THE REGULATION OF GROWTH FACTOR SIGNALING IN *DROSOPHILA* DEVELOPMENT AND DISEASE

A Dissertation

by

JONATHAN RYAN LINDNER

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

December 2010

Major Subject: Biochemistry

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Chair of Committee,	Sumana Datta
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ABSTRACT

The Regulation of Growth Factor Signaling in *Drosophila*

Development and Disease.

(December 2010)

Jonathan Ryan Lindner, B.S., University of Wisconsin

Chair of Advisory Committee: Dr. Sumana Datta

Developmental signaling pathways have many diverse roles throughout the life of an organism. The proper regulation of these pathways is essential for normal development, and misregulation can lead to diseases such as cancer. Heparan sulfate proteoglycans function to modulate growth factor signaling in many biological processes by acting as co-receptors, or by influencing ligand distribution. The heparan sulfate proteoglycan Trol, the *Drosophila* Perlecan homolog, is known to modulate signaling in a population of neuroblasts in the developing *Drosophila* central nervous system. My studies aim to determine the function Trol has in regulating signaling pathways during development. *trol* mutants are examined to determine how various mutant alleles impact signaling in several different developmental contexts. The role growth factor pathways play during induction of a *Drosophila* prostate cancer model is also examined. Gene expression profiles are determined for two types of prostate model overproliferation. Trol is shown to be able to differentially regulate multiple signaling pathways during several developmental processes. The *Drosophila* prostate cancer model is also shown

to have many characteristics similar to those of human prostate cancer, and that signaling and proteoglycan expression are impacted by aberrant overgrowth in the model. My results indicate that Trol is able to specifically modulate different signaling pathways depending on the tissue and developmental context.

DEDICATION

To my friends, family, and to BLF, thank you

ACKNOWLEDGEMENTS

I would like to thank my advisor, Dr. Sumana Datta. Her support and patience through the years is something I appreciate tremendously. Her enthusiasm for science, and life, is an example I have tried to follow every day. She is the type of person I strive to be. I would also like to thank my committee, Dr. Axel Hook, Dr. Arne Lekven, and Dr. Vladislav Panin, for their helpful advice and guidance throughout my graduate career. I would like to thank all the past members of the Datta Lab and all my friends at Texas A&M. Lastly, I want to thank Dr. Brent Ferguson for his help, his friendship, and his ability to keep me sane.

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CHAPTER I

INTRODUCTION

Signaling by growth factors has diverse and essential roles in countless biological processes. Many of the processes affected are conserved across organisms, making the study of their regulation central to a full understanding of how these signaling cues are used in such a wide range of events. During development, growth factors help to regulate the assembly and growth of tissues and organs. Signaling molecules and their downstream pathways play a role in controlling gene expression and function, allowing for the proper assembly and patterning of structures. The same signaling pathways that are critical for development are also important for an organism after maturation. Maintenance of tissues and organs, as well as immunological responses and other biological processes, are coordinated by growth factor signaling. Inadequate regulation of these pathways can lead to problems such as disease or degeneration of an organism's tissues and organs. In fact, misregulation of growth factor signaling has been shown to impact the metastatic potential of different cancers.

The strict regulation of signaling pathways is essential at all times during the life of an organism. Many growth factor signaling pathways are dependent upon the extracellular matrix. Heparan sulfate proteoglycans (HSPGs) are an integral component

This dissertation is written in the style and format of Developmental Biology.

of the extracellular matrix, and many signaling molecules rely on HSPGs for the proper modulation of signaling. Examining how growth factor signaling impacts such a wide range of both positive and negative biological processes will help to better understand ways in which developmental signaling pathways and their regulators can be utilized in treating problems from early development to disease. The conserved, universal nature of these pathways allows for their study in lower organism model systems, such as *Drosophila melanogaster*.

Drosophila Model System

The *Drosophila* system has been used extensively as a model for signal transduction during development. Many components of the signaling pathways that are present in higher organisms exist in *Drosophila* as well. However, the small genome size of *Drosophila* decreases the complexity found in mammalian systems. Due to the long history of research in this model system, a sophisticated set of molecular and genetic tools, as well as numerous mutant lines, have been developed. Furthermore, *Drosophila* has a relatively short life span consisting of distinct morphological stages, making this model system ideal for study of developmental processes. After an egg is fertilized, embryogenesis lasts one day, after which the egg hatches and the first of three larval stages begins. The first instar larval stage lasts one day, followed by a second instar stage for another twenty-four hours, and finally a third instar stage, which lasts two days. The *Drosophila* pupal stage lasts approximately five days, after which an

adult fly emerges. The vast knowledge base of *Drosophila* biology, and specifically growth factor signaling pathway components, transduction, and regulation, make it a valuable model system for the study of signaling in different biological processes.

Drosophila model for development

Drosophila has been widely used as a model for various developmental processes. The presence of a similar set of developmental pathways and molecules to that of other organisms allows for extrapolation of the information gained to other systems. The relatively small genome size of *Drosophila* decreases genetic redundancy in the fly compared to vertebrates. For example, where mammals have three Hedgehog growth factor ligands, Sonic Hedgehog, Indian Hedgehog, and Desert Hedgehog, *Drosophila* contains only one (Ingham and McMahon, 2001; Carpenter et al., 1998). Similarly, mammals contain twenty-two Fibroblast Growth Factor molecules with four different receptors, while *Drosophila* has three Fibroblast Growth Factor molecules and two receptors (Tsang and Dawid, 2004; Gryzik and Muller, 2004; Stathopoulos et al., 2004). The study of these pathways in *Drosophila* has led to the discovery of many roles these growth factors play during development. For example, *Drosophila* Hedgehog (Hh) is important for anterior-posterior patterning, segmental polarity, and central nervous system development (Lum and Beachy, 2004; Tabata and Takei, 2004; Tabata and Kornberg, 1994; Park et al., 2003). Cell migration and tracheal development utilize Fibroblast Growth Factor (Branchless, or Bnl) in the fly (Venkataraman et al., 1999; Powers et al., 2000; Bottcher and Niehrs, 2005; Sutherland et al., 1996). The

Drosophila gene for Wnt, wingless (Wg), is responsible for synapse development in larval stages, as well as patterning as a segment polarity gene (Logan and Nusse, 2004; Wodarz and Nusse, 1998; Bhat, 1996). The Transforming Growth Factor-β like molecule Decapentaplegic (Dpp) has roles in limb patterning and dorsal/ventral polarity during embryogenesis (Tabata et al., 1995; Affolter and Basler, 2007).

All of these signaling molecules have been examined in various developmental systems. In many cases, more than a single pathway is utilized during a developmental event. For example, both Hh and Bnl are important during the reactivation of a particular subset of quiescent neural stem cells in the developing larval brain (Barrett et al., 2008). Understanding the regulation of signaling pathways when multiple pathways are functioning, as well as at any point during a developmental process, is of vital importance.

Drosophila central nervous system development

The *Drosophila* central nervous system utilizes a number of signaling pathways throughout development. Neurogenesis in flies occurs in two distinct phases, an embryonic stage and postembryonic stage. During embryogenesis, the central brain, thoracic, and abdominal neuroblasts are formed when the cells of the ventral and procephalic neuroectoderm are divided into neural equivalence groups (Figure 1.1A). Expression of the segment polarity genes *wingless*, *hedgehog*, and *engrailed*, along with *muscle segment homeobox*, *ventral nervous system defective*, and *intermediate neuroblast defective*, lead to the establishment of the groups containing five to seven

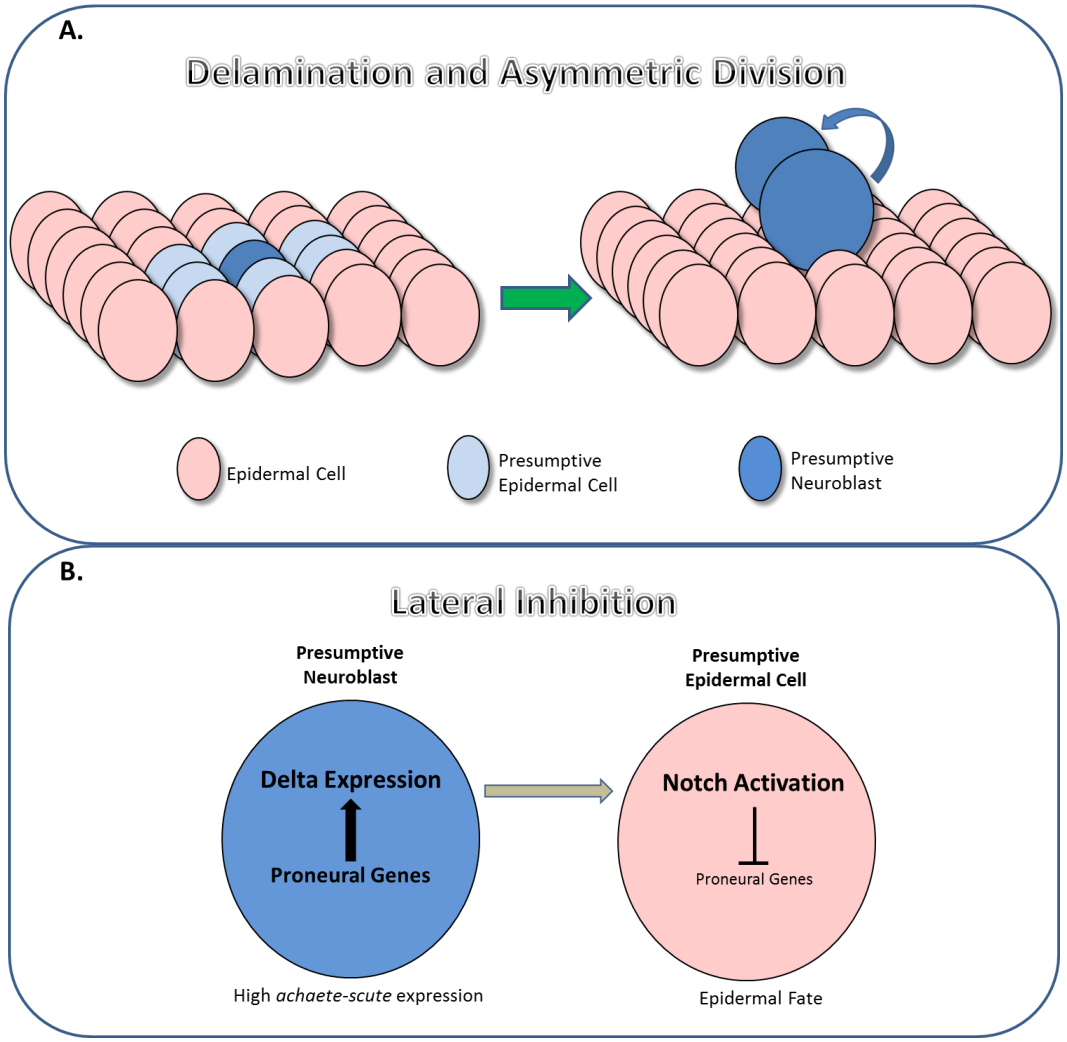

Figure 1.1. Neuroblast specification in the *Drosophila* central nervous system. (A) Neuroblasts arise from neural equivalence groups of five to seven cells established by expression of proneural genes. The presumptive neuroblast delaminates basally, followed by asymmetric division. (B) The cell in the equivalence group with the highest expression of *achaete-scute* becomes the presumptive neuroblast. It expresses Delta, which activates Notch signaling in the adjacent cells, which leads to downregulation of proneural gene expression and an epidermal fate.

neuroectoderm cells (Doe, 1992; Doe, 1996; Urbach et al.,2003; Egger et al., 2007). In the neural equivalence group, all of the cells express the *achaete-scute* complex. The cell that has the highest level of *achaete-scute* expression will be designated to become a neuroblast through lateral inhibition (Egger et al., 2007). Lateral inhibition occurs when the cell fated to become the neural stem cell expresses the transmembrane signaling ligand Delta (Figure 1.1B). This in turn activates the receptor for Delta, Notch, in all of the other cells in the neural equivalence group (Artavanis-Tsakonas and Simpson, 1991). After the Notch pathway is activated in the adjacent cells, proneural gene expression is downregulated. The decrease in proneural gene expression leads these adjacent cells to adopt an epidermal fate. This allows the original, Delta-expressing cell to be the sole neuroblast that forms from that neural equivalence group. The cell fated to become a neuroblast then becomes larger and delaminates. The new neuroblast begins to divide asymmetrically, giving rise to two daughter cells. This asymmetric division results in a new neuroblast and a ganglion mother cell. The ganglion mother cell then divides symmetrically to give rise to two progenitor cells (Figure 1.2). These progenitor cells will differentiate into glial cells or neurons (Hartenstein et al., 1987; Prokop and Technau, 1991; Doe, 1996).

Optic lobe neural stem cells also begin formation during embryogenesis. These neuroblasts are formed from an optic placode dorsolateral to the embryonic brain. After embryonic stage eleven, the optic placode invaginates and fuses to the brain (Green et al., 1993; Hartenstein, 1993; Ebens et al., 1993). After fusion, the optic lobe cells divide symmetrically, in contrast to the other embryonic neuroblasts that divide

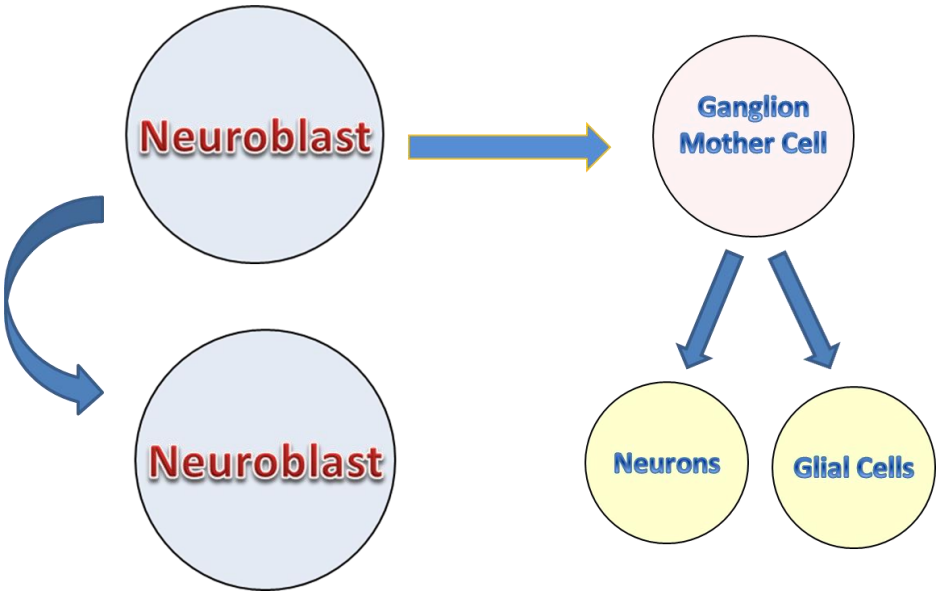

Figure 1.2. Asymmetric neural stem cell division in *Drosophila.* Neuroblasts can divide asymmetrically to give rise to two daughter cells: another neuroblast and a ganglion mother cell. The ganglion mother cell then divides symmetrically to produce two progenitor cells (neurons or glial cells).

asymmetrically. These neuroepithelial optic lobe progenitor cells generate neuroblasts that will divide asymmetrically during the second instar larval stage (Egger et al., 2007). The optic lobe neuroblasts separate into the inner proliferative center and the outer proliferative center, which will eventually give rise to adult optic structures (Hofbauer and Campos-Ortega, 1990; Ebens et al., 1993). At the end of embryogenesis, all of the neural stem cell groups stop dividing and become quiescent, with the exception of the mushroom body and ventral lateral populations (Truman and Bate, 1988; White and Kankel, 1978; Prokop and Technau, 1991).

After embryogenesis, the post-embryonic stage of *Drosophila* CNS development occurs in multiple stages. Most of the cells for the adult central nervous system are generated during this post-embryonic developmental period (Truman and Bate, 1988; Maurange and Gould, 2005). Reactivation of quiescent neuroblasts occurs in a strict spatial and temporal pattern (Figure 1.3). The neuroblasts of the central brain and optic lobe resume division during the late first instar stage (Ito and Hotta, 1991). Two to six hours after the start of the second instar phase, the thoracic neuroblast population exits quiescence and resumes asymmetric division (Truman and Bate, 1988; Datta, 1995).

Neural stem cell regulation

The regulation of neural stem cells in the *Drosophila* larval central nervous system is a complex process. Multiple different populations of quiescent neuroblasts respond to various signals that direct the stem cells to resume proliferation in a very

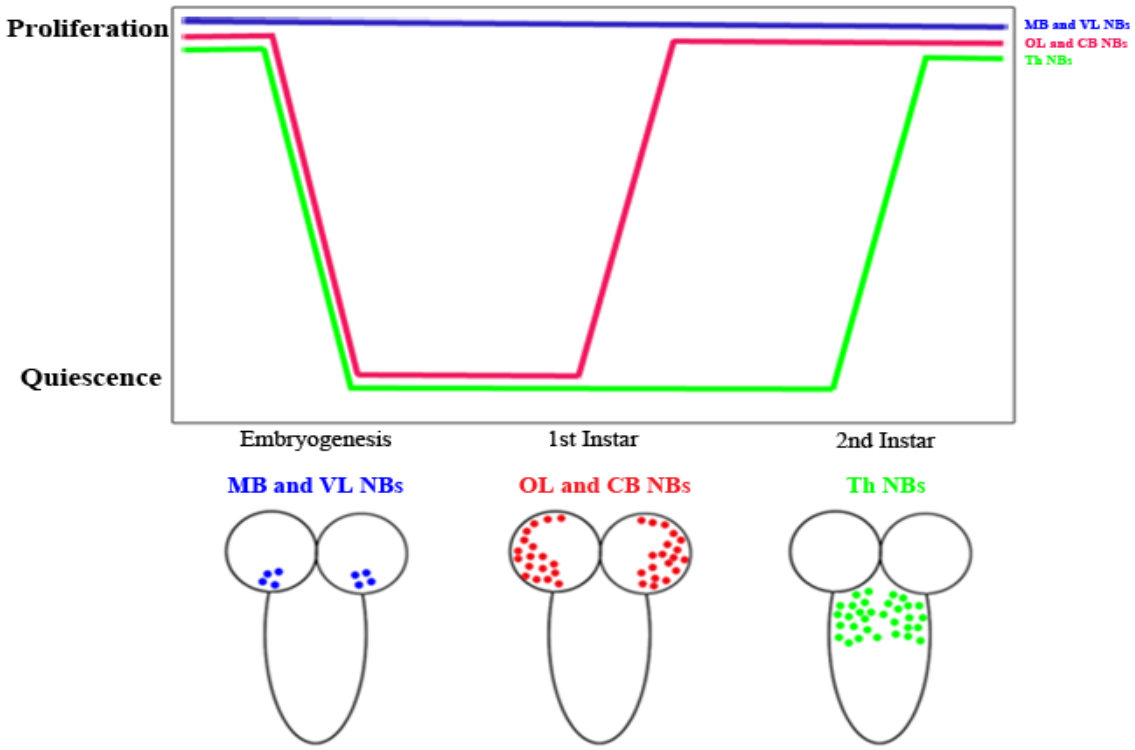

Figure 1.3. Reactivation of quiescent neural stem cells in the *Drosophila* central nervous system. Neuroblasts (NBs) in the *Drosophila* larval brain resume proliferation in a distinct spatial and temporal pattern. The mushroom body (MB) and ventral lateral (VL) neuroblasts continue to proliferate throughout embryogenesis and larval development. After entering quiescence at the end of embryogenesis, the optic lobe (OL) and central brain (CB) neuroblasts resume proliferation during mid-1st instar. The thoracic neuroblasts exit quiescence during early 2nd instar.

distinct pattern. Several genes have been identified that influence the reactivation of these cell populations during the post-embryonic phase of neural development.

terribly reduced optic lobes, or *trol*, was first identified in a screen for mutations that affected *Drosophila* larval brain morphology (Datta and Kankel, 1992). *trol* encodes the *Drosophila* Perlecan, a heparan sulfate proteoglycan (Park et al., 2003; Voigt et al., 2002). Loss-of-function mutations in *trol* have been shown to disrupt the ability of neural stem cells to exit quiescence and resume proliferation (Datta, 1995). Overexpression of cyclin E, which is required for the G1-S transition, is able to rescue the *trol* loss-of-function mutation, which suggests that quiescent neuroblasts are G1 arrested. Ectopic expression of *Drosophila* cdc25, or *string*, is also able to rescue proliferation in the *trol* mutant phenotype (Caldwell and Datta, 1998; Park et al., 2003). Taken together, this data strongly suggests that quiescent neuroblasts are arrested in the G1 phase of the cell cycle.

anachronism encodes a glycoprotein that is secreted by glial cells in the *Drosophila* larval CNS. Loss-of-function mutants for this gene increase the number of proliferating stem cells, which suggests that *anachronism* is important in the maintenance of neuroblast quiescence (Ebens et al., 1993). The epistatic relationship between these two genes can be determined using a double loss-of-function mutant. The double mutant shows that *anachronism* is epistatic to *trol*, which suggests that *trol* functions to inhibit the repressive effect of *anachronism* on cell cycle progression (Datta, 1995).

hedgehog and *branchless* (*Drosophila* Sonic hedgehog and FGF-2, respectively) are both crucial during *Drosophila* CNS development. Expression of these genes is able to partially rescue a *trol* mutant phenotype in the optic lobe and central brain populations of neuroblasts during the first instar larval stage (Park et al., 2003). These growth factor ligands have been shown to act together in a positive feedback loop to help regulate the onset of neuroblast division in a particular subset of cells in the *Drosophila* central nervous system (Barrett et al., 2008). Furthermore, co-immunoprecipitation analysis identified physical interactions between both the Branchless ligand and Trol, and the Hedgehog ligand and Trol (Park et al., 2003). This implicates these growth factors, with their function modulated by the heparan sulfate proteoglycan Trol, as necessary components of the neuroblast regulatory machinery during first instar CNS development. What is less clear is whether Hedgehog and Branchless operate in a similar fashion in other spatially and temporally distinct populations of neuroblasts, or whether other growth factor signaling pathways known to be modulated by Trol also play a role in the reactivation of quiescent neuroblasts.

The distinct spatial and temporal patterns in which quiescent neuroblasts resume division in the developing *Drosophila* central nervous system can be used to address a number of questions. Do the spatially and temporally distinct populations of neuroblasts respond to the same signaling cues when resuming proliferation? How are these signaling cues regulated differently for the different stages of central nervous system development? And is this regulation similar to the regulation of signaling pathways in

other areas of *Drosophila* development? These questions will be addressed in Chapter II of this dissertation

Plasmatocyte and larval disc development

Another system in which to study growth factor signaling during *Drosophila* development is hemocyte production. Prohemocytes are formed in the lymph gland of third instar larvae (Evans et al., 2003). After maturation, hemocytes are released into the hemolymph, and circulate as three different cell types. Cell cycle marker staining reveals that these circulating hemocytes continue to proliferate at a slow rate (Qiu et al., 1998; Asha et al., 2003). The hemocytes continue to respond to growth factor signaling cues, as activation of the Ras-MAPK pathway through expression of a constitutively active form of Ras leads to a 40-fold increase in cell numbers (Asha et al, 2003). Vascular Endothelial Growth Factor (VEGF) and Platelet Derived Growth Factor (PDGF) are two signaling pathways that work through the Ras-MAPK pathway, and decreasing the expression of their receptor in plasmatocytes leads to increased hemocyte cell death (Evans et al., 2003). Furthermore, Perlecan has been shown to modulate signaling by mammalian homologs of both growth factors (Iozzo, 2005). Therefore, hemocyte production and maintenance in *Drosophila* is another intriguing system in which to study growth factor signaling and its regulation during development.

Drosophila larval disc development has been a very successful system in the study of growth factor signaling. Many of the developmental signaling pathways have been widely studied in this system, and numerous insights have been gained on how

these pathways function and interact during development. *Drosophila* imaginal discs are specified by the fifth hour of embryogenesis and can be recognized later as small sacs of epithelial cells that have invaginated from the epidermis (Cohen et al., 1993; Couso et al., 1993; Li et al., 1995). Imaginal disc cells continue to proliferate during larval development to form a folded epithelial sheet that eventually differentiates into various adult structures (Li et al., 1995). The expression of the growth factor *wingless*, which is found only in the ventral region of early leg and wing discs, is required for cells to adopt ventral fates (Baker, 1988; Couso et al., 1993; Williams et al., 1993; Li et al., 1995). In the posterior region of thoracic discs, the signaling molecule gene *hedgehog* is transcribed, and influences the development and patterning of both the anterior and posterior compartments by inducing other growth factor molecules at the anteroposterior boundary (Mohler, 1988; Basler and Struhl, 1994; Li et al., 1995; Lee et al., 1992; Tabata et al., 1992). In the developing *Drosophila* eye disc, several other signaling pathways have also been shown to be active (Kaphingst and Kunis, 1994; Silver and Rebay, 2005). These examples demonstrate the importance of *Drosophila* larval disc development to the understanding of growth factor signaling during developmental processes.

In this section, I have described some of the benefits the *Drosophila* model system gives researchers studying development. I have reviewed *Drosophila* central nervous system development and regulation, as well as several other developmental processes that are useful in the study of growth factor signaling and the modulation of these important pathways. I am interested in gaining a better understanding of how these

same signaling pathways result in such diverse responses during different biological processes, and specifically if the regulation of these pathways plays a role in the different responses. To fully understand these complex regulatory processes, it is important to have an excellent grounding in the mechanisms and components of the signaling pathways themselves.

Signaling Pathways in Development

Growth factor signaling pathways have countless diverse and essential roles throughout the lifetime of an organism. Signaling induced by the Hedgehog, Fibroblast Growth Factor, Wnt, and Bone Morphogenetic Protein groups of ligands affect processes during development as well as later in adult life. These pathways are highly conserved across all species, and knowledge gained from their study in invertebrates can and has been applied to humans (Echelard et al., 1993; Kumar et al., 1996). Signaling by these growth factors has been extensively studied, and much is known about the ligands and other pathway components. However, discovery of new pathway functions, interactions, and components demonstrate the need for further research into how these complex processes work.

Hedgehog pathway

The *hedgehog (hh)* gene was first discovered in *Drosophila* during a genetic screen for defects in segmental patterning (Nusslein-Volhard and Wiesehaus, 1980). The ligand acts as a morphogen, and is capable of both short and long range signaling

(Roelink et al., 1995). Among many other developmental processes, signaling from the Hedgehog pathway influences anterior-posterior patterning, segmental polarity, neural tube development, limb patterning, and reactivation of quiescent neuroblasts (Lum and Beachy, 2004; Park et al., 2003). However, the effects of Hedgehog signaling reach further than early development. Hh signaling has been shown to be involved in tissue homeostasis, and misregulation of the Hedgehog pathway has been implicated in various diseases, including breast cancer, small-cell lung cancer, pancreatic cancer, basal cell carcinomas, and prostate cancer (Watkins et al., 2003; Thayer et al., 2003, Shaw and Bushman, 2007; Roessler et al., 2003; Kubo et al., 2004; Lau et al., 2006). With such wide ranging and potentially deadly roles during development and disease, understanding how this pathway operates, including how it is regulated, is critical.

