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Involvement of JAK/STAT Signaling and a Basement Membrane-Associated Protein during Air Sac Primordium Development in Drosophila Melanogaster

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INVOLVEMENT OF JAK/STAT SIGNALING AND A BASEMENT MEMBRANE-ASSOCIATED PROTEIN DURING AIR SAC PRIMORDIUM DEVELOPMENT IN *DROSOPHILA MELANOGASTER*

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INVOLVEMENT OF JAK/STAT SIGNALING AND A BASEMENT MEMBRANE-ASSOCIATED PROTEIN DURING AIR SAC PRIMORDIUM DEVELOPMENT IN *DROSOPHILA MELANOGASTER*

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I dedicate this thesis to my fellow Biology Graduate students, both current and former. Without their camaraderie, moral support, and inspiration, this thesis would not have been possible.

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Tumor metastasis currently presents the greatest obstacle for effective cancer remediation. Metastatic growth necessitates both degradation of a specialized form of extracellular matrix (ECM) known as the basement membrane (BM) and the invasion of surrounding tissues thereafter. The thoracic air sacs of fruit flies (*Drosophila melanogaster*), which develop and operate in a fashion comparable to the human lung, provide a unique model for identifying and characterizing factors that contribute to its own development as well as tumoral invasion. We investigated the involvement of both Janus kinase (JAK)/Signal transducer and activator of transcription (STAT) signaling and a BM-associated protein during the development of air sac primordia (ASPs), the precursors to *Drosophila* air sacs. We find that JAK/STAT signaling occurs in ASP tip cells and that misexpression of core pathway components via the GAL4/UAS system negatively impacts ASP development. Further, we identify Unpaired 2 (Upd2) as the primary activating ligand for JAK/STAT activity in the ASP. Knockdown of the BM-associated protein using *GAL4* drivers associated with a fibroblast growth factor (FGF) receptor gene, *breathless* (*btl*), and segment polarity gene, *patched* (*ptc*), prevented larval development beyond the second larval instar (L2). Knockdown of the BM-associated protein in the wing also produced bristle defects, but its overexpression did not have an effect anywhere other than in the ASP, where the proportion of mutant phenotypes increased significantly ($p < .0001$) in

response. Finally, we find that collagen IV localization was unaffected by knockdown of the BM-associated protein. Together, our data constitute a significant step forward in understanding the role of both this BM-associated protein and JAK/STAT signaling in the ASP and similar mammalian structures.

1. GENERAL INTRODUCTION

Cancer has proven to be a notoriously difficult problem for researchers and clinicians to solve for a variety of reasons. Among these is tumor heterogeneity, which in part results from cancer cells' capacity to shift between epithelial and mesenchymal states by hijacking normal developmental pathways (Srivastava *et al.* 2007; Hanahan and Weinberg 2011; Terry *et al.* 2017). For example, c-Jun N-terminal kinase (JNK) signaling and matrix metalloproteinases (MMPs) play demonstrable roles in both disc eversion, which is required for proper morphological development in *Drosophila*, and tumor invasion through control of basement membrane (BM) remodeling (Srivastava *et al.* 2007). Border cells, as they migrate from the follicular epithelium of the *Drosophila* ovary toward the oocyte, similarly employ tactics akin to those adopted by tumor cells during metastasis. The two polar cells that lie at the center of this eight-cell cluster produce the activating ligand for Janus kinase (JAK)/signal transducer and activator of transcription (STAT) signaling, Unpaired (Upd), which subsequently initiates the collective migration of the surrounding border cells via JAK/STAT (Beccari *et al.* 2002). JAK/STAT signaling has been heavily implicated in tumor progression (Constantinescu *et al.* 2008; Amoyel *et al.* 2014; Khanna *et al.* 2015), as have receptor tyrosine kinases (RTKs), such as those associated with platelet-derived growth factor (PGDF) (Farooqi and Siddik 2015), vascular endothelial growth factor (VEGF) (Alevizakos *et al.* 2013; Costache *et al.* 2015), and epidermal growth factor (EGF) (Seshacharyulu *et al.* 2012). These latter RTKs are likewise critical for border cell migration by providing directional cues to long cellular extensions that generate the force required to navigate through nurse cells in front of the oocyte (Aman and Piotrowski 2010; Poukkula *et al.* 2011).

The threat of metastasis posed by the ability to acquire migratory properties is severe, as it is the leading cause of all cancer-related mortalities (Hanahan and Weinberg 2000; Tarin 2008; Chaffer and Weinberg 2011). This fact makes preventing the mobilization of cancerous tissue a critically important subject for clinical research. Employing model organisms as an investigative tool has been, and continues to be, a useful approach for the discovery of novel treatment options (Rudrapatna *et al.* 2012). While several models of invasive development in addition to those discussed above have been established previously (Blaser 2005; Aman and Piotrowski 2010; De Graeve *et al.* 2012; Kulesa *et al.* 2013; Gallardo *et al.* 2015), recent evidence suggests that the air sac primordium (ASP) of *Drosophila melanogaster* appears to be a worthwhile addition to this group and has notable unrealized potential. Compared to other, more complex model organisms, *Drosophila* possesses some key advantages, including shorter generation times in addition to well-characterized genetic and developmental profiles. The ASP in particular serves as an interesting developmental model for several reasons. A short developmental period of only about five days is required for visualization of the ASP at its most advanced morphological stage (Sato and Kornberg 2002; Cabernard and Affolter 2005), and established protocols exist for marking proteins, cells, and tissues effectively as a result of the aforementioned advantages inherent to using *Drosophila* as a model system.

A simple, yet powerful, technique for controlling gene expression exists in *Drosophila* as well: the GAL4/Upstream Activation Sequence (UAS) system (Fig. 1). In this system, a transcriptional activator isolated from yeast, GAL4, is expressed in a time- and tissue-specific manner by a "driver" gene in one parental line. In the other parental line, one or more UAS sites are inserted upstream of a target gene or genes. GAL4 has little to no effect

on fly development in the absence of a UAS element, and the same is true for the converse: flies carrying a UAS transgene, but no GAL4, remain wild type, phenotypically. However, when both parental lines are crossed, their progeny both express GAL4 in a pattern determined by its associated driver [e.g. a *Breathless* (*Btl*)-*GAL4* driver would limit expression to the tracheal system and other tissues] and contain a UAS site (Fig. 1). Though all cells contain the UAS site, GAL4 will only induce overexpression of the target gene associated with UAS in the tissue(s) where the driver is expressed (Brand and Perrimon 1993). This system further contributes to the tractability of *Drosophila* as a model organism, with tens of thousands of different *GAL4* and *UAS* lines available from stock centers.

Air sac primordia (ASPs) are also known to exhibit a characteristic bud and stalk structure with a series of actin-rich filopodia protruding from said bud, which makes the distinguishing morphological features of this structure readily identifiable via confocal fluorescence microscopy (Figure 2A). Most importantly, ASP development involves three processes that are also required for tumor metastasis: re-initiation of a proliferative program in quiescent cells (Sato *et al.* 2008), downregulation of adherens junction proteins in tip cells (Dong *et al.* 2015), and extracellular matrix (ECM) remodeling (Srivastava *et al.* 2007; Guha *et al.* 2009; Wang *et al.* 2010; Dong *et al.* 2015). Understanding the complex network of cellular signaling that orchestrates these events may yield new insights regarding metastasis and its prevention.

1.1 ASP Function and Development

ASPs are the precursors to the dorsal thoracic air sacs found in adult *Drosophila*, which act to directly oxygenate the organism's flight muscles via the interdigitation of tracheole bundles associated with the air sacs (Sato and Kornberg 2002). This role makes the ASP functionally analogous to that of the human lung, which similarly provides oxygen to all vascularized tissues. ASPs develop from a group of tracheoblasts in the transverse connective (TC) of the second tracheal metamere (Tr2) during the early third instar (L3) stage of larval development (Cabernard and Affolter 2005). In contrast to the more derived method of development employed by many other holometabolous insects (Švácha 1992; Tanaka and Truman 2005), *Drosophila* seems to utilize a more ancestral method of organogenesis with respect to ASPs (Sato *et al.* 2008). Rather than eliminating larval cells through apoptosis and replacing them with a discrete population of imaginal cells, as proposed initially (Guha and Kornberg 2005), the larval cells of Tr2 remain diploid and do not endoreplicate throughout larval development (Sato *et al.* 2008).

Recent evidence has pointed to the contributions of homeobox (Hox) transcription factors in regulating entry and exit from these endocycles of continuous growth without division prior to metamorphosis (Sato *et al.* 2008; Djabrayan *et al.* 2014). Studying this relationship could be clinically beneficial considering that aberrant Hox gene expression is associated with several cancers and that select candidates are predicted to serve as biomarkers for targeted therapy (McGrath *et al.* 2013; Joo *et al.* 2016). Furthermore, polyploid cells are reportedly less susceptible to traditional apoptotic pathways (Mehrotra *et al.* 2008), which provides another mechanism whereby oncogenic tissues can circumvent cell-cycle checkpoints. This capacity to avoid cell death can become notably more

pernicious if uncoupled from the arrested proliferative program associated with polyploidy. The cell-cycle regulators E2F and String/Cdc25 (Stg/Cdc25) are currently implicated as the driving forces behind the shifts from quiescence to G2 observed during the second larval instar (L2) and G2 to M phase in L3, respectively (Djabrayan *et al.* 2014). However, *stg* transcription is dependent upon the release of ecdysone that coincides with the transition between larval molts, and the process can be disrupted by the expression of Fizzy-related (Fzr) (Djabrayan *et al.* 2014), as is the case in the other tracheal metameres (Figure 3A). The absence of Fzr is necessary and sufficient for the reactivation of mitotic activity in the larval cells of Tr2 through Stg/Cdc25 (Djabrayan *et al.* 2014), making its regulatory role paramount to initiating ASP growth via cell division (Sato *et al.* 2008). Indeed, Fzr has previously been demonstrated to suppress tumor growth in mice (Wang *et al.* 2000) and is currently being investigated in human cell lines as a target for clinical application as well (Crawford *et al.* 2016; Ishizawa *et al.* 2017).

While endocycle exit helps initiate ASP development, morphogenesis is concomitantly directed by the fibroblast growth factor (FGF) homolog Branchless (Bnl/FGF) through the action of filopodia that extend outward from distal tip cells and bind Bnl/FGF via the FGF receptor Breathless (Btl/FGFR) (Sato and Kornberg 2002; Cabernard and Affolter 2005; Roy *et al.* 2014) (Figure 2B). However, recent work indicates that the regulation of Ca^{2+} levels through the action of Sarcoendoplasmic Reticulum Calcium Transport ATPase (SERCA) at the tips of the ASP, trachea, and other tubules controls the timing of migration and branching prior to input from morphogens, including FGF (Bower *et al.* 2017). The researchers found this regulation occurs within all germ layers and in vertebrate tissue as well, suggesting great potential for translating future mechanistic

insights gained from studying the ASP and applying them in the search for novel remediation strategies. SERCA inhibitors have been proposed for the treatment of several cancer types (Arbabian *et al.* 2011; Papp *et al.* 2012), but cell-type specificity remains a challenge that might be overcome by developing and testing drug cocktails on ASPs demonstrating aberrant SERCA expression and searching for off-target effects in other tissues. Indeed, a similar screening approach was recently used to reveal synergy between trametinib and fluvastatin in both suppressing tumor formation and reducing whole-body toxicity associated with trametinib, using ASP and pupal air sac phenotypes to visually assay tumorigenicity (Levine and Cagan 2016).

The cells that secrete Bnl/FGF lie in the columnar epithelium of the wing imaginal disc while the population of tracheoblasts along the TC are embedded in the adepithelium among myoblasts destined to become the adult flight muscles (Sato and Kornberg 2002). Even after a calcium differential between "leading" (distal) and "lagging" (proximal) cells has been established to permit FGF signaling, this situation poses a challenge for the migration of ASPs toward the chemoattractant signal, as the cells must travel through the ECM surrounding the TC and adepithelium before arriving at the source of Bnl/FGF. However, the position of the TC with respect to the wing disc is rather conducive for invasive development, given that the thick layer of lamina densa encapsulating the trachea and wing imaginal disc thins out between the region where the ASP emerges along the TC and the adepithelial layer of the wing disc (Guha *et al.* 2009) (Figure 3B). The relationship between reduced ECM thickness and budding epithelia is also common to the ASP's human analog, the lung, as well as other tissues that rely on ECM remodeling to facilitate branching morphogenesis, such as the intestine and mammary gland (Bonnans *et al.* 2014).

1.2 ECM Remodeling and ASP Invasion

Activated Btl/FGFR facilitates the expression of Matrix Metalloproteinase 2 (MMP2) in ASP tip cells (Guha *et al.* 2009; Wang *et al.* 2010). MMP2 is an important protein for the degradation and remodeling of the ECM surrounding the developing ASP through collagen IV and perlecan turnover (Srivastava *et al.* 2007; Guha *et al.* 2009; Wang *et al.* 2010). It has been hypothesized that MMP2-mediated ECM remodeling not only facilitates the invasive propagation of the ASP, but also releases some inhibitory signal that laterally prevents the expansion of tip cell fate by blocking Bnl/FGF binding sites in middle and stalk cells (Wang *et al.* 2010). Another class of proteases, cathepsins, has been implicated in ECM remodeling around the ASP as well by evidence of hindered migratory capability in lines where Cysteine Proteinase 1 (CP1), an ortholog of Cathepsin-L (CTSL), was knocked down (Dong *et al.* 2015). Interestingly, the ASP appears capable of outward growth despite its inability to penetrate the adepithelial layer of the wing disc, suggesting a role during invasion distinctly different from that of MMP2. Both of these proteolytic enzymes have been reported to serve as prognostic markers in several cancers, including breast (Thomssen *et al.* 1995; Sivula *et al.* 2005), bladder (Yan *et al.* 2010, 2014), ovarian (Fu *et al.* 2015; Sui *et al.* 2016), prostate (Ross *et al.* 2003; Sudhan and Siemann 2013), lung (Ishikawa *et al.* 2004; Cui *et al.* 2016), and pancreatic cancer (Singh *et al.* 2013, 2014). However, the mechanisms by which CP1/CTSL facilitates ASP invasion and MMP2 prevents the lateral expansion of tip cell fate have yet to be investigated further. Regardless, the aforementioned thinning of ECM around the leading edge of the ASP plays another critical role unrelated to ECM remodeling. This thinner lamina densa makes the ECM highly permeable to FGF signaling, which allows for the initiation of a greater signaling

response that regulates cell proliferation and survival (Cabernard and Affolter 2005; Guha *et al.* 2009; Cruz *et al.* 2015), mirroring a similar mechanism required for human lung development (Min *et al.* 1998; Park *et al.* 1998; Abler *et al.* 2008; Volckaert *et al.* 2013).

1.3 Additional Regulators of ASP Development

Several signaling pathways are known to play a role in the development of ASP in addition to FGF. EGF Receptor (EGFR) signaling is arguably the most important of this set for its role in the regulation of cell survival and proliferation (Cabernard and Affolter 2005; Chanut-Delalande *et al.* 2010; Cruz *et al.* 2015). It was recently found that FGF signaling induces Vein (Vn/EGF) expression via the transcription factor PointedP2 (PntP2) (Cruz *et al.* 2015). This is notable because Vn initiates EGFR signaling in the ASP and induces a positive feedback loop that stimulates cell proliferation and survival in the stalk cells proximal to the tip cells (Cruz *et al.* 2015). Interestingly, the mechanism of action for this feedback loop is the activation of the mitogen-activated protein kinase (MAPK) signaling pathway by both Vn/EGF and Bnl/FGF; the key difference between stalk cells and the tip cells that induces their proliferation is that when MAPK is activated by Bnl/FGF, additional transcription factors, including PointedP1 (PntP1) and Escargot (Esg), are expressed as well (Sato and Kornberg 2002; Cabernard and Affolter 2005; Cruz *et al.* 2015) (Figure 4). It has been proposed that Esg inhibits the proliferative genetic program activated by Vn/EGF-induced MAPK signaling and maintains the cell survival program, while PntP1 stimulates cell migration, together contributing to the determination of tip cell fate (Cruz *et al.* 2015). Downregulation of Shotgun (Shg) and Armadillo (Arm), the *Drosophila* homologs of E-cadherin and β-catenin, respectively, has also been observed in

the tip cells of the ASP (Dong *et al.* 2015), but the contributing factors are currently unknown in spite of the role Esg plays in regulating Shg expression elsewhere in the tracheal system (Tanaka-Matakatsu *et al.* 1996). Recently, the *Drosophila* ortholog of Dedicator of cytokinesis (DOCK) family proteins 3 and 4 (DOCK3/4), Sponge (Spg), was reported to promote cell survival through MAPK signaling as well. The authors of the study suggest that this could be accomplished via the intermediate activation of p21-activated kinase (PAK), but this model currently lacks experimental evidence (Morishita *et al.* 2017).

