipitated (IP) a FLAG-tagged Mib2 expressed in transgenic flies under control of its endogenous promotor (Sarov et al., 2016) from ovaries and used tims-Time-of-Flight Mass Spectrometry (timsTOF MS) to identify proteins in this complex. As a comparison and negative control, we separately IPed FLAG-Tagged ImpL2 (Imaginal morphogenesis protein-Late 2, which is also expressed in the follicular epithelial cells (L. Manning et al., 2017). We found 54 proteins that preferentially bound to FLAG-tagged Mib2 compared to controls and 16 that bound only to the FLAG-tagged Mib2. Proteins enriched in the FLAG-Mib2 complexes are represented in a heatmap in Figure 2.4a. These include actin binding or cytoskeletal regulatory proteins like Rho GTPase activating protein at 19D (RhoGAP19D), Supervillin (Svil), Quail (Qua), Wings up A (WupA), β-Spectrin (β-Spec), and Hu li tai shao (Hts), and cell adhesion complex components α-catenin (α-Cat), β-catenin (Arm), and E-cad. While we used whole ovaries in these samples, Mib2 is enriched in follicle cells, so we believe most of the interacting proteins we found are important in follicle

cells. Several of these have been shown to be highly expressed in follicular epithelium at these stages, RhoGAP19D (Fic et al., 2021), Hts (Lin et al., 1994), β-Spec (Tanentzapf et al., 2000)), Qua (Borghese et al., 2006; Mahajan-Miklos & Cooley, 1994)), α-Cat (Pacquelet & Rørth, 2005; Sarpal et al., 2012), Arm (Pacquelet & Rørth, 2005; Peifer M, 1993), E-cad (Niewiadomska et al., 1999b), and others, such as Terribly reduced optic lobe (Trol) have not been characterized in ovary. Among these potential interacting proteins, we confirmed binding of Arm and E-cad with Mib2 using IPs and Western blot analysis (Figure 2.4b). Altogether our biochemical analysis suggests that Mib2 physically interacts with adhesion proteins and actin network regulators, and we propose this increases their stability.

2.3.5) Mib2 associated proteins identify novel border cell migration regulators

Stabilization of E-cad, *α*- and β-catenin complexes could explain *mib2*'s requirement in epithelial organization and also could explain a role in border cell migration, where it is known to be required along with β-catenin (Cai et al., 2014; Niewiadomska et al., 1999b; Pacquelet & Rørth, 2005)). E-cad adhesions are strong and stable between the cells within the border cell cluster but are required more transiently between border cells and nurse cells. Consistent with this, when we knocked down *α-Catenin,* border cell migration often failed (Figure 2.4F). Since adhesions are more rapidly turned over in motile cells than stationary epithelial cells, migrating cells may be very sensitive to destabilization of these complexes.

To determine if any of the other Mib2-interacting proteins could also contribute to its role in border cell migration, we used the UAS-GAL4 system to manipulate the expression of several candidates. We focused on candidates related to cytoskeleton or transcriptional regulation, given that this is known to be critical in border cells (Seetharaman & Etienne-Manneville, 2020). Strikingly, we uncovered roles for three novel genes in border cell migration. One encodes the DNA binding protein Modulo (Mod) (Krejci et al., 1989). RNAi-

mediated reduction of *mod* led to migration defects in about 23% of stage 10 egg chambers (Figure 2.4E). A second new candidate regulator is RhoGAP19D, which was recently shown to regulate Rho family protein Cdc42 and suppress follicle cell invasion (Fic et al., 2021). Interestingly, RhoGAP19D overexpression in anterior follicle cells resulted in severe border cell migration defects (Figure 2.4D). However, *rhogap19D* knockdown in anterior follicle cells did not lead to significant border cell migration defects. We do not know if this is because of insufficient downregulation or if this gene may function redundantly. Finally, reduced expression of *svil*, which encodes a regulator of actin filament dynamics (Gaudet et al., 2011), resulted in poor border cell migration in stage 9 egg chambers (Figure 2.4G). The migration defects for these candidates are quantified in Figure 2.4H. Beside these candidates, we also examined other proteins that we found associated with Mib2 in our immunoprecipitation, namely nuclear proteins JIL-1 anchoring and stabilizing protein (Jasper) and Elys, and cytoskeletal/membrane components Kramer, WupA and β-Spectrin. However, reduction of these using existing RNAi lines did not have any obvious effect on migration of border cell-cluster, suggesting they may not be required or may function redundantly for migration (data not shown). However, these may have other roles in the follicular epithelium. Since E-cad, *α*- and β-catenin, Mod, RhoGAP19D, and Svil (Figure 2.4D-G) are required for border cell migration and physically interact with Mib2, we propose that they are all stabilized and/or regulated by Mib2 function.

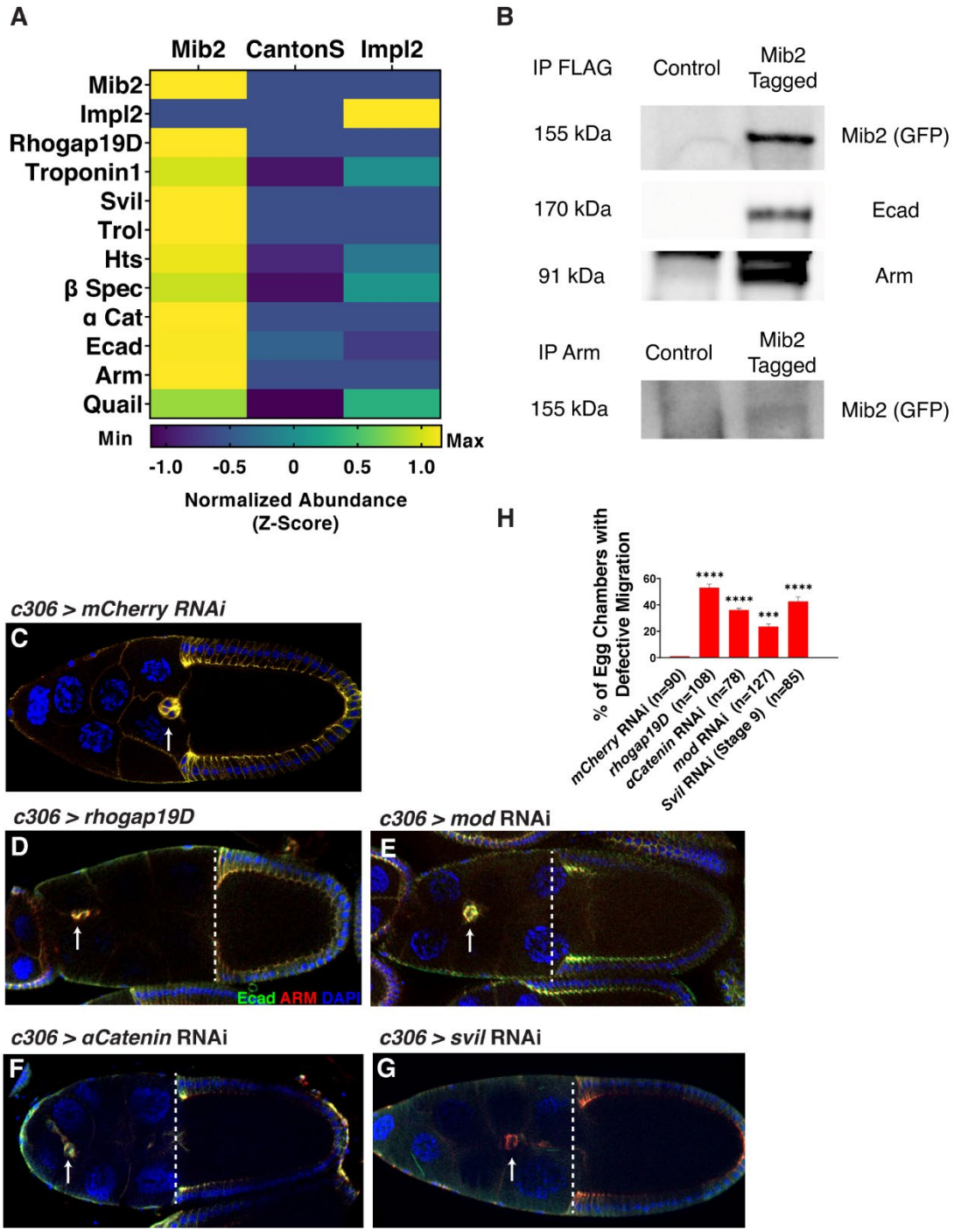


Figure 2.4) Mib2 physically interacts with E-cadherin and cytoskeletal proteins and identifies new proteins required in border cell migration. A) Mass spectrometry data identifies proteins associated with Mib2 in immunoprecipitation from ovary extract. Heatmap shows a selection of interacting proteins according to the relative abundance of each.

Immunoprecipitation with FLAG-tagged Mib2 is compared to the negative controls CantonS and FLAG-tagged ImpL2. B) Western blot analysis shows coimmunoprecipitation of Mib2 with Arm (β-catenin) and E-cad. The control lane contained protein extract from Canton S ovaries, and the Mib2 lane shows the results from extracts from flies with endogenously GFP and FLAG-tagged Mib2. C-G) Egg chambers of the indicated genotypes immunofluorescently stained with antibodies directed against Arm protein (red), E-cad (green); and DAPI (blue). C) The knockdown of *mCherry* in anterior follicle cells serves as wild type control for border cell migration. The c306 drives the anterior follicle cell expression. D) The overexpression of *Rhogap19D* in anterior follicle cells caused severe migration defect shown by the lagging border cell cluster in a stage 10 egg chamber. The arrow points to cluster location; the dotted line indicates the expected location at the oocyte border by this stage. E, F, G) The knockdown of *mod*, *α-catenin*, and *svil,* respectively, in the anterior follicle cells results in defective border cell migration. H) Quantification of the penetrance of migration defects from (C, D, E, F, G) in egg chambers with respective genotypes. Statistical analysis was performed using a one-way ANOVA t-test, **** represents $p \leq 0.0001$; n = number of egg chambers analyzed.

2.4) Discussion

Cell migration is an important aspect of both the development of an organism and disease pathophysiology, and collective cell migration is especially interesting as it must balance stable and dynamic cell adhesions (Friedl et al., 2012; Friedl & Gilmour, 2009; Kraemer, 2000; Mehlen & Puisieux, 2006). Hence, to promote correct developmental cues and to battle against migratory disorders, a deeper understanding of the mechanisms behind cell migration is required. Here, we identified critical roles for Mib2 in both collective cell migration and the maintenance of the epithelial organization.

In *Drosophila* egg chambers, we found that Mib2 acts in follicle cells to regulate adhesion complexes and the actin cytoskeleton by stabilizing them. Loss of *mib2* results in a dramatic loss of E-cad, reduced Arm on the lateral surface of follicle cells, and downregulation of cortical actin.

Notably, *mib2* mutant clones of smaller size do not disrupt follicle cell structure. However, in the larger clones we observe cell extrusion and multilayering of follicular epithelium probably owing to loss of adhesion over time. While these phenotypes resemble those in follicle cell null mutant clones for cadherins or catenins, they are less severe. Thus, we suspect that these adhesion complex proteins are being produced but are destabilized leading to much faster turnover. Additionally, the dramatic reduction of actin in *mib2* clones is distinct from what occurs due to loss of adhesion complex components and may suggest a more direct interaction between Mib2 and the cytoskeleton. Since we never observed border cell mutant clones, we speculate that the loss of *mib2* prevents cluster formation. However, as E-cad based adhesion is a key mediator of border cell migration (Cai et al., 2014), changes in the stability of these adhesion complexes likely explain Mib2's role in the migration process. Rapid turnover of adhesion complexes is known to occur in motile cells, so the border cells may be more sensitive to additional destabilization of adhesion compared to other follicle cells.

Zebrafish, mouse, and human Mib2 orthologs act as E3 ubiquitin ligases (Koo et al., 2005; Nguyen et al., 2007), leading to polyubiquitination of several different targets, but this activity has not been directly shown in *Drosophila*. However, catalytic residues in the ligase domain of fly Mib2 are required for muscle development (Nguyen et al., 2007). Our genetic and physical interaction data suggests that Mib2 directly binds and stabilizes certain adhesion complex components, in particular E-cad. It is possible it does this as a scaffold component, simply by creating a complex or through its ligase activity. While polyubiquitination leads to

protein degradation, mono-ubiquitination can increase protein stability or alter subcellular localization (Sadowski & Sarcevic, 2010), so Mib2 may promote this post-translational modification on its targets. Alternatively, it may act as a scaffolding protein and stabilize particular complexes by association. Interestingly, the defects we found due to loss of *mib2* function are different than those due to changes in Notch signaling, indicating fly Mib2 in oogenesis acts differently than its fish or frog homologs (Koo et al., 2005). It is also possible that Mib2 destabilizes negative regulator(s) of the cytoskeleton proteins, which leads to stabilization of Ecad and Actin. More work will be needed to elucidate the roles of the different Mib2 domains involved in protein-protein interaction and if the RING domain, in fact, confers Ubiquitin ligase activity.