The Hedgehog pathway is highly conserved in vertebrates; although gene families of the ligand, receptor, and transcription factor exist that have partial redundancy. In *Drosophila*, the Hedgehog ligand is an approximately 19 kDa molecule that is produced from the autoproteolytic cleavage of a 46 kDa precursor protein (Lee et al., 1994; Porter et al., 1995). During the autoproteolytic cleavage event, a cholesterol moiety required for proper secretion of the protein is added, after which the 19 kDa ligand is further modified by the addition of a palmitate fatty acid group in the Golgi complex (Porter et al., 1996; Ingham and McMahon, 2001; Ho and Scott, 2002; Micchelli et al., 2002; Miura and Treisman, 2006). Hedgehog is then recognized by its cholesterol moiety and secreted from the cell by Dispatched, a transmembrane protein (Burke et al., 1999). Hedgehog acts as a morphogen, and participates in both short and

long range signaling. The posttranslational modifications help to determine the signal range through multimerization, membrane tethering, and interactions with extracellular proteins (Zeng et al., 2001; Gallet et al., 2003; Lum and Beachy, 2004).

The Hedgehog signal is received by its receptor Patched, a 12-pass transmembrane protein with a sterol sensing domain (Taipale et al., 2002). When the ligand is not present, Patched inhibits the 7-pass transmembrane protein Smoothened (Figure 1.4). This inhibition promotes the formation of complex-1, consisting of the kinases Fused, Protein Kinase A, Glycogen Synthase Kinase-3, Casein Kinase-1, the scaffolding protein Costal 2, and the transcription factor Cubitus Interruptus (Hooper and Scott, 2005). When in complex 1, Cubitus Interruptus is phosphorylated and cleaved into an N-terminal fragment, which translocates into the nucleus and acts as a target gene repressor (Osterlund and Kogerman, 2006, Hooper and Scott, 2005). Suppressor of Fused sequesters the remaining unprocessed Cubitus Interruptus in the cytoplasm (Lum and Beachy, 2004). In the presence of the Hh ligand, Hedgehog binds to the receptor Patched, which then releases its inhibitory influence on Smoothened. The resulting phosphorylation of Smoothened leads to the dissociation of complex 1, which allows unprocessed Cubitus Interruptus to enter the nucleus and act as a transcriptional activator of Hedgehog target genes such as *patched* (Osterlund and Kogerman, 2006). Other components also may play a role in signaling by the growth factor Hedgehog. The transmembrane protein interference Hedgehog (Ihog), a homolog of mammalian CDO, has been shown to interact with the Hedgehog ligand and help modulate its signal (Yao et al., 2006). Genetic interaction studies have shown the extracellular matrix protein

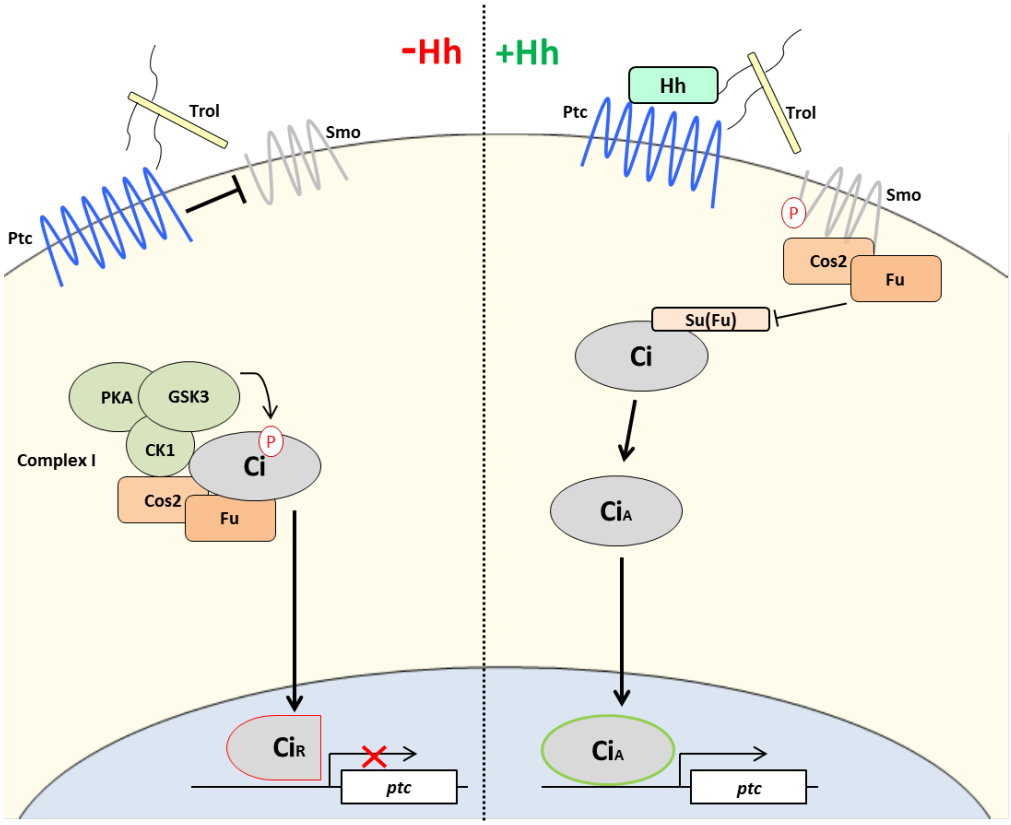

Figure 1.4. The Hedgehog signaling pathway. In the absence of the Hedgehog (Hh) ligand, the receptor Patched (Ptc) inhibits the transmembrane protein Smoothened (Smo), allowing for the formation of Complex I. Complex I can then bind the transcription factor Cubitus Interruptus (Ci), which then is phosphorylated and cleaved into a transcriptional repressor (Ci_R). When Hh is present, it binds to its receptor, releasing the inhibitory effect of Ptc on Smo. Smo is then phosphorylated and can interact with Fused (Fu), preventing formation of Complex I and allowing Ci to act as a transcriptional activator (Ci_A).

Trol (the *Drosophila* Perlecan) to be required for full strength Hedgehog signaling (Park et al., 2003).

Branchless pathway

Basic Fibroblast Growth Factor (FGF-2) was identified in 1973 as a mitogen that could stimulate mouse fibroblast growth (Armelin, 1973; Gospodarowicz, 1974). It quickly became apparent that many different polypeptide growth factors fit into the FGF family, and the role of these growth factors far exceeded inducing fibroblast growth. During development, FGFs influence tracheal development, cell migration, mesoderm induction, and reactivation of mitotically arrested neuroblasts, among many other processes (Sutherland et al., 1996; Venkataraman et al., 1996; Park et al., 2003). FGFs also function after development by impacting homeostasis, angiogenesis, and tumor formation (Venkataraman et al., 1999; Powers et al., 2000; Bottcher and Niehrs, 2005).

The Fibroblast Growth Factor pathway is highly conserved, and many of its components have been identified in a wide range of organisms. Since the first FGF was identified, twenty-two FGFs have been found in vertebrates, while three have been found in *Drosophila*. While all of the mammalian FGFs are smaller than 35 kDa, the FGFs in *Drosophila* are significantly larger at approximately 80 kDa (Ornitz, 2000; Ornitz and Itoh, 2001; Groth and Lardelli, 2002; Stathopoulos et al., 2004 Gryzik and Muller, 2004). FGF proteins contain four domains including a signal peptide, an amino terminus, a conserved core region of 120 amino acids, and a carboxy terminus, of which

the core region is responsible for binding to receptors (Kan et al., 1993; Venkataraman et al., 1999; Thisse and Thisse, 2005, Ornitz, 2000).

The *Drosophila* Basic Fibroblast Growth Factor (FGF-2) is represented by Branchless. Branchless binds to one of two receptors in the fruit fly, Breathless or Heartless (Klambt et al., 1992). FGF receptors have three general domains; an extracellular ligand binding domain itself consisting of three Ig domains and a heparan binding domain, a transmembrane domain, and a cytoplasmic tyrosine kinase domain (Klambt et al, 1992; Bottcher and Niehrs, 2005). Activation of the receptor by an FGF ligand can induce signaling through multiple different pathways including the phospholipase C gamma pathway, the phosphatidylinositol-3 kinase pathway, and the pathway I will focus on, the Ras-Mitogen Activated Protein Kinase (Ras-MAPK) pathway (Sutherland et al., 2006; Powers et al., 2000; Tsang and Dawid, 2004; Bottcher and Niehrs, 2005). When activated by Branchless, the receptors undergo dimerization, which leads to the activation of their intracellular kinase domains by trans-phosphorylation of specific tyrosine sites (Powers et al, 2000). The activation of the receptors results in the phosphorylation of FGF receptor substrate-2, which in turn recruits the adapter molecule Growth factor receptor bound protein-2 (Figure 1.5). A complex is then formed with Son of sevenless, which can initiate the RAS-RAF-MEK-MAPK kinase cascade through GTP exchange with Ras (Tsang and Dawid, 2004; Groth and Lardelli, 2002; Powers et al., 2000). Various transcription factors are then phosphorylated and activated, allowing for transcription of target genes such as *pointed* and *sprouty*. Working through a negative feedback loop, *sprouty* is also an inhibitor of

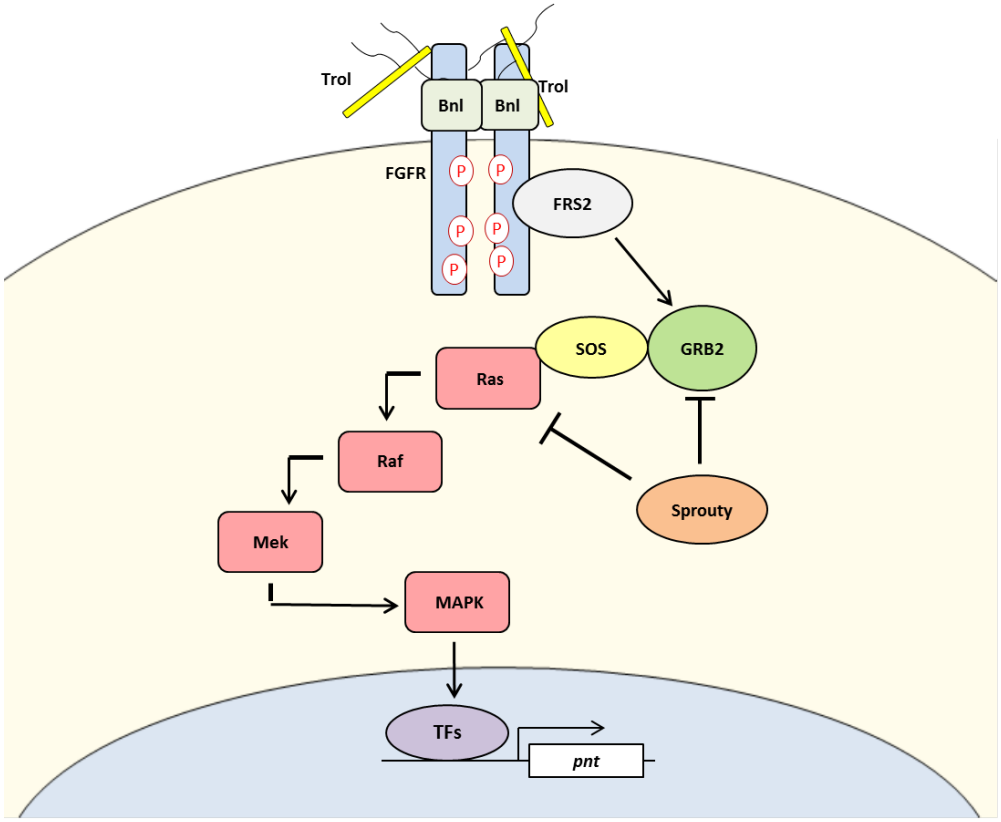

Figure 1.5. The Branchless signaling pathway. The Fibroblast Growth Factor signaling pathway is activated when the ligand Branchless (Bnl) binds and causes dimerization of its receptors (FGFR, Btl or Htl in *Drosophila*). Trans-phosphorylation of the receptors leads to phosphorylation of FGF receptor substrate-2 (FRS2), which in turn recruits the adapter molecule Growth factor receptor bound protein-2 (GRB2). GRB2 then interacts with Son of sevenless, leading to the RAS-RAF-MEK-MAPK kinase cascade. This results in the activation of various transcription factors (TFs), which leads to target gene transcription.

Branchless signaling (Huang and Stern, 2005, Guy et al, 2003). FGF signaling also relies on extracellular matrix proteins such as heparan sulfate proteoglycans, which interact with both ligands and receptors and are required for receptor activation (Yayon et al., 1991; Rapraeger et al., 1991; Lin et al., 1999; Kan et al., 1993; Ornitz, 2000; Volk et al., 1999).

Wingless pathway

wingless was first identified as a mutation that affected wing and haltere development in *Drosophila* (Sharma and Chopra, 1976). Belonging to the Wnt family of secreted signaling molecules, the segment polarity gene *wingless* and its signaling pathway is highly conserved among organisms. In flies, Wingless is required for many processes during embryogenesis, including the proper patterning of the epidermis, limb specification and patterning, and neural tube patterning (Bejsovec and Martinez Arias, 1991; Bejsovec and Wieschaus. 1995; Nusslein-Volhard and Wieschaus, 1980; Wodarz and Nusse, 1998; Bhat, 1996; Patel et al., 1989; Fanto and McNeill, 2004). Wnt signaling is also important beyond development, and misregulation of the signaling pathway has been seen in several human degenerative diseases and cancers (Spink et al., 2000; Logan and Nusse, 2004).

Many of the canonical Wingless/Wnt pathway components were identified by mutations leading to altered cuticle patterning (Noordermeer et al., 1994; Siegfried et al., 1994; Cadigan and Nusse 1997). In the absence of the Wingless ligand, the 7-pass transmembrane G-protein coupled receptors of the Frizzled family are able to inactivate

Dishevelled, a phosphoprotein that helps propagate the signal (Penton et al., 2002; Wang and Malbon, 2004; Wallingford and Habas, 2005). This allows for the formation of the destruction complex, which consists of the scaffold protein axin, the tumor suppressor gene product APC, Glycogen synthase kinase 3 (GSK-3), and the signal transducing protein β-catenin, also known as Armadillo in *Drosophila* (Figure 1.6). When associated with the destruction complex, β-catenin is phosphorylated by casein kinase Iα, which in turn allows GSK-3 to phosphorylate β-catenin on three different serine/threonine residues. This leads to β-catenin being targeted for ubiquitylation and proteolytic degradation. When the Wnt ligand interacts with Frizzled, the receptor is able to bind the transmembrane protein LRP, a low-density lipoprotein receptor-related protein essential for propagation of canonical Wnt signaling. The destruction complex is then inhibited, and β-catenin is no longer degraded. It is then able to translocate to the nucleus where it displaces the transcriptional repressor Groucho from the Tcf/Lef effector complex and induces expression of target genes such as *sloppy paired* in *Drosophila* (Wehrli et al., 2000; Katanaev et al., 2005; Liu et al., 2005a; Liu et al., 2005b; Wallingford and Habas, 2005).

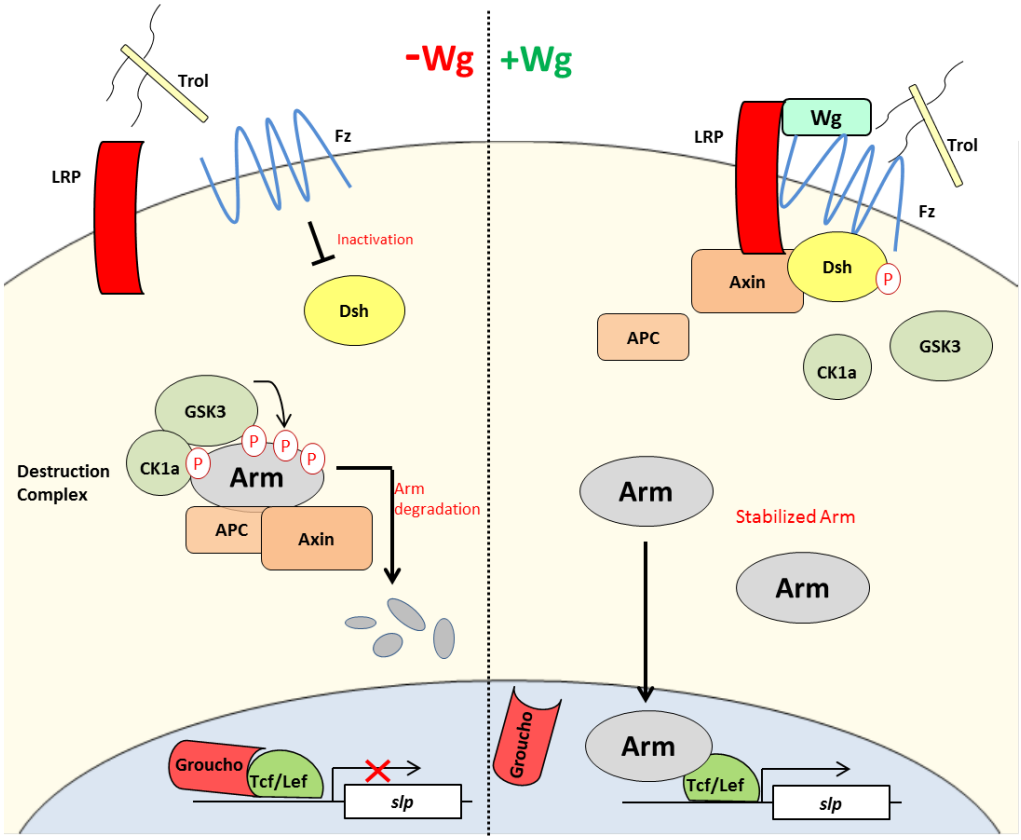

Figure 1.6. The Wingless signaling pathway. In the absence of the ligand Wingless (Wg, *Drosophila* homolog of Wnt), the receptor Frizzled (Fz) is able to inhibit Disheveled (Dsh), leading to the formation of the destruction complex and eventual degradation of Armadillo (Arm, fly homolog of β-catenin). The binding of Wg to Fz results in association with the low-density lipoprotein receptor-related protein LRP. This inhibits the formation of the destruction complex, allowing Arm to be stabilized and translocate into the nucleus where it displaces the transcriptional repressor Groucho from the Tcf/Lef effector complex and promotes transcription.

Decapentaplegic pathway

Several classes of ligands are classified into the Transforming growth factor superfamily of morphogens, including TGF-βs and Bone morphogenetic proteins (BMPs), of which the *Drosophila* signaling molecule Decapentaplegic is one. Like the other growth factor signaling molecules, Decapentaplegic (Dpp) in flies and the TGF-β superfamily of ligands in general are essential for proper development of an organism. During *Drosophila* development, Dpp plays a role in dorsal ventral polarity, establishment of segmental boundaries, and limb development (Nellen et al., 1994; Sekelsky et al., 1995; Tabata et al., 1995; Nellen et al., 1996; Affolter and Basler, 2007). TGF-β like ligands have also been implicated in diseases later in life, including cancer (Zhu and Kyprianou, 2005; Massagué et al., 2000; Padua and Massagué, 2009). Dpp signaling has also been shown to interact with other growth factor signaling pathways. For example, *dpp* cooperates with *hedgehog* and *wingless* during appendage development in *Drosophila*, (Campbell and Tomlinson, 1999; Zecca et al., 1995; Wolpert et al., 1998).

In general, all members of the TGF-β superfamily of signaling molecules are cytokines that contain six conserved cysteine residues (Sun and Davies, 1995). After a TGF-β ligand initiates signaling by binding to and bringing together type I and type II receptor serine/threonine kinases on the cell surface, the type II receptor is able to phosphorylate the receptor I kinase domain (Figure 1.7), which then propagates the signal through phosphorylation of various Smad proteins (Kretzschmar et al., 1997; Shi and Massagué, 2004). There are eight Smad proteins which can be categorized into three

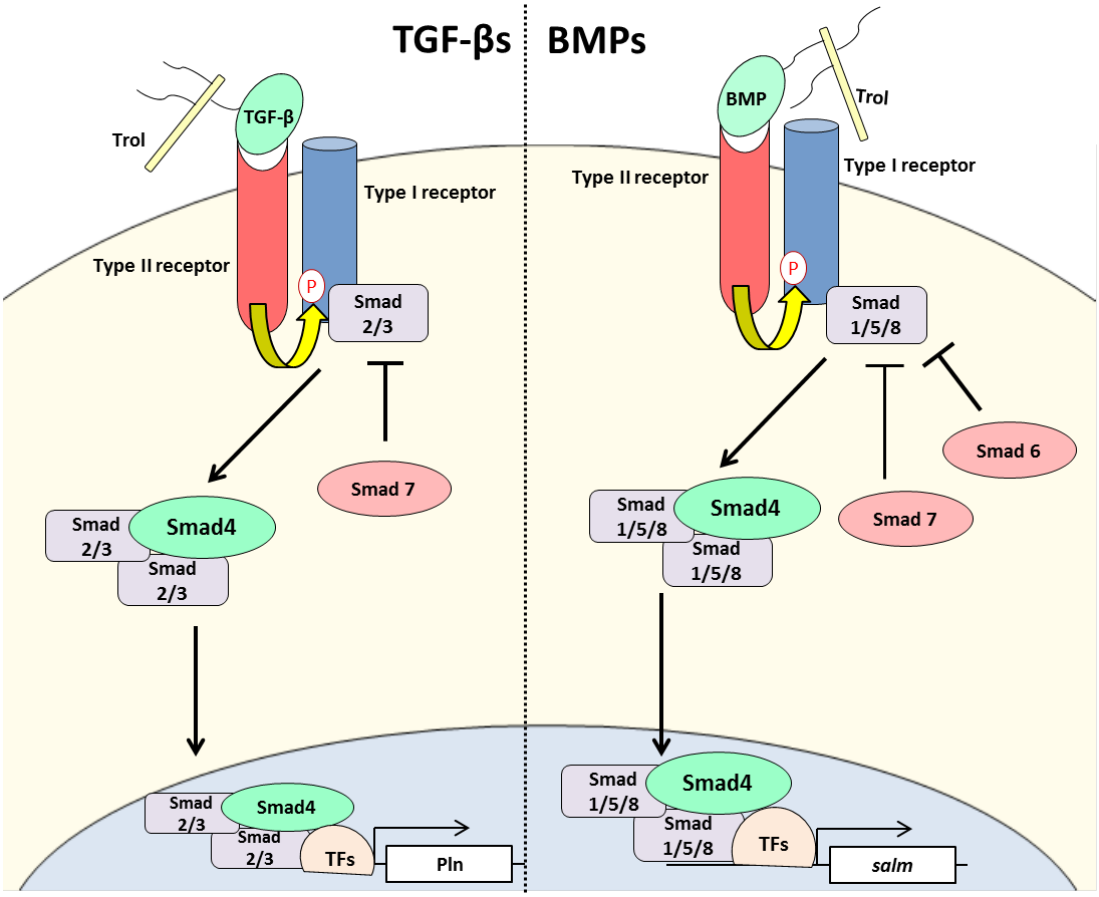

Figure 1.7. The TGF-β signaling pathway. Generalized representation of Smad signaling by TGF-βs (left) and BMPs (right). Signaling ligands bind to Type II receptors, which cause dimerization with and phosphorylation of Type I receptors. The Type I receptors can then phosphorylate receptor-regulated Smads (Smads 2/3 for TGF-β, Smads 1/5-8 for BMP), which allows them to form complexes with the Co-mediator Smad, Smad 4. The complex can then regulate transcription of target genes in conjunction with various transcription factors (TFs). Inhibitory Smads (Smads 6/7) are able to negatively regulate signaling by targeting receptors for degradation or through competitive inhibition.

different classes. Receptor-regulated Smads (Smad1, Smad2, Smad3, Smad5, and Smad8) are phosphorylated and activated by the type I receptor kinases and form complexes with the Co-mediator Smad, Smad4 (Wu et al., 2000; Souchelnytskyi et al., 2001). Various cellular responses are elicited by the different classes of ligands due to the types of Receptor-regulated Smads that are utilized. Whereas Smad2 and Smad3 respond to signaling by the TGF-β subfamily of ligands, Smad1, Smad5, and Smad8 are used primarily by the BMP growth factor molecules (Kirsch et al., 2000). The third class is the inhibitory Smads (Smad6 and Smad 7), which negatively regulate TGF-β signaling by targeting the receptors for degradation or by competing with Receptor-regulated Smads for interaction with the receptor or Co-Smad (Shi and Massagué, 2003). The activated Smad complexes are translocated into the nucleus and, in conjunction with other nuclear cofactors, regulate the transcription of target genes such as *spalt major* in *Drosophila* (Kretzschmar et al., 1999; Shi and Massagué, 2003). Further modulation of Dpp signaling is achieved through HSPGs, which have been shown to regulate morphogen diffusion (Belenkaya et al., 2004). TGF-β signaling is involved in many different developmental processes, and the dichotomous impact the pathway has during diseases such as cancer is still being clarified.

In this section I have reviewed several highly conserved growth factor signaling pathways that are crucial during development. These same pathways play many roles during the adult lives of the organisms they helped to form. Misregulation of these signaling pathways has been implicated in many problems beyond development, including many carcinomas. Understanding the mechanisms by which growth factor

signaling is regulated in both developmental processes and disease will help to prevent some of the complications associated with improper signaling.

Heparan Sulfate Proteoglycans

Signaling by growth factors can be affected through interactions in the extracellular matrix (ECM). The ECM is responsible for providing contextual information to cells and maintaining structure, but also takes an active role in the propagation of growth factor signals (Bissell and Radisky, 2001). The ECM consists of various macromolecules, including integrins, collagen, and heparan sulfate proteoglycans (HSPGs). HSPGs have been shown to play a role in the movement of growth factor ligands through the ECM, as well as in ligand/receptor interactions (Rapraeger et al., 1991; Gallet et al., 2003). Thus, heparan sulfate proteoglycans are good candidates for studying the regulation of signaling pathways during development and disease.

Types of heparan sulfate proteoglycans

Heparan sulfate proteoglycans are basal membrane proteins consisting of a protein core to which heparan sulfate glycosaminoglycans are covalently attached (Nybakken and Perrimon, 2002). In *Drosophila,* three types of HSPGs are present on the cell surface and in the extracellular matrix (Lin and Perrimon, 2000). Glypicans

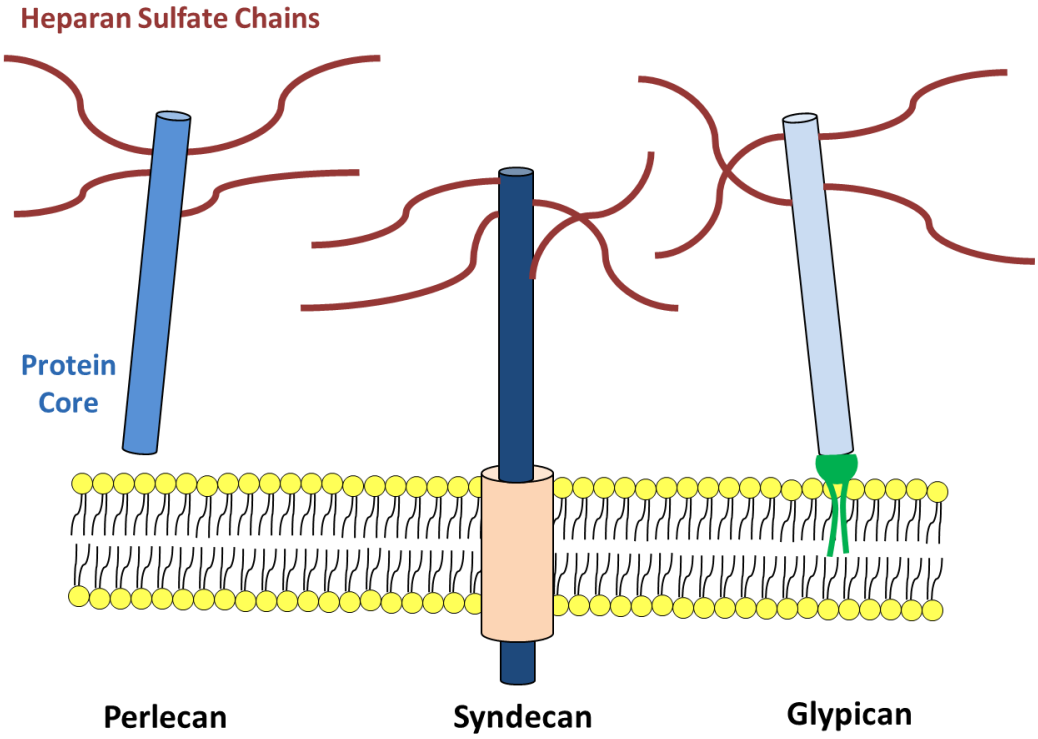

Figure 1.8. The major categories of heparan sulfate proteoglycans. There are three general classes of HSPGs. Perlecan can be secreted into the extracellular matrix or remain associated with the cell surface. The Syndecan protein core spans the cell membrane. Glypicans are attached to the cell membrane by a glycosylphosphatidylinositol anchor.