The localization and turnover of both Btl/FGFR and EGFR are regulated by Hrs and Stam, which together comprise the Endosomal Sorting Complex Required for Transport-0 (ESCRT-0) complex (Chanut-Delalande *et al.* 2010) (Figure 4). While Hrs has been shown to downregulate EGFR signaling in embryonic development, it has been demonstrated that knockouts of Hrs and Stam result in inefficient FGF and EGFR signaling, as well as improperly localized Btl, collectively contributing to impaired ASP development (Chanut-Delalande *et al.* 2010). This research highlighted the importance of endosomes in signal modulation at different points during development, continuing an observed trend in the regulation of RTK signaling (Miaczynska 2013). Filopodia specific for the ortholog of bone morphogenetic proteins (BMPs) 2 and 4, Decapentaplegic (Dpp/BMP), have been identified in the medial region of the ASP as well (Roy *et al.* 2011, 2014; Huang and Kornberg 2016), but whether there are similar models for the regulation of this pathway and its contributions in the context of ASP development have yet to be determined (Figure 4). It has been proposed that two heparan sulfate proteoglycans (HSPGs) of the surrounding ECM, Dally (Dly) and Dally-like (Dlp), operate as co-activators of Dpp and FGF signaling, respectively (Yan and Lin 2007; Dejima *et al.* 2011) (Figure 4). However, another more

recent model expands on this notion to suggest that the stratification of these ECM components also provides instructive cues and structural support for filopodia to facilitate guidance toward their target ligands (Huang and Kornberg 2016). This model parallels reported interactions between filopodia and ECM during neuronal development in avian and mammalian systems (Jacquemet *et al.* 2015) but in cancer as well (Arjonen *et al.* 2014; Cao *et al.* 2014; Makowska *et al.* 2015).

1.4 Conclusions and Future Directions

The dysregulation of proliferative programs has long been a widely recognized hallmark of cancer, and dedifferentiation has increasingly been implicated in the pathology of several cancers, including glioblastoma multiforme (Friedmann-Morvinski *et al.* 2012), colon cancer (Schwitalla *et al.* 2013), and lung cancer (Saijo *et al.* 2016). Indeed, a recent mathematical model of mutation rates leading to carcinogenesis suggests that dedifferentiation of normal cells and loose homeostatic control are critical for hastening the onset of cancer (Jilkine and Gutenkunst 2014). In the interest of identifying key molecular contributors involved in these processes, attaining a greater understanding of how endocycle entry and exit in the *Drosophila* tracheal system are regulated via Hox transcription factors could potentially highlight genes with functional human homologs for further investigation. Though significant differences exist between *Drosophila* and mammals regarding the frequency and nature of polyploidization (Sher *et al.* 2013), modeling the underlying mechanisms in fruit flies could contribute to our current understanding of polyploid giant cancer cells (PGCCs), as these have documented roles in breast (Fei *et al.* 2015) and colon cancers (Lopez-Sánchez *et al.* 2014). Elucidating the

network of interactions required for Hox-mediated control of mitosis in this system could also apply more broadly to the progression of oncogenesis in other tissues, given the diverse roles that these transcription factors play (Bhatlekar *et al.* 2014).

Ras/Raf-MAPK (Santarpia *et al.* 2012), FGF (Huntington *et al.* 2004), and EGFR signaling (Seshacharyulu *et al.* 2012) all have well-documented roles in cancer progression as well, as is the case in ASP development. While the contributions of these signaling pathways are not unique to ASP development, the concurrence of their activities with ECM remodeling could enable the development of a holistic model for invasion that more accurately reflects both ASP migration and tumor metastasis, by extension, compared to other migrating cells. As such, drug screens targeting atypical signaling can reveal the impact of a treatment on processes ancillary to the one(s) targeted more readily when performed in this tissue than others. Of course, further exploration of how these signaling cascades function and cooperate with other pathways within the invasive context of ASP propagation should yield additional insights into what is required for their activity and regulation as well. For example, Notch signaling activity has also been reported to occur in the ASP, as evidenced by the expression of the pathway's ligands, Serrate (Ser) and Delta (Huang and Kornberg 2015; Rao *et al.* 2015). It has been demonstrated that filopodia protruding from myoblasts in the adepithelium make contacts with both the wing imaginal disc and ASP in order to mediate Wingless (Wg) signaling from the former and Notch signaling to the latter (Huang and Kornberg 2015). However, the mechanisms by which such signaling affect ASP development remain unknown. Even less is known about how Ser contributes to this structure's morphogenesis, though Ser is known to be EGF-like and facilitate position-specific cell proliferation in the wing and haltere imaginal discs

(Speicher *et al.* 1994). The transcription factors Cut and Knirps also have documented functions in morphogenesis—apoptosis inhibition (Zhai *et al.* 2012) and Dpp signal mediation (Chen *et al.* 1998), respectively—but their role in ASP development is currently tentative and should be confirmed in order to derive a more complete model for invasive behavior.

The most imperative discoveries to be made regarding both tumor metastasis and ASP development may lie in the identification of novel regulators of ECM remodeling. In the ASP, many factors in this process require further investigation, especially the coupling of invasive migration with ECM degradation. Some contributors to ECM remodeling, including hypoxia (Gilkes *et al.* 2013), have yet to be investigated in the ASP at all. It has been demonstrated that hypoxic conditions promote autonomous sensitization to FGF in the tracheal system in order to vascularize oxygen-deprived tissues (Centanin *et al.* 2008). Assuming the ASP utilizes the same process, manipulating the ASP's expression of the hypoxia-inducible factor (HIF)-α homolog and/or HIF-prolyl hydroxylase that regulate this response could potentially be used to imitate the hypoxic conditions found in malignant tumors. This would provide an additional set of drug targets that could be screened using the ASP as an invasive model.

Our research aims to elucidate the contributions of still other factors, whose significance in the context of ASP development has not been investigated or reported previously. First, we provide evidence for the activity of another signaling pathway (JAK/STAT) within the ASP, identify the primary ligand for said pathway, and propose that elevated MAPK signaling may mediate observed phenotypic effects of JAK/STAT dysregulation via control of cell fate. In addition, earlier work in the Srivastava lab

demonstrated that a vesicular trafficking protein, Surfeit locus protein 4 (Surf4), can associate with Viking (Vkg) (Kapadia and Srivastava 2016), one of two collagen IV chains found in *Drosophila* (Yasothornsrikul *et al.* 1997). Because collagen IV is the primary constituent of the BM (Halfter *et al.* 2013), we explore how Surf4 influences development of the ASP, in addition to various adult structures that also require BM remodeling.

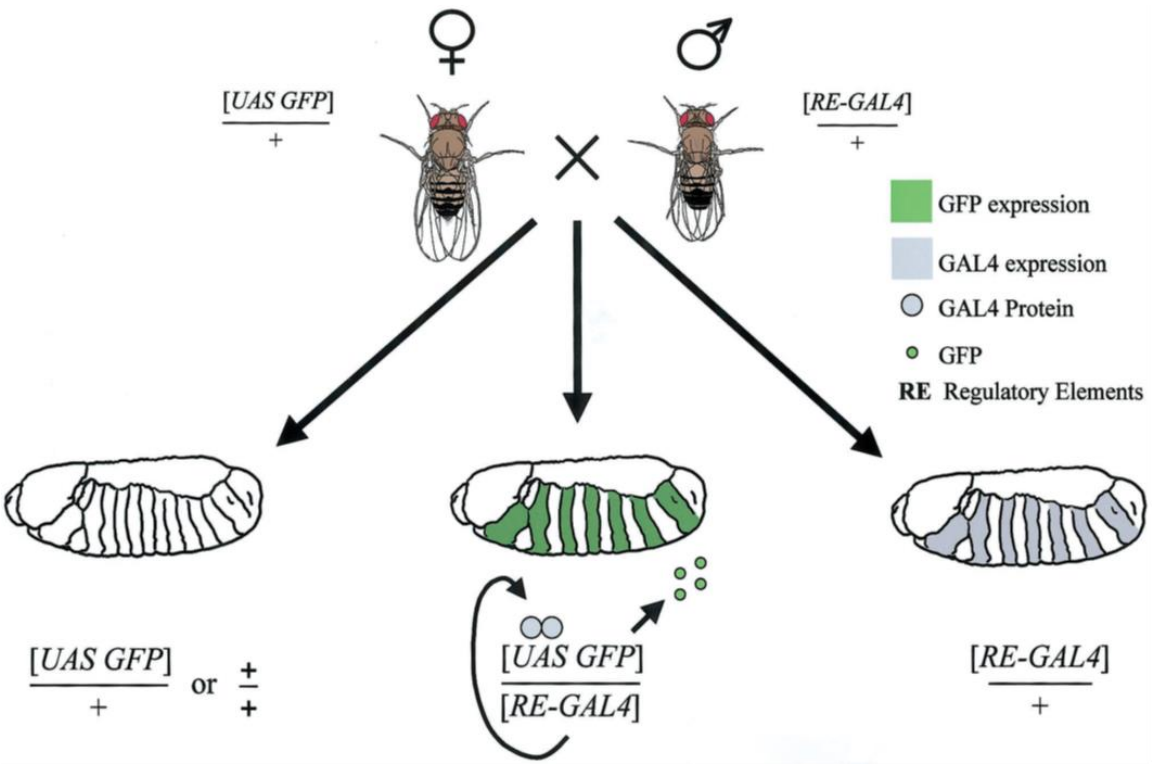

Figure 1: A graphical depiction of the GAL4/UAS system

This figure from (Duffy 2002) demonstrates the lack of target gene expression when one or neither of the GAL4/UAS system's components are present in an individual. However, when a regulatory element (RE) induces GAL4 expression in a specific pattern (striped, in this example) and a UAS element is present upstream of said target gene [green fluorescent protein (GFP), in this example], the target gene is expressed at the same time and in the same region as the RE-GAL4 element.

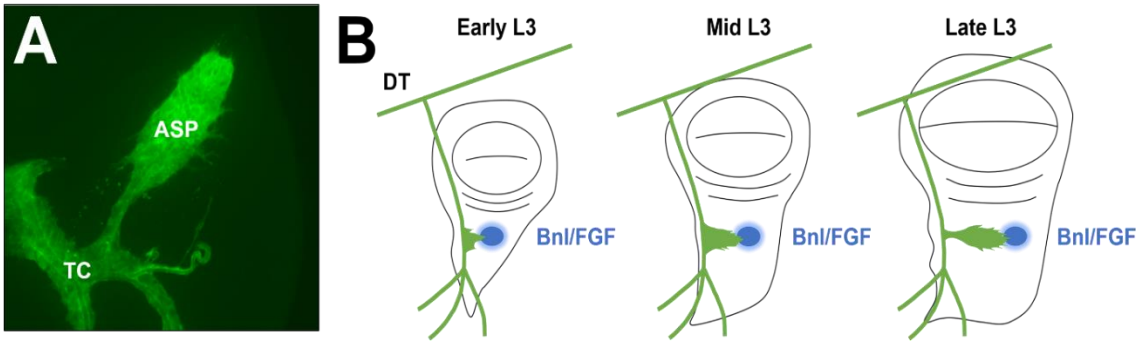

Figure 2: Wild-type ASP morphology and development

(A) and (B) The ASP develops from a population of mitotically active cells located along the transverse connective that divide and migrate toward the morphogen Bnl/FGF throughout L3 stage. Locations of the ASP, transverse connective (TC), dorsal trunk (DT), and Bnl/FGF (blue) are marked.

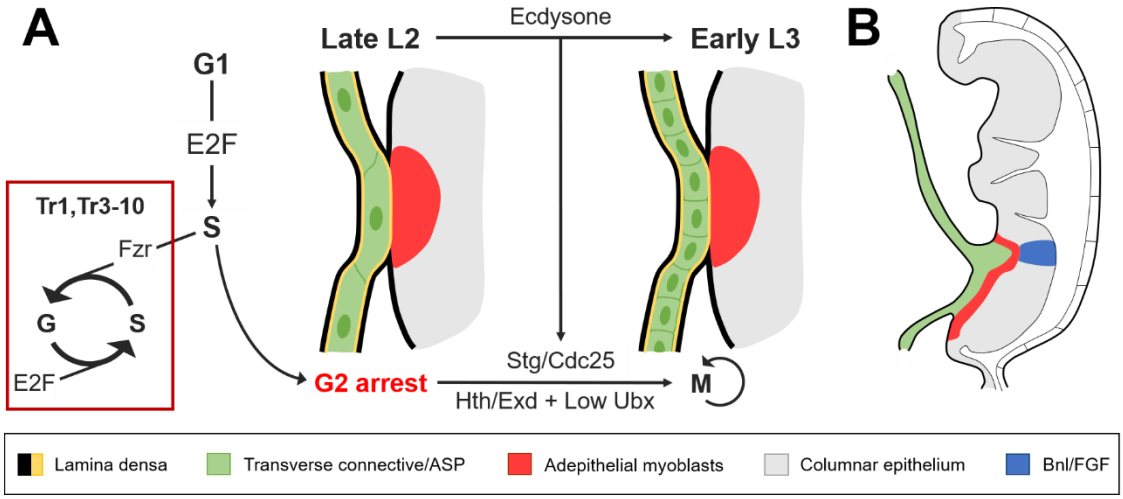

Figure 3: The transverse connective reenters the cell cycle at L3 and is invasively coupled with the wing imaginal disc prior to ASP development

(A) Permissive levels of Homothorax (Hth)/Extradenticle (Exd) and Ultrabithorax (Ubx) are required for ecdysone to activate Stg/Cdc25 expression during the transition to L3. Stg/Cdc25 is necessary for larval cells in the trachea to reenter the cell cycle after the arrest at G2 that occurs during the L2 stage. In a high Hox background, Fzr and E2F cooperate to maintain polyploidy in the other tracheal metameres; (B) Lateral view of the wing imaginal disc during L3. Remodeling of the ECM that separates the ASP from the Bnl/FGF expressed in the columnar epithelium is assisted through the invasive pairing of the transverse connective with the wing disc.