While the direct activity of Mib2 remains to be determined, our biochemical analysis suggests it can interact with a number of candidates. These include cytoskeletal regulatory proteins such as RhoGAP19D, Troponin1, Svil, Hts, β-Spectrin, α-Catenin, and Quail. Among these proteins, α-Catenin has been shown to be involved in border cell migration, while Quail is expressed in border cells and is thought to act redundantly with another actin regulator, Singed (Borghese et al., 2006; Omelchenko, 2012; Pacquelet & Rørth, 2005). Using RNAi analysis, we newly demonstrate that *svil* and *mod* also have a required role in border cell migration regulation. Additional cytoskeletal regulation is likely. In our study, both non-muscle Myosin subunits Spaghetti-squash (Sqh) and Zipper (Zip) as well as cortical f-actin coimmunoprecipitated with Mib2, but these were also detected in the negative controls, and hence not included in our dataset. Prior studies suggest that fly Mib2 binds and stabilizes the non-muscle myosin subunit Zipper, which is also required for border cell migration (Carrasco-Rando & Ruiz-Gómez, 2008). Moreover, Skeletrophin can physically interact with α-actin monomers and act as a novel suppressor for invasion in myeloma cells (Takeuchi et al., 2006).

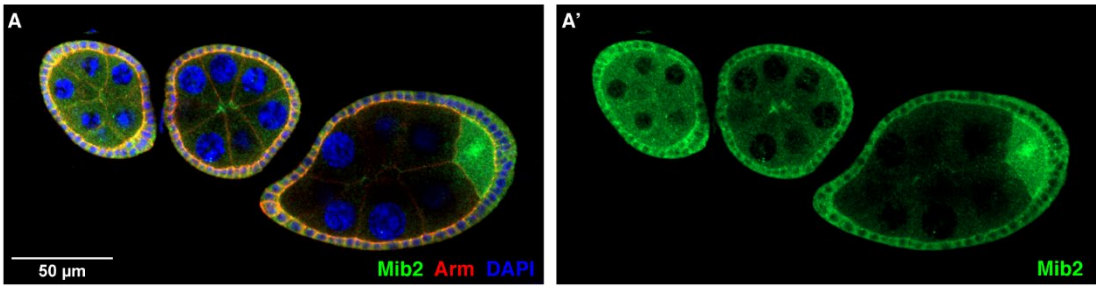
Thus, it is compelling to predict that Mib2's regulation of the actinomyosin network may be a conserved function of Mib2.

In addition to roles in cytoskeleton and adhesion regulation, our study indicates that Mib2 may interact with several transcriptional regulators. Since STAT transcriptional activity is critically required for border cell migration (Hou et al., 2002; Silver et al., 2005; Silver & Montell, 2001)and *mib2* has been shown to negatively regulate STAT activity (Müller et al., 2008), we speculated that *mib2* may be working through JAK/STAT signaling. qPCR analysis showed us that JAK/STAT signaling promotes *mib2* expression in anterior follicle cells (Supp. Figure 2.8) and some but not all STAT target genes had altered expression in *mib2* mutants (data not shown here). Our data suggest that *mib2* could be a part of positive feedback loop with *stat92E* expression and raises the interesting possibility that Mod or other transcriptional regulators that can interact with Mib2 (JASPER or Elys) to modify STAT signaling. Future work will explore this possibility.

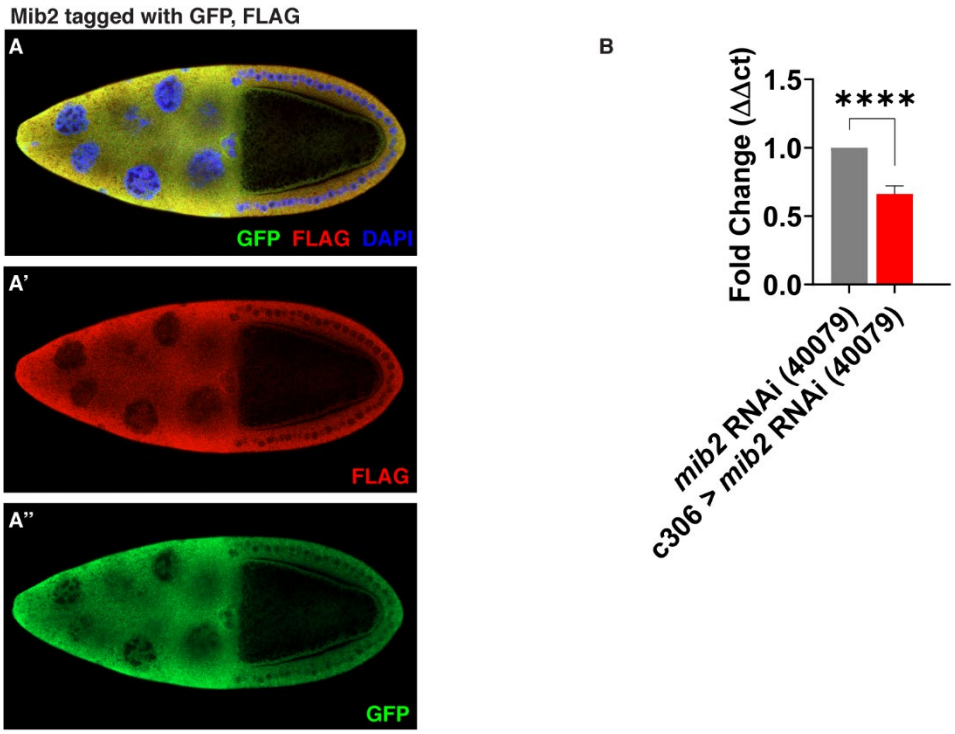
2.5) Acknowledgments

We would like to thank Alexander George and Mallika Bhattacharya for their comments in manuscript preparation. We thank Josh Wilhide and Christina Obriecht from MCAC (Molecular Characterization and Analysis Complex) facility for their Mass spectrometry analysis and Tagide deCarvalho of the Keith Porter imaging facility for assistance with imaging.

2.6) Supplementary figures

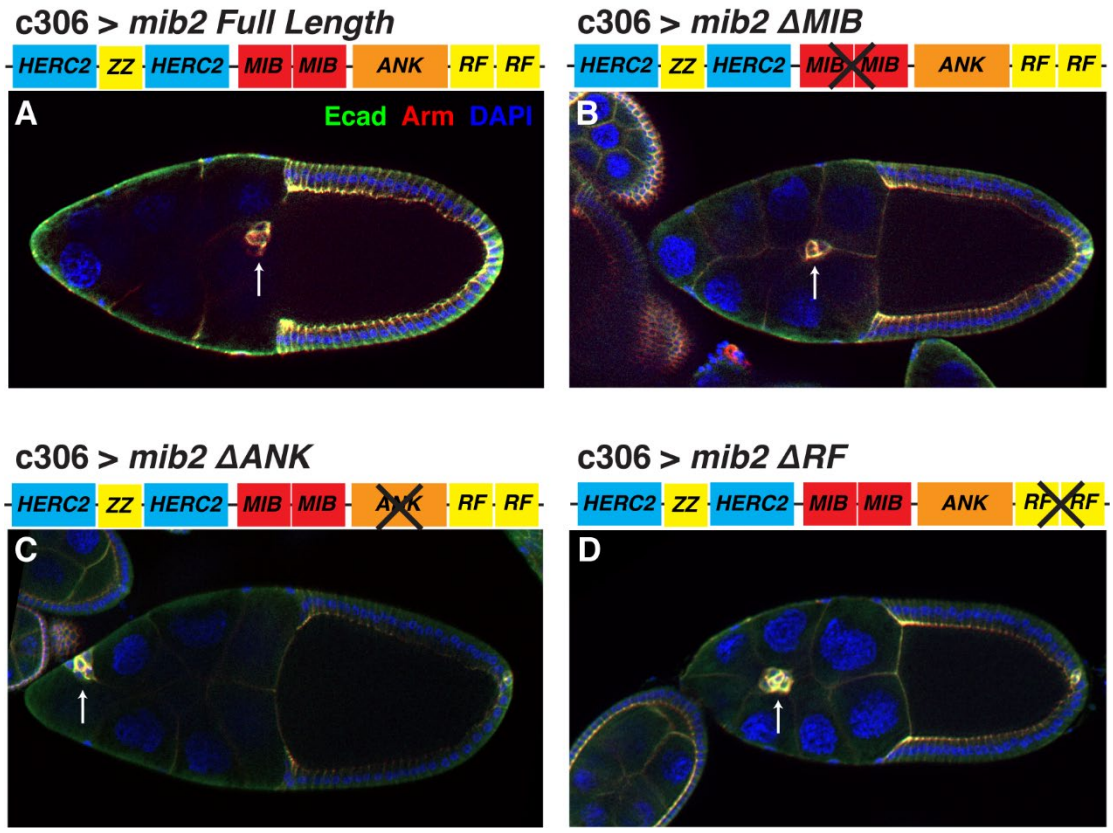


Supp. Figure 2.5) Mib2 expression in younger egg chambers. A) The wild type egg chambers immunofluorescently stained for Mib2, shown in green (A'), and Arm, shown in red; and nuclei are shown with DAPI staining in blue.



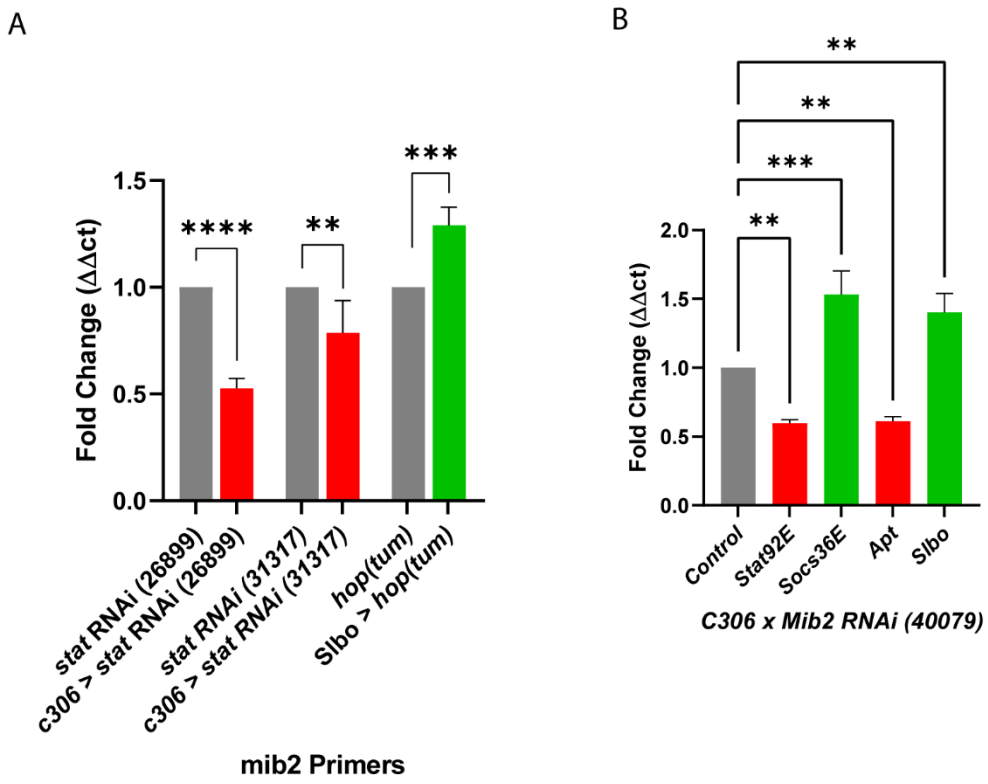
Supp. Figure 2.6) Expression of tagged Mib2 and *mib2* knockdown. A-A'') The Mib2 tagged fly line egg chambers immunofluorescently stained for FLAG, shown in red (A'), and GFP, shown in green (A''); nuclei are shown with DAPI staining in blue. B) The knockdown

of *mib2* RNAi (V40079) in anterior follicle cells is confirmed by significantly lower *mib2* expression compared to the control. Statistical analysis was performed using a one-way ANOVA test, **** represents $p \leq 0.0001$.