(*dally* and *dally-like protein* in *Drosophila*) are bound to the cell membrane by a GPI link (Figure 1.8). The Syndecan protein core spans the cell membrane. Perlecans (*trol* in *Drosophila*) are secreted HSPGs that are part of the extracellular matrix (Perrimon and Bernfied, 2000, Lin and Perrimon, 2000). Heparan sulfate chains are formed from long, unbranched chains of modified glycosaminoglycan (GAG) sugar residues (Nybakken and Perrimon, 2002). The GAG chains are attached at serine residues on the protein core. Following addition of a Xylose residue, two galactose residues are added to the forming GAG chain (Prydz and Dalen, 2000). The tetrasaccharide linker region of the GAG chain is completed following addition of a glucuronic acid. Polymerization of the heparan sulfate chain continues with the addition of repeating disaccharides of N-acetyl glucosamine and glucuronic acid (Nybakken and Perrimon, 2002, Prydz and Dalen, 2000). Modification of the sugar chain is then controlled by the N-deacetylase/N-sulfotransferase enzyme and various sulfotransferases (Nakato and Kimata, 2002). Sulfation can occur at the acetyl group, 2, and 6 positions of N-acetyl glucosamine, as well as the 2 and 3 positions of glucuronic acid. Thus, the heparan sulfate chains of HSPGs are extremely variable. A single heparan sulfate chain can include different regions containing either high or low levels of modification and sulfation (Nakato and Kimata, 2002). This variability gives each HS chain the potential of containing numerous growth factor binding sites. The regions of the HS chains may be modified to target certain growth factors at specific times and locations during development. The diversity both within and among heparan sulfate chains, coupled with their large size, enhances the ability of HSPGs to modulate growth factor signaling.

Heparan sulfate proteoglycan modulation of growth factor pathways

Heparan sulfate proteoglycans have been shown to play several diverse and vital roles in both the transport of growth factor ligands and the transmission of their signals (Hacker et al., 2005). Early studies in *Drosophila* demonstrated the importance of the GAG chains for proper signaling. Disruption of heparan sulfate chain synthesis by mutating the *tout velu (ttv)* enzyme, or altering modification of the sugar chains by sulfotransferase enzyme mutation, leads to improper signaling and development (The et al., 1999, Bellaiche et al., 1998). *ttv* was also shown to be important in long range Hedgehog signaling in the Drosophila wing imaginal disc (Bellaiche et al, 1998). Genetic interaction studies have further shown the importance of HSPGs during growth factor signaling (Desbordes and Sanson, 2003; Borneman et al., 2004). Various signaling pathways, including Hedgehog, Wnt, and TGF-β, are all affected by mutation of HS synthesis or modification enzymes (Giraldez et al., 2002, Selleck, 2001, Tsuda et al., 1999, Jackson et al., 1997). HSPGs also are important in the proper transport of signaling molecules through the ECM. Hedgehog growth factors may rely on HSPGs for appropriate distribution of the morphogen (Gallet et al., 2003). Heparan sulfate proteoglycans have also been shown to act as co-receptors during FGF signal transduction (Yayon et al., 1991, Rapraeger et al., 1991). The presence of heparan sulfate leads to increased dimerization of the FGF receptors (Venkataraman et al., 1999). These examples illustrate how the appropriate synthesis and modification of heparan

sulfate chains, as well as proper structure of the core protein, are essential for the modulation of growth factor signaling.

The *Drosophila* gene *terribly reduced optic lobes* (*trol*) encodes a product of approximately 450 kDa, and contains significant sequence similarity to three of the five domains of human Perlecan (Park et al, 2003). Like other perlecans, Trol is secreted and present in the extracellular matrix. Trol-Hedgehog and Trol-Branchless interactions have been isolated using co-immunoprecipitation (Park et al, 2003). *trol* has been shown to modulate signaling by Hedgehog and Branchless during first instar in the developing *Drosophila* larval brain. Genetic interaction studies show that combining a weak mutant *trol* allele having no phenotype with heterozygous mutants for either *bnl* or *hh*, neither of which display a mutant phenotype, leads to a decreased number of proliferating optic lobe and central brain neuroblasts (Park et al, 2003). This demonstrates that Trol, like other HSPGs, is important for the modulation of growth factor signaling during *Drosophila* development.

In this section, I have reviewed the general types of heparan sulfate proteoglycans. I have given examples of how HSPGs are essential for the proper signaling by growth factors. HSPGs play roles in both the transduction of signals as well as transport of molecules, and can act as co-receptors for growth factor ligands. The modulation of signaling pathways by proteoglycans is not limited to the developmental processes of organisms. Regulation of signaling by HSPGs can also affect processes later in the life of an organism.

Model Systems of Disease

The growth factor signaling pathways that are necessary for the normal development of an organism remain present and active later in life. After the conclusion of development, these signaling pathways are important to many different biological processes, including tissue repair, maintenance of stem cells, and homeostasis (Boutros et al., 2002; Martin and Parkhurst, 2004; Parisi and Lin, 1998; Palma et al., 2005; Potter, 2007). Abnormal signaling by or improper regulation of these developmental pathways has been implicated in a variety of different diseases, including many different cancers (Li et al., 2003; Cronauer et al., 2003; Bale and Yu, 2001). Abnormal expression of signaling ligands and other pathway components or improper receptor activation may allow cells to escape normal regulatory cues. Furthermore, improper signaling by these pathways may aid diseased cells in proliferation and metastasis. The redundancy of growth factor signaling pathways among organisms, as well as the vast knowledge base of their roles in developmental contexts, allows model systems to be invaluable tools in the study of disease.

Many problems are encountered by researchers interested in the study of human disease. Acquiring samples with the appropriate controls, ethical concerns about treatments, and other environmental factors all make examining disease in human patients problematic. Additionally, diagnosing a patient early enough to properly examine the initial causes of a disease is nearly impossible. Model systems allow researchers to control both the environment and genetic background of samples, and also allow for biochemical manipulation and treatments not appropriate outside of Tuskegee.

Thus, the use of model systems is vital to the understanding and eventual prevention of disease.

Countless studies have demonstrated the importance of research in model systems to the understanding of human diseases (Brumby and Richardson, 2005). *In vitro* systems such as cell culture studies allow researchers to perform biochemical and genetic manipulations necessary for the understanding of basic mechanisms in disease. These studies also simplify controlling for environmental differences. However, this simplicity in *in vitro* systems often does not allow for examination of more complex interactions that a cell may encounter in a whole organism. Whole organism model systems have also been utilized extensively to study disease. *In vivo* studies permit researchers to control for environmental and genetic factors while still maintaining biological context for the cells or tissues they are examining. Although many homologous molecules, pathways, and processes exist between model systems and humans, the evolutionary distance between species requires data collected from whole organism models to be validated in the context of human disease.

Drosophila as a model for disease

Drosophila has been used extensively as a model for development. The presence of evolutionarily conserved processes and pathways has led to many advances in knowledge of human biology. In addition to its use as a model system for development, *Drosophila* has emerged as a model for various types of diseases. Recently, the fly has been used to model human neurodegenerative diseases such as Alzheimer's,

Huntington's, and Parkinson's (Feany and Bender, 2000; Shulman et al., 2003; Lu and Vogel, 2009). For example, molecular tools available in *Drosophila* allowed researchers to overexpress a mutant form of human a-synuclein, a gene linked to familial Parkinson's disease, which led to recapitulation of the features of the disease in the fly (Feany and Bender, 2000). The ability to control the genetic background and manipulate expression of detrimental molecules in *Drosophila* results in insights that can be applied to humans suffering from neurodegenerative diseases. Fly models of inherited neuromuscular diseases such as spinal muscular atrophy, myotonic dystrophy, and dystrophinopathies recapitulate many of the key pathologic features of the human diseases (Lloyd and Taylor, 2009). The *Drosophila* model system is also useful in the study of cancer. Many of the same molecules and pathways known to be affected in human cancers are present in the fly. For example, genes known to influence the epithelial to mesenchymal transition, a hallmark of cancer, are able to cause metastasis in *Drosophila* by affecting apical basal-polarity in a similar way to that in the disease (Pagliarini and Xu, 2003). The ability to manipulate the genetic background and expression pattern of a fly, control its environment, and perform genetic screens to uncover the molecules and mechanisms associated with disease make the *Drosophila* model system an invaluable research tool.

Human Prostate Cancer

Prostate Cancer is the most commonly diagnosed form of cancer in men. In 2010 alone, there is estimated to be 217,730 new cases diagnosed, and 32,050 deaths from this disease in the United States (National Cancer Institute). Studies have shown that two out

of every three prostate cancer diagnoses occur in men over the age of 65, making age the single biggest risk factor for this disease. Approximately one out of every six men will be diagnosed with prostate cancer in his lifetime. It is the second leading cause of cancer death in males in the US, accounting for 11% of cancer deaths (American Cancer Society). Despite these horrifying statistics, there is a good prognosis if diagnosed early, with treatment consisting of radiation or prostatectomy. Early prostate cancer tumor growth is androgen-dependent, allowing this to be a treatment target. However, androgen ablation therapies are not useful indefinitely, having a median response time of only one to two years (So et al., 2005). Furthermore, tumors may reoccur after prostatectomy (Feldman and Feldman, 2001). Prostate cancer becomes androgen-independent in later stages, which leads to a much less hopeful prognosis. Androgen-independent prostate cancer is invasive and metastatic, and has no current treatments.

Prostate development

The prostate is an accessory reproductive gland responsible for producing and secreting the proteolytic fluid that supports sperm survival. Normal development of the human prostate begins around the seventh week of embryogenesis, when testosterone signaling by the testes leads to differentiation of the prostate. The prostate remains developmentally inactive throughout childhood, and during puberty exits quiescence and resumes growth. During this period, its adult secretory function is established. The adult prostate surrounds the urethra underneath the bladder (Figure 1.9A). The epithelium of the prostate consists of luminal secretory cells, epithelial cells, and

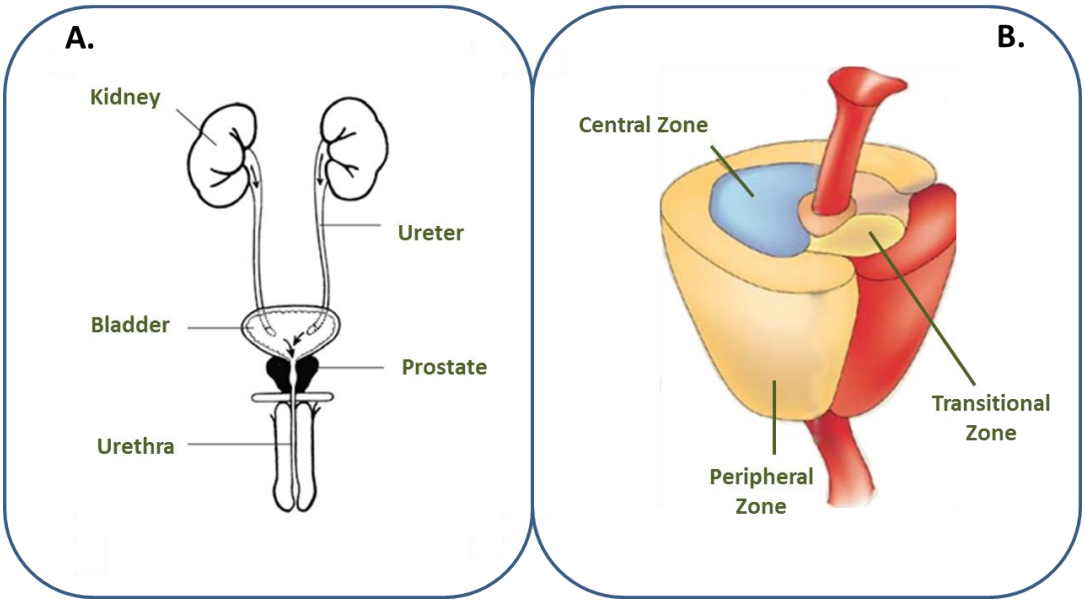

Figure 1.9. Location and zones of the human prostate. (A) The location and anatomical position of the human prostate. (B) The prostate is sub-divided into three main zones: the central zone, the peripheral zone, and the transitional zone. Most prostate cancers originate in the peripheral zone. Modified from (Abel, 2001), (Ellem and Risbridger, 2007), and (De Marzo et al., 2007).

neuroendocrine cells, while the stroma is made of muscle cells, fibroblasts, endothelial cells, and immune system cells (Feldman and Feldman, 2001). The gland is subdivided into a peripheral zone, a central zone, and a transitional zone (Figure 1.9B). It is mainly composed of acini, which secrete into the ejaculatory ducts leading to the urethra. As androgen signaling decreases in the aging male, the prostate begins to atrophy.

The improper expression of signaling pathway components has been observed in many different forms of carcinomas (Cronauer et al., 2003; Bale and Yu, 2001). During normal prostate development, signaling by the Sonic hedgehog ligand is required for epithelial differentiation as well as ductal branching morphogenesis (Freestone et al., 2003; Berman et al., 2003; Berman et al., 2004). In fact, several other signaling pathways, including the FGF, Wnt, and BMP pathways, have been shown to be important during normal prostate development (Settle et al., 2001, Kwabi-Addo et al., 2004; Yardy and Brewster, 2005). However, abnormal signaling by growth factors may play a role in the progression of the disease. Studies have shown that TGF-β acts as a tumor suppressor early in cancer progression, but it is a tumor promoter in late stage carcinomas (Steiner et al., 1994; Wikstrom et al., 1998; Wakefield and Roberts, 2002). TGF-β inhibits proliferation and stimulates apoptosis early, but low levels of receptor expression and changes in other signaling pathways result in TGF-β being upregulated and acting as a tumor promoter (Kyprianou, 1999; Guo et al., 1997; Guo and Kyprianou, 1998; Guo and Kyprianou, 1999; Kleeff, 1999; Festuccia et al., 1999). Wnt signaling is also increased in advanced, metastatic prostate cancer (Chen et al., 2004). Sonic Hedgehog and its signaling pathway modulator Perlecan have been shown to be

upregulated in advanced prostate cancer as well (Sanchez et al., 2004; Datta et al., 2006; Datta and Datta, 2006). The heparan sulfate proteoglycan Perlecan has been shown to regulate signaling by Sonic Hedgehog, FGF2, and Vascular Endothelial Growth Factor in human prostate cancer cell lines (Datta et al., 2006, Savore et al., 2005). Abnormal expression of these growth factor signaling components in diseased tissue suggests that improper regulation or signaling by these developmental pathways may play a role in the progression of prostate cancer later in life. Establishing a model system in which signaling pathways and their regulators can be examined during the onset and progression of prostate cancer may lead to insights on how better to treat the disease.

Drosophila Prostate Cancer Model

Model systems allow researchers to examine aspects of disease that may not be easily or ethically achievable in a human patient. Establishing a model system to study how growth factor signaling affects prostate cancer initiation and progression is crucial in understanding how the disease can be treated. Some systems have already been created to better understand the mechanisms by which a prostate becomes cancerous. However, the complexity and the timing of the disease leads to problems with the model systems already in use.

Multiple cell line models have been established to study the progression of prostate cancer. Prostate and prostate cancer cell lines have been established to simulate the biological progression of the disease. Individual cell lines have been created that have different dependencies on androgen, different invasiveness, and different metastatic

potential. These lines are able to model some of the hallmarks of prostate cancer, and can easily be used to study the basic mechanisms of cancer progression. Cell line studies are able to closely control environmental conditions and genetic background, helping to reduce some of the complexity associated with the disease. As with other cell culture models, prostate cancer lines have some disadvantages. The *in vitro* system may only accurately model a single point in the progression of the disease, and interactions with other cell types or molecules may not be represented. These issues require the use of a whole organism model system.

Several *in vivo* systems have been established for the study of prostate cancer. Several transgenic mouse models have been created that allow researchers to study the progression of the disease. Mouse models that generate invasive and metastatic tumors, and allow for tissue specific manipulation of gene expression, have proven useful in the study of prostate cancer progression (Roy-Burman et al., 2004). Another whole organism model for prostate cancer, the Lobund-Wistar rat model, can form spontaneous, metastatic prostate tumors, and has been valuable in the study of prostate cancer and the genes associated with the disease (Pollard, 1998). Although these animal models have yielded much data on prostate cancer progression, difficulties remain with the systems. The mouse and rat models are costly to maintain, and can be exceedingly time consuming. Prostate cancer is an age dependent disease, and formation of tumors in these animal models can take years (Pugh et al., 1994). Studies have also suggested that older age correlates with more aggressive phenotypes, and the behavior of diseased cells differs between young and old hosts (Alexander et al., 1989; McCullough et al.;

1997). Therefore, an *in vivo* model system is needed that can reproduce the complexity of the disease while still maintaining a practical timeframe for study.

Drosophila as a model

The benefits of establishing a short-lived and cost effective model system for the study of prostate cancer progression are great. *Drosophila* has been used extensively as a model for both development and disease, including cancer (Pagliarini and Xu, 2003; Brumby and Richardson, 2003). Signaling pathway components and regulators that have been implicated in prostate cancer are present in the fly. Hedgehog, Branchless, Wingless, and Dpp signaling is active in *Drosophila*, and the pathways are regulated by HSPGs. The major prostate cancer risk factor is age, and a suitable model system must be able to take this factor into account. *Drosophila* has been used extensively as an aging model (Helfand and Rogina, 2003). The specific expression patterns of various age-dependent genes have been identified in the fly, and the evolutionary conservation of aging pathways has been demonstrated (Rogina and Helfand, 1995; Helfand et al., 1995, Helfand and Rogina, 2003). Furthermore, the human *PERLECAN* gene, which maps to a locus associated with increased risk of prostate and brain cancer, is upregulated in advanced prostate cancer (Gibbs et al., 1999; Datta et al., 2006; Iozzo et al., 1994). The interaction of Perlecan with growth factors and its influence on signaling has been well established in *Drosophila* (Park et al., 2003). Relatively short lifespan, homologous signaling components and regulators, and prior establishment as an aging

and cancer model all indicate *Drosophila* may be useful as a model for prostate cancer progression.

To be developed as a practical model for prostate cancer, the fruit fly must contain an organ with analogous anatomy, function and molecular markers to that of a human prostate. The *Drosophila* ejaculatory bulb fits these criteria (Figure 1.10). Like the human prostate, the fly ejaculatory bulb mixes sperm produced in the testes with seminal fluid secreted from the gland. This mixture is then pumped into the ejaculatory ducts (Lung and Wolfner, 2001; Ludwig et al., 1991). Several fly homologs of human prostate specific markers are also expressed in the fly ejaculatory bulb. *bagpipe* (*Drosophila* Nkx3.1 homolog), α-methylacyl CoA Racemase (AMACR), *acph-1* (Prostate-Specific Acid Phosphatase), *sprouty* (Sprouty1), and *shaven* (Pax2) are all prostate specific markers (Tanaka et al., 1998; Bieberichet al., 1996; Rubin et al., 2002; Vihko et al., 1980; Kwabi-Addo et al., 2004; Khoubehi et al., 2001) that are only expressed simultaneously in the ejaculatory bulb (Hernandez and Datta, unpublished data). Thus, the *Drosophila* ejaculatory bulb has comparable anatomy and function to the human prostate, and the expression pattern of marker genes mimics that in humans.

The importance of growth factor signaling and its modulation by heparan sulfate proteoglycans in prostate cancer progression has been demonstrated (Chen et al., 2004; Sanchez et al., 2004; Datta et al., 2006). For the ejaculatory bulb to function as a model for prostate cancer progression, the signaling pathways and regulators known to affect cancer must also be present in the fly. The Hedgehog and Branchless ligands are indeed expressed in the ejaculatory bulb, and expression levels of target genes associated with

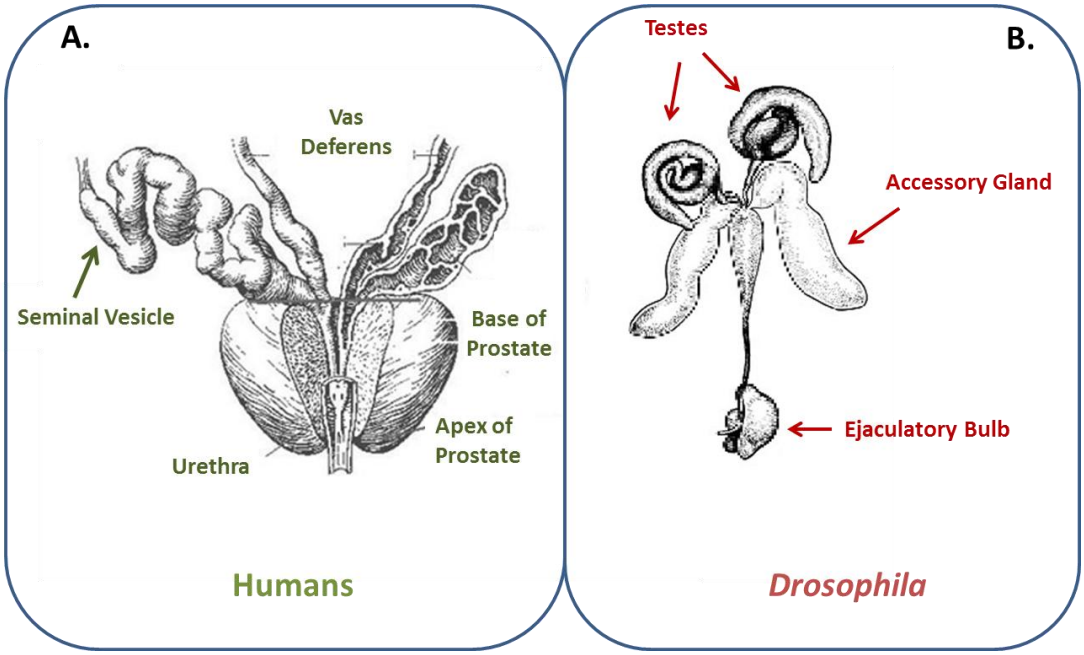

Figure 1.10. The basic anatomy of the human and *Drosophila* male reproductive tracts. (A) The anatomy of the human male reproductive system. The prostate produces seminal fluid, combines sperm from the testes and protein secretions from the seminal vesicles, and pumps semen out. (B) The *Drosophila* reproductive tract resembles that of a human. The ejaculatory bulb combines sperm from the testes with secretions from the accessory glands and other proteins and pumps the mixture into the ejaculatory duct. Modified from the SEER training program (SEER Registry Database, 2010).

the growth factors show that signaling is active and changes with the age of the fly. Furthermore, expression of HSPGs in the bulb is also observed (Hernandez, unpublished data). The *Drosophila* ejaculatory bulb therefore expresses some of the same molecules known to impact prostate cancer progression in humans. The ETS transcription factor ERG is one of the most consistently and highly overexpressed genes associated with prostate cancer (Rostad et al., 2007). The upregulation of this transcription factor is due to a chromosomal rearrangement that places it under the control of an androgen-responsive prostate gene early during prostate cancer progression, and its altered expression may play a role in tumor growth (Iljin et al., 2006; Mehra et al., 2008; King et al., 2009; Carver et al., 2009). The fly homolog of the ERG transcription factor is ETS65A. Another ETS transcription factor in *Drosophila* is Pointed, of which there are two transcripts, P1 and P2. Both isoforms of Pointed contain an ETS oncogene domain, and P2 contains a second evolutionarily conserved domain (Klambt, 1993).

Overgrowth of the ejaculatory bulb

In order to function as an effective model for prostate cancer progression, the ejaculatory bulb must be able to mimic the overproliferation and tumor formation seen in cancer. Ras-MAPK signaling plays an important role in many different carcinomas, and abnormal signaling by FGF and VEGF is critical for progression of prostate cancer. A member of that signaling cascade, Ras, is a known oncogene. Overexpression of Ras in *Drosophila* can produce overgrowth in larval tissues (Brumby and Richardson, 2003; Pagliarini and Xu, 2003).

In *Drosophila*, Ras can be overexpressed, and Ras-dependent signaling upregulated, using a constitutively active form of the molecule, Ras^{V12}. Epithelial overgrowth in the ejaculatory bulb can be induced by driving Ras^{V12} during the adult life of the fly. DAPI staining showed an increase in the number of stained nuclei in overgrown ejaculatory bulbs, with the distribution of cells being much more disorganized than that of control bulbs (Datta, unpublished data). Overexpression of the oncogene Ras increases the signaling activity of the Ras-MAPK pathway, which in turn results in expression and activity of its target genes, including *pointed*. Thus, driving the constitutively active Ras^{V12} may lead to the same result seen in human prostate cancer: upregulation of the ETS family of transcription factors. Engineered overgrowth in the *Drosophila* ejaculatory bulb is a promising model for prostate cancer neoplasia, and may be valuable in the study of how signaling affects prostate cancer progression.

While an engineered overproliferation model can be enormously beneficial, it is still an artificial system. Manipulations made to gene expression may result in unplanned and unseen consequences. Therefore, to study how signaling impacts the onset of prostate cancer, a spontaneous model for hyperproliferation would be extremely valuable. A single fly line with un-engineered overgrowth of the ejaculatory bulb resembling that of Ras^{V12} has been isolated. Enlarged bulbs in this line look similar to the overgrown Ras^{V12} ejaculatory bulbs. Genetic analysis of the spontaneous line showed that the overgrowth did not track with any single chromosome, suggesting the overgrowth phenotype is multigenic, similar to human prostate cancer. The frequency of overgrowth seen in the spontaneous line increases as the flies get older, suggesting there

may be an age component to the phenotype (Datta, unpublished data). The spontaneous overgrowth *Drosophila* line may be an important model for understanding the onset and progression of prostate cancer.

In this section I have reviewed some of the basic hallmarks of prostate cancer. The rationale for establishing a short lived model system for the progression of prostate cancer was explained. I reviewed how the *Drosophila* ejaculatory bulb has similar anatomy and function to that of the human prostate, and how expression of markers and signaling pathway components in the normal ejaculatory bulb resembles that in humans. Furthermore, the presence of spontaneous as well as induced overgrowth in the fly bulb was reviewed. In Chapter III, I will attempt to determine whether ejaculatory bulb overgrowth resembles human neoplasia. I will examine the expression and signaling activity of growth factors associated with carcinomas in both induced and spontaneous overgrowth in the fly bulb. The effect of manipulating HSPG expression on this overgrowth will also be examined. I will examine whether ejaculatory bulb overgrowth has altered cell proliferation, another characteristic of human tumors. An early method of ejaculatory bulb transplantation will also be demonstrated. These studies will serve to help establish the *Drosophila* ejaculatory bulb as a viable model for human prostate cancer and aging.