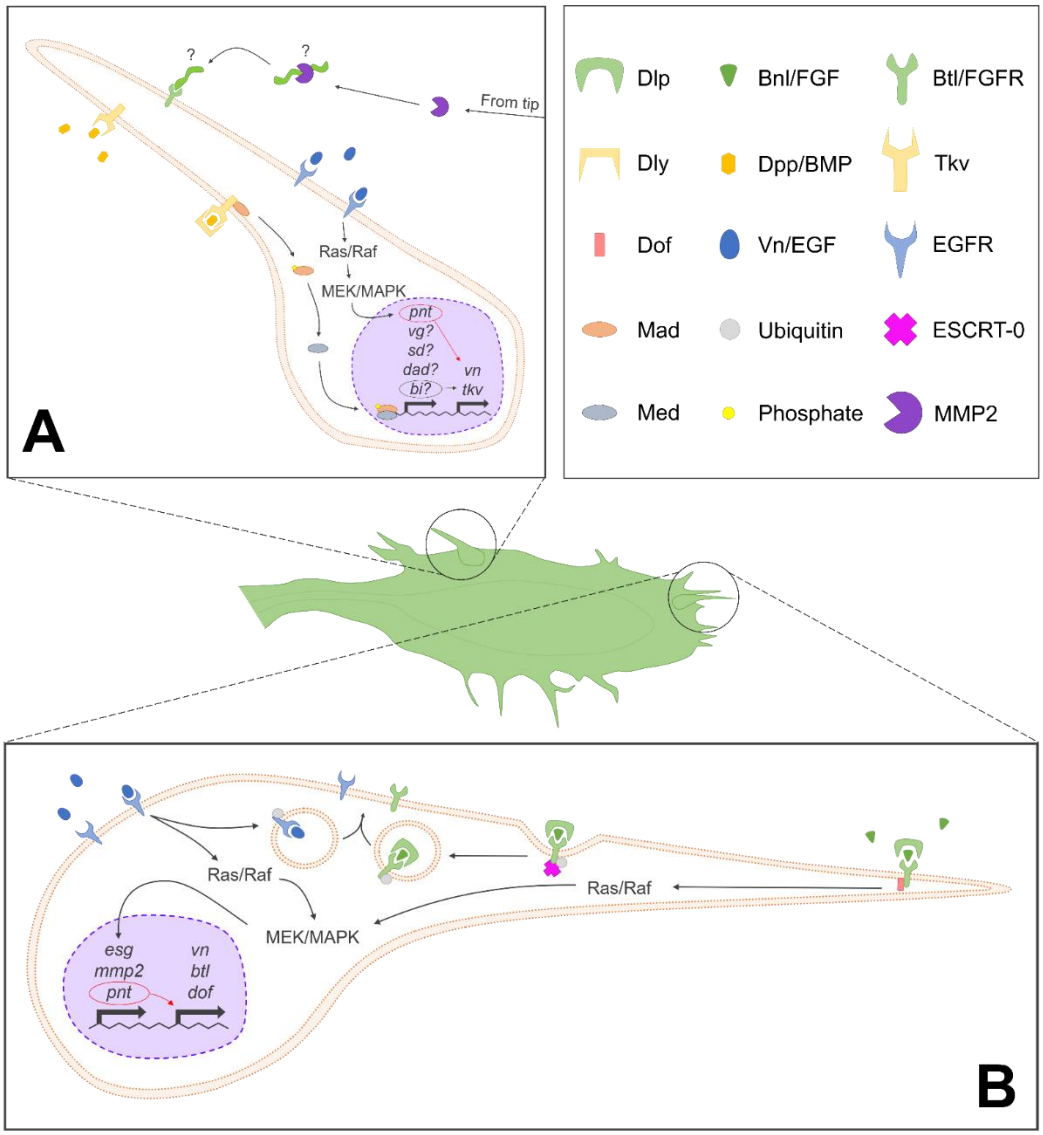

Figure 4: Signaling pathways involved in ASP development

(A) Summary of signals and pathways implicated in the development of lateral ASP cells, with speculative or unknown components denoted with "?". (B) Summary of signals and pathways involved in the determination of tip cell fate and development. ESCRT-0-mediated endocytic recycling is only depicted in (B), but this process occurs in (A) as well. A key with symbols used to denote various molecular actors is depicted in the top right panel, adjacent to (A). Dlp, Dally-like; Bnl/FGF, Branchless/Fibroblast Growth Factor; Btl/FGFR, Breathless/Fibroblast Growth Factor Receptor; Dly, Dally; Dpp/BMP, Decapentaplegic/Bone Morphogenetic Protein; Tkv, Thickveins; Dof, Downstream of FGF; Vn/EGF, Vein/Epidermal Growth Factor; EGFR, Epithelial Growth Factor Receptor; Mad, Mothers Against Dpp; ESCRT-0, Endosomal Sorting Complex Required for Transport-0; Med, Medea; and MMP2, Matrix Metalloproteinase 2.

2. JAK/STAT SIGNALING IS INVOLVED IN ASP DEVELOPMENT

2.1 Introduction

Adult *Drosophila* possess dorsal thoracic air sacs, which develop and function in a manner analogous to human lungs, providing oxygen to nearby flight muscles (Min *et al.* 1998; Park *et al.* 1998; Sato and Kornberg 2002; Guha and Kornberg 2005). During L3, precursory structures called ASPs begin to form within Tr2 (Guha and Kornberg 2005). Throughout L3, ASPs develop from groups of multipotent cells along each TC within Tr2, which intimately associate with each wing imaginal disc, forming characteristic bud and stalk structures by late L3 (Guha and Kornberg 2005). Importantly, ASPs follow a developmental program that requires invasive behavior in order for cells initially embedded in the adepithelial layer of each wing disc to migrate along the disc's anterior-posterior axis into the columnar epithelium, toward a distal source of Bnl/FGF (Sato and Kornberg 2002; Dong *et al.* 2015). While Bnl/FGF signaling controls this pattern of cell migration, it also facilitates activation of EGFR signaling, which regulates cell proliferation in the ASP's medial cells (Cabernard and Affolter 2005; Cruz *et al.* 2015). As a result, these pathways have been studied extensively for their potential to be co-opted by tumor cells in promoting oncogenesis. We have previously suggested that the ASP holds great potential for modeling invasion due to the confluence of signaling pathways and other developmental mechanisms required for both its development and the metastasis of malignant tumors (Powers and Srivastava 2018). However, the potential influence of other pathways on ASP development has either been limited in scope, or entirely unexplored. The involvement of Dpp/BMP signaling has been reported, but research has focused almost entirely on the formation of Dpp-specific cytonemes (Roy *et al.* 2011, 2014). Similarly,

Notch signaling dysregulation is known to negatively impact ASP morphology while the Wg pathway negatively regulates expression of Notch's activating ligand, Delta (Huang and Kornberg 2015), but the underlying molecular mechanisms are not entirely clear. In contrast, a relationship between ASP development and JAK/STAT signaling has not been previously established. However, this pathway might be another regulator of ASP development considering its diverse roles within both developmental and pathological contexts across many metazoan species.

In mammals, these roles are executed by a suite of semi-redundant effectors that can be activated in different combinations by the binding of more than 50 cytokines and growth factors to various transmembrane receptor families (Schindler and Plumlee 2008). Humans and mice possess four JAKs (JAK1-3 and Tyk2) (Yamaoka *et al.* 2004) and seven STATs (STAT1-4, 5A, 5B, and 6) (Levy and Darnell 2002), together mediating processes including hematopoiesis (Ward *et al.* 2000), stem cell maintenance (Tang and Tian 2013), lung development (Liu and Kern 2002; Piairo *et al.* 2018), and immune response (Durbin *et al.* 1996). This pathway's activity is likewise required for the same phenomena in *Drosophila* (Luo *et al.* 1997; Brown *et al.* 2001; Sotillos *et al.* 2010; Xu *et al.* 2011; Osman *et al.* 2012; Sinden *et al.* 2012; Myllymäki and Rämet 2014), among others (Binari and Perrimon 1994; Luo *et al.* 1999; Beccari *et al.* 2002; Tsai and Sun 2004; Tsai *et al.* 2007; Wang *et al.* 2014), though the pathway involves only one transmembrane receptor [Domeless (Dome)], one JAK [Hopscotch (Hop)], one STAT (Stat92E), and three activating ligands (Upd1-3). The role of JAK/STAT signaling in these events is governed by its more general involvement with cell migration, differentiation, and proliferation. Naturally, disruption of this signaling cascade has also been implicated in oncogenesis and

tumor metastasis because of its contributions to these processes [reviewed in (Amoyel *et al.* 2014; Pencik *et al.* 2016)]. Unfortunately, the intricacy of mammalian JAK/STAT signaling creates a barrier to elucidating key molecular targets for the development of novel cancer remediation strategies. The conservation of JAK/STAT functionality across phyla (Arbouzova and Zeidler 2006) combined with a significant reduction in complexity as discussed above makes *Drosophila* a simple but powerful model to further examine the relationships between JAK/STAT signaling, lung morphogenesis, and tumor metastasis.

2.2 Materials and Methods

2.2a Drosophila stocks and culture

All flies were cultured and crossed at 25°C on standard media and in vials according to standard procedures. For misexpression of transgenes, we utilized the GAL4/UAS system in flies (Brand and Perrimon 1993). The following stocks were obtained from the Bloomington Drosophila Stock Center (supported by NIH grant P40OD018537) and used in this study: *w*; Btl-GAL4, UAS-Act5C:GFP/CyO, lacZ* (RRID:BDSC_8807); w^{1118}*, UAS-Dcr-2;* Pin^{1}*/CyO* (RRID:BDSC_24644); w^{1118}*; 10XStat92E-GFP* (RRID:BDSC_26197); *UAS-os RNAi* (RRID:BDSC_28722); *UAS-upd RNAi* (RRID:BDSC_33680); *UAS-os2 RNAi* (RRID:BDSC_33949); *UAS-upd2 RNAi* (RRID:BDSC_33988); *w*, upd2Δ* (RRID:BDSC_55727) (Osman *et al.* 2012); and *hs-FLP;; Act5C>*y^{+}*>GAL4, UAS-GFP* (MacKay *et al.* 2003). The following stocks were gifts from Douglas A. Harrison: *UAS-ET* (FBal0264424); y^{1}*, sc*,* v^{1}*;; UAS-dome RNAi* (RRID:BDSC_32860); y^{1}*,* v^{1}*;; UAS-Stat92E RNAi* (RRID:BDSC_33637); *w*; UAS-hop/CyO* (RRID:BDSC_79033) (Harrison *et al.* 1995); *Upd-GAL4, w**

(RRID:BDSC_26796); *UAS-iupd3* (FBal0177288) (Agaisse *et al.* 2003); *Upd3-GAL4, UAS-upd3-GFP/CyO; Dr/TM6B* (cross between FBal0181583 and FBal0302834); and y^1, *sc*,* v^1*;; UAS-upd3 RNAi* (RRID:BDSC_32859).

2.2b Genotypes used in various figures

Fig. 5

w^{1118}*; 10XStat92E-GFP*

Fig. 6

(Normal) *w*; Btl-GAL4, UAS-Act5C:GFP/CyO, lacZ*

(Short) *UAS-Dcr-2/+; Btl-GAL4, UAS-Act5C:GFP/+; UAS-upd3 RNAi*

(Bud) *upd2Δ/-; Btl-GAL4, UAS-Act5C:GFP/+*

Fig. 7

(A) (from left to right):

w; Btl-GAL4, UAS-Act5C:GFP/CyO, lacZ*

+/+; Btl-GAL4, UAS-Act5C:GFP/UAS-ET

+/+; Btl-GAL4, UAS-Act5C:GFP/UAS-hop

(B) (from left to right):

UAS-Dcr-2/+; Btl-GAL4, UAS-Act5C:GFP/CyO, lacZ

UAS-Dcr-2/+; Btl-GAL4, UAS-Act5C:GFP/+; UAS-dome RNAi/+

UAS-Dcr-2/+; Btl-GAL4, UAS-Act5C:GFP; UAS-Stat92E RNAi/+

Fig. 8

Upd-GAL4/+; +/+; Act5C>y^+*>GAL4, UAS-GFP*

Fig. 9

Upd3-GAL4, UAS-upd3-GFP/CyO; Dr/TM6B

Fig. 10

(A) (from left to right):

w; Btl-GAL4, UAS-Act5C:GFP/CyO, lacZ*

upd2Δ; Btl-GAL4, UAS-Act5C:GFP/+

(B) (from left to right)

UAS-Dcr-2/+; Btl-GAL4, UAS-Act5C:GFP/CyO, lacZ

UAS-Dcr-2/+; Btl-GAL4, UAS-Act5C:GFP/+; UAS-upd RNAi/+

UAS-Dcr-2/+; Btl-GAL4, UAS-Act5C:GFP/+; UAS-upd2 RNAi/+

UAS-Dcr-2/+; Btl-GAL4, UAS-Act5C:GFP/+; UAS-upd3 RNAi/+

UAS-Dcr-2/+; Btl-GAL4, UAS-Act5C:GFP/UAS-iupd3

2.2c Dissections and immunohistochemistry

Larvae were collected at late L3 and dissected in cold 1X phosphate-buffered saline (PBS). Tissues were then fixed with 0.1 M PIPES (pH 7.2) + 4% paraformaldehyde diluted in PBS for 10 minutes and washed for 20 minutes with PBTA (1X PBS + 0.1% Triton X-100 + 1% bovine serum albumin + 0.01% sodium azide) twice before mounting in a drop of Vectashield-DAPI (Vector Laboratories, Burlingame, CA). Antibody staining was performed using the above steps prior to mounting, followed by blocking in a normal goat serum (Abcam Inc., Cambridge, MA) solution (1:20, normal goat serum:PBTA) for 30 minutes and overnight incubation with primary antibody at 4°C. Primary antibody incubation was followed by four 15-minute washes in 1X PBTA, 30 minutes of blocking,

90-minute incubation with secondary antibody at room temperature in dark, and four more 15-minute PBTA washes prior to mounting. The primary antibody, mouse anti-Arm (N27A1, used at a dilution of 1:100), was obtained from the Developmental Studies Hybridoma Bank at the University of Iowa. Goat anti-mouse Alexa Fluor 568 (Invitrogen, Carlsbad, CA) was used as the secondary antibody at a dilution of 1:400. Image acquisition and data analysis were performed in part through the use of the Vanderbilt Cell Imaging Shared Resource (CISR) (supported by NIH grants CA68485, DK20593, DK58404, DK59637 and EY08126). A Zeiss LSM 510 confocal microscope at the CISR was used to obtain the images in Fig. 5, while images in Fig. 6, 8, and 9 were produced using a Zeiss AxioPlan 2 widefield microscope at WKU.

2.2d Statistical analysis

Observed values for normal and "mutant" (short + bud) ASPs were compared to expected values derived from the phenotype distribution of a given experimental group's control (i.e. *Btl-GAL4* or *UAS-Dcr-2; Btl-GAL4*) using a chi-squared test. Microsoft Excel was used to calculate chi-squared and p-values.

2.3 Results

2.3a JAK/STAT signaling is activated in ASP tip cells

Several studies have examined the contributions of JAK/STAT signaling to wing imaginal disc development (Ayala-Camargo *et al.* 2013; Johnstone *et al.* 2013; Recasens-Alvarez *et al.* 2017) and tracheal pit formation (Sotillos *et al.* 2010), but none have investigated the relationship between this pathway and the ASP, a tracheal outgrowth

invasively coupled with the wing disc (Guha *et al.* 2009). To determine whether the JAK/STAT signaling pathway is also involved in ASP development, we monitored expression of *10XStat92E-GFP*, a common JAK/STAT signaling reporter containing ten Stat92E binding sites derived from the *Socs36E* gene (Bach *et al.* 2007). This reporter's expression coincided with the characteristic "five-spot" pattern of *upd* expression within the wing disc (Ayala-Camargo *et al.* 2013; Johnstone *et al.* 2013), but was also detected in ASP tip cells, identified by an apparent downregulation of the *Drosophila* β-catenin homolog, Arm (Fig. 5) (Dong *et al.* 2015). Previous research has demonstrated that these ASP tip cells play a central role in several mechanisms that mediate invasive behavior during normal development.

These tip cells express proteolytic enzymes (Guha *et al.* 2009; Wang *et al.* 2010; Dong *et al.* 2015), remodeling the surrounding ECM in a fashion similar to metastatic tumors. In addition, these tip cells possess actin-rich filopodia that interact with the signaling ligands Bnl/FGF (Sato and Kornberg 2002; Cabernard and Affolter 2005; Roy *et al.* 2011, 2014; Cruz *et al.* 2015) and Dpp/BMP (Roy *et al.* 2011, 2014). Bnl and Dpp are essential proteins for proper morphological development of the ASP (Sato and Kornberg 2002; Cabernard and Affolter 2005; Cruz *et al.* 2015) and wing imaginal disc (Hamaratoglu *et al.* 2014), respectively, which makes ASP tip cells critical sites for the mediation of these processes. The detection of JAK/STAT signaling activity in these tip cells suggests that the pathway plays some role in their development and/or function. We chose to alter expression of core components in the *Drosophila* JAK/STAT system to further investigate the potential link between JAK/STAT signaling and ASP development.

2.3b JAK/STAT dysregulation negatively affects ASP development

In *Drosophila*, the JAK/STAT signaling cascade is initiated by binding any of the Upd ligands to Dome (Harrison *et al.* 1998; Hombría *et al.* 2005; Fisher *et al.* 2016), thereby inducing homodimerization of two Dome monomers. However, another transmembrane protein, Eye Transformer (ET), can negatively regulate this process via heterodimerization with Dome (Kallio *et al.* 2010; Fisher *et al.* 2016). We selected ET to investigate the effects of JAK/STAT downregulation because it essentially restricts intracellular signal transduction by preventing extracellular ligand binding. Another negative regulator, Suppressor of cytokine signaling at 36E (Socs36E), can effectively reduce both basal and stimulated JAK/STAT activity (Stec *et al.* 2013), but Socs36E has also been shown to negatively regulate EGFR signaling (Callus and Mathey-Prevot 2002). We did not alter Socs36E expression because the developmental impact resulting from reduced JAK/STAT activity would likely be obfuscated by concurrent inhibition of EGFR signaling, which plays a central role in ASP cell proliferation (Sato and Kornberg 2002; Cabernard and Affolter 2005; Cruz *et al.* 2015).