Supp. Figure 2.7) The ANK, MIB and RF domains of Mib2 are involved in border cell migration. (Corresponds to results shown in Figure 2.2 panel G.) *Drosophila* Mib2 has eight highly conserved domains namely, 2xHERC2, ZZ (zinc finger), 2xMIB, ANK (ankyrin repeats), and 2xRF (RING Finger). All the egg chambers are stained with antibodies that bind to Arm, shown in red, and E-cad, shown in green; nuclei are shown with DAPI staining in blue. A) An over expression of *mib2* shows no migration defects evident by the cluster reaching to the oocyte by stage 10. B, C, D) Expression of the domain deletions constructs *mib2(ΔMIB)*, *mib2(ΔANK)*, *mib2(ΔRF)* results in significant migration defects in ~20%, ~18%, and ~32% of the stage 10 egg chambers respectively. We believe these defects are due to dominant negative

effects of the remaining domains. Expression of full length Mib2 or expression of constructs with ZZ, HERC2a, or HERC2b deleted did not disrupt border cell migration.



Supp. Figure 2.8) Jak/STAT signaling regulates *mib2* expression. The knockdown of *stat* by expressing *stat* RNAi(s) in anterior follicle cells results in the downregulation of *mib2* expression in *Drosophila* ovaries. Similarly, overactivation of JAK/STAT signaling by expressing *hop(tum)* in border cells causes upregulation of *mib2* expression. B) The *mib2* knockdown in anterior follicle cells reduced the *Stat92E* and *apt* expression however induced the *socs36E* and *slbo* expression. The results indicate complex feedback signaling that needs further study. Statistical analysis was performed using a one-way ANOVA test, **** represents $p \leq 0.0001$.

Chapter 3: The role of miRNA in JAK/STAT signaling and collective cell migration

3.1) Introduction

Micro RNAs (miRNAs) are short non-coding RNAs with a length of ~22 nucleotides that are involved in the regulation of gene expression. Since the discovery of the first miRNA, *lin-4* in *C. elegans*, many more miRNAs have been discovered in various animal models and they appear to be highly conserved (Li et al., 2010; Pasquinelli et al., 2000). To date, over 17,000 miRNAs have been documented among 140 species, revealing their physiological relevance (Kozomara & Griffiths-Jones, 2011). Specifically, in humans, over 2000 microRNAs have been annotated that regulate one-third of the human genome. Similar to messenger RNAs, miRNAs also get transcribed into transcripts that must be processed further. DNA is transcribed into the primary miRNAs, which are usually over 1kb in length with a local stem-loop structure (Ha & Kim, 2014). The RNase III Drosha crops out the stem-loop structure and releases a hairpin RNA of about 65 bp in length called the precursor miRNA. This precursor miRNA is then processed into a ~22 bp long mature miRNA by Dicer and helper protein complex.

miRNAs are known to have a diverse functional portfolio; albeit in most animals miRNAs are known to base pair with the 3`UTR of target mRNAs, leading to their degradation and suppression of gene expression. Since the miRNA seed region, the region that is complementary to its target mRNA, is only ~5-7 nucleotides long, each miRNA can potentially bind to hundreds of mRNAs while each mRNA can be regulated by multiple miRNAs (Lewis et al., 2005). Other lesser observed target interaction regions are the 5`UTR and the coding region of mRNAs (Broughton et al., 2016). Recent studies also indicate that miRNA can be found in the nucleus, cytoplasm, and even extracellularly to mediate cell signaling (Hayes et al., 2014; Makarova et al., 2016). With a dense functional repertoire, miRNAs are implicated in both morphogenesis and pathophysiology (Fu et al., 2013). Such a complex and diverse set

of functional targets for miRNAs makes them challenging to study, but miRNAs remain interesting candidates to help us explain genetic regulation.

There are several modes for miRNA function at a molecular level. One of them is miRNA-based gene silencing with the help of a guide strand and Argonaut (Jo et al., 2015). A fully complementary single-stranded seed sequence of miRNA, called the guide strand, targets the mRNA and promotes catalytic mRNA cleavage induced by Argonaut (Ago) through the RNA-induced silencing complex (RISC). In another mechanism, *let-7* directly binds to the L1-mRNA and acts as a translational repressor in cultured tumor cells (Pillai et al., 2005). On the other hand, several studies showcase the translational activation and gene upregulation by miRNAs (Truesdell et al., 2012; Vasudevan & Steitz, 2007). For example, *let-7* activates its targets during cell cycle arrest, and other miRNAs upregulate gene expression in *Xenopus* oocytes. This increase in translation is dependent on the base-pairing between *let-7* and its targets as well as the presence of Ago2 and associated proteins. Hence, miRNA can up or down-regulate translation to regulate the protein levels of the target mRNA.

miRNAs have been shown to be involved in collective cell migration. One mechanism in Michigan Cancer Foundation 7 (MCF-7) epithelial cancer cells is through regulating Transforming Growth Factor-β (TGF-β) signaling (An et al., 2013). TGF-β promotes *miR-21* expression during epithelial collective cell migration (Dean et al., 2015). During the wound healing process of MCF-7 cells in a culture dish, the leading edge of collectively migrating cells experience an increase in the gradient of *miR-21* expression upon TGF-β treatment. However, the *miR-21* targets are unknown and require further attention. A second example is in primary breast carcinomas where *miR-10b* directly targets and downregulates an mRNA that encodes Homeobox D10 (HOXD10) (Ma et al., 2007). HOXD10 is a transcriptional repressor of genes involved in cell migration and extracellular matrix dynamics. Thirdly, in liver

mesenchymal-like cancer cells, *miR-194* overexpression downregulates the expression of N-cadherin to suppress invasion and cell migration both *in vitro* and *in vivo* (Meng et al., 2010). These and other examples show us that microRNAs are directly or indirectly involved in cell migration and need further characterization to understand their mode of mechanism.

We use *Drosophila* ovaries as our system to understand the roles of miRNAs in gene regulation and collective cell migration. A review on the circuitous genetic regulation of collective cell migration shows that multiple signaling pathways, such as JAK/STAT, Ecdysone, Hippo, Notch, growth factor, and chemokine signaling, are required in the specification and collective migration of border cells (Saadin & Starz-Gaiano, 2016a). These pathways regulate transcription factors that modulate the expression of genes involved in adhesion, cytoskeletal components, and cellular polarity. Notably, these pathways are known to be regulated by several microRNAs in various systems. *miR-279* and *miR989* are two microRNAs shown to regulate border cell migration (Kugler et al., 2013; Yoon et al., 2011). In *Drosophila* egg chambers JAK/STAT signaling specifies non-migrating epithelial follicle cells into migrating border cells (Starz-Gaiano et al., 2008; Xi et al., 2003). *miR-279* directly binds to the *Stat92E* transcripts causing their destruction, and thereby acts as a repressor for STAT activity. It is involved in a feedback loop with other STAT regulators such as Apontic (Apt), Slow border cells (Slbo), and Ken and barbie (Ken) (Yoon et al., 2011). In another instance, *miR-989* is abundantly expressed in the *Drosophila* egg chambers and it is required for the regulation of border cell migration (Kugler et al., 2013); however, its targets have not been determined.

We speculate that there are other microRNAs involved in border cell migration based on our microRNA analysis and the predictions for involvement in migration-related signaling. A mechanistic mathematical model (Berez et al., 2020; Ge, X., Stonko, D., Peercy, B., and

Starz-Gaiano, 2012) shows that microRNAs have a likely role in cross repression between *apt* and *slbo*. In the model, the microRNA(s) requires activation by JAK/STAT signaling and in turn, that microRNA(s) regulates JAK/STAT signaling, thus, microRNAs become a part of the JAK/STAT feedback loop. The data from our laboratory suggests that the seed sequences of *let-7* and *miR-8* have complementarity for the 3'UTR of *slbo,* whereas the 3'UTR of *apt* and *slbo* have two and three potential binding sites for the seed sequence of *miR-315,* respectively. This data presents *let-7, miR-8*, and *miR-315* as interesting candidates to study within the scope of JAK/STAT signaling and collective cell migration.

let-7 has been shown in other tissues to regulate two pathways important in the border cells. Unpaired, an activator of JAK/STAT signaling is expressed in follicle cells and the testes stem cell niche, and there its message is protected from siRNA by its stabilized interaction with IGF-II mRNA binding protein (Imp) (Toledano et al., 2012). However, *let-7* represses Imp in an age-dependent manner, causing Unpaired degradation as well. Additionally, *let-7* is also a known repressor of *abrupt* (Kucherenko et al., 2012). JAK/STAT signaling induces Abrupt loss in the border cell nucleus at stage 9 of oogenesis, which prohibits Abrupt from downregulating Ecdysone signaling and inhibiting border cell migration (Jang et al., 2009). Ecdysone signaling is normally active and required in the timing of border cell migration (Bai et al., 2000). Since Abrupt provides an integral signal in border cell migration, *let-7* may mediate its regulation to allow migration and is a good candidate for our studies.

Among multiple candidates, in this chapter, we focus mainly on *miR-8* as prior studies show that the *miR-8* locus has binding sites that may be activated by the JAK/STAT signaling target Slbo and has a predicted seed sequence with complementarity to Apt (Ge, X., Stonko, D., Peercy, B., and Starz-Gaiano, 2012). Other pathways may also be targeted by *miR-8*. For example, *miR-8* directly regulates a Notch signaling ligand, Serrate, in *Drosophila* eyes

(Vallejo et al., 2011). The *miR-8* targets Serrate to downregulate Notch signaling and block cell proliferation and growth. Conversely, *miR-8* can potentiate growth and cell survival in fly wings by promoting neoplasia and metastasis (Eichenlaub et al., 2016). There, *miR-8* represses Peanut, a pro-apoptotic protein, and promotes cell growth by aiding Epidermal Growth Factor Receptor (EGFR)-driven tumorigenesis. Within glial cells, *miR-8* is co-expressed with and targets *spitz*, which encodes a ligand of EGFR signaling, to regulate cell proliferation rate and cell remodeling (Morante et al., 2013). In fat body cells, *miR-8* downregulates *ImpL2* which is a target of Ecdysone signaling (Honegger et al., 2008). Since both EGFR and Ecdysone signaling are required for proper border cell migration, we were intrigued to investigate the involvement of *miR-8* in border cell signaling.

3.2) Materials and Methods

3.2.1) Fly stocks and Quantitative real-time PCR analysis

Slbo-Gal4 (Rørth, 1998) virgin female flies were crossed respectively with lines: UAS-Hop(tum) (Harrison et al., 1995) to activate STAT signaling, UAS-stat RNAi (Kim LK et al., 2007) to knock down STAT signaling, and UAS-miR-8 (Szuplewski et al., 2012) to overexpress this miRNA in the border cell at 25ºC. The male parental lines were used as a control for all qRT-PCR experiments. All F1 progeny were fattened at 29ºC overnight and ~30 pairs of ovaries were dissected. Total RNA was extracted using the Thermo Fisher Scientific mirVana microRNA isolation kit with DNase I digestion. Using TaqMan microRNA primers, we reverse transcribed the respective microRNA from the isolated RNA fraction. The qPCR reactions (3 biological and 3 technical replicates for each sample) were set up with TaqMan microRNA assay (20x) (1µl), a product from RT reaction (minimum 1:15 dilution) (1.33µl), TaqMan 2x universal PCR master mix (10µl), nuclease-free water (7.67µl) with 20 µl reaction volume. The plates were analyzed using the CFX96 (Bio-Rad) qPCR detection system and fold

change was measured using ΔΔCt value. The primers and probes were part of the Thermo fisher TaqMan assay kits (4427975).

3.2.2) Binding motif analysis

The binding motif analysis of *stat*, *slbo,* and *ecr* was performed using the JASPAR platform (Fornes et al., 2020). The platform used the binding motifs for the transcription factors; *slbo*: ATTGCAAA, *stat*: CGGAATTCCNGGAAA, and *ecr*: GAGTTCATTGACCTT. Many of the bases could have a variation based on the prediction model. Using these binding motifs, we performed the analysis using FIMO (Grant et al., 2011) to identify binding sites upstream of miRNA loci. The TargetScanFly platform (Agarwal et al., 2018) was used to identify seed sequences of miRNAs that may target relevant genes in border cells.