CHAPTER II

PERLECAN MODULATES SIGNALING BY MULTIPLE GROWTH FACTORS DURING *DROSOPHILA* DEVELOPMENT*

Growth factor signaling is essential for the development of every organism. Nearly every biological process is impacted at some level by growth factor signaling pathways. However, the number of signaling molecules and pathways that work to develop and maintain a living organism pale in comparison to the multitude of events they influence. How so few signaling pathways can impact such a diverse and sizeable assortment of processes is an interesting question. Furthermore, the damage that can occur when growth factor signaling goes awry is frightening. One explanation as to how signaling pathways can influence so many different processes throughout a lifetime is through differential regulation.

One way in which developmental pathways can be differentially regulated is by heparan sulfate proteoglycans. HSPGs have been shown to be able to modulate signaling by acting as co-receptors (Yayon et al., 1991; Rapraeger et al., 1991) and by regulating the diffusion of morphogens (Gallet et al., 2003; Belenkaya et al., 2004). The vast number of ways in which HSPGs can be differently modified (Nybakken and Perrimon, 2002) allows them to help growth factors reach that high level of variability in

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function.

The *Drosophila* Perlecan homolog Trol has been shown to modulate signaling by growth factors during development (Park et al., 2003). In this chapter, I will examine the ability of this heparan sulfate proteoglycan to regulate growth factor signaling in a variety of developmental contexts. The ability of Trol to modulate the same signaling pathways in different developmental processes will be assayed. I will examine the role Trol plays in assisting growth factors in impacting various developmental processes in *Drosophila*.

Heparan sulfate proteoglycans modulate signaling by a variety of growth factors. The mammalian proteoglycan Perlecan binds and regulates signaling by Sonic Hedgehog, Fibroblast Growth Factors (FGFs), Vascular Endothelial Growth Factor (VEGF) and Platelet Derived Growth Factor (PDGF), among others, in contexts ranging from angiogenesis and cardiovascular development to cancer progression. The *Drosophila* Perlecan homolog *trol* has been shown to regulate the activity of Hedgehog and Branchless (an FGF homolog) to control the onset of stem cell proliferation in the developing brain during first instar. Here we extend analysis of *trol* mutant phenotypes to show that *trol* is required for a variety of developmental events and modulates signaling by multiple growth factors in different situations. Different mutations in *trol* allow developmental progression to varying extents, suggesting that *trol* is involved in multiple cell-fate and patterning decisions. Analysis of the initiation of neuroblast proliferation at second instar demonstrated that *trol* regulates this event by modulating signaling by Hedgehog and Branchless, as it does during first instar. Trol protein is

distributed over the surface of the larval brain, near the regulated neuroblasts that reside on the cortical surface. Mutations in *trol* also decrease the number of circulating plasmatocytes. This is likely to be due to decreased expression of *pointed*, the response gene for VEGF/PDGF signaling that is required for plasmatocyte proliferation. Trol is found on plasmatocytes, where it could regulate VEGF/PDGF signaling. Finally, we show that in second instar brains but not third instar brain lobes and eye discs, mutations in *trol* affect signaling by Decapentaplegic (a Transforming Growth Factor family member), Wingless (a Wnt growth factor) and Hedgehog. These studies extend the known functions of the *Drosophila* Perlecan homolog *trol* in both developmental and signaling contexts. These studies also highlight the fact that Trol function is not dedicated to a single molecular mechanism, but is capable of regulating different growth factor pathways depending on the cell-type and event underway.

Background

Heparan sulfate proteoglycans (HSPGs) are a family of cell-surface and extracellular proteins modified by the attachment of glycosaminoglycan chains. The general structure of the protein core determines the family the HSPG belongs to: Syndecans contain a transmembrane domain, Glypicans are tethered to the cell surface via a GPI linkage and Perlecans are secreted components of the extracellular matrix. Both the protein core and glycan chains play important roles in HSPG function through protein-protein and sugar-protein interactions. Genetic studies, first in Drosophila and later in mouse and zebrafish, demonstrated the importance of the heparan sulfate chains

on all three types of HSPGs for signaling by multiple growth factors such as the Fibroblast Growth Factors (FGFs), Hedgehogs, Wnts and Transforming Growth Factors (TGFβs) (reviewed in Lin, 2004).

Perlecan is the largest member of the HSPG family with a core protein of approximately 450kD in size. Perlecan has been linked to signaling by the heparan-dependent growth factors FGF2, Vascular Endothelial Growth Factor (VEGF) and Sonic Hedgehog (SHH) in mammalian systems (reviewed in Datta et al., 2006; Iozzo, 2005). Studies of Perlecan knock-out mice have demonstrated roles for Perlecan in vascular development and chondrogenesis as well as maintenance of basement membrane integrity (Arikawa-Hirasawa et al., 1999; Costell et al., 2002; Costell et al., 1999; Gonzalez-Iriarte et al., 2003). Additional mammalian studies have revealed Perlecan's functions in angiogenesis and carcinogenesis (Datta et al., 2006; Mongiat et al., 2003; Sharma et al., 1998; Zhou 2004; reviewed in Datta et al., 2006; Iozzo and San Antonio, 2001). Mutation of Perlecan in humans leads to the muscle tone symptoms of Schwartz-Jampel syndrome, possibly through altered excitability of the neuromuscular junction and the skeletal abnormalities of Silver-Handmaker syndrome, presumably through effects on chondrogenesis (Arikawa-Hirasawa et al., 2002; Arikawa-Hirasawa et al., 2001; Nicole et al., 2000).

Studies of Perlecan in invertebrate model systems have led to additional insights into Perlecan function. The single Perlecan gene in C. elegans is encoded by the unc-52 locus (Rogalski et al., 1993). Mutations in unc-52 result in embryonic or adult paralysis due to defects in body wall muscle cells (Rogalski et al., 1993; Brenner, 1974; reviewed

in Rogalski et al., 2001). Mutations in unc-52 also enhance cell migration defects caused by decreased netrin, FGF, TGFβ or Wnt signaling. In Drosophila, Perlecan is encoded by the *trol* gene on the X chromosome (Park et al., 2003; Voigt et al., 2002), which was initially implicated in the control of stem cell division in the developing larval brain (Datta, 1995; Datta and Kankel, 1992). In the larval brain, trol promotes the cell cycle progression of mitotically arrested neuroblasts (Caldwell and Datta, 1998; Park et al., 2003) through modulation of FGF and Hedgehog signaling (Park et al., 2003). These Drosophila studies were the first to link Perlecan to Hedgehog signaling. More recently, studies of oogenesis in Drosophila have uncovered a role for Perlecan in the maintenance of epithelial cell polarity through interactions with the extracellular matrix receptor Dystroglycan (Schneider et al., 2006).

The many signaling pathways associated with HSPGs in general and Perlecan in particular led us to ask what other biological processes may require Perlecan function. We used a series of *trol* mutants to investigate several phenotypes ranging from overall developmental progress to specific alterations of stem cell division and hemocyte production. Furthermore, analysis of signaling pathway response genes revealed that while mutations in Perlecan decrease signaling in multiple pathways, at least some of these effects are tissue specific.

Results and Discussion

Development and lethal phase

We had previously shown that the viable *trol*^{b22} and the lethal *trol*^{8}, *trol*^{4}, and *trol*^{sd} alleles form an allelic series of increasing severity based on their onset of neuroblast proliferation phenotype in first instar larval brain lobes (Park et al., 2003). Identification (Park et al., 2003) and phenotypic analysis of a fifth *trol* allele, *trol*^{7}, revealed that *trol*^{7} is the strongest allele with respect to the first instar proliferation phenotype (Figure 2.1A). Unexpectedly, *trol*^{7} mutant larvae appeared healthier overall than other *trol* mutant larvae, suggesting that the order of allelic severity determined by analysis of first instar brain lobes would be different from one based on developmental progression. To test this hypothesis, we examined the lethal stage and developmental progression of larvae mutant for *trol*^{b22}, *trol*^{8}, *trol*^{4}, *trol*^{sd} and *trol*^{7}. In all the experiments, crosses were designed to use sibling controls in order to minimize the effects of genetic background, which can be significant in fly stocks kept in reproductive isolation from each other for years in our laboratory. For the lethal *trol* alleles, *y trol*^{x} */Binsn* stocks were used as the source of mutant and control larvae. At this stage of first instar, mutations in *y* produce one of the few reliable phenotypic markers. Thus *trol* mutant animals were identified as *y* mutant larvae that are *y trol*^{x} hemizygous males and sibling controls were a mixed population of y^{+} animals: *y trol*^{x}*/Binsn* heterozygotes,

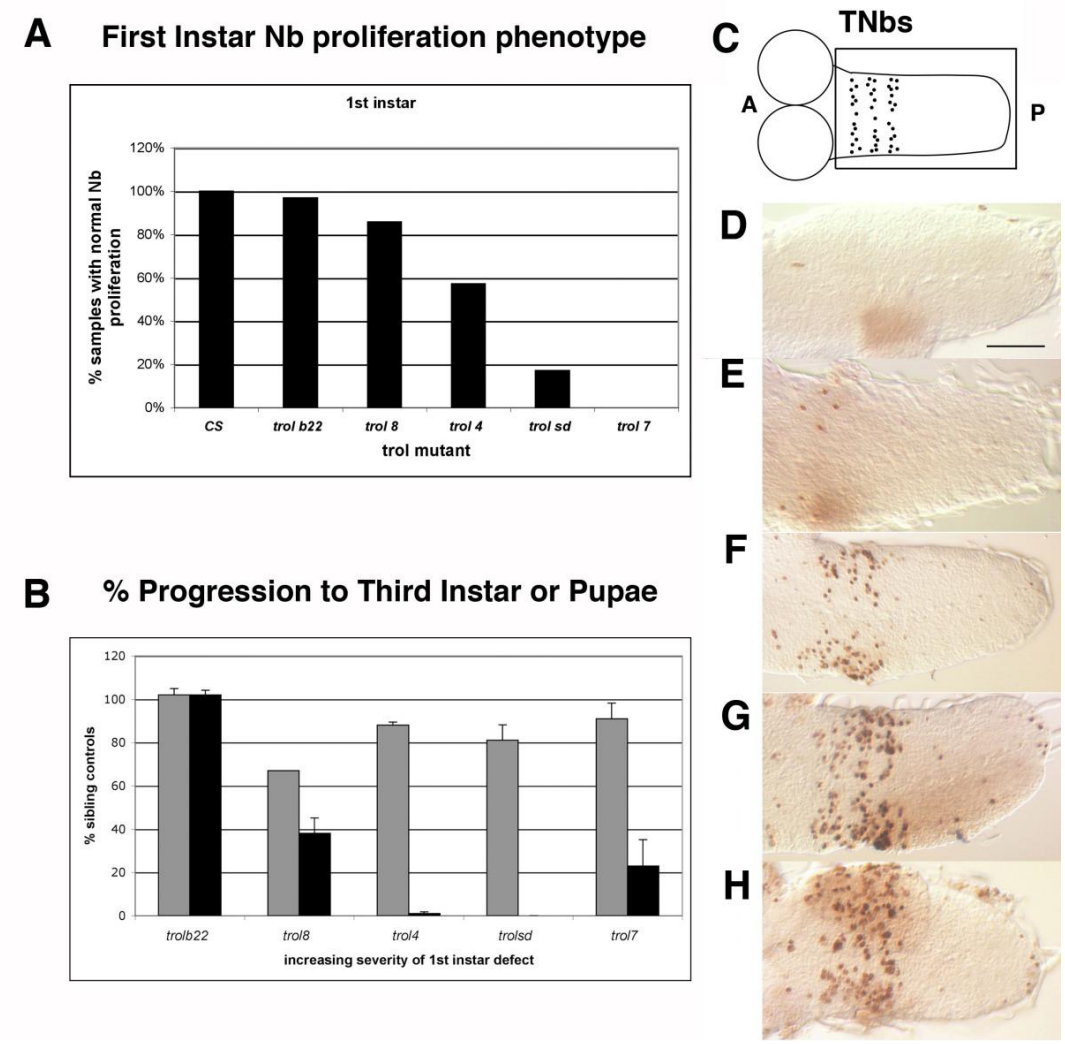


Figure 2.1. Phenotypic series of *trol* alleles. (A) First instar neuroblast proliferation phenotype presented as % of samples with numbers of BrdU labeled neuroblasts falling within the control range, some data originally published in Park et al, 2003a. (B) Lethal phase phenotype presented as the percentage of *trol* mutant animals capable of survival and development to third instar (grey bars) or to pupal formation (black bars) compared to sibling controls. Error bars indicate s.e.m. (C) Cartoon of second instar larval brain with dividing TNBs in ventral ganglion. Boxed area indicates portion of brain shown in panels D-G below. A = anterior, P = posterior. (D-G) Examples of the five classes of BrdU incorporation into TNbs are shown. In all panels anterior is to the left, posterior is to the right. Scale bar in panel D indicates 25 um. (D) None (class 1). (E) Few (class 2). (F) Segmentally repeated lines with few extra neuroblasts (class 3). (G) Segmentally repeated lines with several scattered neuroblasts (class 4). (H) Heavily populated segmental pattern (class 5).

Binsn homozygous females and *Binsn* hemizygous males. Note that while *Binsn* homozygous females and hemizygous males can become viable adults, not all *Binsn/Binsn* or *Binsn/Y* larvae reach adulthood. Thus our comparison provides a measure of developmental progression and lethal phase that will err on the side of minimizing the *trol* mutant phenotype. For analysis of the viable $trol^{b22}$ allele, additional crosses were required to produce wild-type sibling controls from the homozygous *y* $trol^{b22}$ stock. *y* $trol^{b22}$ animals were crossed to the wild-type strain Canton Special (CS) to produce $trol^{b22}$/CS heterozygous females. These females were mated to CS males to generate hemizygous *y* $trol^{b22}$ male larvae and y^{+} sibling control larvae (a mixture of heterozygous *y* $trol^{b22}$*/CS* female, homozygous *CS* female and hemizygous *CS* male) for the developmental studies. One hundred mutant and sibling control animals for each allele were collected at early first instar and monitored at 24 hour intervals for developmental progression and viability. Of these, only 1 mutant $trol^{4}$ and no $trol^{sd}$ animals pupariated. However, when the same numbers of $trol^{b22}$, $trol^{8}$ and $trol^{7}$ mutant larvae were analyzed and compared to sibling controls, 102%, 38% and 23% of the animals were able to pupariate, respectively (Figure 2.1B). The pupariation assay resulted in shifts of perceived functional severity for both $trol^{4}$ and $trol^{7}$, with $trol^{4}$ appearing stronger and $trol^{7}$ appearing weaker.

Why would animals mutant for $trol^{7}$ (that has a strong effect on neuroblast proliferation) be able to progress further in development than animals mutant for $trol^{4}$ which causes a weaker neuroblast proliferation phenotype? One possibility is that *trol* modulates the activity of different signaling pathways in different tissues. For example,

a mutation that affects the ability of Trol to function in the Hh pathway would have a severe effect on developmental decisions that require Hh activity and very little effect on decisions that do not require Hh signaling. To address this possibility we investigated the impact of *trol* mutations on two distinct developmental events and several signaling pathways.

Effects of trol mutations on TNb proliferation

trol was initially identified as a mutation on the X chromosome that affected the proliferation pattern of neuroblasts in the brain lobes and ventral ganglion (Datta, 1995; Datta and Kankel, 1992). Since neuroblasts in the thoracic region of the ventral ganglion begin proliferation in early second instar (Datta 1995; Truman and Bate, 1988; Prokop and Technau, 1991), we evaluated the ability of thoracic neuroblasts (TNbs) to enter S phase in *trol* mutant animals. We adapted the idea of phenotypic classes to produce a scale for the extent of TNb proliferation at four hours post molt (Figure 2.1C-G). Five TNb classes were defined as follows: Class 1, no neuroblasts labeled; Class 2, a small number of labeled neuroblasts with no distinct segmental pattern; Class 3, labeled neuroblasts in a segmentally repeated lines with very few labeled neuroblasts in between the lines or in the medial region of the ventral ganglion; Class 4, labeled neuroblasts in a segmentally repeated line with some labeled neuroblasts in between the lines or in the medial region of the ventral ganglion; and Class 5, labeled neuroblasts in heavily populated segmental pattern with many labeled neuroblasts in the medial portion of the ventral ganglion. When both sides of a ventral ganglion did not conform to a

single class, the sample was scored as the higher class. This will have a conservative effect of scoring a partial loss-of-proliferation TNb phenotype as more wild-type. Thus we can have greater confidence in the significance of TNb proliferation phenotypes observed compared to controls.

We first examined the onset of TNb proliferation in wild-type sibling controls to determine the time point at which to assay the *trol* mutants (data not shown). In our hands, high levels of 5-Bromodeoxyuridine (BrdU)-labeled TNbs were first observed in control samples between 2–5 hours post molt depending on genetic background. This timing is slightly earlier than the previous observation that TNb mitosis begins between 28–34 hours post hatching, or 4–10 hours post molt (pm) to second instar (Truman and Bate, 1988). To evaluate the TNb proliferation phenotype produced by the different *trol* alleles, at least twenty samples for each mutant and sibling control (generated as described above) were allowed to incorporate BrdU from 4–5 hours pm and scored for TNb class. The average score and standard error of the mean were calculated for each group of sibling controls. The control value for each study was set to a value of 4 to control for genetic background effects between experiments. Setting controls to a value of 4 on our 5 point scale was chosen to allow evaluation of over-proliferation (>4) as well as under-proliferation (<4) mutant phenotypes. To obtain the TNb phenotype score for each mutant allele we normalized the score for each sample to the respective sibling control and calculated the average and standard error of the mean (Table 2.1). Surprisingly, $trol^{b22}$ mutants had a significantly higher than normal level of TNb proliferation (TNb score >4) while the

Table 2.1. TNb BrdU incorporation phenotype of *trol* mutants at 4–5 hours pm. Each mutant allele scored for thoracic neuroblast proliferation and compared to the respective sibling control.

***trol* allele**	**TNb score***	**S.E.M.**
Control	4.00	0.27
$trol^{b22}$	4.55	0.16
$trol^{8}$	3.37	0.21
$trol^{4}$	3.00	0.27
$trol^{7}$	2.95	0.2
$trol^{sd}$	2.45	0.21

* All scores significantly different from controls at $p < 0.05$.

remaining *trol* mutants showed decreased TNb cell division compared to controls. The differences between mutant and control BrdU incorporation were statistically significant ($p < 0.05$) for each mutant allele. Comparison between mutants showed a phenotypic trend from $trol^{b22}$ having hyperactive TNb proliferation to $trol^{sd}$ as the mutant with the fewest labeled TNbs. In this assay $trol^{7}$ mutants appear to have a weaker phenotype than $trol^{sd}$.

The relatively minimal differences in severity observed between the first and second instar neuroblast proliferation phenotype in the various *trol* alleles contrasts with the more dramatic differences observed in the developmental assays. One possible explanation is that the two spatially and temporally distinct classes of neuroblasts might have similar requirements to activate their proliferation. Trol is able to modulate both Hh and Bnl signaling, and first instar neuroblasts require signaling by these growth factors to exit quiescence (Park et al., 2003; Barrett et al., 2008). To determine if similar cues are used by the two different populations of neural stem cells, we asked if the same pathways are also involved in the activation of TNb proliferation in second instar.

hh and bnl signaling in the second instar ventral ganglion

We used *lacZ* insertions in *bnl* (bnl^{06916}) and *hh* (hh^{P30}) to follow the spatial and temporal pattern of expression of these two growth factors in the ventral ganglion during early second instar (Figure 2.2A-E). ß–galactosidase activity staining revealed that both ligands are expressed in the ventral ganglion from the time of molting to second instar through 4 hours pm, the period during which the TNbs are resuming proliferation. ß–

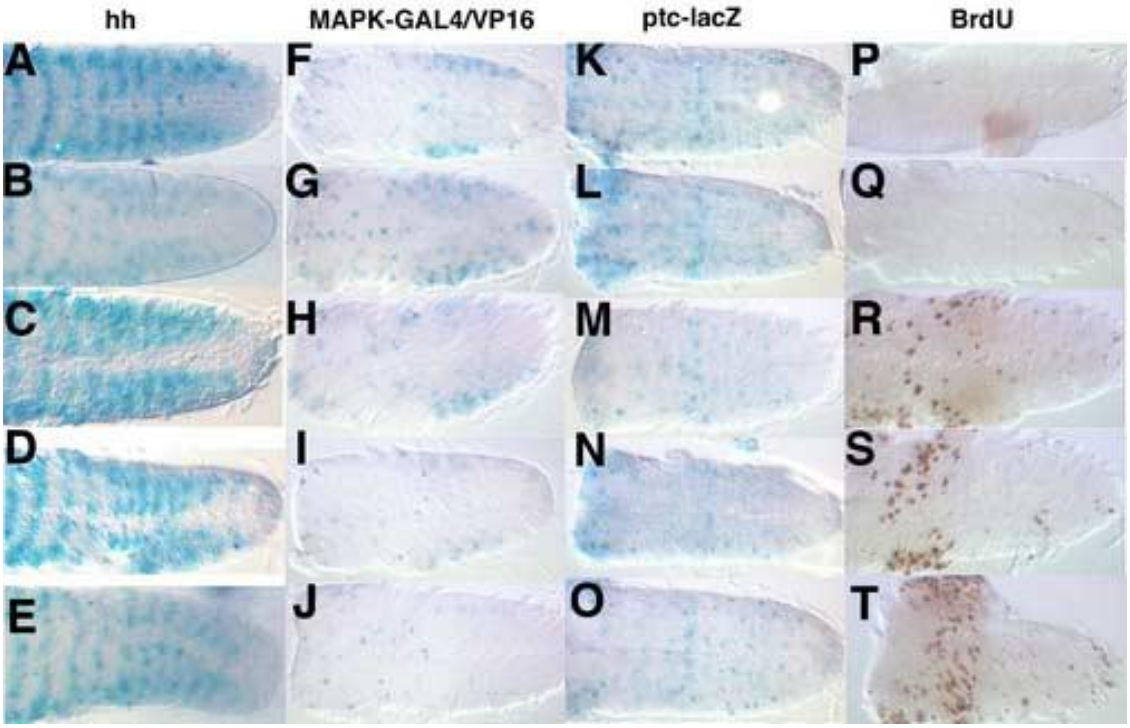


Figure 2.2. Timecourse of *hh* expression, MAPK and Hh signaling activity and BrdU incorporation in the ventral ganglion. *hh* expression as monitored by β-galactosidase staining using the hh^{P30} allele at (A) 0 hours pm; (B) 1 hour pm; (C) 2 hours pm; (D) 3 hours pm and (E) 4 hours pm. The pattern of MAPK activity as assayed using the MAPK-GAL4/Vp16 system in the ventral ganglion at (F) 0 hours pm; (G) 1 hour pm; (H) 2 hours pm; (I) 3 hours pm and (J) 4 hours pm. The pattern of Hh signaling activity as monitored by *ptc-lacZ* in the ventral ganglion at (K) 0 hours pm; (L) 1 hour pm; (M) 2 hours pm; (N) 3 hours pm and (O) 4 hours pm. BrdU incorporation in the ventral ganglion at (P) 0-1 hour pm; (Q) 1-2 hours pm; (R) 2-3 hours pm; (S) 3-4 hours pm and (T) 4-5 hours pm.

galactosidase activity staining showed expression of both *hh-lacZ* and *bnl-lacZ*, although *bnl-lacZ* was considerably lighter (data not shown). Neither the spatial pattern nor the intensity staining representing expression of either growth factor changed appreciably during this time period.

To determine if expression of the *bnl* and *hh* ligands resulted in signaling activity, we used reporter constructs to follow Bnl and Hh pathway activation. Bnl signaling occurs through the Ras-MAPK pathway, and we have shown in first instar that inhibition of MAPK activation inhibits neuroblast proliferation (Park et al., 2003). We followed MAPK activity in the ventral ganglion with a MAPK-GAL4/Vp16 fusion that can transcribe a UAS reporter gene only upon phosphorylation of MAPK and subsequent translocation of the fusion protein into the nucleus (Kumar et al., 2003). ß–galactosidase activity produced by a nuclear localized *UAS-GFPlacZ.nls* reporter gene was assayed since ß–galactosidase activity can be detected in significantly less time than GFP using this system. Our timecourse shows that nuclear MAPK activity is high within the thoracic region of the ventral ganglion from 0 to 2 hours pm, followed by a decline. MAPK activity peaks at 1 hour pm, approximately 3-4 hours prior to the onset of maximal TNb proliferation in controls (Figure 2.2F-J). This is in general agreement with culture studies in first instar where addition of a MAPK inhibitor 6 hours prior to BrdU incorporation assays greatly diminished the number of neuroblasts that labeled with BrdU (Park et al., 2003). While MAPK is also activated by other growth factors such as EGF, our genetic studies indicate a functional role for Bnl in the activation of TNb proliferation. To examine Hh pathway activity, we used a *lacZ* reporter gene for

the Hh response gene *ptc* (*ptc-lacZ*). ß–galactosidase activity driven by *ptc-lacZ* was also high in the ventral ganglion from 0-1 hrs pm, and then decreased at later timepoints (Figure 2.2K-O). Peak *ptc-lacZ* expression in the thoracic region of the ventral ganglion was observed at 1 hour pm, similar to the MAPK activation reporter. Taken together, our data indicate a dynamic pattern of signaling activity as monitored by MAPK and *ptc* response during early second instar, in contrast to the relatively constant pattern of expression of the presumed ligands Bnl and Hh. In the case of MAPK, it is possible that some of the changes in pathway activity are due to the presence of other activating ligands. In the case of *ptc-lacZ*, it is clear that another mechanism besides transcriptional regulation of *hh* must be acting to control pathway activity. Our ligand expression and signaling activity data suggest that Bnl and Hh signaling are occurring in the thoracic region of the ventral ganglion, but the question of whether the signaling is required for control of TNb proliferation remained.