We overexpressed ET and visualized ASPs using *UAS-ET* and *UAS-Act5C:GFP* transgenes, respectively, which were both under the control of a *Btl-GAL4* driver. Although ET is not known to interact with proteins other than Dome despite putative JAK and STAT binding sites (Fisher *et al.* 2016), we also downregulated *dome* expression using RNA interference (RNAi), enhanced by *Dicer-2* (*Dcr-2*) overexpression (Dietzl *et al.* 2007), to support any conclusions that may be drawn regarding the effects of ET overexpression. Similarly, Stat92E RNAi was employed to investigate whether signal modulation at this point in the pathway induced morphogenetic changes of a similar or disparate nature.

Canonical JAK/STAT activity can regulate other signaling pathways (Rawlings *et al.* 2004; Tsai *et al.* 2007; Lopez-Onieva *et al.* 2008), but individual components, especially STATs, mediate signal input from RTKs in several contexts as well (Ruff-Jamison *et al.* 1995; David *et al.* 1996; Yang *et al.* 2009; Dudka *et al.* 2010), including EGFR. We also used a *hop* overexpression construct (*UAS-hop*) in order to model JAK/STAT hyperactivation.

Three distinct phenotypic categories emerged across each line following image capture of ASPs. As previously described (Dong *et al.* 2015), ASPs were classified as "normal" (wild type bud and stalk structures), "short" (short, rounded buds, sometimes paired with shortened stalk), or "bud" (cells clustered near TC with no identifiable stalk) (Fig. 6). The numbers of short and bud ASP were consolidated into one mutant phenotypic class for statistical comparisons between wild type and JAK/STAT dysregulation lines (Fig. 7). Compared to the 5.3% (7/133) of ASPs observed in our control larvae, 24.0% (30/125) of ASPs in our *UAS-ET* overexpression line presented a mutant phenotype, demonstrating a significant ($p < .0001$) increase from wild type levels (Fig. 7A). A similar trend was observed across the other JAK/STAT inhibition lines, wherein 30.7% (31/101) and 42.3% (44/104) of *UAS-dome RNAi* and *UAS Stat92E RNAi* ASP, respectively, appeared to be mutants as opposed to 19.0% (19/100) of our other control, where *UAS-Dcr-2* was also overexpressed (Fig. 7B). Because knocking down the expression of JAK/STAT components appears to restrict ASP development, one might reason that increasing pathway activity would promote ASP invasion to a greater degree. Interestingly, no observable indication of such activity (e.g., increased number of filopodia) emerged during data collection (data not shown). On the contrary, in larvae where we increased *hop* expression, 33% (33/100) of the resultant ASPs demonstrated mutant phenotypes

comparable to those observed among ASPs where JAK/STAT signaling was diminished (Fig. 7A).

2.3c JAK/STAT signaling in the ASP is facilitated by the ligand Upd2

Our data provides strong evidence that JAK/STAT signaling contributes to ASP development in some capacity, but we also wanted to determine which of the three Upd ligands activate(s) the pathway in ASP tip cells. We began by driving GFP expression using an *Upd-GAL4* driver (Fig. 8) to see whether ASP tip cells are positioned to make contact with Upd-expressing cells in the presumptive wing hinge. This does not appear to be the case at late L3, though we did not examine Upd expression with respect to the ASP prior to this stage. We then took a similar approach to observe the distribution of Upd3. In the wing disc, Upd3 appears to be expressed in discrete pockets along the anterior half of the wing disc, localizing in the wing pouch and wing hinge regions, seemingly along the border between the wing hinge and presumptive ventral body wall (Fig. 9). Of most interest for the current study, however, was the finding that Upd3 appears to be expressed ubiquitously throughout the ASP and the TC from which it emerges (Fig. 9). Unfortunately, we were unable to determine the expression pattern of Upd2 in the wing disc or trachea at late L3. Transgenic fly stocks have been created for observing Upd2 expression with the GAL4/UAS system (Hombría *et al.* 2005), but we are unable to use them because none are publicly available at the time of writing. Further, a previous attempt at Upd2 detection in the wing disc via RNA *in situ* reportedly failed to detect its expression (Ayala-Camargo *et al.* 2013), though this was the case for Upd3 in the same study as well. Together, this

information led to some surprising results when we knocked down expression of each individual ligand.

We used at least two means of knockdown for each ligand to assess their individual contributions to ASP development: two different RNAi lines under control of the GAL4/UAS system for each of the three ligands and one viable knockout line. For Upd, we used two Transgenic RNAi Project (TRiP) lines (Ni *et al.* 2009, 2011): one generated double-stranded RNA (dsRNA) from all three exons of the *upd* transcript (*UAS-os RNAi*), while the other targeted a portion of the 3'-untranslated region (UTR) (*UAS-upd RNAi*). For Upd2, we used two additional TRiP lines (*UAS-os2 RNAi* and *UAS-upd2 RNAi*), each targeting a different exon of the *upd2* transcript, and an Upd2 knockout line (*upd2Δ*), where the entire *upd2* transcription unit was deleted on the X chromosome. Finally, for Upd3, we used two more TRiP lines (*UAS-upd3 RNAi* and *UAS-iupd3*) that each target a different exon of the *upd3* transcript. The same controls in Fig. 6 were used for comparison of their phenotypic distributions with the stated knockdown and knockout lines via chi-squared test (Fig. 10).

Our *Btl>Dcr-2, os RNAi* and *Btl>Dcr-2, os2 RNAi* lines did not yield any viable offspring with detectable GFP. The former result is not surprising, given the prominent role of Upd in tracheal placode development in *Drosophila* embryos (Brown *et al.* 2001; Sotillos *et al.* 2010), but the latter is more perplexing. Upon Upd2 knockout, we were able to examine many male L3s that both lacked Upd2 and contained the *Btl>Act5c:GFP* element, albeit with 50.0% (51/102) of observed ASPs exhibiting aberrant development (Fig. 10A). Furthermore, the online program Updated Targets of RNAi Reagents (UP-TORR) (Hu *et al.* 2013) did not identify any potential off-target sites with a ≥15 bp match

that *UAS-os2 RNAi* might affect. The remaining RNAi lines, excluding *UAS-upd2 RNAi*, demonstrated no significant differences from the phenotype distribution of our *UAS-Dcr-2; Btl-GAL4* control when they were overexpressed (Fig. 10B). These results suggest that Upd2 is expressed in the ASP and that it is the primary contributor to JAK/STAT activation along the ASP's leading edge. Nevertheless, we cannot conclusively reject the potential contributions of Upd to JAK/STAT signaling in the ASP, given the likely weaker impact of dsRNA generated from the *upd* transcript's 3'-UTR and the fact that we drove *UAS-upd RNAi* overexpression in the trachea, where no Upd was detected via the *Upd>GFP* protein trap. In addition, Upd2 may be capable of compensating for Upd3 knockdown in the same way that Upd has been proposed to compensate Upd2 loss during tracheal placode development (Hombría *et al.* 2005).

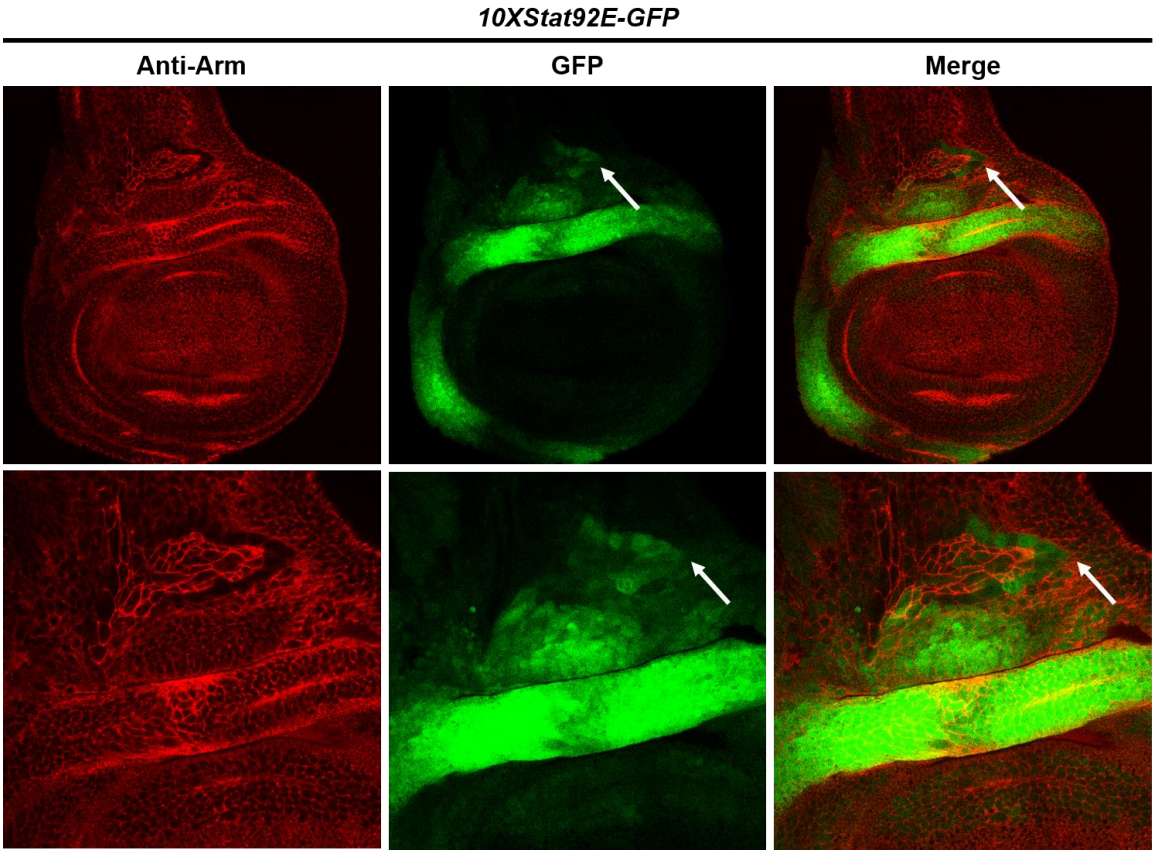

Figure 5: JAK/STAT signaling is activated in ASP tip cells

Anti-Armadillo antibody staining (Anti-Arm) was employed to mark adherens junctions (red) and visualize cell-cell boundaries in the wing imaginal disc. Note that these boundaries are absent along the ASP's outer edge. Phosphorylated Stat92E protein is detected and reported by a *10XStat92E-GFP* transgene (green). ASP tip cells exhibiting JAK/STAT activity are indicated by an arrow. Panels in the bottom row are magnified views of the ASP associated with the wing disc in the top row.

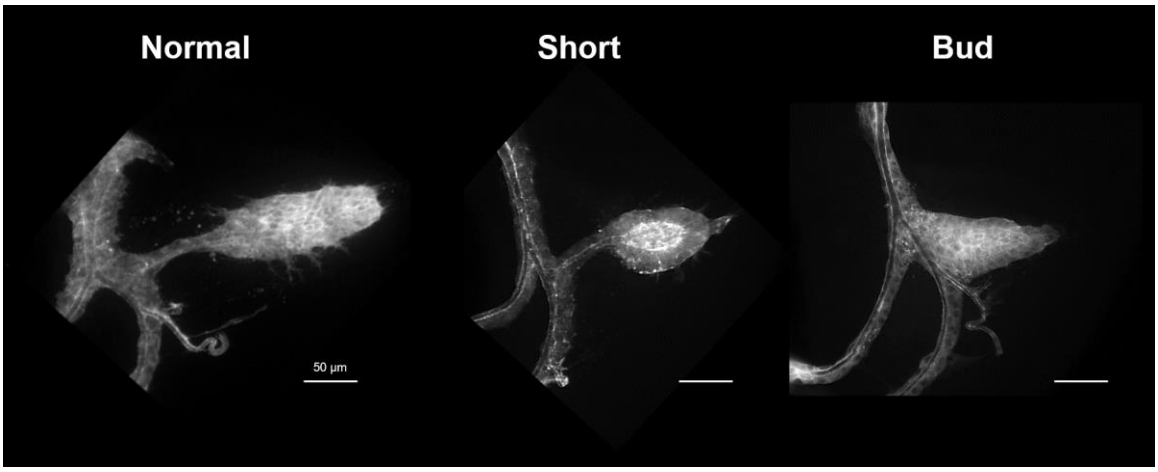

Figure 6: Representative examples of ASP phenotype categories

"Normal" ASPs are similar to or indistinguishable from the wild type structure, which has distinct bud and stalk features. "Short" ASPs are identified by a short, round bud that may also be accompanied by a reduced stalk. "Bud" ASPs exhibit the most severe developmental defect, wherein cells remain clustered near the TC and no stalk forms. ASPs were visualized by using *Btl-GAL4* to drive expression of a *UAS-Act5c:GFP* construct. Scale bar lengths are indicated under the "Normal" ASP.

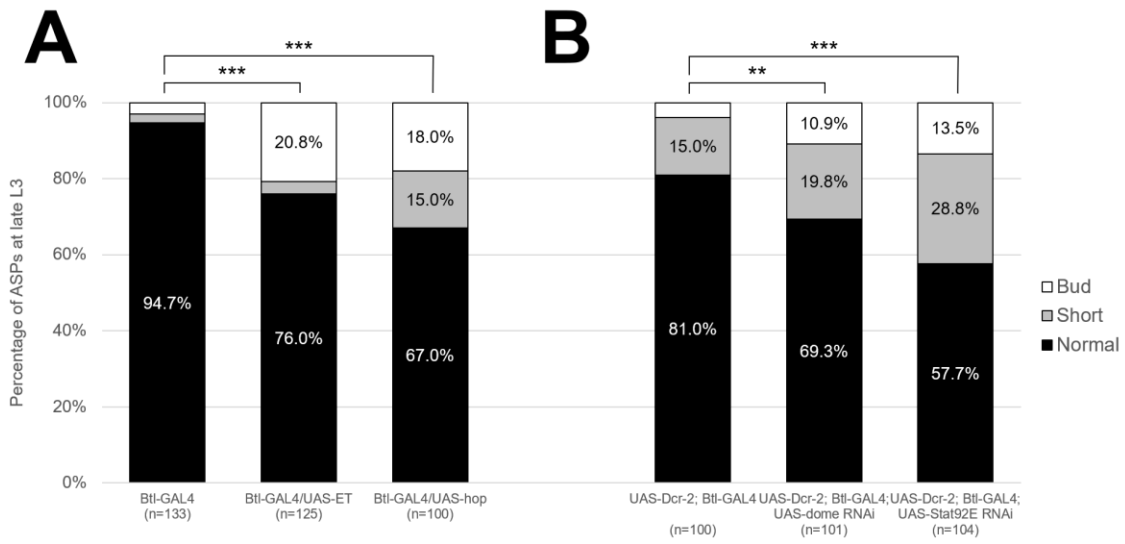

Figure 7: ASP phenotype proportions in response to JAK/STAT dysregulation

(A) Overexpression of *UAS-ET* and *UAS-hop* driven by *Btl-GAL4* yields a significant reduction in the percentage of normal ASP phenotypes observed compared to that found for *Btl-GAL4* ASPs. (B) RNAi-mediated knockdown of Dome and Stat92E bolstered by concurrent overexpression of Dcr-2 also significantly reduces the percentage of normal ASP phenotypes observed compared to our *UAS-Dcr-2; Btl-GAL4* control. A chi-squared test was used to compare the number of normal and "mutant" (short + bud) ASPs observed among experimental lines to expected values derived from their respective controls (** is $p < .005$, *** is $p < .0001$). n = number of late-stage ASPs.