3.2.3) Immunohistochemistry and microscopy

The fly stocks were maintained at 25ºC and were fattened at 29ºC, 16 hours for optimum UAS-Gal4 expression. For our experiments, we used *slbo*-Gal4 (Rørth, 1998), UAS-*hop*$^{(tum)}$ (Harrison et al., 1995), UAS-*stat* RNAi (Kim LK et al., 2007), and UAS-*miR-8* (Szuplewski et al., 2012). We crossed the *slbo*-Gal4 with the respective UAS lines to achieve the cell-specific expression. Ovarioles were extracted from fattened flies into dissection media (1x Schneider's *Drosophila* medium by Thermo Fisher Scientific (21720-001), 10% FBS, 0.6% Pen/Strep) and fixed with 4% paraformaldehyde (0.1M KPO4 buffer) for 10 mins at RT. Fixed ovarioles were rinsed and washed 3 times with NP40 buffer (0.05 M Tris HCl, pH 7.4, 0.15 M NaCl, 0.5% Nonidet P-40 (Igepal CA-630, Sigma-Aldrich), 1 mg/ml BSA) (McDonald et al. 2006). For staining, ovarioles were incubated with the respective primary antibody in NP40 buffer overnight at 4ºC. Ovarioles were rinsed and washed 3 times with NP40 buffer then incubated with Alexa Fluor secondary antibodies in NP40 buffer overnight at 4ºC followed by

DAPI staining for 10 mins. Ovarioles were rinsed and washed 3 times with NP40 buffer and incubated then mounted in 70% Glycerol. Samples were observed using either the Zeiss LSM 900 confocal with Airyscan 2 or Carl Zeiss AxioImager Z1 and captured using the AxioVision acquisition system. Post image processing was performed using ImageJ and Adobe Illustrator.

3.3) Results

3.3.1) JAK/STAT signaling regulates the expression of microRNAs in the *Drosophila* egg chamber

To establish if the candidate miRNAs are downstream of JAK/STAT signaling, we performed qPCR analysis. Since *miR-279* has been fairly characterized (Yoon et al., 2011), we also used it in our analysis as a positive control. To confirm that our probe and primers for qPCR works, we optimized and detected the miRNAs in the ovary using the TaqMan assays. We overactivated JAK/STAT signaling by expressing $hop^{(tum)}$ in *slbo*-GAL4 expressing (motile border cells and centripetal cells) cells of the egg chamber (Luo et al., 1995). We isolated microRNA from the ovaries and performed reverse transcription followed by qPCR using TaqMan assays. The qPCR data showed us a significant overexpression of *miR-279* upon overactivation of JAK/STAT signaling (Figure 3.1). We saw a similar uptrend of *miR-8* and *let-7.* Although this difference was not statistically significant compared to the control, we believe it is noteworthy since JAK/STAT signaling was only activated in a subset of ~20 cells in a tissue of over 1000 cells. Thus, it is important that we could detect a change and is worth further investigation. We also see a dramatic downregulation of *miR-315* when STAT signaling is activated. Conversely, we downregulated JAK/STAT signaling by expressing *stat* RNAi in motile cells. Expression of each microRNA (*miR-8, miR-279, miR-315,* and *let-7*) was reduced upon downregulation of JAK/STAT (Figure 3.1). While this change was not statistically significant, again, the detectable reduction when only a subset of ~20 cells of the whole tissue

had signaling changes could be relevant and should be further investigated. Our data suggest that JAK/STAT signaling regulates the expression of several microRNAs in follicle cells.

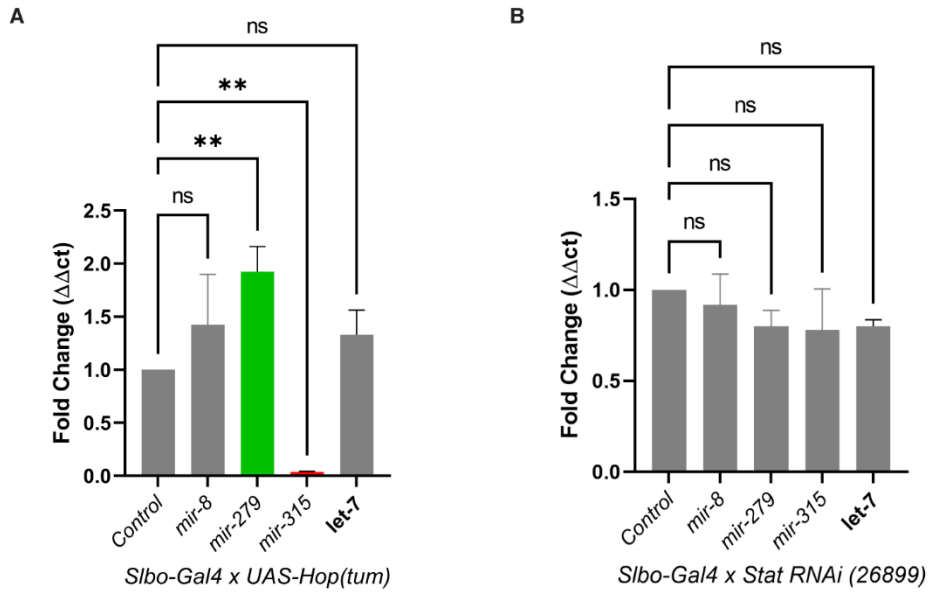


Figure 3.1) JAK/STAT signaling regulates microRNA expression. A) The overexpression of activated Jak by expressing Hop$^{(tum)}$ in border and centripetal cells result in changes of microRNA expression, detected by qRT-PCR from whole ovaries. The expression of *miR-8* and *let-7* are elevated, although the difference is not statistically significant; *miR-279* is significantly enriched, and *miR-315* is significantly downregulated. We used the parent driver line Slbo-Gal4 as a control. The control was normalized as 1 and other samples were read relative to it. B) The downregulation of JAK/STAT signaling by expressing *stat* RNAi in border and centripetal cells results in the downregulation of *miR-8, miR-279*, *miR-315* and *let-7* expression. Statistical analysis was performed using a one-way ANOVA test, ** represents $p \leq 0.0001$.

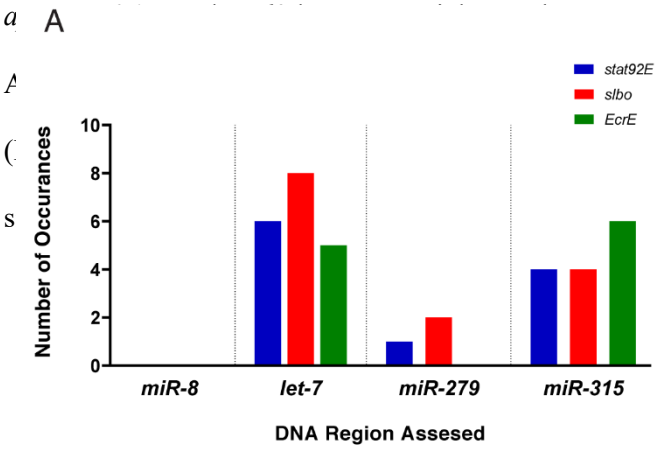
3.3.2) Binding motif analysis suggests that key border cell transcription factors may regulate expression of miRNAs

We also performed transcription factor and miRNA binding site analyses with the help of an undergraduate student, David Waldron, and Mallika Bhattacharya. For our binding site analysis, we used Find Individual Motif Occurrences (FIMO) (Grant et al., 2011) and examined if the transcription factors STAT, Slbo, or EcR had conserved binding site motifs within the genomic loci of the miRNAs of interest (including the transcribed region and 1kb upstream). As expected from the literature, we identified one STAT and one Slbo binding site in the *miR-279* upstream region (Yoon et al., 2011). In the *miR-315* locus upstream of the transcription start site, we found four STAT, four Slbo, and six EcR consensus binding sites. In the *let-7* locus, we found six STAT, eight Slbo, and five EcR consensus binding sites. This is consistent with our model that these may be regulated by STAT signaling. In contrast to (Ge, X., Stonko, D., Peercy, B., and Starz-Gaiano, 2012), who used a different analysis program, we did not find any relevant binding sites in the *miR-8* locus using FIMO, but the sites may be outside of the range we analyzed. Since STAT and EcR activate multiple downstream transcription factors, it is also possible they may have an indirect effect on *miR-8.*

3.3.2) Binding motif analysis suggests miRNAs may regulate genes that encode important border cell transcription factors

For our miRNA target analysis, we choose to analyze the mRNA sequences for *stat92E*, *slbo*, and *EcrE* and their possible complementarity with microRNAs (Figure 3.2). Using the TargetScanFly platform (Agarwal et al., 2018), we identified several microRNAs that have predicted binding to the 3'UTR region of relevant JAK/STAT target genes based on their seed sequences. We also include the gene encoding the negative JAK/STAT regulator Suppressor of Cytokine Signaling 36E (Socs36E) in our analysis as it is involved in the feedback loop

along with *apt* and *slbo* (Monahan & Starz-Gaiano, 2013). Our data shows that the 3'UTRs for



B

Gene Name	Conserved Sites
miR-8	
upd2	2
socs36E	1
apt	1
let-7	
apt	1
EcR	1

DNA Region Assessed
A
stat92E
slbo
EcrE
Number of Occurances
10
8
6
4
2
0
miR-8
let-7
miR-279
miR-315
DNA Region Assessed
B

Gene Name	Conserved Sites
miR-8	
upd2	2
socs36E	1
apt	1
let-7	
apt	1
EcR	1

Figure 3.2) miRNAs regulatory sites and conserved target sites A) A graphical representation of the binding sites upstream of *miR-8*, *let*-7, miR-*279*, and *miR-315* for transcription factors such as Stat, Slbo, and Ecr. B) Analysis of *miR-8* and *let-7* targets using TargetScanFly shows potential conserved targets which are involved in the border cell

migration pathway. We chose the ones potentially activated (and more abundantly expressed) miRNAs.

3.3.3) *miR-8* is involved in JAK/STAT modulation in the *Drosophila* egg chamber

Next, we wanted to understand if the microRNAs also have a regulatory effect on JAK/STAT signaling components or border cell migration. We began investigating *miR-8* because several genetic lines were available. We first verified and overexpressed *miR-8* in motile cells (Szuplewski et al., 2012) A qPCR analysis reveals that *miR-8* overexpression results in a reduction of *stat*, *apt*, and *socs36E* (Figure 3.3). This reduction opens the possibility of a feedback loop between JAK/STAT signaling, expression of *miR-8,* and potential change of STAT activity.

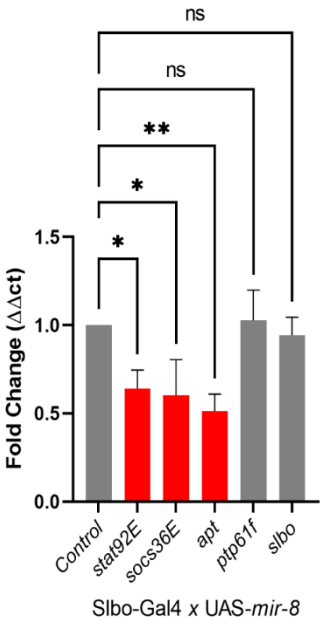


Figure 3.3) *miR-8* regulates the expression of JAK/STAT signaling components. The overexpression of *miR-8* in border and centripetal cells results in changes of JAK/STAT

component expression, as detected by qRT-PCR from whole ovaries. The expression of *stat92E*, *socs36E*, and *apt* are reduced but *ptp61f, mib2,* and *slbo* expression are not affected to a statistically significant extent. Statistical analysis was performed using a one-way ANOVA test, **** represents $p \leq 0.0001$.

3.3.4) The *miR-8* is a border cell migration regulator

To see if *miR-8* has a phenotypic role in collective cell migration, we turned our focus towards border cell migration under *miR-8* modulation. Overexpression of *miR-8* using *slbo*-Gal4 resulted in defective border cell migration in about 24% of 178 stage 9 egg chambers (Figure 3.4). We also performed *miR-8* knockdown in motile cells, but it did not show us any significant migration defects (data not shown). We propose to use the *miR-8* deficiency line to study its knockout effect. We conclude that *miR-8* may be involved in the regulation of border cell migration, possibly via JAK/STAT signaling, but further experiments are necessary. Additionally, it is imperative to analyze whether the overexpression or knockdown of other microRNAs has functional relevance to JAK/STAT signaling and collective cell migration.