To ask if Hh and Bnl signaling activated TNb proliferation, we first examined the effect of ligand over-expression or decreased ligand activity on TNb proliferation. When a *hs-hh* line was reared at 25C to over-express *hh*, more TNbs incorporated BrdU at 0-1 hrs pm compared to control animals (Figure 2.3A-B). In contrast, homozygous hh^{ts2} animals raised at 18C through embryogenesis and then transferred to 25C upon larval hatching clearly showed a diminished number of BrdU labeled TNbs at 2-3 hrs pm compared to control heterozygous individuals (Figure 2.3C-D). To allow over-expression of *bnl*, animals carrying *hs-GAL4* and *UASbnl* were raised at 18C during embryogenesis and moved to 25C upon larval hatching. Over-expression of *bnl* resulted

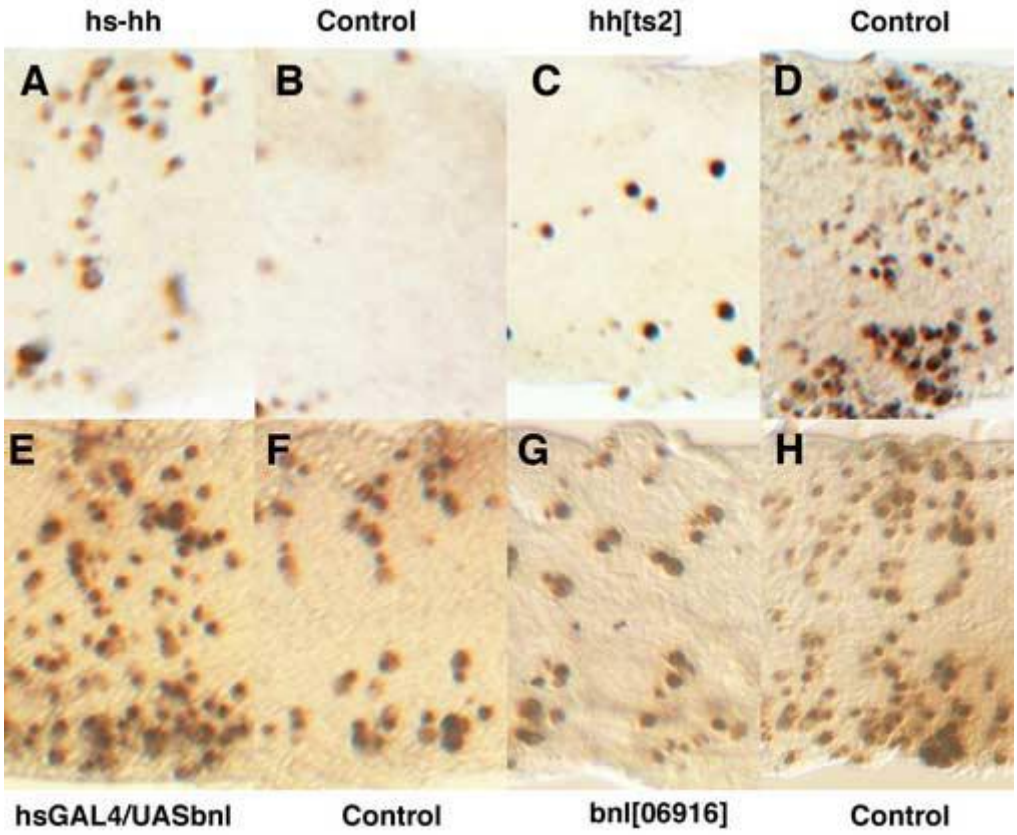


Figure 2.3. Hh and Bnl signaling affect TNb proliferation. One hour BrdU incorporation in TNbs in (A) a *hs-hh* brain from 0-1 hour pm; (B) a control CS brain from 0-1 hour pm; (C) hh^{ts2} brain from 2-3 hours pm; (D) a control hh^{ts2} / + brain from 2-3 hours pm; (E) a *hs-GAL4* / +; + / *UAS-bnl* brain at 23-24 hours ph first instar; (F) a control *hs-GAL4* brain from 23-24 hours ph first instar; (G) a bnl^{06916} brain from 2-3 hours pm and a H) control bnl^{06916} / + brain from 2-3 hours pm.

in excess BrdU labeled TNbs as early as 23-24 hrs ph or late first instar compared to hs-GAL4 controls (Figure 2.3E-F). Conversely, animals homozygous for the partial-loss-of-function allele *bnl*^{06916} showed decreased numbers of BrdU labeled TNbs at 2-3 hrs pm compared to heterozygous controls (Figure 2.3G-H). This data indicate that both Bnl and Hh signaling are required for the normal onset of TNb proliferation during second instar. *trol* mutations also affect TNb proliferation, suggesting that *trol* may modulate Bnl and Hh signaling in the second instar brain as it does during first instar.

To test whether the neuroblasts themselves are responding to the Hh and Bnl signaling, or whether they are receiving the signals indirectly from other cells in the ventral ganglion, we performed double labeling experiments during early second instar. We used a FLP-out system which allowed the cells responding to Hh or Bnl signaling to be permanently labeled. To do this, UAS-*FLP* recombinase enzyme was driven by either MAPK-*GAL4* or *ptc-GAL4*, which targeted a FRT *actin-lacZ* reporter. Any cell in which MAPK or Hh signaling had occurred would then be permanently marked by the expression of *lacZ* under the actin promoter. Labeling with BrdU from 4-5 hrs pm and staining for both BrdU and *lacZ* showed *lacZ* expressing cells near but not overlapping with BrdU incorporated cells (Figure 2.4). Therefore, cells adjacent to proliferating neuroblasts are responding to Hh and Bnl signaling, and both act indirectly to regulate re-activation of thoracic neuroblasts.

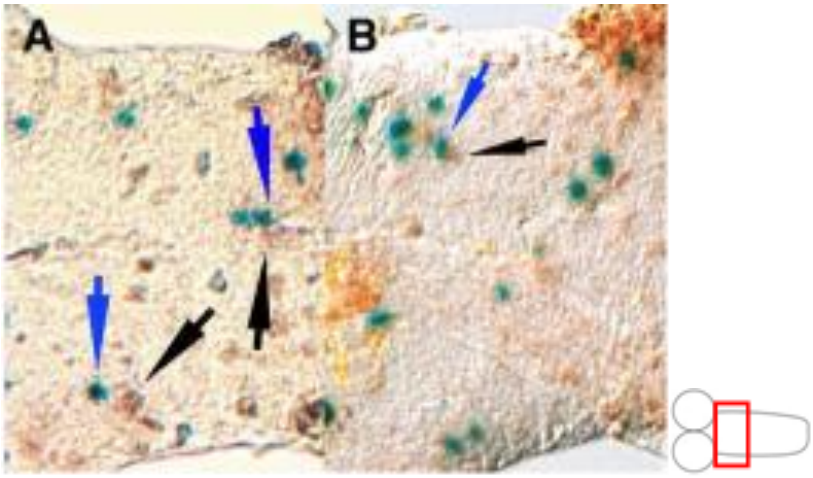


Figure 2.4. Hh and Bnl signaling occurs in cells adjacent to thoracic neuroblasts. (A) Proliferating cells labeled with BrdU (black arrows) are adjacent to MAPK signaling cells, labeled with *lacZ* (blue arrows). (B) Proliferating neuroblasts labeled with BrdU are adjacent to *ptc* expressing cells labeled with *lacZ.* Cartoon shows region of the larval brain pictured.

trol affects Bnl and Hh signaling in the ventral ganglia

To determine if *trol* affects TNb proliferation through modulation of Bnl and Hh signaling, we used genetic interaction studies with the weak *trol* allele $trol^{b22}$. As we have shown, $trol^{b22}$ animals have over proliferation of TNbs compared to sibling controls (Table 2.1). For the genetic interaction assay, *y* $trol^{b22}$ females were crossed to $bnl^{06916}/TM3y^{+}$ males to generate *y* larvae that were y $trol^{b22}$; bnl^{06916}/+ and y^{+} sibling controls that were a combination of *y* $trol^{b22}$ *; +/TM3*y^{+} males, *y* $trol^{b22}$/+ *; +/TM3*y^{+} females and $trol^{b22}$/+ *;* bnl^{06916}/+ females. None of the sibling controls had TNb proliferation scores outside of the normal (CS) range at this timepoint. *y* $trol^{b22}$ males carrying a single copy of the bnl^{06916} allele had fewer BrdU labeled TNbs at 2–3 hours post molt to second instar (pm) compared to siblings that were hemizygous or heterozygous for $trol^{b22}$ alone or heterozygous for both $trol^{b22}$ and bnl^{06916} (Figure 2.5). The decreased TNb proliferation in samples heterozygous for bnl^{06916} in a $trol^{b22}$ background compared to controls versus the increased proliferation in $trol^{b22}$ animals wild-type for *bnl* compared to controls suggests that the $trol^{b22}$ mutation affects signaling by Bnl in the ventral ganglion at second instar.

We also used genetic interactions to evaluate the possibility that *trol* might affect Hedgehog signaling in the ventral ganglion. For this study *y* $trol^{b22}$ females were crossed to $hh^{AC}/TM3y^{+}$ males to generate *y* larvae that were y $trol^{b22}$; hh^{AC}/+ and y^{+} sibling controls that were a combination of *y* $trol^{b22}$ *; +/TM3*y^{+} males, *y* $trol^{b22}$/+ *; +/TM3*y^{+} females and $trol^{b22}$/+ *;* hh^{AC}/+ females. $trol^{b22}$ animals carried a single copy of the hh^{AC} allele, also had fewer dividing TNbs at 2–3 hours pm compared to sibling controls

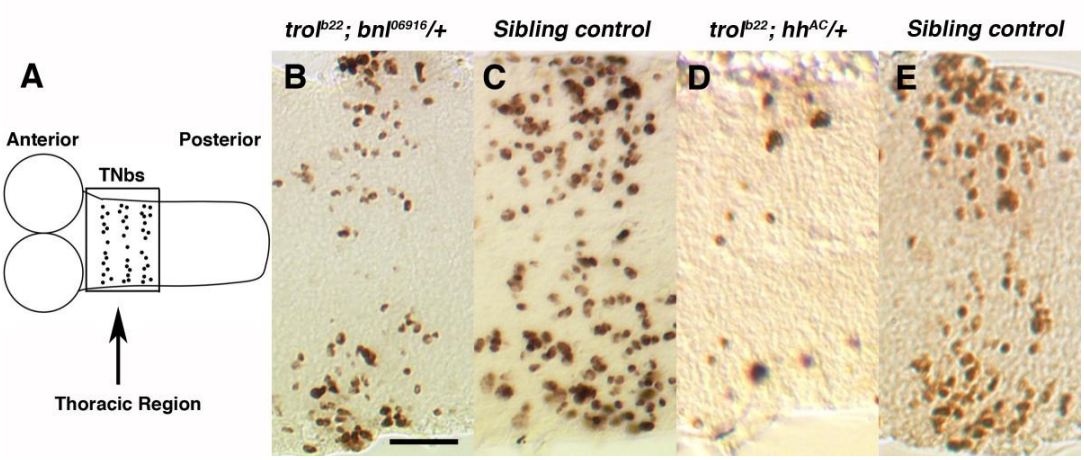


Figure 2.5. Trol modulates Hh and Bnl signaling in the ventral ganglion. (A) Cartoon of second instar brain indicating location of TNbs. Boxed area outlines thoracic region shown in panels B-E. One hour BrdU incorporation in TNbs in (B) a *y trol*^{b22}; *bnl*^{06916}/+ brain from 2–3 hours pm; (C) a sibling control *y trol*^{b22}/+ ; +/*TM3y*$^{+}$, *y trol*^{b22} ; +/*TM3y*$^{+}$ or *y trol*^{b22}/+ ; *bnl*^{06916}/+ brain 2–3 hours pm (see text); (D) a *y trol*^{b22}; *hh*^{AC}/+ brain from 2–3 hours pm and a (E) sibling control *y trol*^{b22}/+ ; +/*TM3y*$^{+}$, *y trol*^{b22} ; +/*TM3y*$^{+}$ or *y trol*^{b22}/+ ; *hh*^{AC} /+ brain from 2–3 hours pm (see text). Scale bar in panel A indicates 10 um.

(Figure 2.5). The decrease in the number of BrdU labeled TNbs in *trol*^{b22} hemizygotes upon heterozygosity for *hh*^{AC} suggest that mutations in *trol* also weaken the signaling action of Hh in the ventral ganglion. To further test our hypotheses, we examined the signaling activity of Bnl and Hh in *trol* mutants directly by quantitative RealTime PCR (qRT-PCR) in the central nervous system (CNS). To avoid interfering signals from the lobes of the second instar brain that might overwhelm differences in signal in the ventral ganglion, we isolated ventral ganglia from second instar *trol* mutant and sibling control brains at one hour post molt. First instar brains were dissected at 20 hours post hatching which correlates with the end of the BrdU labeling period used to assess neuroblast proliferation in first instar (Park et al., 2003; and this manuscript). RNA was isolated, cDNA synthesized and amplified and the level of expression of the Hh response gene *ptc* (Figure 2.6) and the Bnl response gene *pnt* (Figure 2.6) assayed. Our qRT-PCR data demonstrate that mutations in *trol* affect the strength of signaling by both Hh and Bnl in the larval ventral ganglion and in first instar larval brains (data not shown).

Trol localization in the larval brain

Previously we had isolated complexes containing either Trol and FGF2 or Trol and Hh by co-immunoprecipitation (Park et al., 2003). In combination with our genetic studies, these complexes suggested that the Trol protein regulates neuroblast division by binding growth factors that stimulate neuroblast proliferation in a manner similar to Perlecan-mediated promotion of ligand-receptor binding described in mammalian systems (Aviezer et al., 1997). This model predicts that Trol protein should be localized

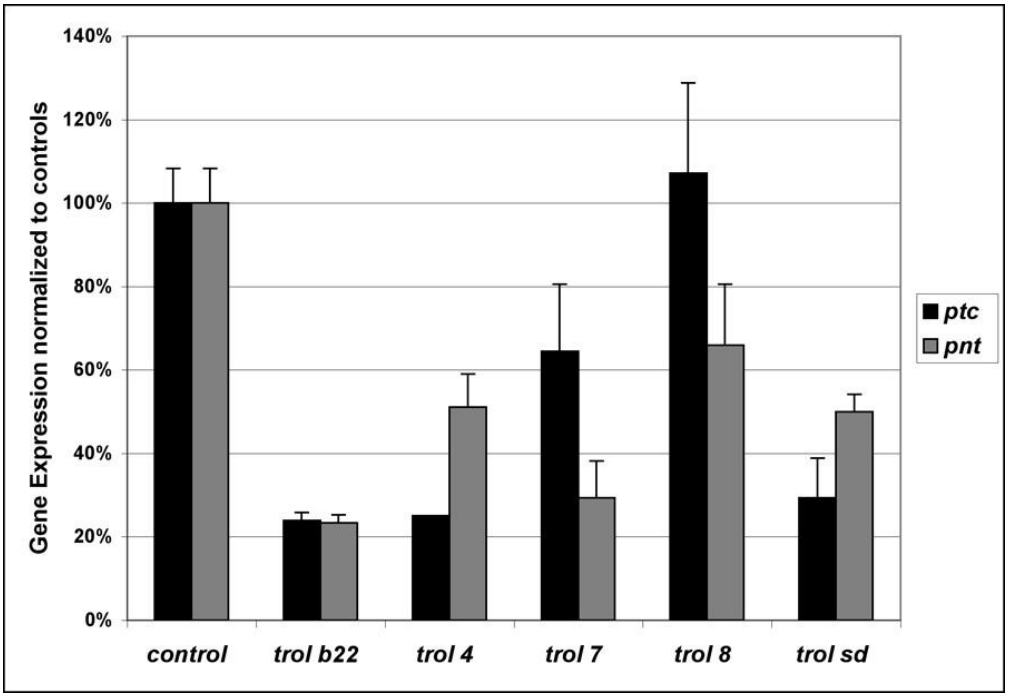


Figure 2.6. Hh and Bnl signaling activity in the ventral ganglion of *trol* mutant animals. Quantitative RT-PCR analysis of the expression of the Hh response gene *ptc* (black bars) and the Bnl response gene *pnt* (grey bars) in the ventral ganglia of *trol* mutant normalized to controls at one hour post molt to second instar. β-*actin* was used to as an internal control to normalize message levels. All analyses were done in triplicate and three different concentrations to ensure samples were within linear range of amplification. Error bars indicate standard deviation.

near the regulated neuroblasts, i.e. the optic lobe and central brain neuroblasts of the first instar brain (Park et al., 2003; Voigt et al., 2002; Datta, 1995; Datta and Kankel, 1992; Caldwell and Datta, 1998; Park et al., 2003; Park et al., 1998) and the thoracic region of the second instar brain (Datta and Kankel, 1992; and Figure 2.7A). In contrast, *in situ* hybridization studies in the third instar larval brain by Voigt et al (Viogt et al., 2002) had revealed that only a few isolated cells at a distance from the optic lobe proliferation centers express *trol*. This led the authors to suggest that Trol is unlikely to regulate neuroblast proliferation by promoting binding of FGF-type ligands to their receptors since this would require Trol protein localization near the responding cells. However, since Trol is a secreted protein with a long half-life, mRNA expression patterns may not accurately portray protein localization. In addition, Voigt et al conducted their *in situ* analysis at late third instar, 2–3 days after the activation of neuroblast division at late first or early second instar. Thus the expression pattern observed for *trol* message at late third instar may not reflect expression of *trol* at earlier larval stages. Furthermore, a study of *trol* mRNA localization by *in situ* hybridization in embryos showed either no obvious staining in the CNS (Friedrich et al., 2000) or expression in a small subset of glial cells in the CNS (Viogt et al., 2002). However, analysis of Trol protein localization with an anti-Trol antibody in embryos revealed localization to the basement membrane of the CNS (Friedrich et al., 2000). This evidence further suggests that *trol* message patterns may not reflect Trol protein localization. To address the conflicting models, we took advantage of a Trol protein trap in which the GFP gene is inserted within the endogenous *trol* locus (Medioni and Noselli, 2005). Analysis of GFP localization in

larval brains demonstrates that Trol-GFP is found in a layer, presumably the basal lamina, encompassing the entire outer surface of the larval brain with little to no signal detectable at internal sites within the brain (Figure 2.7B-E). Trol-GFP was also observed in the basal lamina surrounding nerves emanating from the larval brain. The distribution of Trol over the entire brain was further verified by immunohistochemistry using an anti-Trol antibody (Figure 2.7F). This localization of the Trol protein is consistent with the model that Trol binds Bnl and Hh and facilitates their signaling to promote neuroblast proliferation, as the regulated neuroblasts are found at the surface of the cellular cortex in both the brain lobes and the ventral ganglion (Datta, 1995; Truman and Bate, 1998; White and Kankel, 1978). To determine if the localization of Trol-GFP to the basal lamina was unique to the larval brain, we examined Trol-GFP in the salivary glands. As in our larval brain studies, Trol-GFP is found on the surface of the gland, presumably as a component of the basal lamina (Figure 2.7G).

Effects of trol mutations on larval hemocyte number

A second system where we thought *trol* might have an effect on development is the production of hemocytes during larval life. A number of studies have elegantly shown that the larval lymph gland is the source of larval hemocytes (Evans et al., 2003). In the primary lobe of the third instar lymph gland prohemocytes arise in the medullary zone while maturing hemocytes are found in the adjacent cortical zone. Hemocytes are

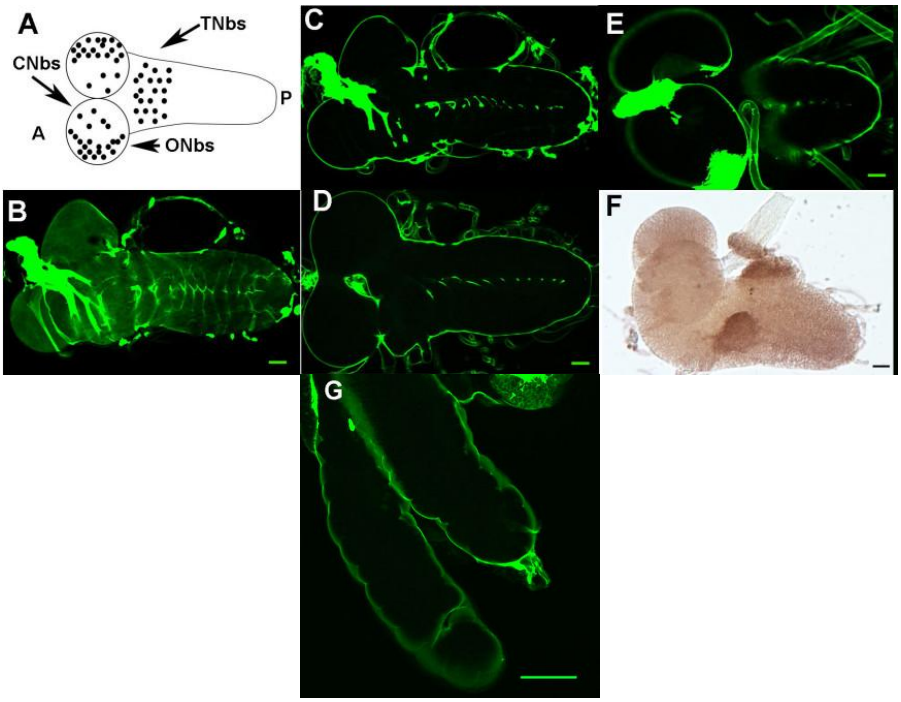


Figure 2.7. Localization of Trol-GFP in the larval brain. (A) Schematic of neuroblast position in the larval brain. A = anterior, P = posterior, ONbs = optic lobe neuroblasts, CNbs = central brain neuroblasts, TNbs = thoracic neuroblasts. In panels B-F, Anterior is to the left, Posterior is to the right. (B) Optical section of Trol-GFP brains at first instar, brain surface, scale bar indicates 10 um for both panels B and C. (C) Trol-GFP localization in first instar internal section. (D) Trol-GFP localization in second instar internal section, scale bar indicates 15 um. (E) Trol-GFP localization in third instar internal section, scale bar indicates 25 um. (F) First instar brain stained with anti-Trol antibody, showing staining over the entire surface of the brain. Scale bar indicates 10 um. (G) Trol-GFP localization in internal section of third instar salivary gland. Scale bar indicates 25 um.

then released into the hemolymph and are present as three types of circulating cells: plasmatocytes (95%), lamellocytes (1–5%) and crystal cells (rare). Each cell-type has characteristic morphology and can be easily identified under a compound microscope. Mature circulating larval hemocytes are still undergoing cell division, albeit at a low rate, as shown by staining of hemocytes with phosphohistone H3, an M phase marker (Asha et al., 2003; Qiu et al, 1998). Expression of an activated Ras (Ras^{v12}) in circulating hemocytes increases the percentage of circulating hemocytes that stain for phosphohistone H3 and results in a 40-fold increase in the number of hemocytes through activation of the Ras-MAPK pathway (Asha et al., 2003). The Ras-MAPK pathway is activated by Vascular Endothelial Growth Factor (VEGF) and Platelet Derived Growth Factor (PDGF) among others. Signaling by mammalian homologs of both growth factors has been linked to mammalian Perlecan (Iozzo, 2005). Furthermore, studies of PDGF/VEGF receptor (*PVR*) in *Drosophila* revealed that *PVR* is expressed in plasmatocytes and that decreased PVR function leads to increased hemocyte cell death (Evans et al., 2003). Thus it seemed likely that mutations in *trol* could decrease PDGF/VEGF signaling in circulating plasmatocytes, resulting in decreased numbers of circulating plasmatocytes in *trol* mutants. To address this hypothesis, we determined the relative number of circulating plasmatocytes in third instar $trol^{b22}$ or $trol^{7}$ and sibling control larvae (Figure 2.8A). Our analysis demonstrates a significant ($p < 0.05$) drop in the number of plasmatocytes in *trol* mutant versus sibling control larvae.

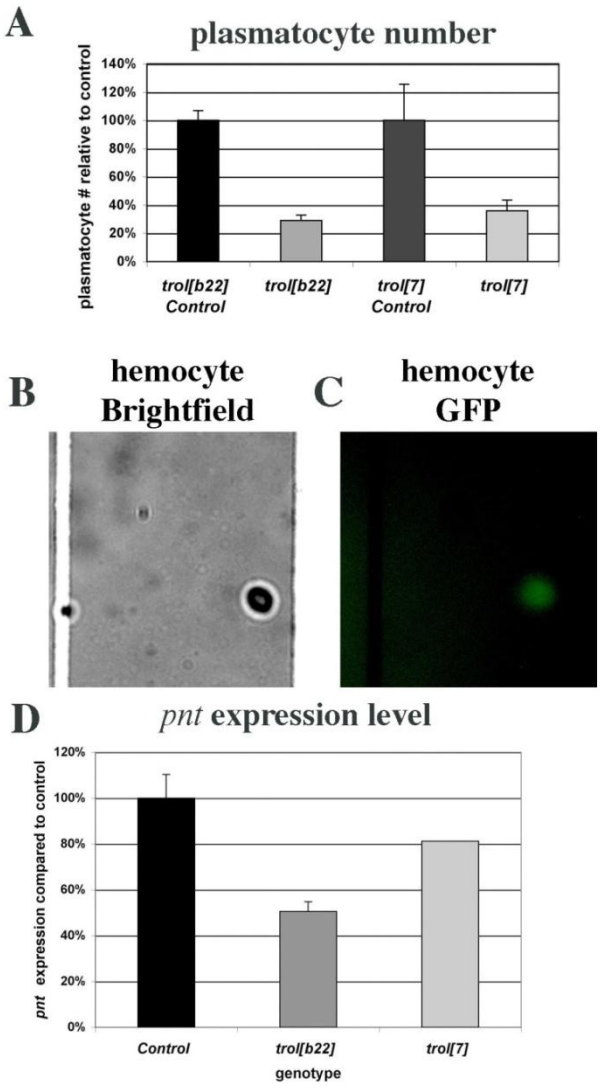


Figure 2.8. Mutations in *trol* decrease circulating plasmatocyte number and *pnt* expression. (A) Quantification of circulating plasmatocytes in $trol^{b22}$ and $trol^{7}$ mutants compared to controls. Each sample consisted of hemolymph pooled from three third instar larvae. Five squares were counted for each sample. Each genotype was analyzed in triplicate. (B) Brightfield image of plasmatocytes from Trol-GFP stock. (C) Fluorescence image of plasmatocytes from Trol-GFP stock demonstrating presence of Trol on plasmatocytes. (D) Expression of the VEGF/PDGF response gene *pnt* in $trol^{b22}$ and $trol^{7}$ mutant hemocytes compared to sibling controls by qRT-PCR. Samples of hemolymph from three third instar larvae of each genotype were pooled, RNA extracted, amplified and analyzed. All reactions were carried out in triplicate at three different template concentrations to ensure amplification was in the linear range. β-*actin* was used as an internal normalization control.

Trol localization and function in hemocytes

The decrease in the number of circulating plasmatocytes in *trol* mutants versus controls suggested that *trol* might indeed function to promote Ras-MAPK signaling by PDGF/VEGF in circulating plasmatocytes. This predicts that Trol protein would be localized on these plasmatocytes. We used Trol-GFP protein trap to examine the plasmatocytes for the presence or absence of Trol protein. Fluorescence microscopy revealed that Trol-GFP is indeed found on circulating plasmatocytes in third instar larvae (Figure 2.8B-C), but not in the lymph gland (data not shown).

This result is consistent with the requirement for Ras-MAPK activation in plasmatocytes for plasmatocyte proliferation and for PVR in plasmatocytes to avert apoptosis, and supports the hypothesis that Trol modulates PVR-Ras-MAPK signaling in plasmatocytes. The ETS-transcription factor *pnt* is a MAPK-response gene and will drive plasmatocyte proliferation (Zettervall et al., 2004). Therefore we asked if *trol* mutant plasmatocytes show decreased levels of *pnt* compared to controls. Plasmatocytes were collected by bleeding third instar $trol^{b22}$ and $trol^{7}$ mutant larvae and sibling controls, RNA was extracted and amplified, and subjected to qRT-PCR analysis. qRT-PCR studies demonstrated that plasmatocytes isolated from either $trol^{b22}$ or $trol^{7}$ mutants show decreased expression of *pnt* compared to controls, further evidence that *trol* modulates Ras-MAPK signaling in plasmatocytes (Figure 2.8D).