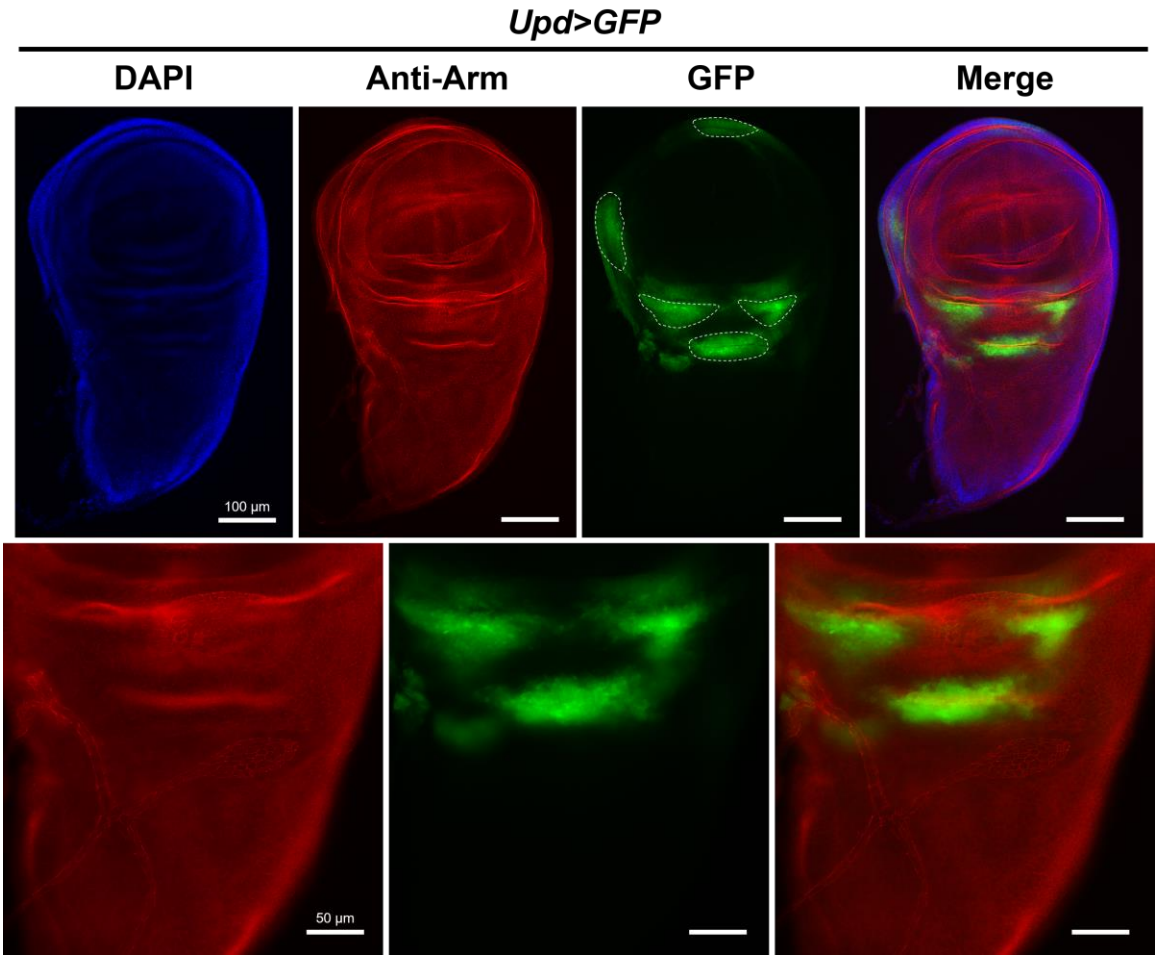

Figure 8: Upd distribution in the wing imaginal disc at late L3

Top row: nuclear staining (DAPI), Armadillo staining (Anti-Arm), and Upd expression (GFP) in the wing disc. The characteristic five spots of Upd expression are circled with dotted white lines. Bottom row: Anti-Arm and GFP channels are displayed at a higher magnification to show the location of the ASP with respect to the nearest source of Upd in the presumptive wing hinge. The edge of the ASP can be identified by the crescent-shaped space where Arm is downregulated. Scale bars lengths are indicated in the bottom right of the leftmost panel in each row.

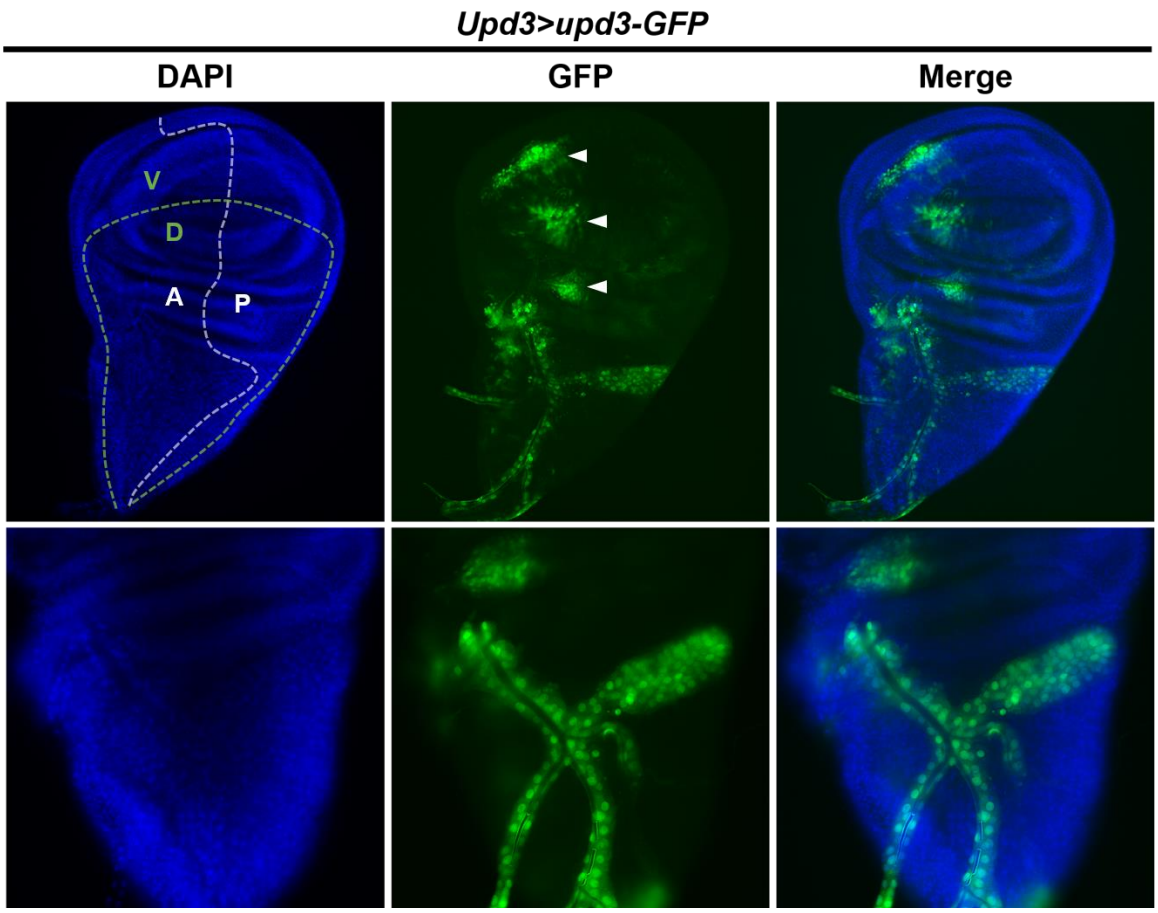

Figure 9: Upd3 distribution in the wing imaginal disc and Tr2 at late L3

Upd3 localizes in the anterior half of the wing disc as three clusters within, moving dorsoventrally, the presumptive wing hinge, wing pouch, and along the border between the presumptive wing hinge and ventral body wall (arrowheads). Upd3 is also expressed throughout the transverse connective (TC) and ASP associated with the wing disc, as shown in the bottom panels, where the ASP from another wing disc is displayed at a higher magnification. A/P = anterior/posterior; V/D = ventral/dorsal.

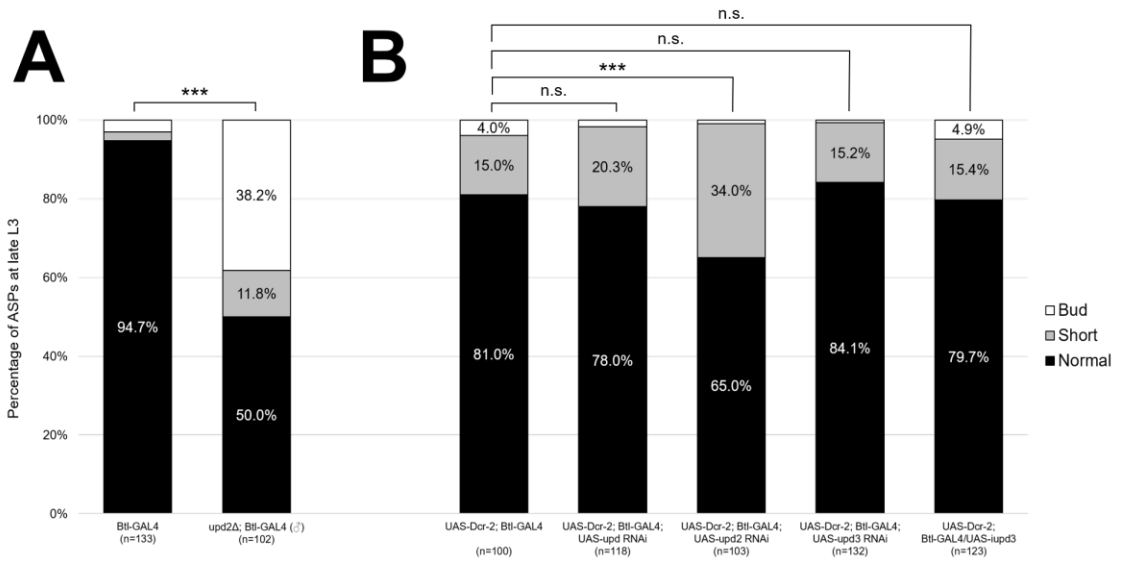

Figure 10: ASP phenotype proportions in response to JAK/STAT ligand knockdown

(A) Compared to the Btl-GAL4 control, a significantly higher percentage of ASPs exhibit a mutant phenotype, and most of those mutants are the more severe variant ("Bud"). Only ASPs from male *upd2Δ; Btl-GAL4* L3s were observed because the X chromosome not carrying the upd2 deletion might become inactivated in females. (B) Differences in normal/mutant phenotype ratios were not significant (n.s.) when comparing the *UAS-Dcr-2; Btl-GAL4* control to lines where Upd RNAi ($p = .4009$), Upd3 RNAi ($p = .3653$), and iUpd3 ($p = .7079$) were overexpressed. In contrast, the proportion of mutant ASPs that emerged in response to Upd2 RNAi overexpression was significantly higher than the control. A chi-squared test was used to compare normal and mutant phenotype proportions between control and experimental lines (*** is $p < .0001$). n = number of late-stage ASPs.

3. THE ROLE OF SURF4 DURING DEVELOPMENT OF THE AIR SAC PRIMORDIUM AND OTHER *DROSOPHILA* TISSUES

3.1 Introduction

The BM is a specialized ECM that is critical for providing structural support, delineating tissue boundaries, and a host of other activities throughout development in multicellular eukaryotes, including morphogenesis (Srivastava *et al.* 2007; Pastor-Pareja and Xu 2011; Isabella and Horne-Badovinac 2015a) and cell-cell signaling (Folkman *et al.* 1988; Isabella and Horne-Badovinac 2015a). The role of BMs in maintaining the structural integrity and compartmentalization of epithelial, endothelial, fat, muscle, and peripheral nerve tissues (Isabella and Horne-Badovinac 2015b) makes BM degradation obligatory for the progression of tumor metastasis (Glentis *et al.* 2014). BMs contain many minor, tissue-specific components including fibulin, SPARC, and collagen VI, XV, and XVIII, but are more broadly composed of four major proteins: laminin, nidogen, perlecan, and collagen IV (Glentis *et al.* 2014; Isabella and Horne-Badovinac 2015b). However, because collagen IV comprises about 50% of the BM (Halfter *et al.* 2013), understanding what, how, and where other proteins associate with it may be most helpful in explaining overall BM development and function. In addition to the multitude of known benefits in using *Drosophila* as a model organism (Hales *et al.* 2015), only two genes in fruit flies code for collagen IV chains [*vkg* and *Collagen at 25C* (*Cg25C*)] (Yasothornsrikul *et al.* 1997) as opposed to the six found in mammals (Khoshnoodi *et al.* 2008). However, it should be noted that only the two "classical" collagen IV chains homologous to Vkg and Cg25C, α2(IV) and α1(IV), respectively (Pastor-Pareja and Xu 2011), are found in all BMs throughout development, while the others are more restricted (Khoshnoodi *et al.* 2008). A

former graduate student in the Srivastava lab, Mayank Kapadia, identified six unique Vkg-associated proteins using immunoprecipitation and mass spectrometry analysis (Kapadia and Srivastava 2016). Of these six, this study seeks to begin characterizing Surfeit locus protein 4 (Surf4), which has been implicated in vesicle trafficking between the endoplasmic reticulum (ER) and Golgi apparatus (Mitrovic *et al.* 2008; Emmer *et al.* 2018).

3.2 Materials and Methods

3.2a Drosophila stocks and culture

All flies were cultured and crossed at 25°C on standard media and in vials according to standard procedures. For misexpression of transgenes, we utilized the GAL4/UAS system in flies (Brand and Perrimon 1993). The following stocks were obtained from the Bloomington Drosophila Stock Center (supported by NIH grant P40OD018537) and used in this study: *w*; Btl-GAL4, UAS-Act5C:GFP/CyO, lacZ* (RRID:BDSC_8807); w^{1118}*, UAS-Dcr-2;* Pin^{1}*/CyO* (RRID:BDSC_24644); *w*;; Surf4:GFP/TM3,* Ser^{1}*,* Sb^{1} (RRID:BDSC_51567); w^{1118}*, Bx-GAL4; UAS-Dcr-2* (RRID:BDSC_25706); *Elav-GAL4; UAS-Dcr-2* (RRID:BDSC_25750); *UAS-Dcr-2,* w^{1118}*; En-GAL4, UAS-GFP* (RRID:BDSC_25752); w^{1118}*;; UAS-Surf4/TM6B,* Tb^{1} (RRID:BDSC_19202); y^{1}*, sc*,* v^{1}*; UAS-Surf4 RNAi* (RRID:BDSC_57471). The following *GAL4* stocks are from the Srivastava lab, with GAL4 insertions listed in parentheses: *Pnr-GAL4/TM6B,* Tb^{1} (FBal0291787); *Cg25C-GAL4, UAS-RFP* (FBal0244165); *Vg-GAL4, UAS-RFP* (FBal0047077); *GMR-GAL4/CyO, Act5C:GFP* (FBal0093304); *Ey-GAL4/CyO* (FBal0093300); *Ubx-GAL4/TM6B,* Tb^{1} (FBal0264894); and *Ptc-GAL4, UAS-RFP, vkg:GFP/CyO; TubGal80*^{ts}*/TM6B,* Tb^{1} (Srivastava *et al.* 2007).

3.2b Genotypes used in various figures

Fig. 11

w;; Surf4:GFP/TM3, Ser^{1}, Sb^{1}*

Fig. 12

UAS-Dcr-2/+; Btl-GAL4/UAS-Surf4 RNAi

Fig. 13

w; Btl-GAL4, UAS-Act5C:GFP/CyO, lacZ*

+/+; Btl-GAL4, UAS-Act5C:GFP/+; UAS-Surf4

Fig. 14

(A-C) *UAS-Dcr-2, w^{1118}; En-GAL4, UAS-GFP* (♀)

(D-G) *UAS-Dcr-2; En-GAL4, UAS-GFP; UAS-Surf4 RNAi* (♀)

Fig. 15

(A-D) *w^{1118}, Bx-GAL4/+; UAS-Dcr-2* (♀)

(E-H) *w^{1118}, Bx-GAL4/+; UAS-Dcr-2/UAS-Surf4 RNAi* (♀)

Fig. 16

(Top row) *Ptc-GAL4, UAS-RFP, vkg:GFP/CyO*

(Bottom row) *Ptc-GAL4, UAS-RFP, vkg:GFP/UAS-Surf4 RNAi*

Fig. 17

(Rows 1 & 2) *Ptc-GAL4, UAS-RFP, vkg:GFP/CyO*

(Rows 3 & 4) *Ptc-GAL4, UAS-RFP, vkg:GFP/UAS-Surf4 RNAi*

3.2c Viability assays

Flies were placed on grape agar plates made with premix from Genesee Scientific (San Diego, CA), and left at 25°C to lay eggs over the course of 24 hours. Embryos demonstrating green fluorescence were collected, transferred to fresh grape agar plates, and observed at 24-hour intervals to determine the number of surviving progenies at each larval stage.