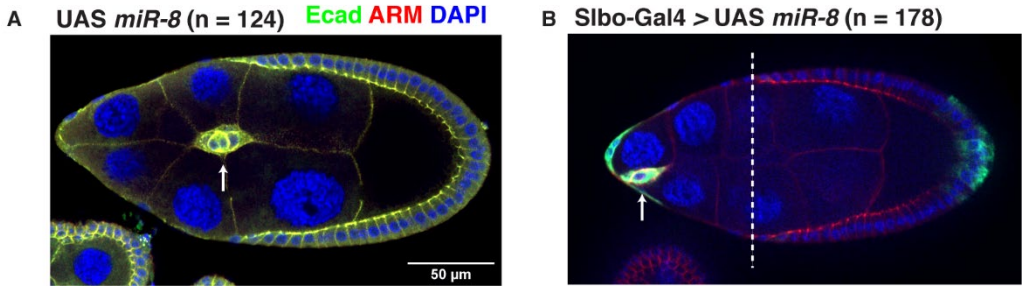


Figure 3.4) *miR-8* is involved in border cell migration. A) Egg chambers immunofluorescently stained for Arm protein, shown in red, and E-cad, shown in green; nuclei are shown by DAPI staining in blue. In the control egg chamber, without Gal4 induction, the border cell cluster moved alongside the cuboidal-squamous follicle cell boundary, which suggests timely migration at stage 9. B) The overexpression of *miR-8* in border cells results in

defective border cell migration at stage 9 with cells remaining close to their anterior starting point (arrow).

3.4) Discussion

Our data suggest that several microRNAs may have a significant role in JAK/STAT signaling as well as collective cell migration in *Drosophila* ovaries. We have found that JAK/STAT signaling impacts the expression of microRNAs *miR-8*, *miR-279*, *miR-315*, and *let-7*; although the differences in expression due to loss or gain of STAT signaling were small, we believe they are important since we only modulated signaling in a subset of cells. Further experiments could be done to extend this analysis to a larger subset of cells. Additionally, our bioinformatics analysis suggests that JAK/STAT signaling not only regulates the miRNA expression but also it is likely to be impacted by miRNAs as a part of a feedback loop involving *upd2, apt* and *Socs36e* regulation. More research will be needed to determine if these predictions are correct *in vivo*.

We focused our phenotypic studies on *miR-8*. We found that *miR-8* has a regulatory role in STAT signaling and in particular, promotes the downregulation of *Stat92e, apt,* and *Socs36e* expression in the ovary. Consistent with this, overexpression also disrupts border cell migration. Since *miR-8* knockdown in motile cells did not show us any significant migration defects, we cannot conclude it is strictly required in this process, but instead, it may act redundantly to fine-tune signaling components. We would also use the miR-8 RNAi line to knockdown its expression in anterior follicle cells. We aim to continue our studies on other microRNAs in border cell migration. Since the endogenous *miR-315* expression in egg chambers is quite low, and it is further repressed by STAT signaling, this may not be the easiest candidate to investigate. Thus, we propose *let-7* should be our next candidate to examine phenotypically.

Evidently, miRNAs are a very powerful component of gene regulation and translational activation. We are interested in identifying the microRNAs that may add a regulatory layer in the JAK/STAT pathway and cell migration. However, further in vivo studies are required to understand their biochemistry and involvement in cell signaling.

3.5) Acknowledgements

STEM BUILD team undergraduate student Joel Hayford helped with genetic crosses and immunofluorescent stainings. The binding motif analysis was performed by David Waldron with the help of Mallika Bhattacharya. The project was supported by an NSF grant (IOS 1656550 to MSG)

Chapter 4: Conclusion and Future Directions

Certain cells can collectively migrate in a specific direction and speed guided by chemotactic gradients, signaling pathways, and mechanical cues (Friedl & Gilmour, 2009). Such a collective cell migration is distinctly observed in embryonic morphogenesis, wound healing, and cancer metastasis. It is crucial that we discover and understand the biomolecules that regulate signaling pathways involved in cell migration since they have implications in development and disease. One of the many pathways involved in this intricate system is JAK/STAT signaling, which is well conserved and provides regulation for many biological processes (Rawlings et al., 2004). Since *Drosophila* border cells are well-studied and tractable, they serve as an excellent system to perform genetic, biochemical, and molecular studies to address how their movement is controlled. We can also take advantage of the relative genetic simplicity of *Drosophila* where for example, only one STAT and one JAK is present in the genome. This can help us understand the signaling pathway and border cell migration more easily. Still, there are multiple JAK/STAT pathway candidates involved in border cell migration like Apt, Socs36E, Slbo, and more to be discovered (Saadin & Starz-Gaiano, 2016b). Our work attempts to further our understanding of collective cell migration via identifying new required genes, including potential JAK/STAT signaling components.

Muller et al. identified major regulators of JAK/STAT signaling and found that *Drosophila mindbomb2 (mib2)* acts as a negative regulator of STAT activity in cell culture (Boutros et al., 2006; Müller et al., 2008). We explore the function of this gene in chapter 2. Our qPCR study shows that JAK/STAT signaling has a positive correlation with *mib2* expression in anterior follicle cells. Moreover, the knockdown of *mib2* has a significant effect on the expression levels of JAK/STAT components in egg chambers such as *Stat92E*, *socs36E*, *apt*, and *slbo* (data not shown). However, the changes in expression of these components are not conclusive since they do not point to clear negative regulation of STAT activity. Thus, we directed our focus towards the cytoskeletal proteins. We speculate that Mib2, an E3 ubiquitin

ligase, might have a role in the regulation of border cell migration via cytoskeletal maintenance, independent of JAK/STAT signaling. A large pool of studies shows that the cytoskeleton network that shapes a cell also has biochemical control over the regulation of collective cell migration (Lucas et al., 2013; Niewiadomska et al., 1999a; Peifer M, 1993; Seetharaman & Etienne-Manneville, 2020; Sekerkova et al., 2004). Many cytoskeletal proteins such as β-catenin (Arm), actin, and actin dynamic regulators are crucial for collective cell migration in border and other cells, and we propose Mib2 as a regulator of these. Cell-cell adhesion is also critical for migration, and it is often mediated by cadherins. In *Drosophila* egg chambers, we found that loss of *mib2* results in a dramatic loss of E-cadherin, reduced Arm on the lateral surface of follicle cells, and downregulation of cortical actin. Our physical interaction study also revealed novel Mib2 binding partners that seem to be regulating collective border cell migration. It would be very interesting to explore these candidates further (including cdc42 regulator RhoGAP19D, actin-associated proteins α-catenin, and Supervillin, and the transcription factor Modulo) and to see if they help to explain the requirement for Mib2.

There are multiple experimental strategies that would expand our understanding of Mib2 in egg chambers. For example, this study can be advanced by students in the future by focusing on genetic interaction tests between these candidates and *mib2* mutants. The individual double heterozygous mutants or RNAi knockdown of both *mib2* and its binding partners could be analyzed for border cell migration defects. A more-than-additive or a rescue of migration defects would shed some light on the mode of mechanism for Mib2 and the pathways it may be involved in. Another important next step is to explore a potential switch of Mib2's function between a role in protein stabilization and/or in target protein degradation. Stabilization sometimes occurs by the addition of a signal ubiquitin, whereas polyubiquitination triggers degradation of proteins by the proteosome. Since our studies suggest a stabilization role for Mib2, the possibility of target monoubiquitination can be

assessed by Western blot. Mib2 can be coimmunoprecipitated with its binding partners in the presence of a proteosome inhibitor and the proteins would be separated by SDS-PAGE. The Western blot analysis using the FK2 antibody, which recognizes ubiquitin, would tell us about the state of ubiquitination. If individual discrete bands are observed, it suggests monoubiquitination of the substrate(s) but if a long smear is observed, it points towards the polyubiquitination. However, this would not determine what the target proteins are, so additional antibodies could be used separately in blots to assay the candidates. Performing *in vitro* ubiquitination assays can also confirm the substrates for Mib2 and the state of ubiquitination. We can keep Ecad, Arm, and αCat at the forefront of future studies as they are well-characterized and related molecular and genetic tools are available.

Several recent studies have suggested that microRNAs are involved in the regulation of collective cell migration (An et al., 2013; Dean et al., 2015; Yoon et al., 2011). Additionally, some studies suggest the involvement of microRNAs in JAK/STAT signaling (Kucherenko et al., 2012; Toledano et al., 2012; Yoon et al., 2011), including in border cell migration. We investigated more about the potential regulation by miRNAs of the STAT signaling feedback loop involving Slbo and Apt signaling. This regulation is predicted by our computational models (Berez et al., 2020). An *in silico* analysis showed us that *miR-8*, *miR-279*, *miR-315*, and *let-7* have seed sequences that could bind within the 3`UTR of *apt* and *slbo*. As expected, we found that their expression is positively correlated with the JAK/STAT signaling, although this will need to be confirmed. Diving further, we also found that *miR-8* is a regulator of collective cell migration. For a future direction, it will be interesting to determine if *miR-8* is regulating collective cell migration via JAK/STAT signaling. We can perform genetic interaction tests between the *miR-8* and JAK/STAT components to observe the migration defect penetrance. A rescue or synergistic effect could point toward the genetic interaction of *miR-8* with the JAK/STAT pathway. Additionally, the 10XSTAT activity reporter(Bach et al., 2007) could be

examined in combination with *miR-8* overexpression or knockdown. Other open questions are if other miRNAs (such as *miR-4974*, *miR-4909*, *miR-9387*, etc., and our candidates *miR-8*, *miR-279*, *miR-315*, and *let-7*) are involved in STAT regulation at the transcript level, and are these miRNAs acting only via transcript regulation or rather by a non-canonical mechanism such as epigenetic regulation? Since different miRNAs usually have multiple targets (Lewis et al., 2005), it is possible that our candidate miRNAs could be involved in multiple pathways. To address this query, we can modulate the candidate miRNAs and look at the expression of a few components of signaling pathways involved in collective cell migration (for example, Notch, Hippo, chemokine signaling, and polarity pathways) (Saadin & Starz-Gaiano, 2016a). miRNA expression is only in part governed by transcription factors. So, it is also very important to look broadly at miRNA regulation, including their biogenesis, stability, and localization in the cell can influence their activity (Gebert & MacRae, 2019). A lot remains to be uncovered on the microRNA front and identifying their mode of mechanisms. This and their functional relevance make them very interesting candidates to explore further with a wide scope in developmental biology.

We are fascinated by not just genetic signaling regulation but by also the role of tissue architecture. Our studies show that an asymmetric tissue landscape could have a significant effect on how morphogen signaling occurs and on subsequent cell activation. Morphogen signaling is one of the many known biological mechanisms in cell fate determination, and is critical for proper animal development (Briscoe & Small, 2015; Müller et al., 2013). While multiple factors govern morphogen transport, we know very little about the effect of tissue architecture on signal distribution. Morphogen signaling has been heavily investigated in the genetically tractable organism *Drosophila*. In *Drosophila* egg chambers the anterior polar cells secrete Unpaired (Upd), which when received by the surrounding follicle cells, activates JAK/STAT signaling and specifies follicle cells into border cells (Beccari et al., 2002;

Ghiglione et al., 2002; Montell, 2003; Montell et al., 2012; Silver et al., 2005; Xi et al., 2003). Surprisingly we observed asymmetries in the STAT activation pattern around the polar cells, and our experiments and modeling suggested this was due to uneven Upd distribution among the follicle cells due to tissue contour (L. A. Manning et al., 2015). To understand the activation pattern and Upd diffusion in between the subcellular domains in a biological system, we created an Upd-fusion with a photoswitchable protein, Dendra2, which is described in the appendix, to examine Upd in vivo. We can detect the fusion protein, and it is able to signal in follicle cells. This study requires multiple future research directions, including determining the kinetics of morphogen distribution, the role of heparin sulfate proteoglycans, and the possible involvement of structures such as cytonemes. A lot of these questions can be answered by optimizing a live imaging analysis that can track the Upd-Dendra2 distribution and its photoconversion. Understanding how morphogen transport is constrained via biophysical parameters and how downstream signaling is regulated can open a whole new aspect of understanding extracellular signaling cues and their impacts on developmental disorders.