Trol and other growth factor signaling pathways

Two other growth factor signaling pathways that have been linked to HSPGs are the *wingless* (*wg*/Wnt) and *decapentaplegic* (*dpp*/TGFβ) signaling pathways. Both of these pathways are active in the developing *Drosophila* eye disc and/or third instar brain along with Hh and Ras-MAPK signaling (Kaphingst et al., 1994; Silver and Rebay, 2005). To ask if Trol might modulate the Dpp and Wg pathways we evaluated the expression of *dpp* and *wg* and their target genes *spalt major* (*salm*, de Celis and Barrio, 2000) and *sloppy paired* (*slp*, Bhat et al., 2000), respectively, in second instar ventral ganglia and third instar brains and eye discs from *trol* mutant larvae by qRT-PCR (Figure 2.9A-B). We also assayed expression of *hh* and its response gene *ptc* in third instar brains and eye discs. In the $trol^{b22}$ second instar ventral ganglion we observed a significant drop in the level of both *dpp* and *wg* compared to controls. The $trol^{b22}$ mutation also resulted in diminished signaling efficiency by both growth factors as indicated by a larger drop in the level of their response genes *salm* and *slp* compared to the ligands themselves. In contrast, the $trol^{sd}$ mutation decreased only *dpp* expression, but the efficiency of both *dpp* and *wg* signaling was impaired. Thus in the second instar ventral ganglion, wild-type function of *trol* appears to be required for normal signaling by *hh*, *bnl*, *dpp* and *wg* (Figures 2.6 and 2.9A). The decreased expression of *dpp* and *wg* in $trol^{b22}$ mutants and of *dpp* in $trol^{sd}$ mutants may be due to secondary effects on *dpp* and *wg* expression caused by the changes in Hh and Bnl signaling in trol mutants. Alternatively, decreased expression of *dpp* and *wg* could be due to positive feedback between Dpp signaling and *dpp* expression and Wg signaling and *wg* expression,

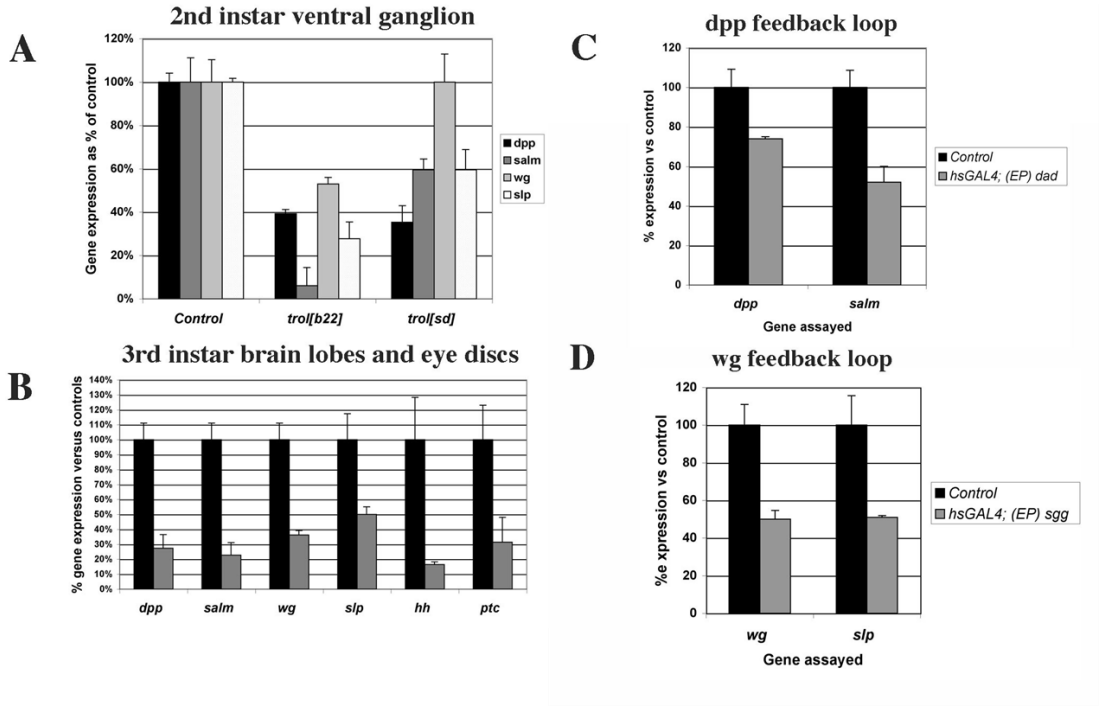


Figure 2.9. Dpp, Wg and Hh signaling are affected in *trol* mutants in second instar brains but not in third instar brain lobes/eye discs. qRT-PCR analysis of (A) the expression levels of *dpp*, its response gene *salm*, *wg* and its response gene *slp* in the ventral ganglia of second instar larvae. Data for the Hh response gene *ptc* is shown in Fig. 2.3. (B) Expression levels of *dpp*, its response gene *salm*, *wg* and its response gene *slp*, *hh* and its response gene *ptc* in *trol*^{sd} mutant third instar brain lobes/eye discs. (C) Expression of *dpp* and *salm* in *hsGAL4/+; +/(EP)dad* second instar ventral ganglia and *hsGAL4* controls. (D) Expression of *wg* and *slp* in *hsGAL4/+; +/(EP)sgg* second instar ventral ganglia and *hsGAL4* controls. In all panels, error bars indicate standard error. All reactions were carried out in triplicate at three different template concentrations to ensure amplification was in the linear range. β-*actin* was used as an internal normalization control.

respectively. To test the latter possibility, we blocked Dpp signaling by over-expression of *daughters against dpp* (*dad)* (Tsuneizumi et al., 1997), and assayed for *dpp* message levels (Figure 2.9C). *(EP)dad* females were crossed to *hsGAL4* males to drive expression of *dad*. Embryogenesis and first instar larval development were carried out at 18°C to limit expression of *dad* and inhibition of Dpp signaling at early stages. Upon molt to second instar, larvae were moved to 25°C for one hour to induce expression of *dad*. Larval brains were dissected and the ventral ganglia harvested for RNA isolations. Inhibition of Dpp signaling was confirmed by analysis of *salm* mRNA levels. Similarly, we inhibited Wg signaling by over-expression of *shaggy* (*sgg*), and assayed for *wg* message levels (Figure 2.9D). Decreased Wg signaling was verified by analysis of *slp* expression levels. As shown by our qRT-PCR analysis, inhibition of Dpp signaling by over-expression of *dad* resulted in a drop in expression of the *dpp* ligand itself. Inhibition of Wg signaling by over-expression of *sgg* also produced a drop in the expression of *wg*. As these studies were conducted in flies wild-type for *trol*, they eliminate the possibility that the decreased expression of *dpp* and *wg* in *trol* mutants was due solely to reduced Trol-mediated signaling by Hh and/or Bnl. These data indicate the presence of a positive feedback loop for Dpp and Wg in the ventral ganglion.

To determine if Trol is necessary for growth factor signaling in other tissues at other stages we assayed for *dpp*, *wg* and *hh* expression and activity in $trol^{b22}$ and $trol^{sd}$ third instar brain lobes and eye discs. No significant changes in either growth factor expression or signaling were observed in $trol^{b22}$ samples (data not shown). In $trol^{sd}$ samples, expression of all three growth factors decreased by 65–85%, as did the

expression of their response genes (Figure 2.9B). The sole exception is *wg/slp*, where *wg* expression decreased about 65% and *slp* expression decreased only about 50%. These data indicate that mutations in *trol* do not dramatically decrease the signaling efficiency of Dpp, Wg or Hh in third instar brain lobes and eye discs, unlike the effect of those same *trol* mutations in second instar.

Conclusion

trol and Drosophila development

We have previously demonstrated that mutations in *trol* prevent the onset of neuroblast division in the first instar brain and that most *trol* mutations are lethal. Mutations in a second gene, *anachronism*, also affect the onset of neuroblast proliferation but in the opposite manner: in *anachronism* mutants, mitotically regulated neuroblasts begin cell division too early (Ebens et al., 1993). However, when a lethal *trol* mutation was combined with a viable allele of *anachronism*, the lack of neuroblast division was rescued (double mutants exhibited the *anachronism* phenotype of premature neuroblast division) but lethality was not (Datta, 1995). This outcome suggested that *trol* function is required for other developmental events necessary for survival. Further analyses revealed that *trol* modulates Hh and Bnl signaling in the first instar brain (Park et al., 2003). Here we have demonstrated that *trol* function is required for developmental progression to third instar and for pupariation. Analogous to its function in the first instar brain, *trol* is required to initiate the division of a second, independent and spatially distinct population of neuroblasts in the second instar brain

(Table 2.1, Figure 2.1). This initiation of division is also dependent on Bnl and Hh signaling (Figure 2.3). Both growth factor ligands are present and signaling in the thoracic region at the proper time (Figure 2.2), and Trol is required to modulate their signals during neuroblast reactivation (Figure 2.5). We have also demonstrated that the Trol protein is localized to the surface of the brain at all larval stages, which places it in close proximity to the regulated neuroblasts. This localization is consistent with our model where Trol regulates Bnl and Hh signaling to cells adjacent to the regulated neuroblasts (Figure 2.4) by binding the growth factors directly (Park et al., 2003). Trol protein localization to the basal lamina is not limited to the larval brain, as Trol-GFP studies also showed Trol protein in the basal lamina surrounding the salivary glands (Figure 2.7G). *trol* function is not limited to the nervous system, as mutations in *trol* also diminish the number of circulating plasmatocytes by decreasing expression of *pnt*, a PVR response gene in plasmatocytes (Figure 8). We speculate that *trol* may be necessary for signaling by the *Drosophila* PDGF and/or VEGF growth factor, just as mammalian Perlecan has been shown to function during angiogenesis (Iozzo, 2005). Our studies of Dpp and Wg indicate a positive feedback between *dpp* expression and Dpp signaling and *wg* expression and Wg signaling in the second instar ventral ganglion. Signaling by Dpp and Wg is also dependent on *trol* in the second instar brain, but not (or very little) in the third instar brain lobes and eye discs (Figure 2.9), despite the fact that Dpp and Wg signaling are taking place in those tissues. In fact, even Hh signaling appears to be independent of *trol* in this context. These results highlight an important concept in *trol*, and indeed, in proteoglycan function: that the Trol protein will be used at

different times and places to regulate the signaling of different growth factors. Deciphering the role of *trol* in different developmental decisions will require that we examine each event individually, as *trol* will not necessarily mediate the same molecular mechanism each time.

Involvement of HSPGs in growth factor signaling

The requirement for heparan sulfate proteoglycans in signaling by different families of growth factors is well established (Hacker et al., 2005), but what is not yet clear is why different organs and tissue types use different HSPGs to modulate these signaling pathways. One possibility is that the specific mechanism(s) through which these molecules modulate signaling activity allows for site-specific variations in the regulation of signaling activity. HSPGs with varied amino acid sequence can act in the same signaling pathway, such as Syndecan-4 and Perlecan for FGF2 (Aviezer et al., 1997; Tkachenko et al., 2005) or Glypicans, Syndecan-3 and Perlecan for Hh (Park et al., 2003; Hacker et al., 2005; Shimo et al., 2004). Mutations that affect heparan sulfate synthesis or modification strongly affect FGF2 and Hh signaling (Hacker et al., 2005). Furthermore, Perlecan isolated from various endothelial cell sources has different binding affinities for FGF2 (Knox et al., 2002). These data initially suggested that the protein core of the HSPG might have little to do with signaling specificity and that the main functional domain of HSPGs is concentrated in the sequence of the heparan sulfate chains.

The carbohydrate-centric view is being challenged by studies that indicate a role for the protein-protein interactions of HSPGs with growth factors and other signaling molecules. For example, expression of chimeric molecules has shown that the cytoplasmic tail of Syndecan is specifically required for FGF2 signaling in addition to its heparan sulfate chains (Volk et al., 1999). Perlecan protein-protein interactions include the ability of Perlecan to bind growth factors and extracellular matrix molecules at various sites on its protein core. Further mechanisms that allow for differential regulation include processing of HSPGs. These studies suggest a reason for the use of a particular HSPG during an individual developmental decision – the flexibility of combining both carbohydrate-based regulation and protein-based regulation of cell-cell signaling may make a specific HSPG uniquely suited for a given situation.

In the context of combined carbohydrate and protein inputs into HSPG function, it becomes clear that a given HSPG may be expressed and function in very specific contexts that take advantage of its unique regulatory abilities. It is interesting to note that we have connected Perlecan with FGF and Hh signaling in the developing fly brain while mouse studies have shown that Perlecan knock-out mice have cerebral cortex abnormalities (Costell et al., 1999; Park et al., 2003; Datta, 1995). *trol* mutant larvae have decreased numbers of circulating hemocytes that are likely due to decreased Ras-MAPK signaling by VEGF/PDGF. Perlecan knock-out mice also have defects in chondrogenesis and cardiovascular development and mammalian studies have demonstrated a role for Perlecan in angiogenesis driven by FGFs, VEGF and PDGF (Iozzo, 2005). Finally, we have shown that Perlecan is required for SHH signaling

during human prostate cancer growth (Datta et al., 2006), which reveals a new system for the investigation of the mechanism of Perlecan action. Further analysis of the ability of HSPGs to substitute for each other in cell fate decisions and the means by which they individually regulate cell-cell communication will lead to a clearer understanding of the inputs necessary for cells to carry out a developmental or disease progression.

Methods

Fly stocks

Stocks of the viable *trol*^{b22} allele and the lethal *trol*^{4}, *trol*^{7}, *trol*^{8} and *trol*^{sd} alleles have been described previously (Park et al., 2003; Datta, 1995; Datta and Kankel, 1992; Park et al., 1998). All *trol* mutant stocks with the exception of *trol*^{b22} are *y trol*^{x}*w/Binsn* where the chromosome carrying the *trol* mutation is marked with *y* to facilitate identification of *y trol* mutant versus *y*$^{+}$control larvae. The *trol-GFP* protein trap was obtained from Dr. Stephane Noselli. The *bnl*^{06916} and *hh*^{AC} were obtained from the Bloomington stock center and used to construct *y ; bnl*^{06916}*/TM3y*$^{+}$ and *y ; hh*^{AC}*/TM3y*$^{+}$ stocks for genetic studies. The *bnl*^{P1}, *hh*^{P30}, *hs-hh*, *hh*^{ts2}, *ptc-lacZ*, *UAS-GFPlacZ.nls* and *Canton S* were obtained from the Bloomington stock center, Dr. Joan Hooper, Dr. Alan Michelson, Dr. Bruce Baker or Dr. Ginger Carney and used as described previously (Park et al., 2003).

Lethal phase

Early first instar larvae were collected and placed on apple juice plates with yeast. Each plate initially had 50 mutant or control animals per plate, segregated to prevent competition between mutant and wildtype siblings. Two plates of each genotype were examined. The number and stage of larvae still present on each plate were assayed every 24 hours and the survivors transferred to a fresh plate. Since none of the *trol* mutants with the exception of $trol^{b22}$ produce viable adults, individual animals were followed only until pupariation.

Developmental staging

Developmental synchronization was carried out as previously described (Park et al., 2003; Datta, 1995; Caldwell and Datta, 1998; Park et al., 2001). Flies were allowed to lay eggs on apple juice agar plates with fresh yeast overnight or for about 24 hours. For staging of synchronized first instar larvae, the plate was first cleared of any larvae and newly hatched larvae collected in one hour windows and placed on new apple juice plates with yeast at 25°C for aging. For staging of second instar larvae, late first instar larvae were placed on fresh apple juice plates with yeast. Newly molted second instars were collected in one hour windows and placed on apple juice plates with yeast at 25°C for aging or dissected immediately.

Proliferation assay

BrdU assays were carried out as previously described (Park et al., 2003; Datta, 1995; Caldwell and Datta, 1998; Park et al., 2001). Briefly, animals were fed BrdU-containing artificial medium for one hour, dissected in PBST and fixed with Histochoice (Amresco) for 10 minutes. Brain samples were denatured in PBST-HCl for 30 minutes, washed and blocked in PBNT for one hour. Primary anti-BrdU antibody (Becton-Dickinson) was added at 1:200 overnight at 4°C. Samples were washed and incubated with HRP-conjugated secondary antibody at 1:400 for 2–4 hours at room temperature. Signal was developed using a DAB substrate (Sigma).

Larval hemocyte assay

Hemocytes from three third instar larvae were harvested using a Pasteur pipette pulled to generate a capillary end, pooled and counted on a standard hemacytometer slide. Five 16-square regions were counted for each pooled sample. Three replicates were assayed for each genotype.

Quantitative RealTime PCR

Whole first instar brains or ventral ganglia dissected from the brains of second instar larvae were used for RNA isolation. For first instar brain samples, total RNA was isolated using Trizol (Invitrogen) following manufacturer's directions. Samples were DNAsed and reverse transcribed using oligo dT primers. The resulting cDNA was used to perform quantitative Real Time PCR with SYBR Green dye. For ventral ganglia

isolated during second instar RNA was extracted and the sequences amplified as described in (Klebes et al., 2002; Klebes et al., 2005). Hemocyte studies were carried out on pooled hemolymph from three third instar larvae per sample. RNA was extracted and amplified as for ventral ganglia. All qRT-PCR reactions were carried out in triplicate at three different template concentrations to ensure that we were within linear template range. Primer sequences are available upon request. *β-actin* expression was used as an internal control. Data were analyzed using the delta-delta calculation method to yield fold change compared to controls.

Statistics

Determination of significance was accomplished by use of Student's t test or ANOVA, depending on the design of the study.

Proper regulation of developmental pathways is essential both during development as well as throughout the lifetime of an organism. Abnormal signaling can have many negative consequences, including lethality during embryogenesis and disease later in life. In this chapter, I demonstrated that the heparan sulfate proteoglycan Trol is able to differentially regulate multiple signaling pathways in various developmental contexts. I showed that Trol can modulate the signaling of specific growth factors similarly in different developmental processes during *Drosophila* larval life. However, Trol also has a much different impact on the same signaling pathways in another developmental context in the larvae. Thus, regulation of developmental pathways in *Drosophila* by the heparan sulfate proteoglycan Trol helps to add to the ability of growth factors to influence such a wide range of biological processes. In the next chapter, I will examine how aberrant signaling can lead to neoplasia in the *Drosophila* ejaculatory bulb, and how this can model human prostate cancer.

CHAPTER III

THE *DROSOPHILA* EJACULATORY BULB IS A MODEL FOR PROSTATE CENCER

Background

This year, over 30,000 men will die from prostate cancer in the United States. The American Cancer Society estimates over 215,000 men in this country will be diagnosed with the disease in 2010, and the number rises every year. One out of every six men will be told they have prostate cancer sometime in their lifetime. If caught early, prostate cancer has a relatively good prognosis. In its early stages, the disease is androgen-sensitive, and there are several different effective treatment options available. However, prostate cancer becomes androgen-independent in later stages, and this invasive, metastatic form of the disease has a significantly lower survival rate. In fact, prostate cancer not identified until later stages has a five year survival rate of 31% (ACS). It is therefore tremendously important that the illness be caught early in its treatable stages. Consequently, understanding the steps that lead up to the onset and early stages of the disease is invaluable.

The biggest risk factor for prostate cancer is age. Greater than two-thirds of all cases diagnosed will be men over the age of 65. This factor makes the study of prostate cancer initiation difficult. One way in which to study the role of aging in the onset and progression of the disease is through the use of model systems. Mouse and rat model

systems that are able to generate spontaneous and metastatic tumors have been used to examine some of the features of prostate cancer progression (Roy-Burman et al., 2004; Pollard, 1998). However, these systems are costly and time consuming, taking years to form tumors (Pugh et al., 1994; Quinn, 2005). A short lived model system that is able to recapitulate the hallmarks of prostate cancer would be immensely beneficial.

Drosophila has been widely used as a model for aging. The fly has become an important aging model system due to its quick lifespan and high number of progeny. To go along with the short lifespan, *Drosophila* also has many of the same aging mechanisms as higher organisms. For example, like other animals, calorie restriction is able to increase the *Drosophila* lifespan by inhibiting the Insulin/Insulin like Growth Factor Pathway (Haigis and Guarente, 2006; Tatar et al., 2003). The availability of mutants that alter the lifespan in the fly, like *chico*, which similarly inhibits the IGF pathway, help make *Drosophila* a relatively simple and useful aging model system (Helfand and Rogina, 2003; Bauer et al., 2004). Furthermore, the lifespan of the fly can be precisely altered by adjusting temperature. Specific expression patterns of various age-dependent genes have been identified in the fly, and the evolutionary conservation of aging pathways has been demonstrated (Rogina and Helfand, 1995; Helfand et al., 1995). The ability to quickly and cost-effectively investigate aging processes similar to those in higher organisms, combined with the ability to manipulate gene expression, make *Drosophila* an excellent candidate to study age-related changes leading to the initiation of prostate cancer.

Many signaling pathways, such as the Sonic Hedgehog, Fibroblast Growth Factor, Wnt, and Transforming Growth Factor-β pathways, have been shown to be misregulated in prostate cancers. Curiously, studies have shown that the ability of tissues to respond to growth factor signaling diminishes with age (Cowan et al., 2003). Yet, in prostate cancer, increases in growth factor signaling occur despite the disease occurring in men of advanced age. One possibility for the differences in the response of diseased tissues to these signaling pathways is heparan sulfate proteoglycans (HSPGs). It is possible that altered expression of signaling regulators such as HSPGs may modify a cell's ability to receive growth factor signals. In the previous chapter, I demonstrated how the fly Perlecan homolog Trol is able to regulate signaling by various pathways in several different developmental contexts. The ability of Trol to differentially modulate signaling in multiple biological contexts during development suggests that it most likely has many roles during processes later in life, such as the initiation and progression of prostate cancer. In fact, Perlecan has been shown to be upregulated in advanced prostate cancer samples (Datta et al., 2006). In this chapter, I will examine the changes in heparan sulfate proteoglycan and signaling pathway expression in a short-lived model for prostate cancer, the *Drosophila* ejaculatory bulb.

Results and Discussion

The ejaculatory bulb has similar anatomy, function, and molecular markers as the male prostate

The benefits of establishing a short-lived and cost effective model system for the study of prostate cancer initiation and progression are great. *Drosophila* has been used extensively as a model for both development and disease, including cancer (Pagliarini and Xu, 2003; Brumby and Richardson, 2003; Igaki et al., 2009). To be an effective model for prostate cancer, *Drosophila* must have an organ with similar anatomy and function to that of the human prostate. In the prostate, sperm produced in the testes is mixed with fluid from the seminal vesicles. The mixture is then pumped out by the prostate upon ejaculation. The *Drosophila* ejaculatory bulb (EjB) has a very similar function and anatomy (Fig 3.1A). The EjB mixes sperm produced in the testes with seminal fluid secreted from the gland and pumps the fluid into the ejaculatory ducts (Lung and Wolfner, 2001; Ludwig et al., 1991). To be a good prostate candidate, the EjB should also express similar molecular markers to those found in the human prostate (Figure 3.1B). Dr. Anita Hernandez has shown that the EjB expresses fly homologs of human prostate specific markers *bagpipe* (*Drosophila* Nkx3.1 homolog), *acph-1* (Prostate-Specific Acid Phosphatase), and *shaven* (Pax2), as well as the prostate cancer biomarkers α-methylacyl CoA Racemase (AMACR) and *sprouty* (Sprouty1) (Tanaka et al., 1998; Biebericheet al., 1996; Rubin et al., 2002; Vihko et al., 1980; Kwabi-Addo et al., 2004; Khoubehi et al., 2001) (Hernandez and Datta, unpublished data). Taken together, this suggests that the fly ejaculatory bulb is the analog of the human prostate.

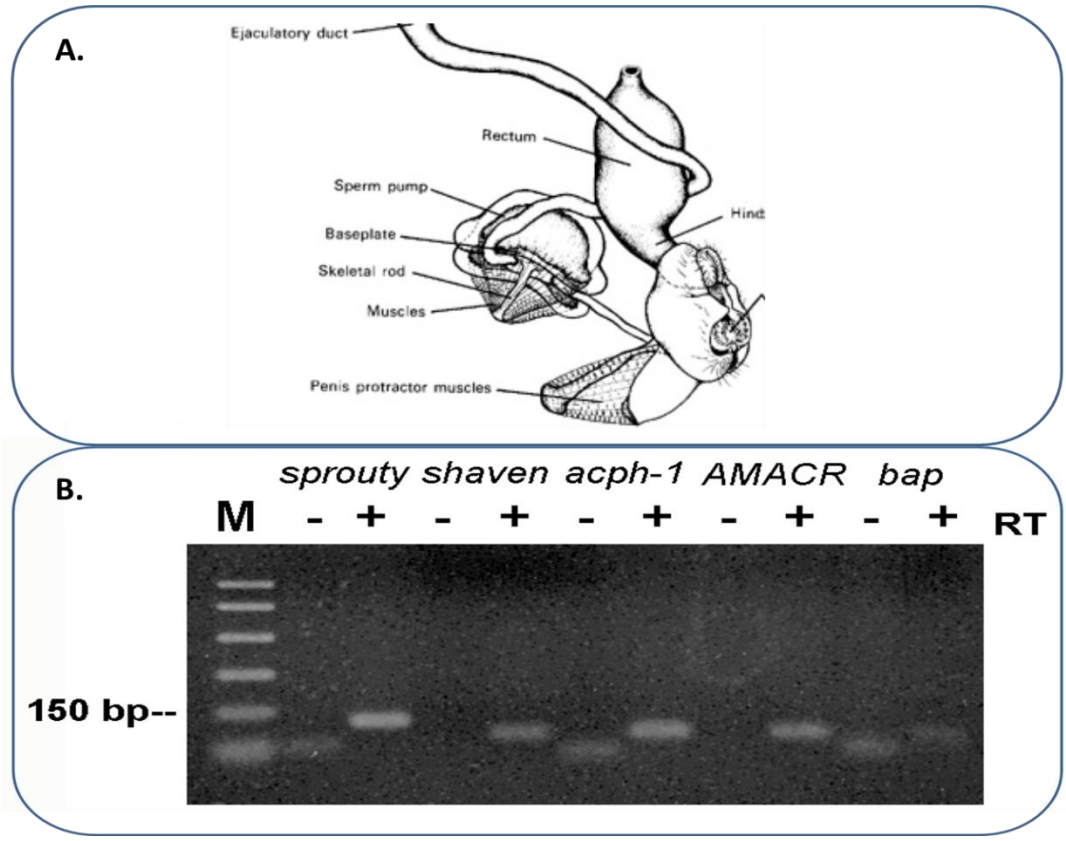


Figure 3.1. The ejaculatory bulb has similar anatomy and markers to the human prostate. (A) The detailed anatomy and connections of the *Drosophila* ejaculatory bulb. (B) PCR amplification off fly cDNA shows expression of the prostate biomarkers *shaven*, *acph-1*, and *bagpipe*, and also the prostate cancer biomarkers *sprouty* and *AMACR*. (Data from Dr. Anita Hernandez).

Growth factor signaling and heparan sulfate proteoglycans are important during prostate cancer progression (Datta et al., 2006; Chen et al., 2004; Sanchez et al., 2004). To reasonably function as a model for prostate cancer, the bulb should also express signaling pathway components and modulators known to function in the progression of prostate cancer. Both Branchless and Hedgehog are expressed in the EjB, as well as the HSPGs that are known to regulate growth factor signaling (Hernandez, data not shown). Signaling by Wnts has been implicated in various cancers, and TGF-β is known to act as a tumor-promoter in advanced prostate cancer (Logan and Nusse, 2004; Zhu and Kyprianou, 2005). To examine whether these ligands are present in the normal ejaculatory bulb, I looked at expression of the fly Wnt homolog, *wg*, and *dpp*, the *Drosophila* TGF-β. β-galactosidase activity staining on both *wg-lacZ* bulbs and *dpp-lacZ* bulbs 24 days post hatching confirmed the expression of the ligands in EjB (Figure 3.2). Therefore, the various signaling pathways that are known to be important in prostate cancer, as well as the HSPG's that modulate their signaling, are present in the normal ejaculatory bulb. We next wanted to determine if the *Drosophila* EjB could recapitulate another hallmark of prostate cancer, neoplasia.