3.2d Dissections and imaging

Larvae were collected as either late or wandering L3s and dissected as described previously (see Chapter 2.2c). A Zeiss AxioPlan 2 fluorescent microscope was used for acquisition of images used in Fig. 11, 14, and Fig. 15D and 15H. Images used in Fig. 18 were taken from z-stacks obtained with a Zeiss LSM 510 confocal microscope at Vanderbilt University's CISR. Adult wings were removed from flies anesthetized with CO_2, mounted in a drop of Permount Mounting Medium (Fisher Scientific, Fair Lawn, NJ), and left on a slide warmer set to approximately 48°C overnight prior to imaging. Images of adult flies in Fig. 15A-C and 15E-G were obtained with a Leica MZ16 stereomicroscope, Schott KL 2500 LCD light source, and Nikon Digital Sight-L1 camera after freezing the flies at -20°C for 2 hours and mounting them on 1% agarose plates.

3.2e Statistical analysis

Statistical analysis was performed as described in Chapter 2.2d.

3.3 Results

3.3a Surf4 is upregulated in the ASP and is required for larval development

We first examined the distribution of Surf4 expression in several larval tissues. As expected, based on its documented role in vesicular transport, Surf4 was found in the cytoplasm of all tissues via detection of a GFP reporter construct (Fig. 11). However, the expression was not always uniform, and this is especially true within the ASP. Upon closer examination of the wing imaginal disc, it appears as if Surf4 is upregulated in the ASP's bud region (Fig. 11F). Observing this, we investigated whether Surf4 played a role in ASP development by knocking down and overexpressing this protein throughout the tracheal system via the GAL4/UAS system (Brand and Perrimon 1993). We find that Surf4 plays a significant role in larval development due to the lethality we observed in response to Surf4 knockdown under various conditions.

We first drove knockdown of Surf4 by crossing *UAS-Surf4 RNAi* flies with *UAS-Dcr-2; Btl-GAL4, UAS-Act5C:GFP* flies to drive knockdown throughout the tracheal system and bolster the effect with Dcr-2 overexpression (Dietzl *et al.* 2007). We did not observe any L3s positive for our GFP reporter, indicating that all those carrying the desired elements to investigate Surf4 knockdown did not reach this developmental stage. We then assayed the viability of progeny from the aforementioned cross to determine at what point in development these larvae become inviable. We found that despite an average hatch rate of 59.9% (121/202) among control embryos, almost all hatched larvae reached L3. In contrast, the furthest stage of development our experimental larvae reached was L2, with many dying at L1 (Fig. 12). This failure of Surf4 knockdown offspring to reach L3 persisted even when culturing the flies at 18°C (i.e. when the temperature-sensitive

GAL4/UAS system performs more sub-optimally than at 25°C) and without the *UAS-Dcr-2* construct. We also saw a significant ($p < .0001$) increase in mutant ASP phenotypes compared to our *Btl-GAL4* control when we overexpressed *UAS-Surf4* (Fig. 13). Interestingly, these data stand in contrast to our finding that Surf4 overexpression had seemingly no effect when driven by other GAL4 drivers.

3.3b Phenotypic analysis of Surf4 overexpression

Seeing the impact of Surf4 misexpression on ASP development, we sought to evaluate the role of Surf4 in the development of several other *Drosophila* tissues where the BM, and/or its remodeling, plays a significant role in morphogenesis. Evagination of the wing, haltere, and leg discs are required for the proper formation of their respective adult structures, and this process requires BM remodeling (Hammonds and Fristrom 2006; Srivastava *et al.* 2007; De las Heras *et al.* 2018). As such, we drove overexpression of Surf4 throughout the haltere disc and metathoracic (i.e. third) leg disc with *Ubx-GAL4*, and along the dorsoventral boundary of the wing imaginal disc with *Vestigial* (*Vg*)-*GAL4*. In both cases, no phenotypic defects were observed in the adult structures associated with their respective GAL4 drivers. Similarly, Surf4 overexpression in the eye driven by two different GAL4 drivers [*Glass Multiple Reporter* (*GMR*)-*GAL4* and *Eyeless* (*Ey*)-*GAL4*] did not yield any detectable abnormalities. Little is known about the mechanisms that dictate BM remodeling in eye development, but it is known that BMs coat the outer surface of eye imaginal discs, form the basis of the adult retina, and interact with integrins that contribute to ommatidial organization during metamorphosis (Zusman *et al.* 1993).

We also drove Surf4 overexpression with *Pannier* (*Pnr*)-*GAL4* and *Cg25C-GAL4* drivers. While *pnr* is not directly involved with BM remodeling, it is expressed in a defined portion of the notum, where mechanosensory bristles develop. The elongation of these bristles during metamorphosis requires protease activity to assist in BM remodeling (Hammonds and Fristrom 2006), suggesting that Surf4 overexpression may influence this dynamic in light of the protein's association with collagen IV. Because Cg25C is the other collagen IV chain in *Drosophila*, we thought that Surf4 overexpression overlapping the Cg25C distribution might also affect BM establishment or remodeling. Neither this supposition, nor that regarding bristle formation, were supported by the phenotypes of *Pnr-Gal4* x *UAS-Surf4* and *Cg25C* x *UAS-Surf4* progenies. Noting the discrepancy between the severity of Surf4 knockdown and overexpression phenotypes driven by *Btl-GAL4*, we wondered if Surf4 might have additional roles in development obscured by the lack of effects observed in response to Surf4 overexpression.

3.3c Surf4 knockdown suggests a role in wing development

We used three other "RNAi driver" lines (i.e. lines containing both *UAS-Dcr-2* and *GAL4* elements) aside from our *UAS-Dcr-2; Btl-GAL4* line to determine the effects of Surf4 knockdown. *Beadex (Bx)-GAL4; UAS-Dcr-2* drove overexpression of *UAS-Surf4 RNAi* throughout the wing; *Embryonic lethal abnormal vision* (*Elav*)-*GAL4; UAS-Dcr-2* drove overexpression throughout the eye; and *UAS-Dcr-2; Engrailed* (*En*)-*GAL4* drove overexpression throughout the posterior wing compartment. As with Surf4 overexpression, no phenotype was observed in the eye in response to *Elav-GAL4*-driven knockdown of Surf4. Though Surf4 overexpression in the wing did not produce defects either, Surf4

knockdown did. When *UAS-Surf4 RNAi* was overexpressed in the posterior wing compartment via *En-GAL4*, this portion of the adult wing became wrinkled with bristles adopting a somewhat whorled phenotype, contrasting the planar, uniform nature of wild-type wings (Fig. 14). Likewise, when this Surf4 knockdown was expanded to the entire wing, this slight warping of bristle orientation characterized the entire wing epithelium, conferring a curved wing phenotype as well (Fig. 15). A summary of all discussed phenotypes (and lack thereof) are recorded in Table 1.

3.3d Collagen IV distribution in response to Surf4 knockdown

In an attempt to establish a direct link between Surf4 expression and collagen IV distribution, we knocked down Surf4 expression along the anterior/posterior (A/P) axis of the wing imaginal disc in the salivary gland and throughout the salivary gland with a *Ptc-GAL4, UAS-Red Fluorescent Protein* (*RFP*), *vkg:GFP* construct. As was the case with *Btl-GAL4*-driven knockdown, *Ptc-GAL4*-driven knockdown proved lethal, even in the absence of Dcr-2 overexpression at 18°C. Unlike the *Btl>Surf4 RNAi* line, however, we were able to collect and dissect several "escaper" larvae at wandering L3 from the *Ptc>Surf4 RNAi* line. In the wing imaginal disc, Ptc is expressed in a well-defined stripe, thereby allowing quick identification of discrepancies in collagen IV levels from surrounding tissue. Our lab has previously used *Ptc-GAL4* to assay collagenase activity of CP1/CTSL (Dong *et al.* 2015), demonstrating the efficacy of this approach. Regardless, no discernible difference in collagen IV levels was detected in response to Surf4 knockdown (Fig. 16). Likewise, collagen IV distribution in the salivary glands of control and knockdown lines did not differ in an appreciable way, extracellularly or intracellularly (Fig. 17).

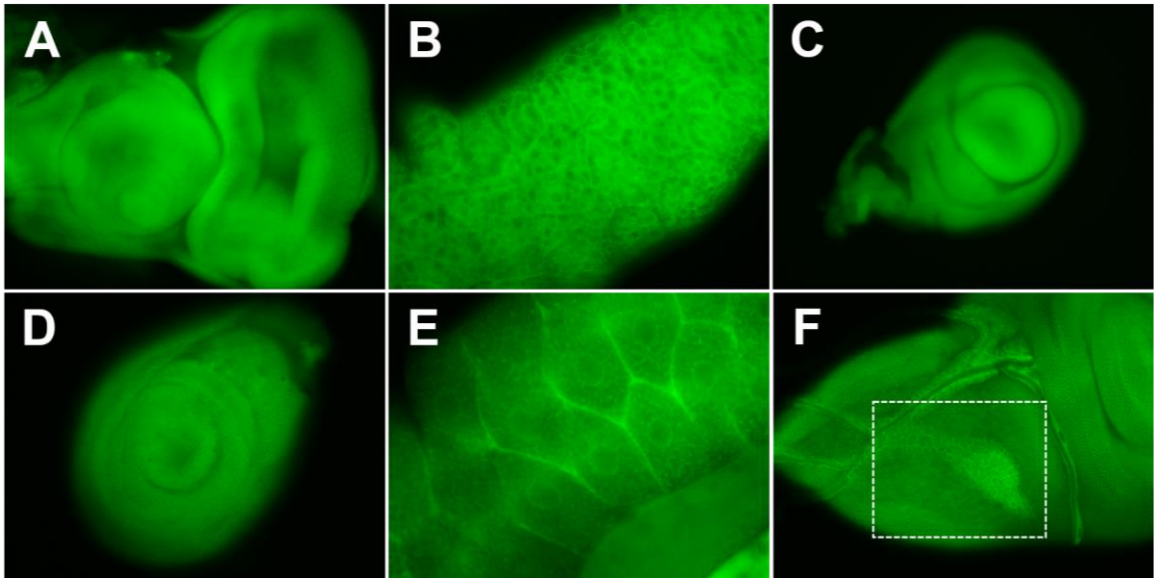

Figure 11: Surf4 expression in various *Drosophila* tissues

(A) Surf4 expression in the eye and antennal imaginal discs. (B) Surf4 expression in the fat body. (C) Surf4 expression in the haltere imaginal disc. (D) Surf4 expression in the leg imaginal disc. (E) Surf4 expression in the salivary gland. (F) Surf4 expression in the wing imaginal disc and ASP (boxed region). Note the elevated Surf4 expression in the mediodistal portion of the ASP.

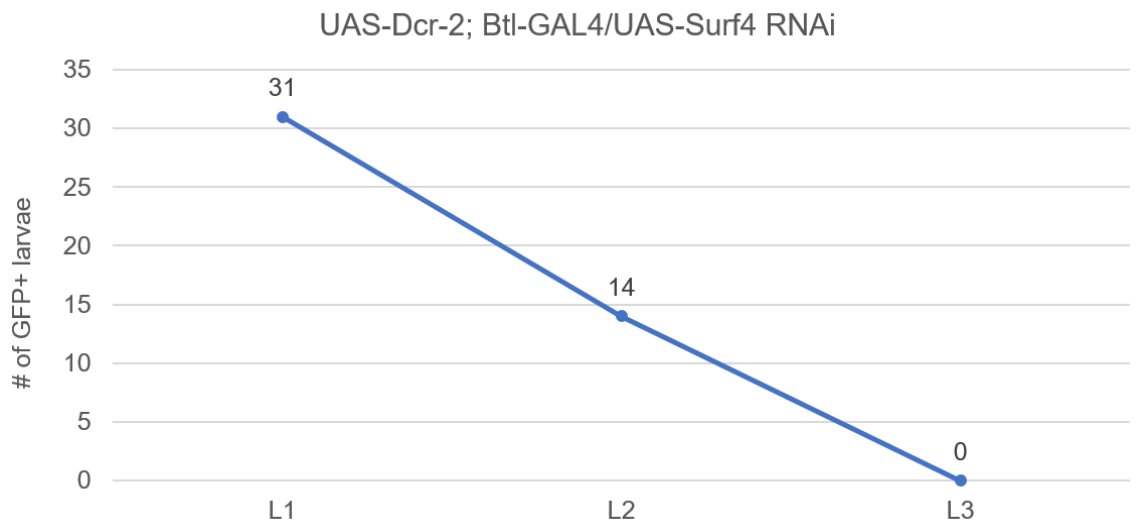

Figure 12: Surf4 knockdown driven by *Btl-GAL4* is lethal at late L2.

When we overexpressed *UAS-Surf4 RNAi* with a *Btl-GAL4* driver, over half of assayed larvae (n = 31) died before reaching the second larval instar (L2), and all died before reaching L3, though some larvae reached late L2.

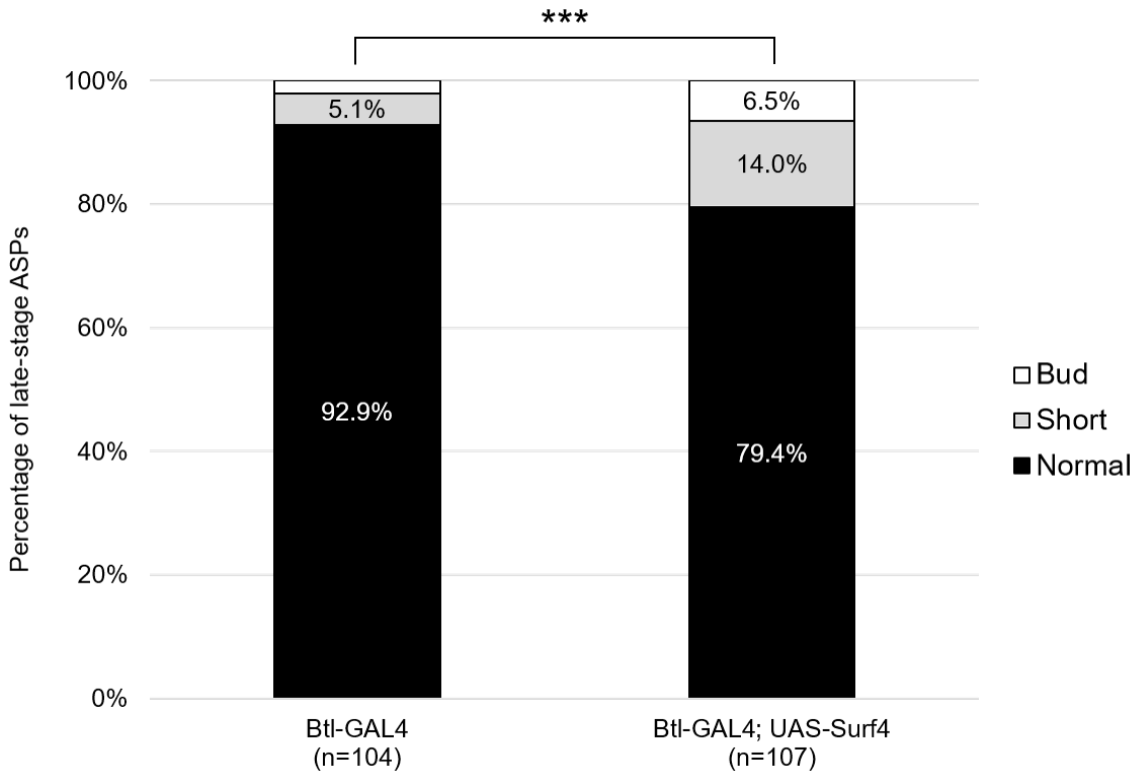

Figure 13: Surf4 overexpression throughout the tracheal system induces ASP defects

The proportion of mutant ASPs observed in response to *Btl-GAL4*-driven overexpression of *UAS-Surf4* nearly tripled with respect to the *Btl-GAL4* control, representing a significant (***, $p < .0001$) increase.