It is crucial that we discover and understand signaling biomolecules and their role in signaling pathways in their in vivo contexts, as this has implications in disease. My dissertation added to this research area by identifying a new regulator of cell migration and epithelial cell maintenance, Mib2, and by exploring novel ways JAK/STAT signaling may be modulated by miRNAs and tissue structure. The functional characterization of signaling pathways, biomolecules, and biophysical attributes can lead to a better understanding of collective cell migration and its involvement in morphological and pathophysiological processes.

Appendix Chapter: Tissue Landscape - A Conceptual Regulator of *Drosophila* Ovary Cell Fate Specification

A.1) Introduction

A.1.1) Morphogen Signaling and Transportation

One of the fascinating aspects of developmental biology is that a single cell can divide numerous times to form a highly complex functional organism containing billions of cells with their respective defined roles (Briscoe & Small, 2015). After every successive division, the daughter cells are arranged spatially and temporally to form intricate tissues leading up to the development of an adult organism. What would dictate how the cells adopt their respective fates, one may ask. One of the important mechanisms is communication between cells via signaling molecules known as morphogens. Morphogens, typically proteins, are secreted from a source and received at target sites via traveling through intermediating biochemical environments. Morphogens, when received at the target, usually lead to gene regulation and determine the fate of the cell in a concentration-dependent manner. The scientific community is interested in studying various morphogens and their expression patterns, including

Decapentaplegic (Dpp), Hedgehog (Hh), Fibroblast growth factor (FGF), and Nodal in various organisms (Briscoe, 2015). Morphogens can signal intracellularly and extracellularly and can be transported via different routes. Their transportation could be via simple diffusion or can be influenced by various biochemical and/or biophysical factors. It is very important to understand how morphogen distribution is regulated so that we can control or modulate cell fates.

Several theories have been put forth to explain the mode of morphogen transport, which is important given their concentration-dependent effects (Müller et al., 2013). In this study, we focus on two of the multiple models proposed: free (simple) diffusion and hindered diffusion (Figure A.1). One model postulates that the morphogens are spread through free diffusion. However, if the molecules were to simply diffuse, after a while there would be a uniform distribution of molecules. To create a characteristic gradient of signaling, in addition to diffusion, the morphogens must be constantly degraded due to their short half-life, and some are affected by the presence of other repressive signals (Wartlick et al., 2009). A second model explains the extracellular movement of signaling molecules hindered by obstacles, which may or may not have a binding affinity for the morphogens. Degradation also must occur in this model. This model explains the effect of cellular arrangements and the effect of the tortuosity of available space for morphogens to pass by (Sawala, Sutcliffe, and Ashe, 2012). This model is very important for the hypothesis that we are postulating about the asymmetric distribution of a morphogen due to the hindrance of extracellular space (L. A. Manning et al., 2015). Under this mode of transport, morphogens may take longer to move across a similar distance compared to the free diffusion model and may concentrate in different places (Thorne & Nicholson, 2006).

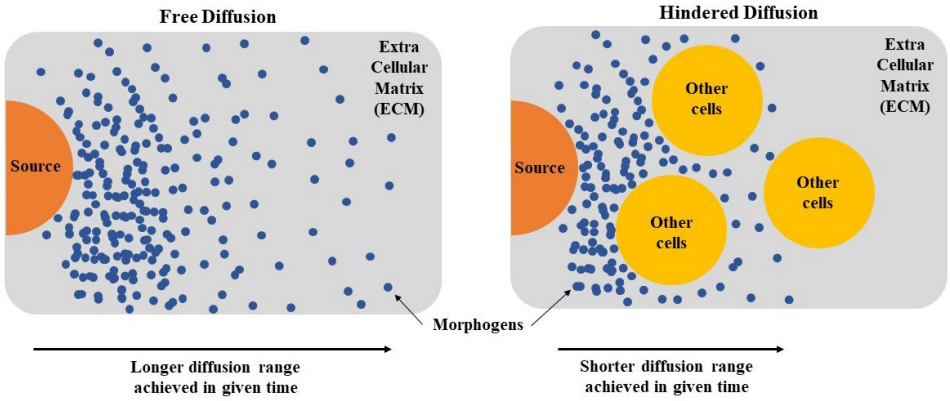


Figure A.1) Different models of morphogen transport. Adapted from Muller et al., 2013: A) The morphogens diffuse from source cell/s (actively or passively) through the extracellular matrix freely without any hindrance. B) The morphogens secreted by the source cells face hindrance from the other cells or molecules present in the tissue. This hindered diffusion can lead to a longer time before morphogens reach their target(s). Here the target cells are present along the bottom of the grey domain.

Given that it has been difficult to track morphogens in the extracellular environment, computational and mathematical models can help us understand the distribution and predicted outcomes of patterning. Molecular simulations translate complex mathematical models of morphogen diffusion kinetics into the predicted cellular responses. Spatiotemporal analysis with the help of recently improved technological tools allows us to study patterns through simulation platforms. To inform these models, it is important to determine the molecular kinetics and dynamics of morphogen diffusion along with the domain sizes through which they move, on the order of 10-100µm, the size of morphogens, half-life, and clearance rates. By tracking the diffusion of any molecule in the given action field through time and space, we can generate the kinetic data for that molecule, and using mathematically derived equations, we can more accurately predict the dynamics of signaling in the given system. The goal of this

work was to create tools that would help us track the distribution of morphogens like Upd in egg chambers.

A.1.2) Unpaired and STAT activation

Janus Kinase/Signal Transducer and Activator of Transcription (JAK/STAT) is an intracellular signaling cascade essential for optimal tissue growth and maintenance, notably in blood stem cell and immune cell functions in mammals. Loss or constitutive activation of the cascade is shown to be associated with unresponsiveness to interferons and hormones, immune deficiency disorders, and leukemia (Darnell, Kerr, and Stark, 1994). The key components of this pathway are JAK and STAT. JAK has four mammalian members and is comprised of two similar kinase domains with a carboxy-terminus involved in tyrosine phosphorylation, whereas, STAT has seven mammalian members and, when activated, is involved in transcriptional regulation by binding to specific enhancer sequences and activating gene expression. Some of the crucial ligands for this pathway are cytokines and growth factors, which bind to their receptors on the cell membrane to initiate the cascade (Ihle, 1996). The pathway is activated when the ligand binds to the transmembrane receptor and the receptor is multimerized. The receptor multimerization event brings the JAK molecules, which stay bound to the intracellular part of the receptor, closer together to facilitate their transphosphorylation. The phosphorylated JAK molecules create binding sites for STAT and inactive STAT molecules from the cytoplasm towards the inner cell surface for their activation. The activated, phosphorylated STAT molecules dimerize, translocate to the nucleus, and bind to particular DNA sequences, and promote their transcription (Rawlings, Rosler, and Harrison, 2004).

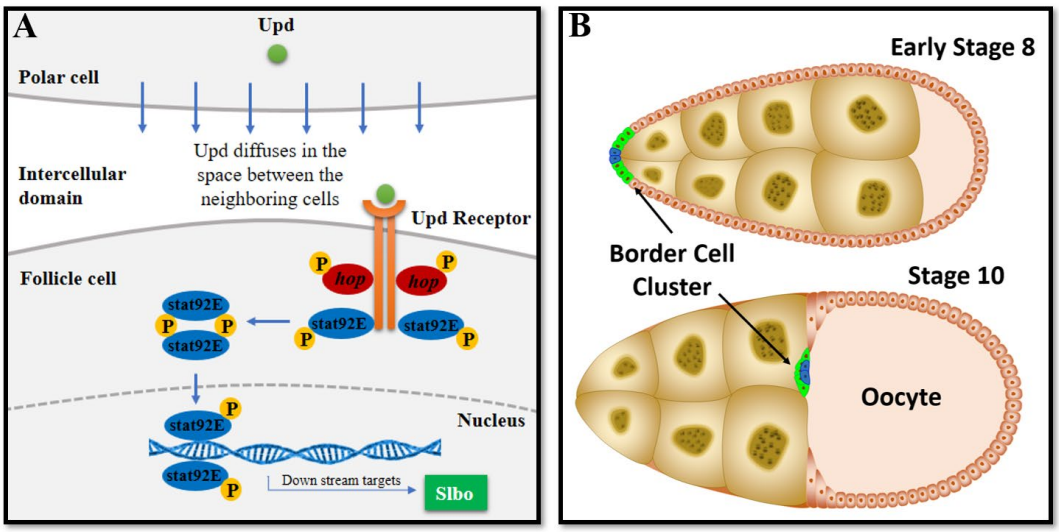


Figure A.2) STAT activation and border cell migration. A) The JAK/STAT equivalent cascade in *Drosophila* shows Upd signaling followed by Stat92E activation, resulting in the expression of Slbo, which promotes motile cell identity. B) After border cell specification in stage 8, the border cells along with the polar cells migrate to the oocyte in a highly controlled manner by stage 10.

Simultaneously with the characterization of the mammalian system, in the past few decades, the JAK/STAT pathway in *Drosophila* has caught the attention of many researchers due to its extensive involvement in tissue patterning and organogenesis. The *Drosophila* homologs for JAK and STAT are encoded by *hopscotch (hop)* and *Stat92E* (also called *marelle*) respectively, and many components of the pathway are conserved between flies and mammals but with fewer family members. In flies, this pathway is involved in blood cell proliferation, stem cell maintenance, embryonic patterning, and eye and wing development. (Luo & Dearolf, 2001). Like cytokines in mammals, a ligand was found to be associated with extracellular matrix and activation of the JAK-STAT pathway, which was termed Unpaired (Upd) by Harrison in 1998 (Harrison et al., 1998). The mutant phenotypes of all three genes, *hop, stat92E,* and *upd* are similar, which initially suggested their involvement in the same molecular

cascade. Although *upd* does not have sequence similarity to any known vertebrate genes or cytokines, the general structure may be conserved to address its functional requirement of receptor binding (Harrison et al., 1998). Upd has three family members, Upd, Upd2, and Upd3, all of which activate the JAK/STAT pathway, but Upd2 is observed to signal at greater distances (Rajan & Perrimon, 2012). For Upd to be received by the target cells, they require a receptor, Domeless (Dome), a membrane protein homologous to human cytokine I receptor.

JAK/STAT signaling occurs in many cell types in flies, but our focus is during the border cell specification event in *Drosophila* ovaries (Figure A.2). In this context, Upd1 has the main role. Upd1 is a 47kD glycoprotein localized at the apical boundary of polar cells, next to the border cell precursors (van de Bor et al., 2011). Presumably, the native Upd is glycosylated and secreted at the endoplasmic reticulum followed by cleavage into the mature form releasing the signal sequence via post-translational modification. A study shows that the secreted Upd tightly associates with the ECM via heparan sulfate proteoglycans (HSPGs) such as Dally and Dally-like (Harrison et al., 1998; Hayashi et al., 2012). Upd can be released from the association with these HSPGs by the addition of heparin to modulate JAK/STAT signaling activity. While Upd is expressed only in polar cells at the anterior and posterior ends of the egg chamber, *dome* is expressed in all follicle cells of the ovary. In contrast to other cell types, Dome undergoes Upd dependent internalization in follicle cells (Ghiglione et al., 2002). Upd is a morphogen and is secreted by the polar cells at the anterior part of the egg chamber, which is received by neighboring follicle cells to initiate the JAK/STAT cascade, resulting in border cell fate specification (Silver and Montell, 2001; Xi, McGregor, and Harrison, 2003; Silver, Geisbrecht and Montell, 2005; Starz-Gaiano et al., 2008). JAK/STAT regulation is crucial for the activation of an optimal number of border cells and their migration in the egg chambers. Downregulation of JAK/STAT pathway components leads to fewer activated border cells

whereas, ectopic activation leads to overpopulation of border cells, both of which results in delayed or no border cell migration.

A.1.3) End on imaging and mathematical model

As discussed earlier, morphogen distribution can be regulated by many factors, one of which is the tissue landscape. The obstruction by a physical barrier in the morphogen transport route could affect morphogen spread and result in different activation patterns of the receiving cells. A previous graduate student, Dr. Manning, from Dr. Starz-Gaiano`s lab, along with collaborators in the mathematics department showed that the activation pattern of target cells could be severely affected due to the tissue contour via alteration of Upd morphogen concentration. A combined result of all the models suggests that the tissue architecture of nurse cells could affect the resultant concentration of Upd from polar cells that is received by the anterior follicle cells (L. A. Manning et al., 2015).