Engineered and spontaneous models of bulb overgrowth

In order to function as an effective model for prostate cancer progression, the ejaculatory bulb must be able to mimic the overproliferation and tumor formation seen in cancer. Abnormal signaling by FGF and VEGF is critical for progression of prostate

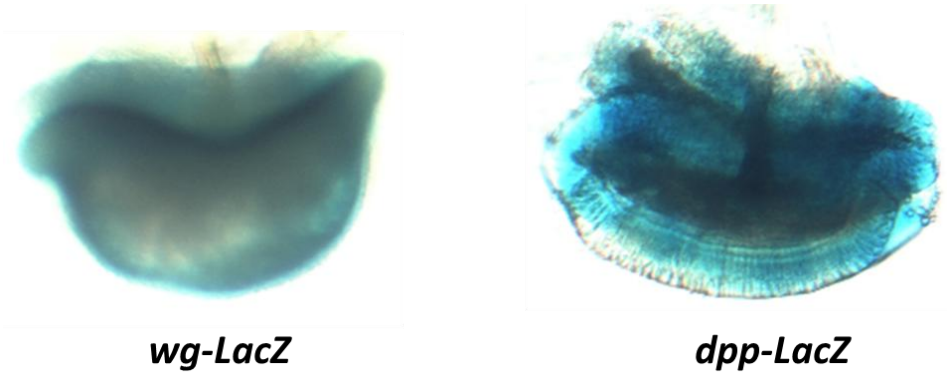


Figure 3.2. Expression of *wg* and *dpp* in the *Drosophila* EjB. ß–galactosidase activity staining on *wg-lacZ* bulb (left) and *dpp-lacZ* bulb (right). EjBs were dissected from males 24 days post hatching.

cancer, and both pathways signal through the Ras-MAPK cascade. The oncogene Ras is a member of that signaling cascade, and overexpression of Ras in *Drosophila* can produce overgrowth in larval tissues (Brumby and Richardson, 2003; Pagliarini and Xu, 2003).

In *Drosophila*, Ras can be overexpressed, and Ras-dependent signaling upregulated, using a constitutively active form of Ras, Ras^{V12}. Driving Ras^{V12} during the adult life of the fly causes epithelial overgrowth in the ejaculatory bulb (Figure 3.3A, Dr. Suma Datta). Inducing *UAS-Ras^{V12}* for 7 days with a heat shock-*Gal4* (hs*Gal4*) driver leads to a 100% frequency of overgrowth (data not shown). To test whether the overgrowth was due to a greater number of cells being present, DAPI staining was performed to determine nuclear distribution. Staining showed an increase in the number of nuclei in overgrown ejaculatory bulbs, with the distribution of cells being much more disorganized than that of control bulbs (Figure 3.3B, Dr. Suma Datta). Although Ras is not induced before the adult hatches, it is possible that the additional cells were generated during embryogenesis. To test whether the additional cells were proliferating during adult life, I performed 5-bromo2'-deoxy-uridine (BrdU) incorporation and staining on the hs*Gal4* Ras ^{V12}-induced flies. BrdU was applied topically once a day to adults undergoing Ras induction. After 3 days induction/ BrdU application, bulbs were stained to visualize nuclei that had undergone S-phase. BrdU incorporation showed cells were proliferating during Ras induction (Figure 3.3C, Lindner). Overexpression of oncogenic Ras increases the signaling activity of the Ras-MAPK pathway, which in turn results in expression and activity of its target

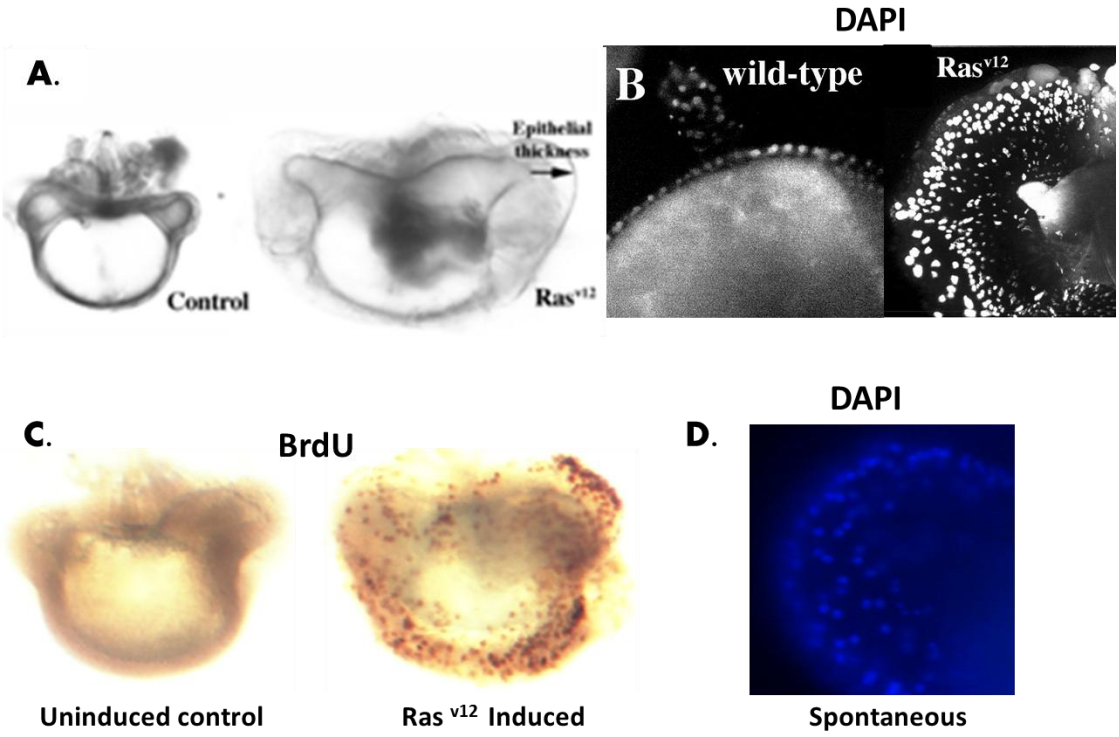


Figure 3.3. Ras induced and spontaneous overgrowth in EjBs. (A) Control ejaculatory bulb (left) and Ras^{V12} induced overgrowth after 7 day induction (right) (Data from Dr. Suma Datta). (B) DAPI staining to visualize nuclei in control and Ras induced bulbs (Data from Dr. Suma Datta). (C) Control 3 day BrdU incorporation with no Ras induction (left) and 3 day Ras^{V12} induction/BrdU incorporation (right, Lindner). (D) DAPI nuclear staining on spontaneous overgrowth EjB.

genes, including *pointed*. Therefore, overexpressing Ras may lead to a similar endpoint to that inhuman prostate cancer: upregulation of the ETS family of transcription factors. Engineered overgrowth in the *Drosophila* EjB is a promising model for prostate cancer neoplasia, and may be valuable in the study of how signaling affects prostate cancer progression.

Although an engineered overproliferation model can be enormously beneficial, it is still an artificial system. Manipulations made to gene expression may have unintended and unseen consequences. To study how signaling impacts the onset of prostate cancer, a spontaneous model for hyperproliferation would be extremely valuable. A single fly line with un-engineered, spontaneous overgrowth of the EjB resembling that of Ras^{V12} has been isolated. Enlarged bulbs in this line look similar to the overgrown Ras^{V12} bulbs. Genetic analysis of the spontaneous line showed that the overgrowth did not track with any single chromosome, suggesting the overgrowth phenotype is multigenic, similar to human prostate cancer. The frequency of overgrowth seen in the spontaneous line increases as the flies get older, suggesting there may be an age component to the phenotype (Datta, unpublished data). DAPI staining on spontaneous overgrowth EjBs 24 days after eclosion results in a similar pattern of nuclei seen in Ras^{V12} induced overgrowth (Figure 3.3D, Lindner), suggesting overgrowth in this line is also due to overproliferation of cells in the epithelial layer of the bulb. The spontaneous overgrowth *Drosophila* line may be an important model for understanding the onset and progression of prostate cancer.

Signaling in engineered and spontaneous overgrowth EjBs

Signaling by the Wnt, Transforming Growth Factor-β, Sonic hedgehog, and Fibroblast Growth Factor families of growth factors is critical for the proper development of the normal prostate (Freestone et al., 2003; Settle et al., 2001, Kwabi-Addo et al., 2004; Yardy and Brewster, 2005). However, misregulation and improper signaling by these pathways has been seen in different cancers (Li et al., 2003; Cronauer et al., 2003; Bale and Yu, 2001). For example, TGF-β acts as a tumor suppressor early in cancer progression, but it is an oncogene in later stage carcinomas (Steiner et al., 1994; Wikstrom et al., 1998; Kyprianou, 1999; Guo et al., 1997; Wakefield and Roberts, 2002). Sonic hedgehog and its signaling pathway modulator Perlecan have been shown to be upregulated in advanced prostate cancer as well (Sanchez et al., 2004; Datta et al., 2006; Datta and Datta, 2006). Signaling by Wnt is also increased in advanced, metastatic prostate cancer (Chen et al., 2004).

To determine if signaling is misregulated in our ejaculatory bulb prostate cancer models, I examined gene expression levels of four signaling ligands and their pathway response genes. Newly eclosed hs*Gal4*;*UAS-Ras*^{V12} males were induced for 7 days at 30^{o}C, followed by isolation of the EjB. Bulbs were also collected from 7 day aged but uninduced control flies. EjBs from newly eclosed (hatched) flies from the spontaneous overgrowth line were collected, as well as overgrown EjBs at 8 days, 16 days, and 24 days post hatching. RNA was isolated from the bulbs, cDNA generated, and expression levels determined by quantitative real time PCR (qPCR). Gene expression in the Ras^{V12} induced overgrowth bulbs was compared to the uninduced control EjBs (Figure 3.4A),

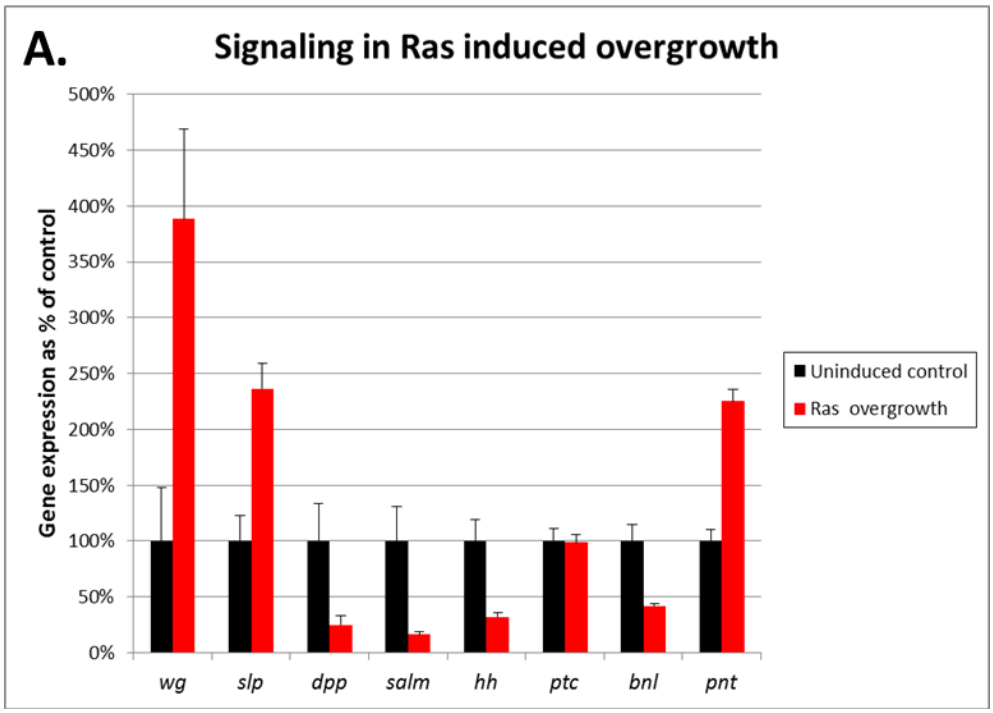


Figure 3.4. Signaling in Ras induced and spontaneous overgrowth EjBs. Gene expression levels in ejaculatory bulbs of four signaling pathway ligands and their pathway response genes as determined by quantitative real time PCR. (A) Expression in Ras^{V12} induced overgrowth bulbs compared to same-aged uninduced bulbs. (B) Expression in spontaneous overgrowth bulbs compared to newly eclosed flies from the same line. (C) Expression in the spontaneous line compared at 0, 8, 16, and 24 days to same-aged isogenic *yw* control flies. Error bars indicate standard deviation.

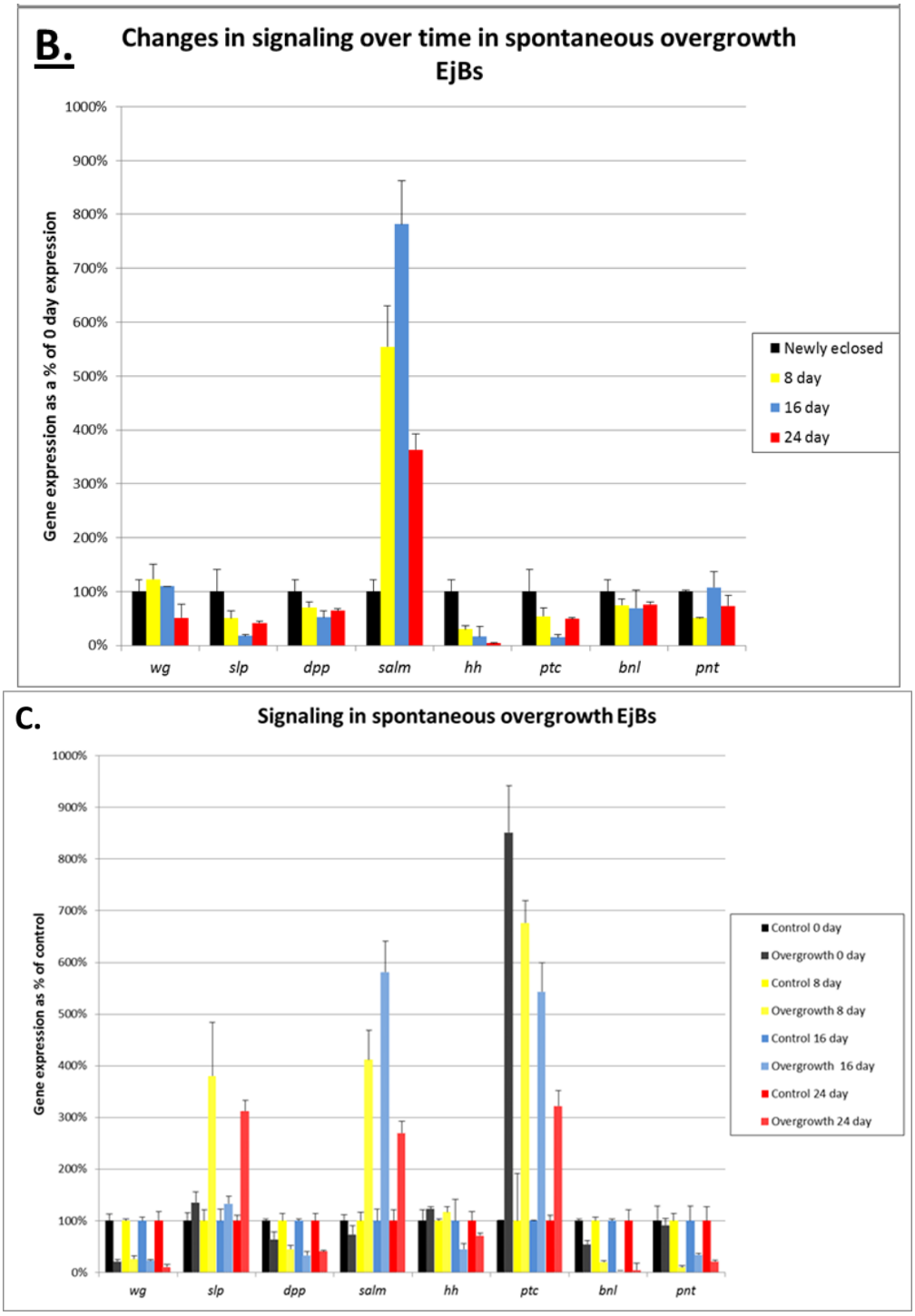


Figure 3.4. Continued.

while expression levels in the spontaneous overgrowth bulbs were compared to either newly eclosed spontaneous line bulbs (Figure 3.4B) or isogenic *yw* control EjBs the same age as the overgrown bulbs (Figure 3.4C). Ras^{V12} induced overgrown bulbs showed a 4-fold increase in *wg* expression, with a 2.5-fold increase in its response gene *slp*. *dpp* and its signaling readout *salm* both showed a sharp decrease in expression. Expression of the ligands *hh* and *bnl* were also down, but expression of the Hh signaling pathway readout *ptc* was not affected. *pnt* expression increased, but that is to be expected as *pnt* responds to the Ras-MAPK signaling cascade. Therefore, induction of Ras results in the stimulation of Wg and its signaling pathway, and upregulation of other pathways is not necessary for overgrowth in this model.

Examining the data from the spontaneous overgrowth bulbs compared to newly eclosed 0 day bulbs from the same line shows that expression of most of the signaling components either remains the same or decreases as the flies age (Figure 3.4B). The sole exception is the readout for Dpp signaling, *salm.* Expression of this gene increases approximately 4 to 8 fold over levels at the zero time point. Although TGF-β acts as a tumor suppressor in early prostate cancer, it changes roles and becomes an oncogene (Kyprianou, 1999; Guo et al., 1997; Wakefield and Roberts, 2002). Therefore, this upregulation in Dpp signaling resembles what is seen in human tumors. The decrease in Dpp ligand expression while signaling increases suggests that either Dpp signaling is more efficient, or another factor is influencing *salm*. When compared to *yw* control bulbs of the same age, the pathway response genes for the Wg, Dpp, and Hh pathways increase significantly at most timepoints. Expression of their ligands decreases,

suggesting again that another regulator of signaling is positively influencing these pathways (Figure 3.4C). The relative increase in expression for *slp* and *ptc* when compared to same aged controls suggests that these pathways are being downregulated in control EjBs as the flies age. In the spontaneous line, these pathways remain at the same level of signaling, possibly influencing overgrowth. It is also important to note that when compared to same age controls, *slp* expression is increased at 8 and 24 days. Wnt signaling is increased in advanced prostate cancers (Chen et al, 2004). Therefore, the increase in *slp* observed in both the Ras and spontaneous overgrowth EjBs resemble the Wnt upregulation observed in prostate cancer.

The differences in expression patterns between each model and the increases in pathway readouts independent of ligand expression indicates other factors are affecting signaling in the bulbs. To investigate what other factors might be influencing cell proliferation in these prostate cancer models, I looked at other known regulators of growth factor signaling.

HSPG expression in engineered and spontaneous overgrowth EjBs

Growth factor signaling relies heavily on regulation by heparan sulfate proteoglycans. HSPGs can impact distribution of ligands, as well as act as co-receptors in ligand/receptor interactions (Rapraeger et al., 1991; Gallet et al., 2003). In the previous chapter, I demonstrated the role one HSPG, Trol, plays in regulating signaling in various developmental processes. The human Trol, Perlecan, has been shown to be upregulated in advanced prostate cancers (Datta et al., 2006; Sanchez et al., 2004), and

other HSPGs are also known to impact cancer progression. Thus, heparan sulfate proteoglycans are good candidates for studying the regulation of signaling pathways during ejaculatory bulb overgrowth.

I examined heparan sulfate proteoglycan expression in a similar fashion as I did the signaling pathways. Overgrowth was induced in Ras^{V12} flies as explained in the previous section. Expression in 7 day Ras induced overgrowth bulbs was compared to uninduced controls. Expression in spontaneous overgrowth bulbs was compared to newly eclosed (0 day) same line controls to determine change in expression over time, or to *yw* same-age controls to determine overall changes in gene expression. In the Ras^{V12} induced overgrowth line, only *trol* expression goes up, increasing approximately 40% compared to the uninduced controls (Figure 3.5A). The other HSPGs, dally, dally-like protein (dlp), and syndecan (sdc), all showed a significant decrease in gene expression. HSPG expression in the spontaneous overgrowth bulbs showed a sharp decrease in expression for all four proteoglycans over the 24 days assayed (Figure 3.5B). However, when compared to same aged *yw* controls, the expression of *dally* at three of the four time points, and most notably *syndecan*, increased significantly (Figure 3.5C). *syndecan* expression reached a maximum of approximately an 8-fold increase over controls. This correlates with previous, unpublished data from Dr. Anita Hernandez that showed large decreases in HSPG expression in normal ejaculatory bulbs as they aged. The decrease in *sdc* expression in normal EjBs as they age more than offsets the decrease seen in the spontaneous line itself. The net result is more *syndecan* being expressed in the spontaneous overgrowth line compared to normal aging bulbs.

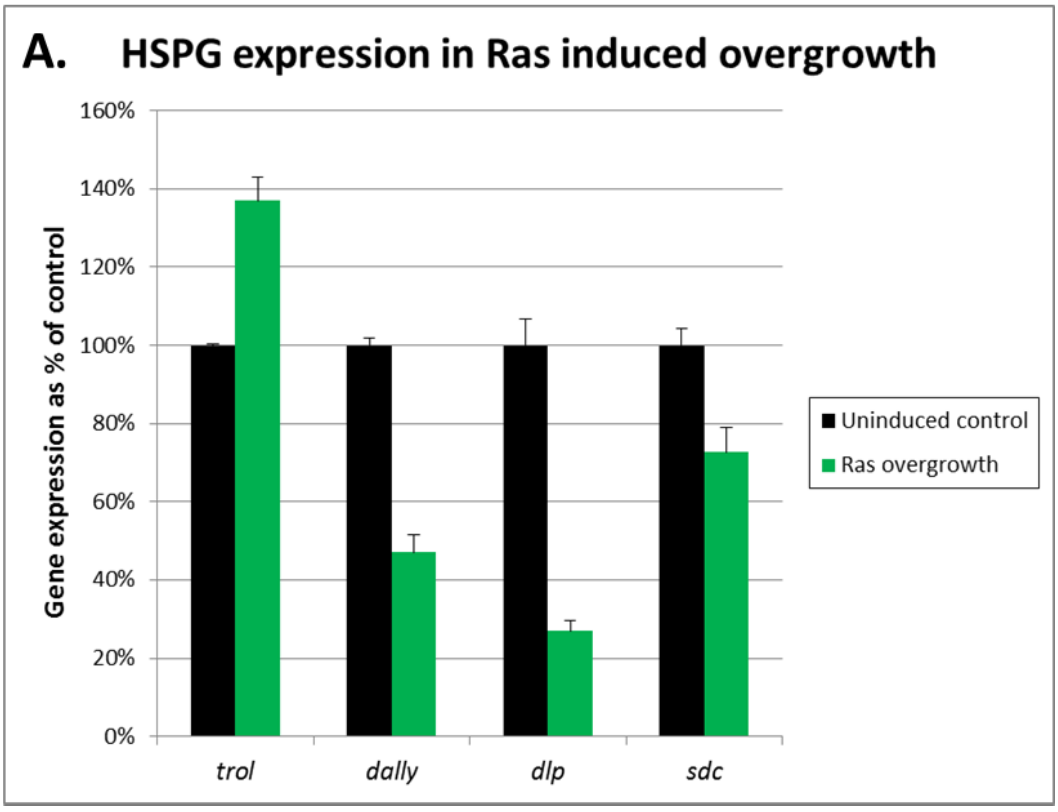
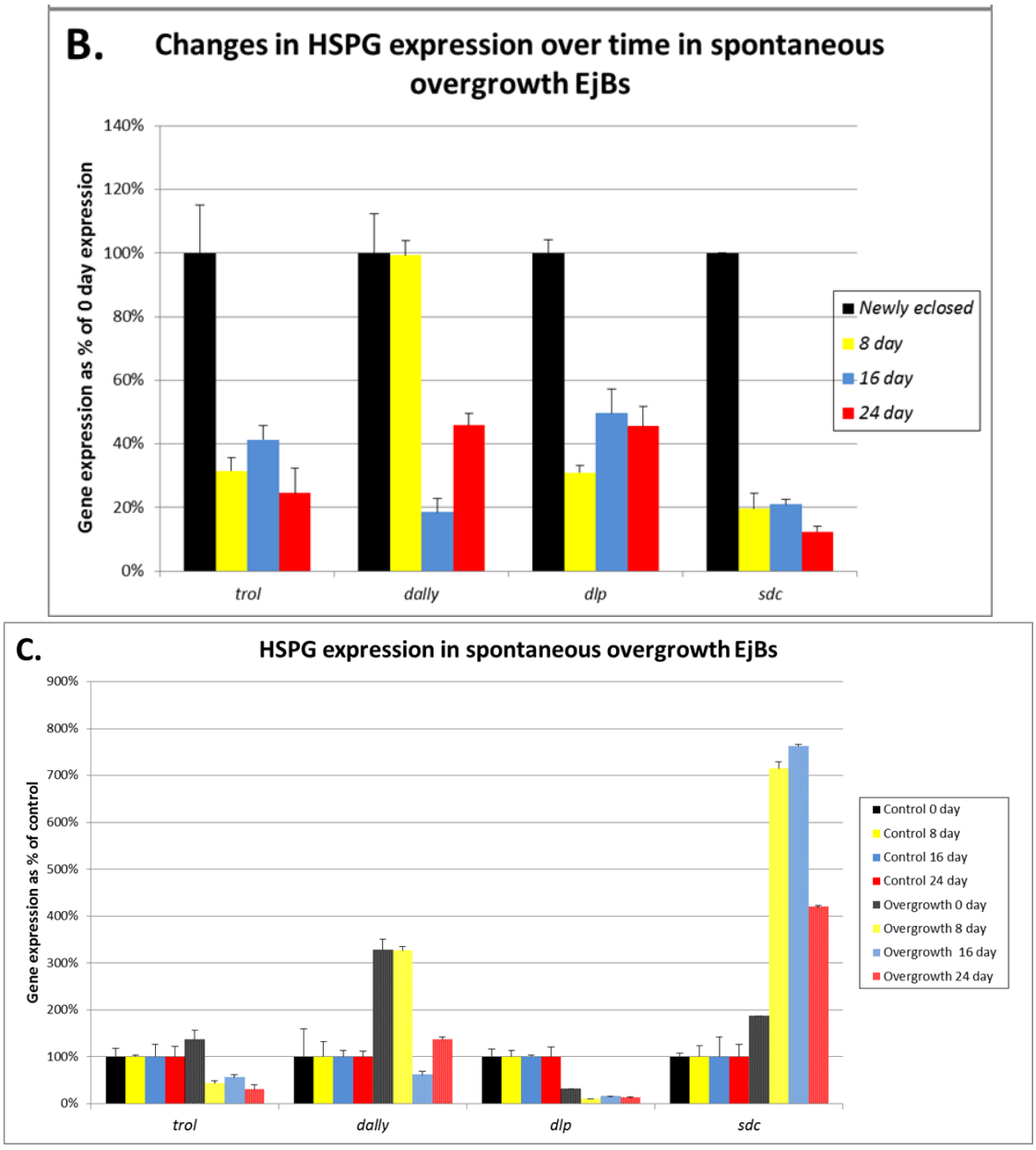


Figure 3.5. HSPG expression in Ras^{V12} induced and spontaneous overgrowth EjBs. Gene expression levels in ejaculatory bulbs of four HSPGs as determined by quantitative real time PCR. (A) Expression in Ras^{V12} induced overgrowth bulbs compared to same-aged uninduced bulbs. (B) Expression in spontaneous overgrowth bulbs compared to newly eclosed flies from the same line. (C) Expression in the spontaneous line compared at 0, 8, 16, and 24 days to same aged isogenic *yw* control flies. Error bars indicate standard deviation.