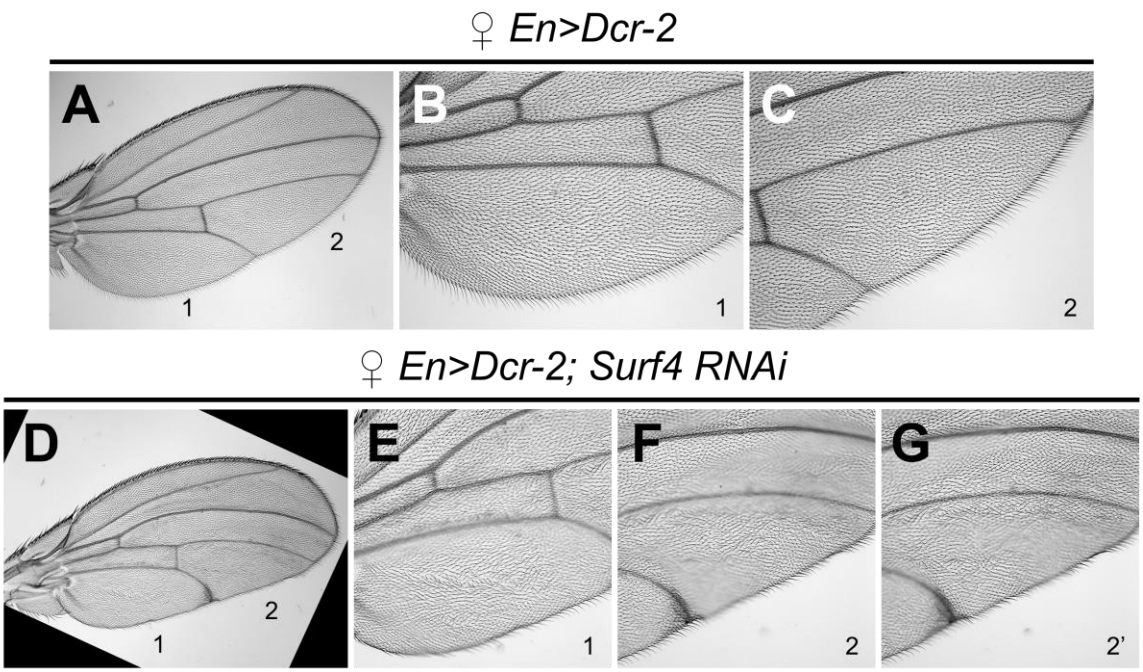

Figure 14: Surf4 knockdown driven by Engrailed (En) induces bristle defects in the posterior half of the adult *Drosophila* wing

(A) Wing from a female fly driving overexpression of a *UAS-Dcr-2* construct with an *En-GAL4* driver. "1" and "2" denote regions magnified in (B) and (C). (D) Wing from a female fly driving overexpression of *UAS-Dcr-2* and *UAS-Surf4 RNAi* constructs with an *En-GAL4* driver. "1" and "2" denote regions magnified in (E-G). (G) This panel depicts the same region in (F) at a different focal plane, emphasizing the uneven surface of the wing tissue.

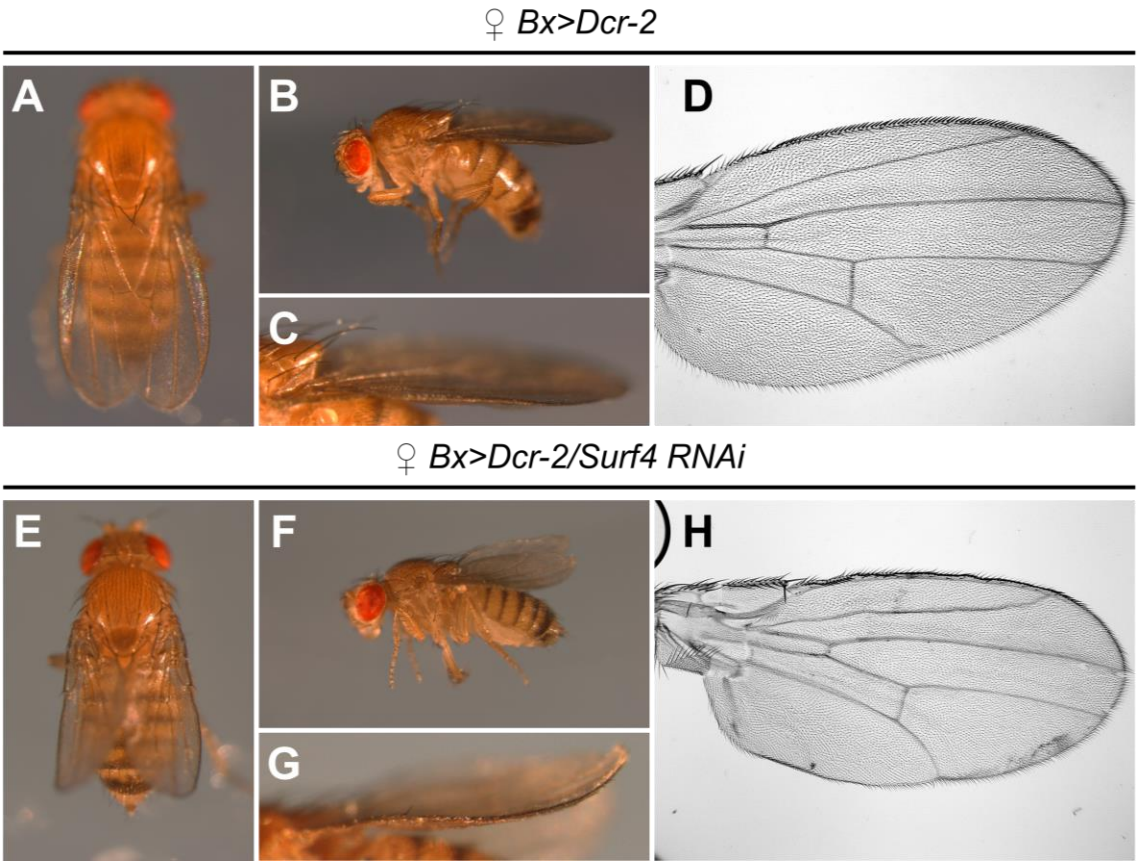

Figure 15: Surf4 knockdown driven by Beadex (Bx) induces bristle defects throughout the adult *Drosophila* wing

(A-C) A female fly where *Bx-GAL4* is driving overexpression of *UAS-Dcr-2*. (A) Dorsal and (B) lateral views demonstrate the straight, planar nature of the wings. (C) Magnified view of the wing in (B). (D) Wing from another female *Bx-GAL4; UAS-Dcr-2* fly, exhibiting uniform distribution and orientation of wing bristles. (E-G) A female fly where *Bx-GAL4* is driving overexpression of both *UAS-Dcr-2* and *UAS-Surf4 RNAi*. (E) Dorsal and (F) lateral views show the curled nature of the wing in response to Surf4 knockdown. (G) Magnified view of the wing in (F) at a slightly different angle. (H) Wing from another female *Bx-GAL4; UAS-Dcr-2/UAS-Surf4 RNAi* fly exhibiting aberrant wing bristle orientation.

GAL4 Driver	x *UAS-Surf4 RNAi*	x *UAS-Surf4*
Bx-GAL4; UAS-Dcr-2	Wrinkled wings due to apparent bristle defects	---
UAS-Dcr-2; En-GAL4	Wrinkled wings due to apparent bristle defects	---
Elav-GAL4; UAS-Dcr-2	No effect	---
Pnr-GAL4	---	No effect
Ubx-GAL4	---	No effect
Cg25C-GAL4	---	No effect
Vg-GAL4	---	No effect
GMR-GAL4	---	No effect
Ey-GAL4	---	No effect
Btl-GAL4	Lethal at late L2	Impaired ASP development

Table 1: Summary of phenotypes observed in response to Surf4 misexpression

The columns "x *UAS-Surf4 RNAi*" and "x *UAS-Surf4*" list the phenotypes resulting from Surf4 knockdown and overexpression, respectively, driven by the adjacent GAL4 drivers. Instances where no cross was conducted between the listed GAL4 driver and UAS construct are indicated by a "---".

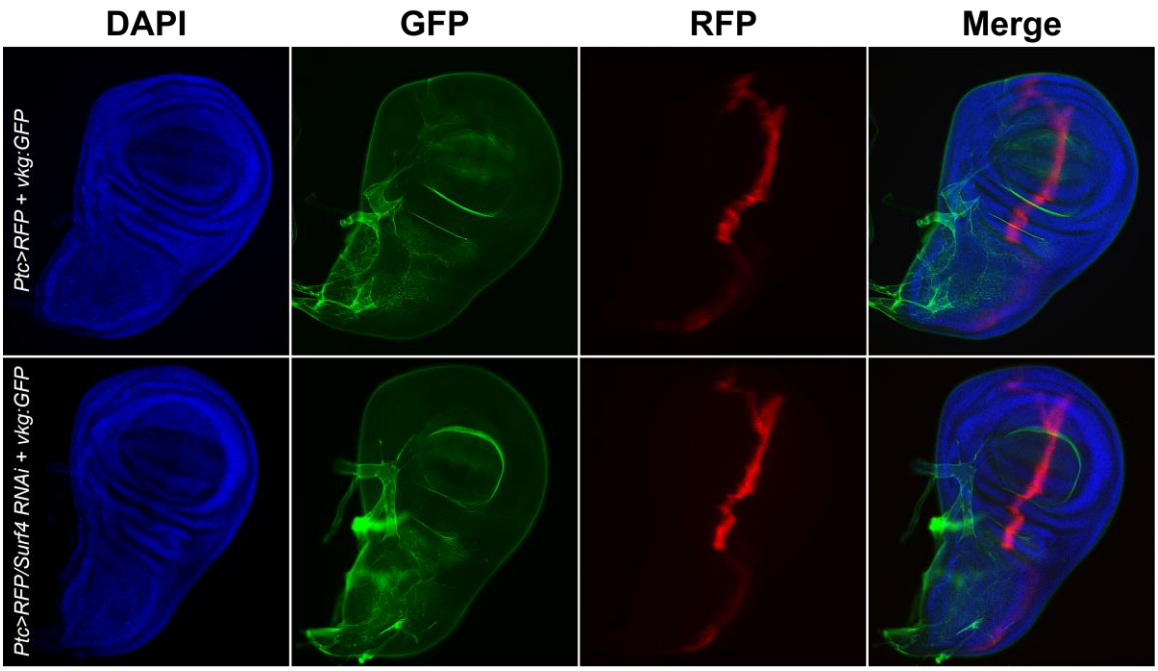

Figure 16: Surf4 knockdown along the A/P wing axis has no apparent effect on collagen IV distribution.

No clear difference exists between control (*Ptc>RFP + vkg:GFP*) and Surf4 knockdown (*Ptc>RFP/Surf4 RNAi + vkg:GFP*) wing discs with respect to collagen IV distribution along the anterior/posterior (A/P) axis. DAPI = cell nuclei; GFP = Vkg; RFP = Ptc.

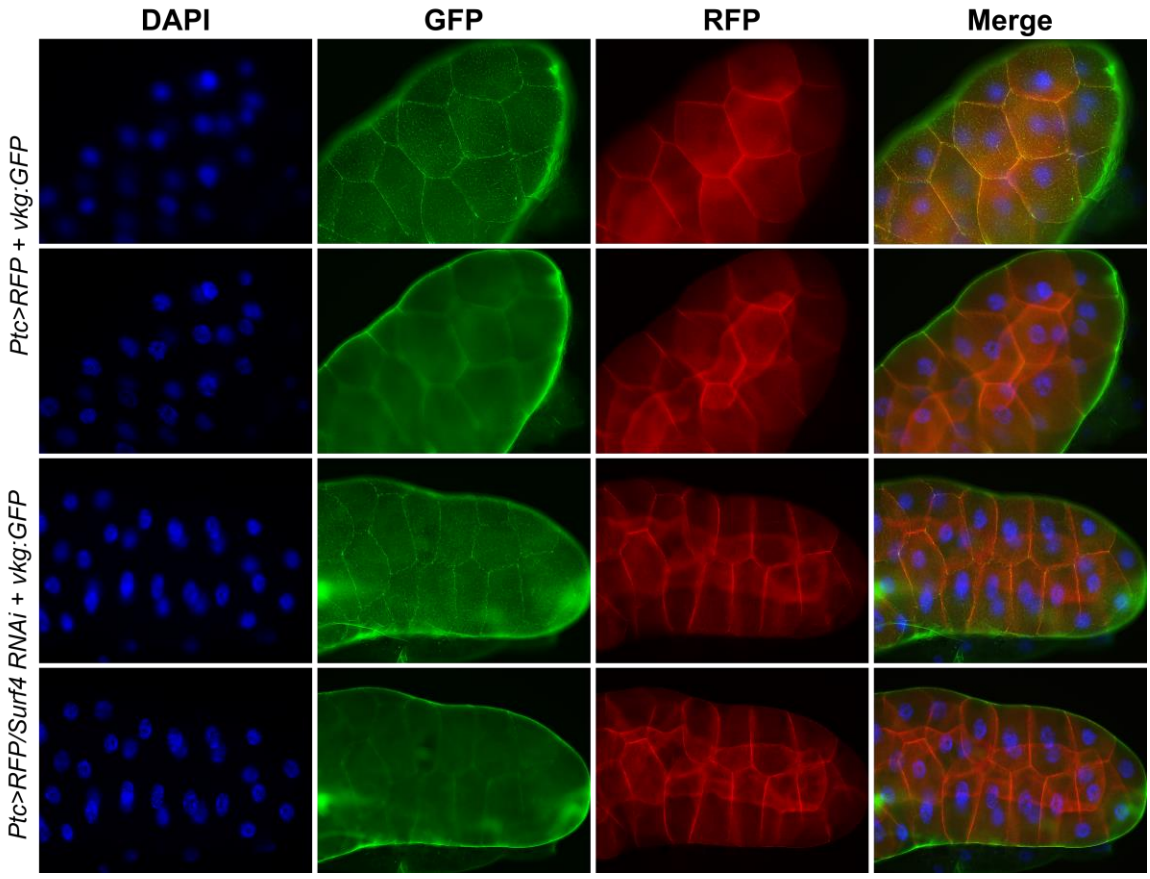

Figure 17: Surf4 knockdown in the salivary gland has no apparent effect on collagen IV distribution.

From top to bottom: (Row 1) Collagen IV distribution in the salivary gland of a control (*Ptc>RFP + vkg:GFP*) specimen. This focal plane shows wild-type collagen IV expression on the outer surface of the salivary gland. (Row 2) The salivary gland from Row 1 viewed at a lower focal plane. Here, several faint, diffuse puncta can be found along the edges of salivary gland cells. (Row 3) Collagen IV distribution in the salivary gland of a Surf4 knockdown (*Ptc>RFP/Surf4 RNAi + vkg:GFP*) specimen. The pattern of collagen IV expression at this focal plane is indistinguishable from that observed in (Row 1). (Row 4) The salivary gland from Row 3 viewed at a lower focal plane. The puncta observed in (Row 2) are present, though slightly out of focus. DAPI = cell nuclei; GFP = Vkg; RFP = Ptc.

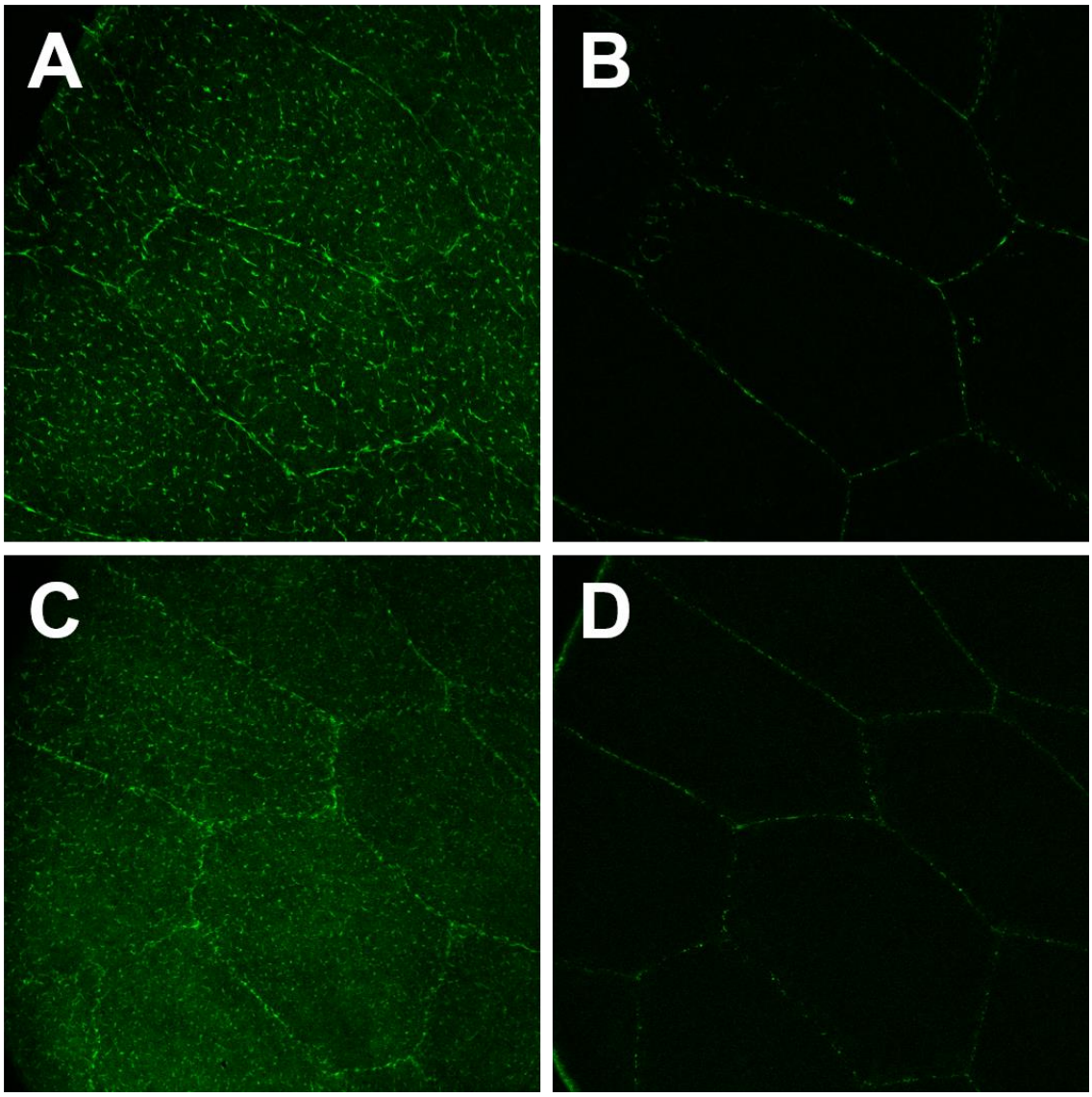

Figure 18: Confocal scans documenting collagen IV distribution in salivary glands in response to Surf4 knockdown.