To investigate a possible role of tissue architecture, it is necessary to adopt a 3D representation of tissues rather than only opting for a lateral view. To achieve the end-on view of the egg chamber, all the egg chambers were embedded into a gel in such a manner that the anterior-posterior central axis remains vertical (L. A. Manning et al., 2015). As shown in Figure A.3, Upd is secreted by the polar cells and likely moves to the nearby follicle cells via free and/or hindered diffusion. In absence of an influence of tissue contour, such as the structure of the nurse cells, Upd is expected to diffuse to all the follicle cells adjacent to the polar cells radially. Strangely, in most cases, there is an asymmetric activation of cells that is proposed to be the result of interference of diffusion by tissue landscape.

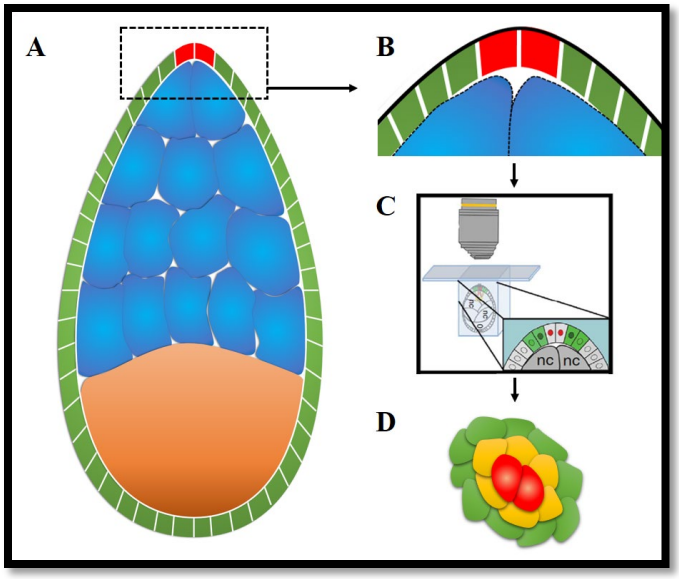


Figure A.3) Imaging via "end on" view and analysis of STAT activation patterns. Adapted from (L. A. Manning et al., 2015): A) Shows the morphology of egg chambers; blue cells are nurse cells, follicle cells are shown in green, the oocyte is shown in brown color and polar cells are shown as red cells. B) Anterior egg chamber zoomed-in and C) "end on" viewing approach to facilitate analysis from the top (D). D) The cellular arrangement description from the end on view where yellow cells are potential STAT-positive border cells.

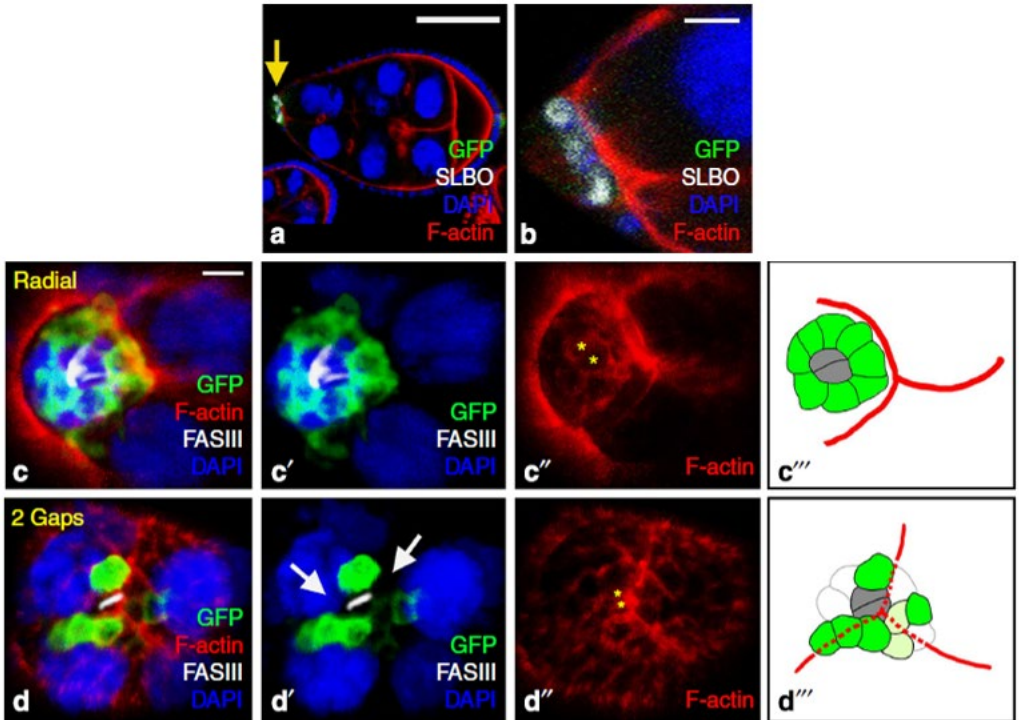


Figure A.4) Cell activation pattern with respect to nurse cell positions (L. A. Manning et al., 2015): A, B) Shows the presence of subcellular space between nurse cells and epithelial cells in a lateral view. C, C`, C") One nurse cell is present underneath polar cells correlating with the radial activation pattern in an end on view. D, D`, D") Presence of multiple nurse cells beneath polar cells show asymmetry in the border cell activation pattern in an end on view.

To study the possible influence of tissue architecture on cell activation patterns, Manning et al. examined the contours of the nurse cells next to the follicle cell epithelium. As shown in Figure A.4b, there is the presence of an extracellular domain between the apical surface of polar cells and the anterior surface of nurse cells, and where two nurse cells come together, a cleft is formed. After secretion of Upd from the apical surface of polar cells, the uneven tissue contour could create high concentration patches of Upd adjacent to some polar cells and sub-threshold levels adjacent to others. Because nurse cells do not possess any receptors, they would not be participating in the reception of the signal, but they can create

pockets of space where Upd could sink and be nonuniformly distributed. Thus, in three dimensions, the adjacent cells lying directly above the nurse cells will receive above-threshold signal but at the same time, the cells lying above the cleft between nurse cells would not receive as much because most of the Upd would have moved into that sink. The cell activation patterns, and position of the cleft were correlated in all the egg chambers. As predicted, in the case of a nurse cell present directly next to polar cells, follicle cells showed radial symmetry of STAT activity, whereas the presence of a cleft beneath the polar cells correlated with asymmetry of activation. Since the existing fluorescent reporter of Upd was not bright enough to track its localization (Rørth et al., 1998), Manning et al. defined a mathematical model which could predict the outcomes for different cellular arrangements. The computer simulation predicts the spatial and temporal Upd distribution and possible cell activation patterns as shown in Figure A.5. The model accurately shows the radial activation pattern in the case of no cleft beneath polar cells. In addition, it also shows the uneven distribution of Upd when the cleft domain is present to the side of the polar cells. The simulation correlated the localization of the extracellular domain space between nurse cells with the high occurrence of asymmetrical STAT activation patterns. To test this model more directly, we aimed to create a genetically labeled, photoswitchable-fluorescent Upd that could be tracked in vivo, which is described in this appendix.

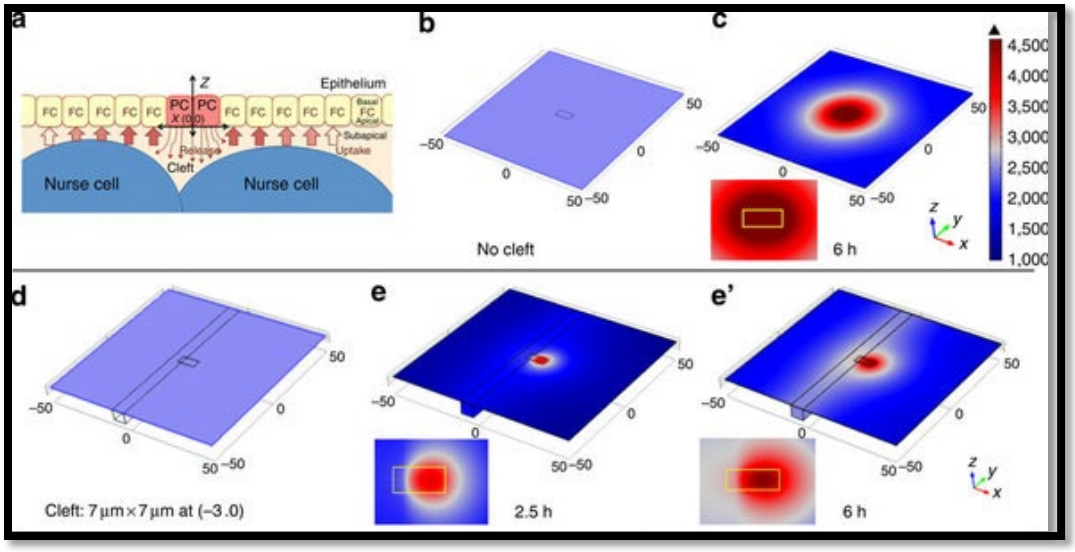


Figure A.5) Nurse cell position and cell activation patterns. Modified after (L. A. Manning et al., 2015): A) Shows the cellular arrangement at the anterior tip of the egg chamber. B, C) shows the radial activation pattern in the case of no cleft. D) shows the one-sided activation when cleft is present underneath one of the polar cells, although at a later time (E), a different activation pattern emerges.

A.2) Materials and Methods

A.2.1) Construct generation and expression

We isolated the total mRNA from *Drosophila* egg chambers using the Qiagen RNeasy mini kit with DNase I digestion. Using BioRad iScript, ~1µg/µl cDNA was synthesized from total RNA. We amplified and added restriction sites to *upd* CDS, *upd* 3`UTR, and *dendra2* (amplified from commercially available vector) using their specific primers. We then ligated all the fragments into a shuttle vector, pBluescript with restriction sites as follows: pBluescript vector with Not1 and Kpn1, *upd* CDS with Not1 and EcoR1, *dendra2* with EcoR1 and Xba1, and *upd* 3'UTR with Xba1 and Kpn1 restriction enzyme sites. We cloned the full construct from the shuttle vector into the pUAST expression vector followed by sequence analysis. The

final plasmid was sent to Bestgene for microinjection and P-element insertion mediated by transposase and received the stable, transgenic flies that carry the Upd-Dendra2 under UAS control, which we mapped and tested genetically by crossing with Upd-Gal4.

A.2.2) Immunostaining

Ovarioles were extracted from fattened flies into dissection media (1x Schneider's *Drosophila* medium by Thermo Fisher Scientific (21720-001), 10% FBS, 0.6% Pen/Strep) and fixed with 4% paraformaldehyde (0.1M KPO4 buffer) for 10 mins at RT. Fixed ovarioles were rinsed and washed 3 times with NP40 buffer (0.05 M Tris HCl, pH 7.4, 0.15 M NaCl, 0.5% Nonidet P-40 (Igepal CA-630, Sigma-Aldrich), 1 mg/ml BSA) (McDonald et al. 2006). For staining, ovarioles were incubated with Dendra2 antibody (1:100) from Origene (TA180094) in NP40 buffer overnight at 4ºC. Ovarioles were rinsed and washed three times with NP40 buffer then incubated with Alexa Fluor anti-mouse secondary antibodies in NP40 buffer overnight at 4ºC followed by DAPI staining for 10 mins. Ovarioles were rinsed and washed three times with NP40 buffer and incubated then mounted in 70% Glycerol. Samples were observed using either the Zeiss LSM 900 confocal with Airyscan 2 or Carl Zeiss AxioImager Z1 and captured using the AxioVision acquisition system. Post image processing was performed using ImageJ and Adobe Illustrator.