(A) Collagen IV distribution on the surface of a salivary gland collected from a control specimen (*Ptc>RFP + vkg:GFP*). (B) A confocal scan from the same tissue in (A), approximately 5 μm deeper. Note the numerous green puncta along the borders of each cell. (C) Collagen IV distribution on the surface of a salivary gland collected from a specimen where Surf4 was knocked down (*Ptc>RFP/Surf4 RNAi + vkg:GFP*). (D) A confocal scan from the same tissue in (C), approximately 5 μm deeper. Neither the puncta at this depth, nor the surface, appear to differ in a meaningful way from those observed in the control panels (A & B).

4. GENERAL DISCUSSION & FUTURE DIRECTIONS

To the outside observer, the penetrance of our mutant phenotypes in response to JAK/STAT dysregulation may appear low, even though the proportional difference from our control is highly significant. However, our rates of mutant phenotype penetrance may be due, in part, to the temperature-sensitive nature of the GAL4/UAS system, which can produce a broader range of phenotypic outcomes when flies are cultured at temperatures lower than the optimal 29°C (Duffy 2002). Another contributing factor is the qualitative nature of phenotype delineation in this study. That is, ASPs exhibiting a phenotype of an ambiguous nature (i.e. is it "bud" or "short?"; "short" or "normal?") were recorded as the less severe of the two options. Consequently, all phenotypic proportions are likely to understate the developmental impact of JAK/STAT dysregulation to a certain degree. Regardless of the frequency at which ASP growth was negatively impacted, the character of the impact was relatively consistent independent of how core components were misexpressed, which surprised us.

We initially suspected that the invasive capacity of ASPs would be either enhanced or stymied depending on how JAK/STAT activity was modulated, given its activation along the ASP's leading edge. Instead, we found that both hyperactivation and inactivation of JAK/STAT signaling leads to comparably irregular development at similar, though slightly variable, rates of penetrance. These data suggest an alternative model, wherein JAK/STAT activity serves as a mediator for another signaling cascade, which is certainly not without precedent. For example, JAK/STAT and EGFR signaling have been shown to facilitate intestinal stem cell maintenance and proliferation alongside Wg during *Drosophila* midgut development (Xu *et al.* 2011). STAT5A, the closest homolog of Stat92E after STAT5B

[per http://www.flyrnai.org/diopt (Hu *et al.* 2011)], has also been shown to be an effector of FGF signaling (Yang *et al.* 2009). The effects of JAK/STAT dysregulation might also be mitigated by another pathway instead, as is the case for Notch signaling in intestinal stem cells, which limits JAK/STAT-induced proliferation (Liu *et al.* 2010). Determining the exact relationship between JAK/STAT and these other signaling pathways is challenging because current evidence demonstrates the importance of each of the above signaling networks during ASP development (Sato and Kornberg 2002; Cabernard and Affolter 2005; Cruz *et al.* 2015; Huang and Kornberg 2015).

We propose that a regulatory threshold with upper and lower limits of activity exists for JAK/STAT signaling in the ASP, such that the extracellular signal-regulated kinase (ERK) pathway is activated as a response to both JAK/STAT hyperactivation and inactivation via phosphorylation of ERK's threonine and tyrosine residues. We further suggest that the elevation of diphosphorylated ERK (dpERK) levels in cells where this response is initiated drives a cell fate switching program such that cells from the medial and proximal regions of the ASP adopt a tip cell-like phenotype (Fig. 18). Thus, "short" and "bud" phenotypes represent the manifestation of a cell fate switching continuum, wherein short ASPs may indicate cell fate switching of medial cells alone and bud ASPs indicate a more widespread tip cell fate expansion that includes the proximal cells comprising the stalk. The exact mechanism by which dysregulated JAK/STAT activity initiates an upregulation of dpERK remains a subject for further investigation. We attempted to examine dpERK levels during L3 for both of our controls as well as our two most severe JAK/STAT mutant lines, *Btl>hop* and *Btl>Dcr-2; Stat92E RNAi*, but the data

were inconclusive, presumably due to poor binding efficiency of the primary anti-dpERK antibody (data not shown).

An alternate means to test our model would be through antibody staining directed against Esg, which has been previously used as a marker of tip cell fate in the ASP (Wang *et al.* 2010). Experimental evidence from this study shows that MMP2 knockdown in the tip cells driven by an *Esg-GAL4* driver is still capable of inducing tip cell fate expansion, but concomitantly producing a much subtler difference in ASP morphology that is comparable to the description of our "short" phenotype. This finding makes the link between the expansion of tip cell fate and dysregulation of JAK/STAT activity, which is limited to ASP tip cells, far more plausible in lieu of direct experimental evidence. Western blots and antibody staining directed against dpERK may also be repeated, but perhaps with a different antibody and/or more specialized protocols.

The lethality of Surf4 knockdown driven by *Btl-GAL4* may be a consequence of failure to traffic proteins necessary for tracheal development, as the role of Btl is often investigated within this context. However, Btl is an FGF receptor, and it is also expressed in midline glial cells (Klambt *et al.* 1992) as well as the midgut primordium (Shishido *et al.* 1993). It seems possible that Surf4 may have a role in the trafficking of proteins associated with one of these tissues, specifically, if not the trachea. For example, Surf4 was very recently identified as a diagnostic biomarker predictive of a favorable response to imatinib mesylate treatment of gastrointestinal stromal tumors (Atay *et al.* 2018), suggesting a potential role in a resilient digestive system. Translating this to the observed larval lethality, the Surf4 knockdown may interfere with food processing and/or nutrient absorption in some capacity due to improper development of the midgut primordium. Nevertheless, it is possible that

Surf4 is involved in vesicular traffic of the apical surface protein Uninflatable (Uif)—which is lethal primarily in larvae (Zhang and Ward IV 2009)—or something similar, if the lethality is more related to tracheal development. This could be tested by more closely examining *Btl>Surf4 RNAi* larvae to see if this setup phenocopies *uif* defects (i.e. crushed and twisted tracheal tubules). Regardless, it is also a distinct possibility that the lethality of Surf4 knockdown results from a failure to transport of more generalized cargo as well.

The lethality of *Ptc>Surf4 RNAi* is a little more difficult to parse, but it does not preclude the notion of Surf4's involvement with general cargo transport. Though *En>Surf4 RNAi* induced wing bristle defects of an indeterminate—but likely polarity-related—nature, these individuals were still viable. This is important to note because Engrailed is a transcription factor that functions upstream of Ptc, which is a component of the Hedgehog signaling pathway. Perhaps Surf4 has less to do with Hedgehog signaling as a whole, and more to do with Ptc, specifically, as *Bx>Surf4 RNAi* flies were also viable. Alternatively, the potency of the *Ptc*-, *En*-, and *Bx-GAL4* drivers may vary to a degree that negates direct comparison of developmental outcomes. It would be useful to conduct another exploratory co-immunoprecipitation and mass spectrometry study like the one that identified the association between Surf4 and Vkg in order to identify other Surf4-associated proteins. In doing so, said proteins could be organized by gene ontology (GO) terms to reveal interactions that may potentiate some of the phenotypic outcomes documented in the current study, especially those related to neural and midgut development if those were indeed causal factors in the *Btl*-driven lethality.

The complete impotency of Surf4 knockdown on collagen IV distribution is even more curious. In part, this result can be explained by a very limited sample set, which only

emerged under rather permissive conditions. Out of at least 192 L3s from *Ptc>Surf4 RNAi* lines, only 14 individuals (7.3%) screened were positive for the *Ptc-GAL4* line reporters (RFP and GFP). The lack of a collagen IV phenotype may in fact be the reason that these individuals survived long enough to be dissected in the first place, and perhaps those that died would have a dramatically different phenotype upon reaching L3 were it possible. It would be worth investigating the impact of Surf4 on collagen IV distribution in the fat body as well with another *GAL4* driver specific to that tissue because collagen IV secretion plays a distinct role in shaping the fat body (Pastor-Pareja and Xu 2011). If this also proves lethal, one could perform more viability assays to determine the point of lethality and examine the fat body at this stage, if feasible.

It would also be interesting to investigate whether Surf4 co-localizes with CP1/CTSL and if CP1/CTSL expression is affected by Surf4 knockdown. These are distinct possibilities, especially in light of a very recent study that demonstrated knockdown of the *C. elegans* Surf4 homolog, SFT-4, resulted in accumulation of the CP1 homolog, CPL-1 (Saegusa *et al.* 2018). The Srivastava lab previously generated our own anti-CP1 antibody that could be used in tandem with our *Surf4:GFP* line to investigate the former possibility. One simple way to examine the latter relationship would be to drive Surf4 knockdown with *En-GAL4* as done previously and perform antibody staining with the same CP1-antibody.

Finally, based on lack of effects observed in response to Surf4 overexpression, we think the efficacy of the *UAS-Surf4* construct used in this work should probably be evaluated. To do so, real-time quantitative PCR (qPCR) should be performed on larval tissue samples isolated from progenies expressing both a *GAL4* and *UAS-Surf4* construct and compared to qPCR analysis data generated from both parental lines as controls. If the construct is

actually effective in increasing *Surf4* transcript copies, a logical explanation does exist as to why overexpression would have an effect limited to the ASP. Simply put, overexpression of a cargo receptor without an equivalent or comparable increase in cargo shouldn't have much of an effect other than increasing the efficiency of cargo transport, though not from the standpoint of energy expenditure in producing the cargo receptor proteins. We are unsure how this would still induce a significant effect on ASP development, but it is clear that much remains to be done to begin painting a more comprehensive picture of Surf4's involvement in general. Overall, despite some gaps in experimental evidence, this work lays the groundwork for many additional experiments to come regarding the roles of both JAK/STAT signaling and Surf4 in *Drosophila* development.

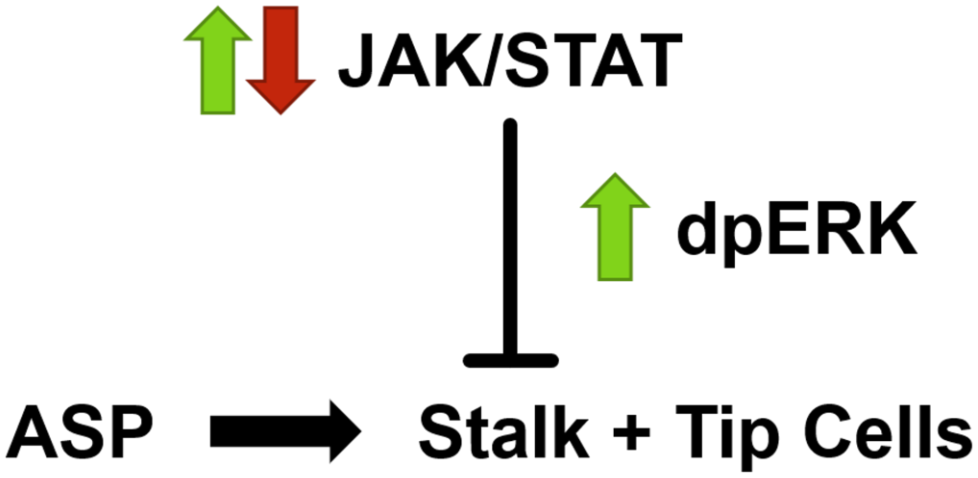

Figure 19: A proposed model for how JAK/STAT dysregulation impacts ASP development

Whenever a core component of the JAK/STAT signaling cascade exceeds an upper or lower threshold for pathway activity, ERK signaling is activated. The consequent increase in ERK's active form, dpERK, promotes tip cell differentiation in cells that normally comprise the ASP's medial and proximal regions, which are generally considered to adopt a "stalk cell" fate.

5. REFERENCES

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6. ABBREVIATIONS

Abbreviation	Definition
Arm	Armadillo
ASP/ASPs	Air sac primordium/air sac primordia
BM	Basement membrane
BMP	Bone morphogenetic protein
Bnl	Branchless
BSA	Bovine serum albumin
Btl	Breathless
Bx	Beadex
Cg25C	Collagen at 25C
CISR	Cell Imaging Shared Resource
CP1	Cysteine Proteinase 1
CTSL	Cathepsin-L
CyO	Curly O
Dcr-2	Dicer-2
Dlp	Dally-like
Dly	Dally
DOCK	Dedicator of cytokinesis
Dof	Downstream of FGF
Dome	Domeless
dpERK	Diphosphorylated ERK
Dpp	Decapentaplegic
dsRNA	Double-stranded RNA
DT	Dorsal trunk
ECM	Extracellular matrix
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
Elav	Embryonic lethal abnormal vision
En	Engrailed
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinase
ESCRT-0	Endosomal Sorting Complex Required for Transport-0
Esg	Escargot
ET	Eye Transformer
Exd	Extradenticle
Ey	Eyeless
FGF	Fibroblast growth factor
FGFR	Fibroblast growth factor receptor
Fzr	Fizzy-related
GMR	Glass Multiple Reporter
HIF	Hypoxia-inducible factor
Hop	Hopscotch
Hox	Homeobox
HSPG	Heparan sulfate proteoglycan
Hth	Homothorax
JAK	Janus kinase
L1/L1s	First larval instar/first instar larvae

L2/L2s	Second larval instar/second instar larvae
L3/L3s	Third larval instar/third instar larvae
Mad	Mothers against Dpp
MAPK	Mitogen-activated protein kinase
Med	Medea
MMP	Matrix metalloproteinase
PAK	p21-activated kinase
PBS	Phosphate-buffered saline
PBTA	PBS + 1% BSA + 0.1% Triton X-100 + 0.01% sodium azide
PDGF	Platelet-derived growth factor
PGCC	Polyploid giant cancer cell
PIPES	Piperazine-N,N′-bis(2-ethanesulfonic acid)
Pnr	Pannier
PntP1	PointedP1
PntP2	PointedP2
Ptc	Patched
qPCR	Quantitative PCR
RFP	Red fluorescent protein
RNAi	RNA interference
RTK	Receptor tyrosine kinase
Ser	Serrate
SERCA	Sarcoendoplasmic reticulum calcium transport ATPase
Shg	Shotgun
Socs36E	Suppressor of cytokine signaling at 36E
Spg	Sponge
STAT	Signal transducer and activator of transcription
Stg/Cdc25	String/Cdc25
Surf4	Surfeit locus protein 4
TC	Transverse connective
Tkv	Thickveins
Tr2	Tracheal metamere 2
TRiP	Transgenic RNAi Project
UAS	Upstream Activation Sequence
Ubx	Ultrabithorax
Upd	Unpaired
Upd2	Unpaired 2
Upd3	Unpaired 3
UP-TORR	Updated Targets of RNAi Reagents
UTR	Untranslated region
VEGF	Vascular endothelial growth factor
Vg	Vestigial
Vkg	Viking
Vn	Vein
Wg	Wingless