A.3) Results

A.3.1) A construct to track Upd distribution in *Drosophila* egg chambers

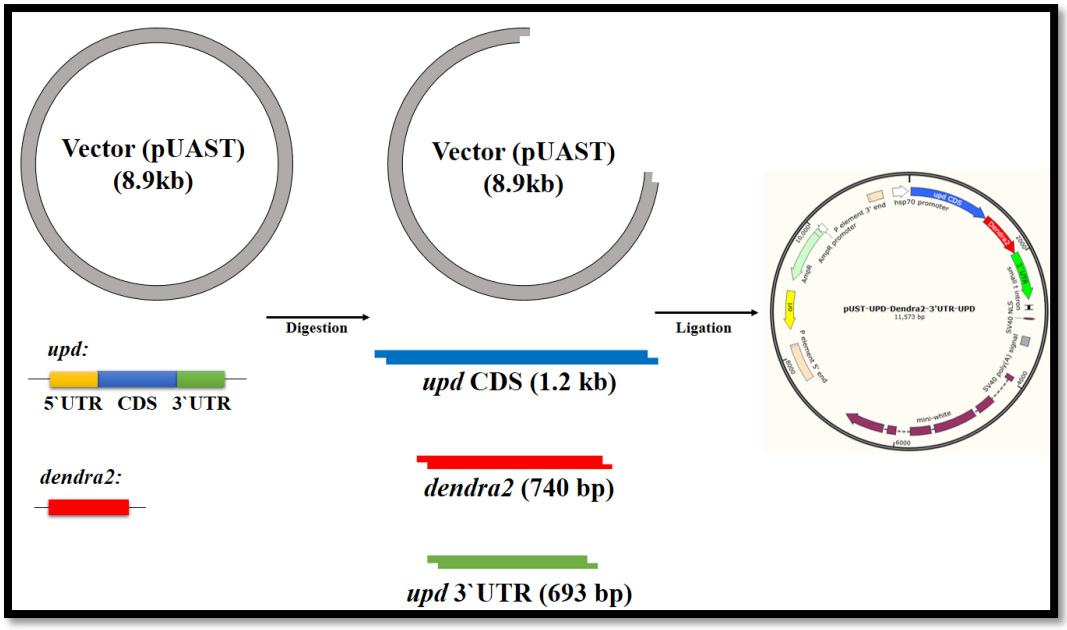


Figure A.6) The cloning strategy. The *upd* CDS, *upd* 3'UTR, and *dendra2* fragments were generated using sequence-specific primers and were digested along with pUAST vector to create complementary overhanging DNA strands. We ligated all the fragments into the vector to generate a plasmid that has all three components as shown here, which was used to create transgenic *Drosophila* by P-element mediated insertion.

Using a conventional cloning strategy, we made a construct comprising of the coding sequence of *upd*, the coding sequence of *dendra2* and 3`UTR sequence of *upd* in the same order as shown in Figure A.6. It has been reported that the 3`UTR of *upd* has four ATTTA motifs which are also found in the many cytokines (Harrison et al., 1998). Also, the A/TTTGTA motif from *ftz*, a pair-rule gene, is in common with the 3`UTR of *upd*. The presence of these motifs points towards the possible role of 3`UTR in *upd* mRNA localization, and its

apical localization has been shown to be important in polar cells (van de Bor et al., 2011). To minimize disruption of Upd signaling and promote its correct subcellular localization, we included the 3`UTR of *upd* in the construct. The construct was cloned into pUAST, a transgenic expression vector for *Drosophila* that contains binding sites for the Gal4 transcription factor, called Upstream Activation Sequences (UAS). Dendra2 is a green to red photoswitchable protein converted by UV exposure (Chudakov et al., 2007). This fluorescent protein can be expressed in flies when crossed to a Gal4 line, which will help us track Upd diffusion in the egg chamber.

Lane: 1 2 3 4 5 6

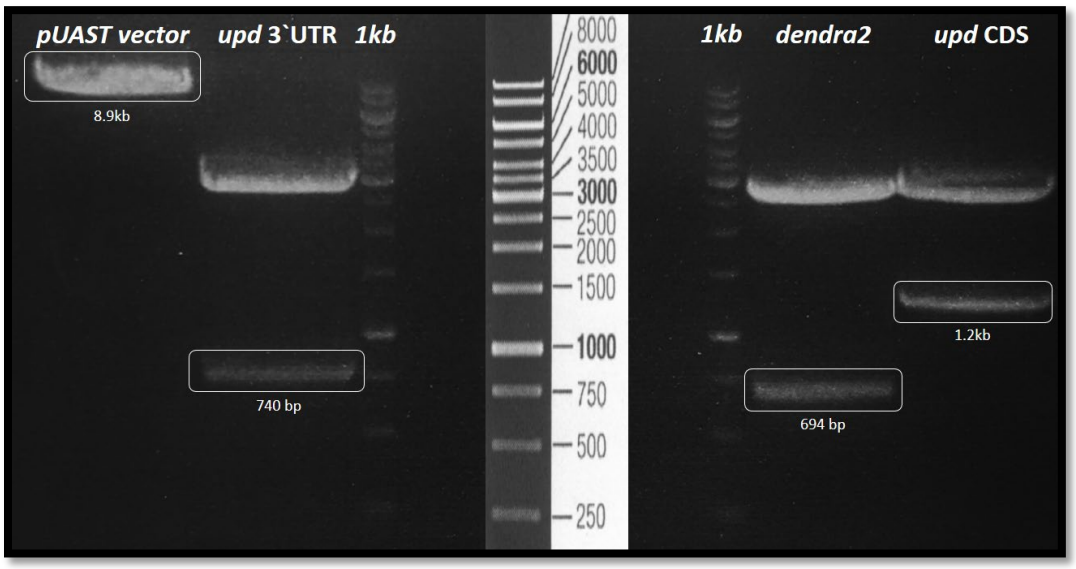


Figure A.7) Fragment size analysis using agarose gel electrophoresis. The lanes show the successful cloning of the fragments after digesting them from the vector and the 1kb ladder. Lane 1 shows linearized pUAST vector, lane 2 shows *upd* 3'UTR and pBluescript vector, lanes 3 and 4 are 1kb ladder, lane 5 shows *dendra2* fragment and pBluescript vector, and lane 6 shows *upd* CDS fragment and pBluescript vector. The samples were run on 1% agarose gel

The band size corresponding to the expected size of the DNA fragments (Figure A.7) following sequencing analysis (data not shown here) indicates successful cloning of the components of the final construct. Sequence alignment of sample plasmid with the reference construct shows 100% identity suggesting that we have designed the construct of interest. Once the construct was generated by insertion of the three fragments into pUAST, with the help of Bestgene inc, it was micro-injected into fly embryos along with transposase for random integration in the genome. After selective genetic crosses, we established nine transgenic fly lines that stably harbor the constructs on the first, second, and third chromosomes individually and can inducibly express Upd-Dendra2.

A.3.2) To analyze the Upd-Dendra2 construct for its expression

We crossed the transgenic flies that contain UAS-*upd-dendra2* with the c306-Gal4 to express the Upd-Dendra2 in anterior follicle cells. The immunofluorescent staining of egg chambers using an antibody directed against Dendra2 shows us the Upd-Dendra2 expression pattern (Figure A.8). The data illustrates that the Upd-Dendra2 expresses in the border cell cluster, as it should, during both specification and migration stages. We also see some non-specific expression of the Dendra2 antibody, which requires further attention. Although this may indicate ectopic expression, we may pre-absorb the antibody to reduce the noise to signal ratio as well as titrate the antibody concentrations to determine if this staining accurately captures the expression pattern.

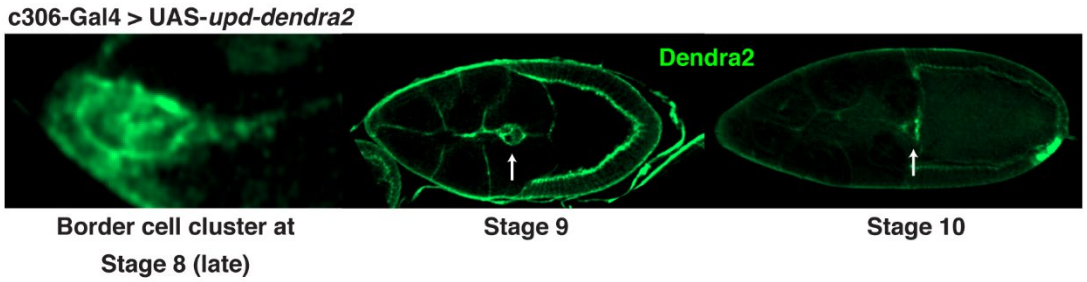


Figure A.8) Expression of Upd-Dendra2 in *Drosophila* egg chambers. The egg chambers of stage late 8 (border cell cluster enlarged), 9, and 10 showing Dendra2 expression (in green) especially in the specified and migrating cluster (shown by arrows).

A.4) Discussion

Morphogen signaling is one of the many known biological mechanisms in cell fate determination, and therefore is critical for proper animal development. While multiple factors govern morphogen transport, we know very little about the effect of tissue architecture on signal diffusion. Morphogen signaling has been heavily investigated in the genetically tractable organism *Drosophila*. In *Drosophila* egg chambers the polar cells secrete Unpaired (Upd), which when received by the surrounding follicle cells, activates JAK/STAT signaling and specifies anterior follicle cells into border cells. A previous study observed asymmetries in the STAT activation pattern around the polar cells, suggesting uneven Upd distribution among the follicle cells (L. A. Manning et al., 2015). This work developed a three-dimensional computer simulation to predict the role of tissue contour in morphogen distribution. The simulation data concurred with the *in vivo* activation data, supporting the influence of tissue arrangements on morphogen spread.

It is now our goal to understand the activation pattern and Upd diffusion in between the subcellular domains in a biological system. We have created an Upd fusion with a photoswitchable protein, Dendra2, to examine Upd kinetics *in vivo*. We are able to detect this protein by antibody staining, although the expression levels of Upd-Dendra2 have been quite low in the egg chamber, which makes it very difficult to track. We plan to increase the Upd-Dendra2 levels by expressing an extra copy of it on other chromosomes and by expressing multiple Gal4 constructs to overcome this issue. Notably, Alexander George in the laboratory has demonstrated that the Upd-Den2 fusion protein is functional: it can activate

STAT signaling and is sufficient to induce motile cell fate (unpublished data). To address our hypothesis, we plan to express the *upd-dendra2* construct in anterior follicle cells to high levels. If we observe accumulation of Upd-Dendra2 in the nurse cell cleft, we can conclude that Upd diffuses into the cleft, which would support the model that tissue contour is indeed playing a role in cell activation patterns (Figure A.9). For this, we would cross the UAS-*upd-dendra2* with the c306-Gal4 to drive the Upd-Dendra2 expression in anterior follicle cells. Additionally, with the help of the photo switching ability of Dendra2 we can study the kinetics of Upd-Dendra2 diffusion in an extracellular space. We would require Fluorescence Correlation Spectroscopy (FSC) to analyze the kinetic parameters and the diffusion rates. We also plan to analyze Upd-Dendra2 in mutants that have a reduced number of nurse cells in egg chambers(van Buskirk & Schüpbach, 2002). Prior work suggests that when there is a lesser number of nurse cells, sometimes the extracellular space between follicular epithelium and nurse cells gets bigger and this can have an effect on the radially symmetrical cell activation. Understanding how morphogen transport is impacted via biophysical parameters and how downstream signaling is regulated can open a whole new aspect of understanding extracellular signaling cues and their involvement in developmental disorders.

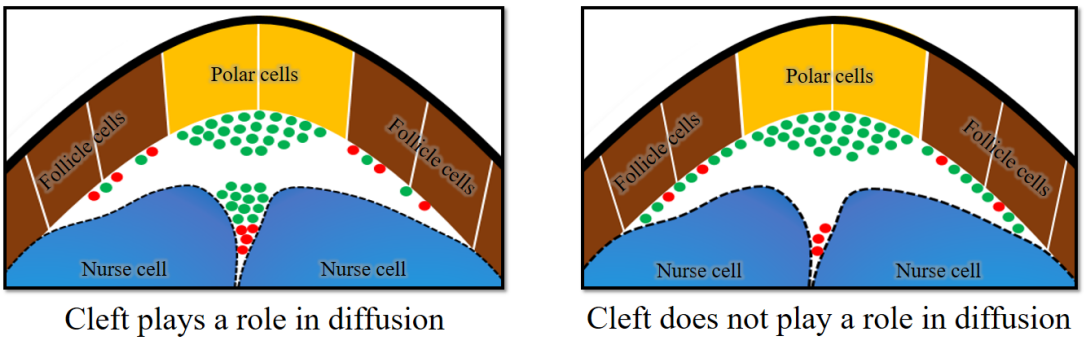


Figure A.9) Model of Upd distribution in extracellular space. The polar cells (yellow cells) secrete the Upd-Dendra2 (shown in green) in the extracellular space between the follicular epithelium (brown cells) and nurse cells (blue cells). These Upd-Dendra2 molecules can be

received by nearby follicle cells as well as accumulate in the space between nurse cells. Upon photoconversion, the green Upd-Dendra2 molecule can photoswitch into the red so the movement overtime can be assessed.

A.5) Acknowledgments

The premises of the work were postulated by myself and Dr. Manning, Dr. Peercy and Dr. Starz-Gaiano. The work was supported by an NSF grant (IOS 1656550 to MSG).

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