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Prostate specific marker expression in engineered and spontaneous overgrowth EjBs

The three prostate specific markers *acph-1, bagpipe,* and *shaven*, as well as two prostate cancer biomarkers, *sprouty* and *AMACR*, are all expressed in the *Drosophila* ejaculatory bulb. To further investigate how closely our two overgrowth models resemble human prostate cancer, I wanted to examine expression of these markers, and specifically the two prostate cancer biomarkers, in both the Ras^{V12} and spontaneous overgrowth EjBs.

I determined gene expression levels of the prostate markers by dissecting and collecting ejaculatory bulbs from Ras^{V12} induced flies, uninduced controls, spontaneous overgrowth flies at 0, 8, 16, and 24 days post hatching, and aged *yw* control flies. In the Ras^{V12} induced EjBs, expression of *acph-1* decreases slightly, while *bagpipe* and *shaven* saw a 1.5 and 2-fold increase in gene expression, respectively (Figure 3.6A). Expression of the prostate cancer marker *AMACR* was halved after a 7 day Ras^{V12} induction. The prostate cancer marker *sprouty* had a 2-fold increase, although this is most likely because *sprouty* can act as a Ras-MAPK response gene. In the spontaneous overgrowth line, all three prostate markers (*acph-1, bagpipe,* and *shaven*) generally decrease over the 24 days assayed (Figure 3.6B). The prostate cancer markers are expressed at higher levels after 24 days, and *AMACR* was markedly higher throughout the time assayed. This suggests that as the flies in the spontaneous overgrowth line age, the overgrown EjBs' increased expression of prostate cancer biomarkers and decreased prostate marker expression allows them to more closely resemble cancerous human prostates than normal glands. When compared to aged *yw* controls, expression of *sprouty*

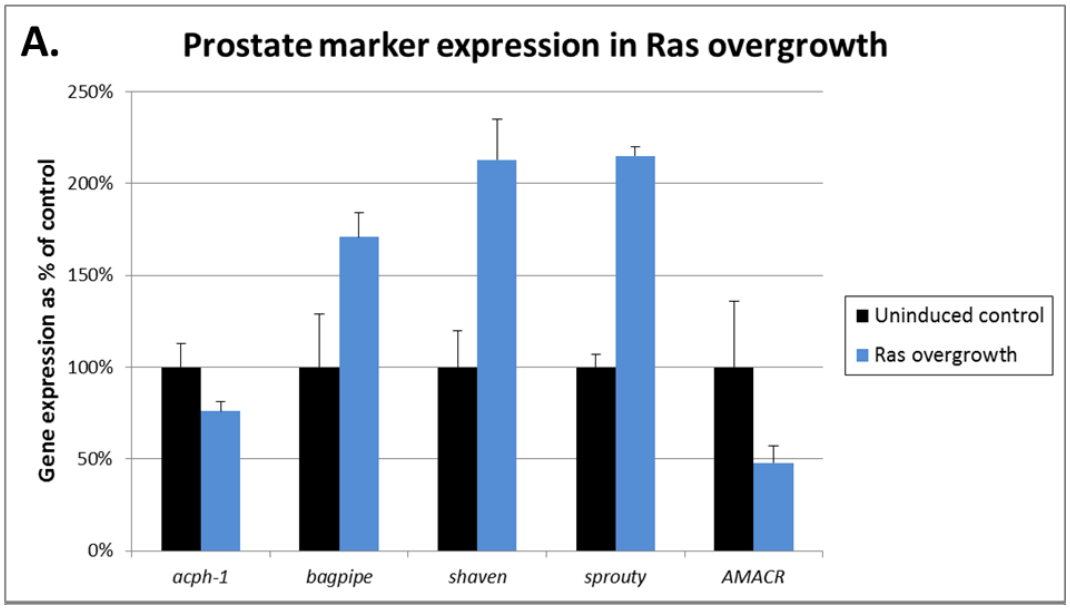
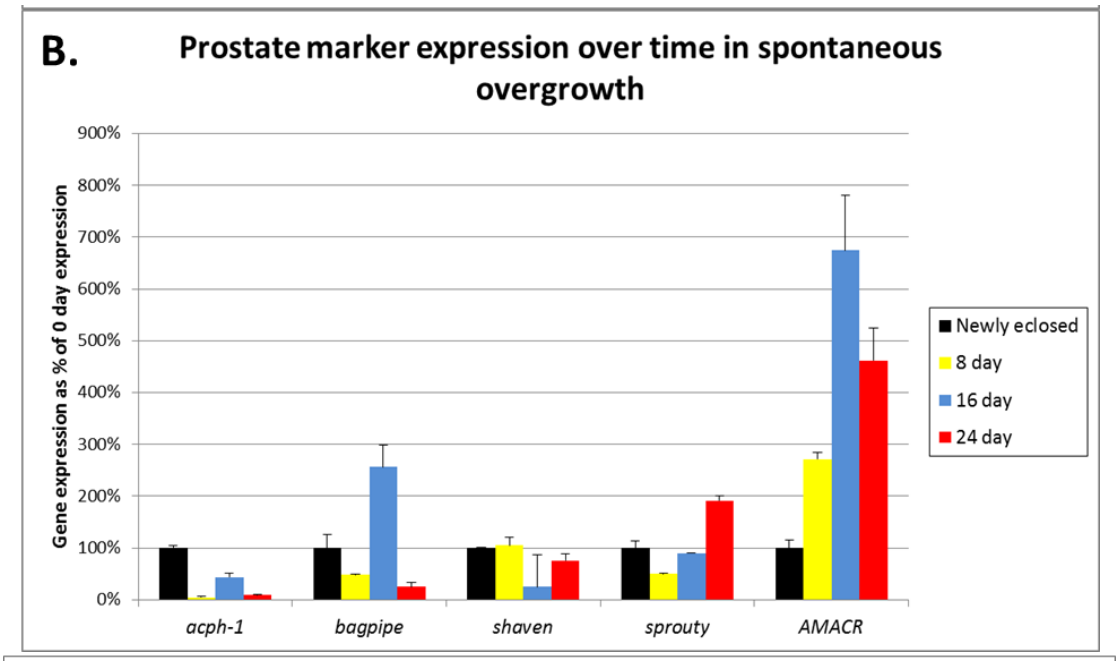
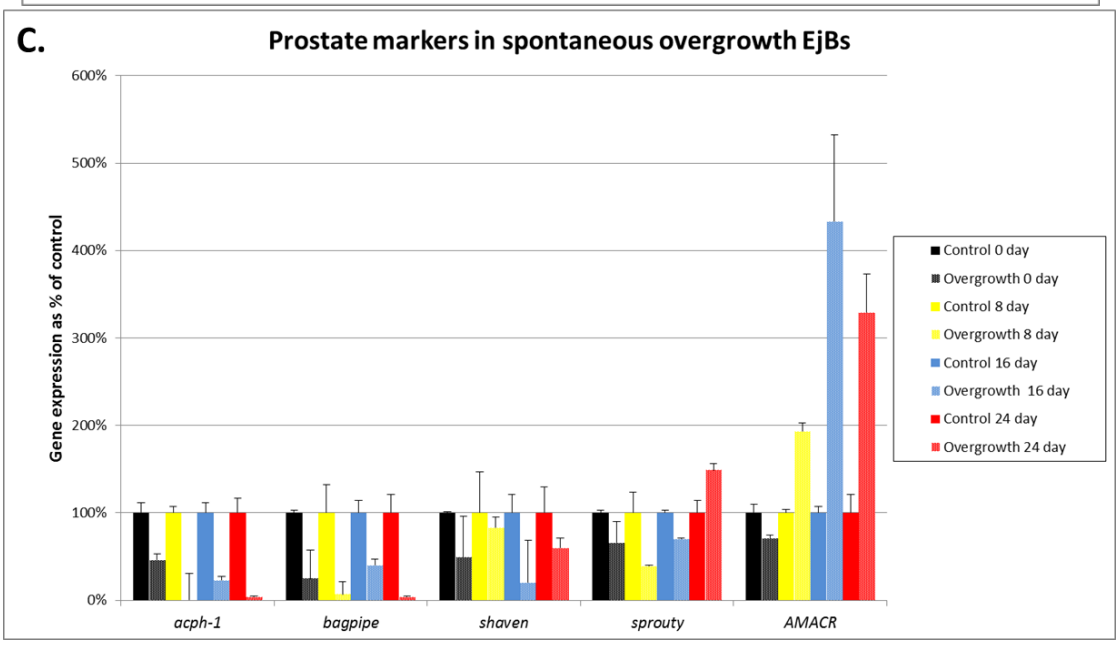


Figure 3.6. Expression of prostate and prostate cancer markers in overgrowth EjBs. Gene expression levels in ejaculatory bulbs of three prostate markers and two prostate cancer markers as determined by quantitative real time PCR. (A) Expression in Ras^{V12} induced overgrowth bulbs compared to same-aged uninduced bulbs. (B) Expression in spontaneous overgrowth bulbs compared to newly eclosed flies from the same line. (C) Expression in the spontaneous line compared at 0, 8, 16, and 24 days to same-aged isogenic *yw* control flies. Error bars indicate standard deviation.





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at 24 days and *AMACR* throughout the timecourse remains higher in overgrown bulbs than in controls (Figure 3.6C). Therefore, as the overgrown EjBs begin to resemble prostate cancer with an overproliferation phenotype, they also increase expression of biomarkers related to the disease.

ETS65A expression in prostate cancer models

One of the most consistently and highly overexpressed genes associated with prostate cancer is the ETS transcription factor ERG (Rostad et al., 2007). Upregulation of ETS transcription factors stems from a chromosomal rearrangement that places them under the control of androgen-responsive promoters active in the prostate early during cancer progression (Iljin et al., 2006; Mehra et al., 2008; King et al., 2009; Carver et al., 2009). Furthermore, altered expression of ETS transcription factors may play a role in tumor growth. In normal tissue, ETS transcription factors respond to Ras-MAPK signaling. Oncogenic Ras therefore leads to increased expression of the ETS genes. However, the chromosomal rearrangement alleviates the need for Ras to promote ETS expression, which may lead to cancer progression independent of Ras. The *Drosophila* homolog of the ERG transcription factor is ETS65A. I wanted to investigate whether this transcription factor that is highly overexpressed in prostate cancer is also upregulated in our overgrowth models.

I examined gene expression levels of the Drosophila ERG transcription factor *ETS65A* by qPCR. EjBs were collected from the same lines and at the same timepoints as described earlier. The expression of the transcription factor was five times higher in

hs*Gal4*; *UAS-Ras*^{V12} induced EjBs, which is to be expected since ETS factors are upregulated by Ras-MAPK signaling (Figure 3.7A). More interesting is the expression levels in the spontaneous overgrowth line. *ETS65A* was upregulated at every time point compared to newly eclosed EjBs, almost reaching the same level of relative expression seen in the Ras^{V12} induced bulbs (Figure 3.7B). This is exciting because neither *bnl* nor *pnt*'s expression is upregulated at any time I assayed in these bulbs, suggesting that *ETS65A* is being positively influenced by other factors in the spontaneous overgrown EjBs. The expression of the transcription factor is also upregulated in later ages when compared to same aged control bulbs (Figure 3.7C).

Another ETS transcription factor in *Drosophila* is Pointed. There are two different transcripts of pointed, P1 and P2. Both isoforms of Pointed contain an ETS oncogene domain, and P2 contains a second evolutionarily conserved domain (Klambt, 1993). To examine whether the ETS transcription factor Pointed could cause overgrowth without expression of the constitutively active Ras, I crossed both *UAS-pnt P1* and *UAS-pnt P2* separately to a hs*Gal4* driver. After a three day induction, overgrowth was seen in 27% of Pnt P1 induced ejaculatory bulbs, and 78% of Pnt P2 bulbs (Figure 3.8). After a seven day induction, 54% of Pnt P1, and 100% of Pnt P2 bulbs were overgrown compared to controls. At both time points, the thickness of epithelial overgrowth was greater in the Pnt P2 samples. Pnt-induced increases in epithelial thickness indicate that EjB overgrowth can be achieved without the overexpression of oncogenic Ras, similar to the way in which chromosomal

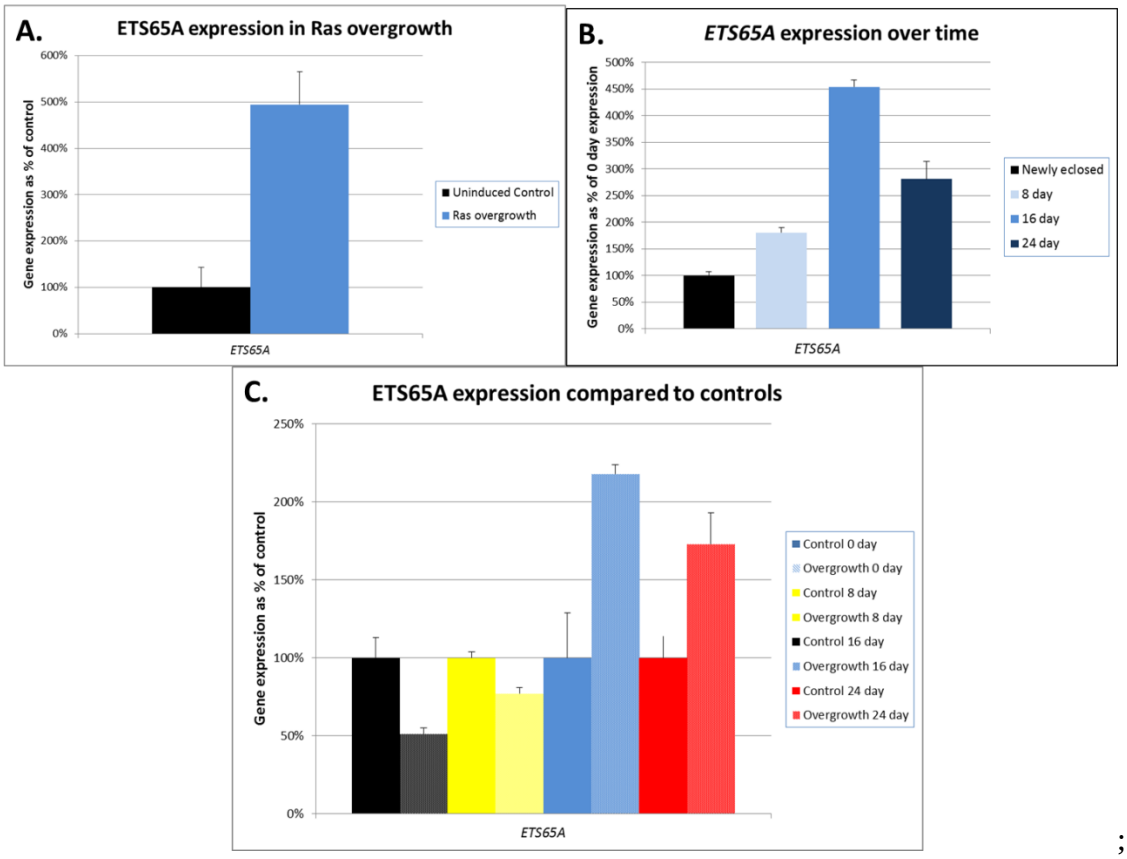

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Figure 3.7. ***ETS65A*** **expression in engineered and spontaneous EjB overgrowth.** Gene expression levels in ejaculatory bulbs of the ERG transcription factor *ETS65A* as determined by quantitative real time PCR. (A) Expression in Ras^{V12} induced overgrowth bulbs compared to same-aged uninduced bulbs. (B) Expression in spontaneous overgrowth bulbs compared to newly eclosed flies from the same line. (C) Expression in the spontaneous line compared at 0, 8, 16, and 24 days to same-aged isogenic *yw* control flies. Error bars indicate standard deviation.

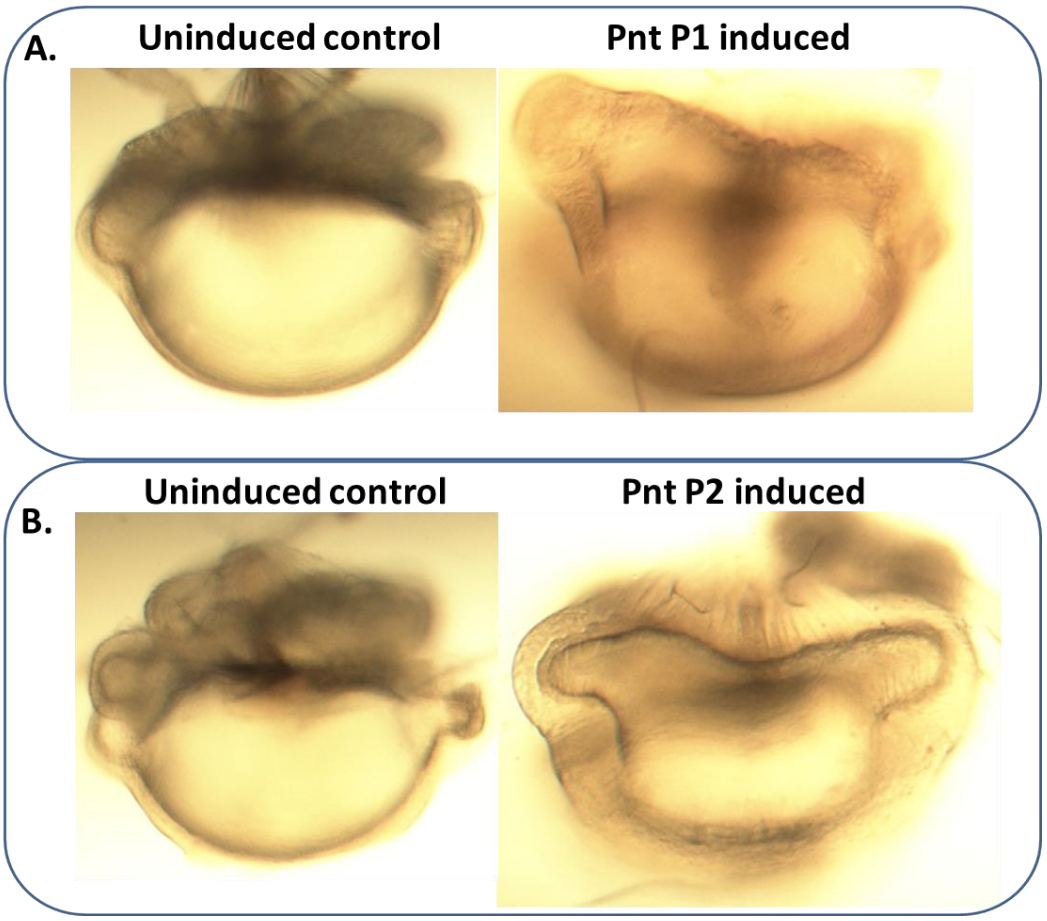


Figure 3.8. Pointed induced overgrowth in the EjB. hs*Gal4* flies were crossed to either *UAS-Pnt P1* (A) or *UAS-Pnt P2* flies to overexpress the ETS transcription factor. After a 3 day induction at 30^{o}C, 27% of Pointed P1 induced and 78% of Pointed P2 induced bulbs were overgrown. After 7 days of induction, 54% of P1 induced and 100% of the P2 induced bulbs showed overgrowth compared to uninduced controls. The PntP1 overgrowth was generally less severe than in the PntP2 induced EjBs.

rearrangements allow ETS transcription factors to influence prostate cancer progression independent of Ras in humans.

Age affects the morphology of Ras induced overgrowth

Induction and overexpression of Ras^{V12} in newly eclosed to 1 week old flies invariably results in uniform overgrowth of the epithelial layer. However, in bulbs where hs*Gal4* driven *UAS-Ras*^{V12} induction occurs after 2 weeks of non-induced aging, the EjBs begin to show signs of uneven overgrowth. Aging non-induced flies for 21 or 28 days prior to the onset of induction increases frequency and extent of irregular overgrowth, leading to a drastic variance between the epithelial thickness on either side of the ejaculatory bulb (Figure 3.9A). To examine what may be causing uneven overgrowth in flies aged before induction, I used qPCR to assay the expression of several different genes in EjBs that had been aged 7, 14, 21, and 28 days before Ras^{V12} induction, comparing expression levels to bulbs that were not aged before Ras induction began (7 day induction starting at 0 days). I tested the expression of *wg* and its signaling pathway response gene *slp*. *slp* expression is upregulated in both the Ras^{V12} engineered overgrowth (Figure 3.4A) as well as the spontaneous overgrowth (Figure 3.4C). While *wg* expression in the aged samples decreases compared to un-aged induced bulbs, expression of the gene remains above levels seen in uninduced controls. Thus, there remains a higher level of Wg ligand available in any Ras induced animal. Expression of the Wg response gene *slp* is increased when flies are aged before induction, compared to flies induced at eclosion. This upregulation is similar to expression seen in both the

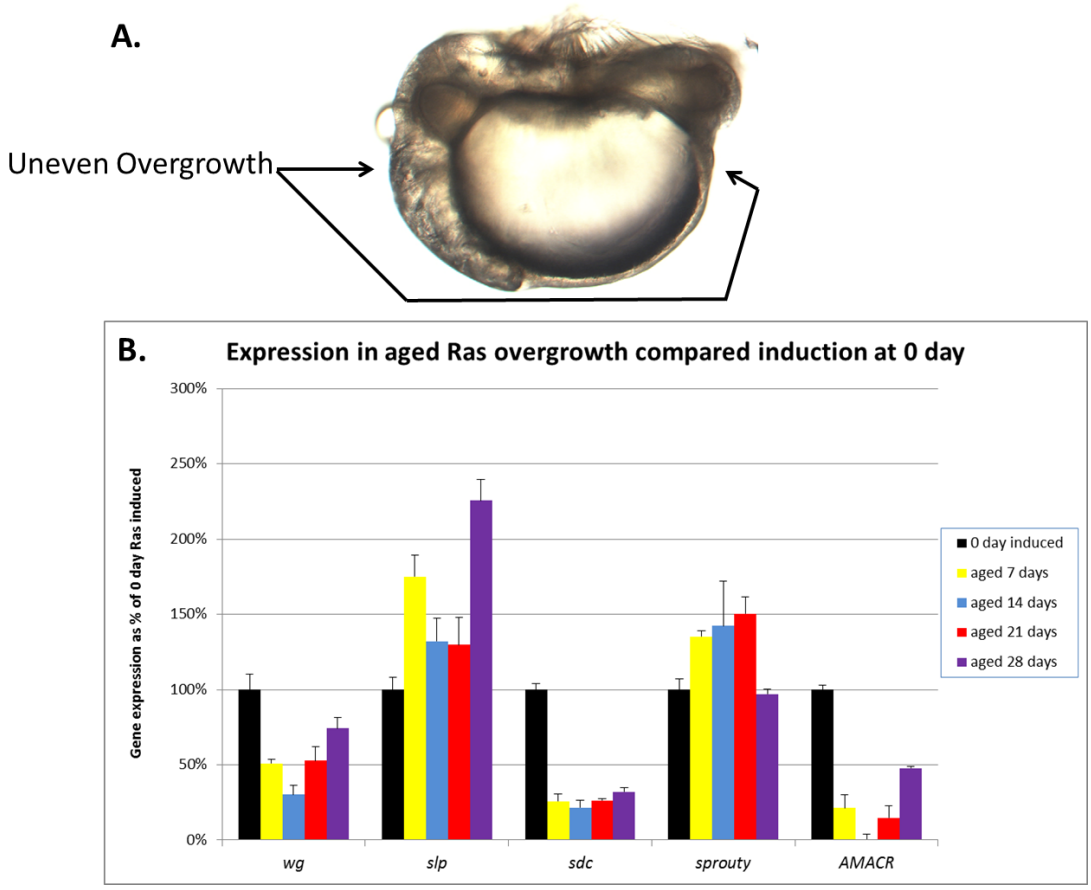


Figure 3.9. Overgrowth variability in EjBs aged before Ras induction. (A) EjB overgrowth in a fly aged 28 days prior to a 3 day Ras^{V12} induction. A difference in thickness of the epithelial overgrowth on each side of the bulb is seen. (B) Expression of *wg*, *slp*, *sdc*, *sprouty*, and *AMACR* in bulbs aged before Ras^{V12} induction compared to un-aged Ras induced bulbs. Error bars indicate standard deviation.

un-aged RasV12 induced and spontaneous overgrowth (Figure 3.9B). This suggests that the asymmetric overgrowth seen in flies aged prior to induction is not due to differential Wg signaling in older animals. I also assayed *sdc* expression, which is highly upregulated in the spontaneous but not the un-aged Ras^{V12} induced overgrowth (Figure 3.5). Similar to what is seen in un-aged engineered overgrowth, expression of the heparan sulfate proteoglycan is decreased. This continued downregulation of *sdc* suggests that the HSPG is not important for Ras induced ocergrowth at any age. I also tested the two prostate cancer biomarkers *sprouty* and *AMACR*. Both genes are upregulated in the aged spontaneous model (Figure 3.6C), but only *sprouty* expression is increased in the Ras^{V12} induced overgrowth (Figure 3.6A). Compared to the expression level in the un-aged Ras^{V12} induced flies, *sprouty* expression is upregulated in the 7, 14, and 21 day timepoints. This increase in expression compared to uninduced controls in all Ras induced bulbs regardless of age is most likely due to *sprouty* responding to Ras-MAPK signaling. *AMACR* expression is downregulated in all aged time points compared to the un-aged induction (Figure 3.9B), which itself had only half the expression levels of uninduced controls. In contrast with the spontaneous overgrowth model, the Ras induced model does not appear to resemble human prostate cancer in its expression of the *AMACR* prostate cancer marker. Taken together, this data suggests that flies aged before Ras^{V12} induction have similar expression patterns in the EjBs to flies induced immediately after eclosion. Although still higher than uninduced samples, aged bulbs have decreased *wg* expression, but are able to respond to Wg signaling slightly better than un-aged bulbs. This increase is not due to *sdc*, as expression of that

HSPG declines with age and is not influenced by Ras overexpression. This indicates another untested factor must be responsible for the irregular, uneven overgrowth seen in animals aged before Ras induction.

Transplantation of overgrown ejaculatory whether bulbs

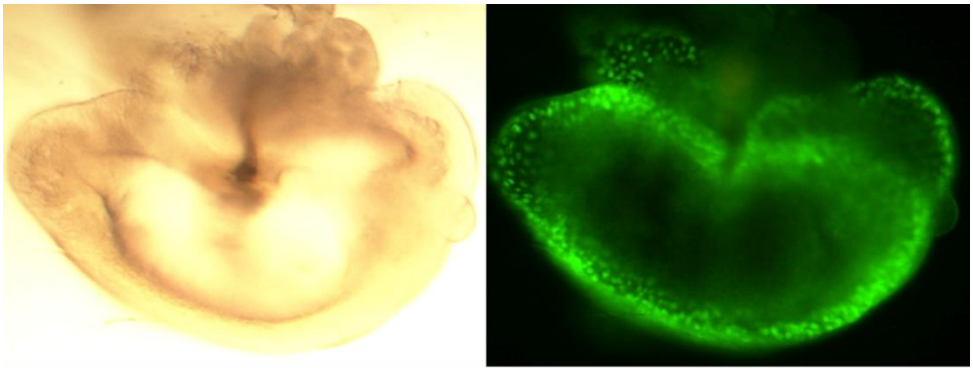
In order to be a complete model for prostate cancer, the *Drosophila* ejaculatory bulb should be able to model not only neoplasia, but also metastasis. Studies have shown that knocking down the apical-basal polarity genes *bazooka* and *scribbled,* combined with expression of a constitutively active form of the oncogene Ras, can cause metastasis in *Drosophila* larval tissue (Pagliarini and Xu, 2003; Brumby and Richardson, 2003). In order to follow metastasis from a tissue, the original tissue must be labeled. Unfortunately, an ejaculatory bulb specific Gal4 driver does not exist, and in the absence of a bulb specific driver, we cannot specifically target gene expression, or a GFP reporter, to the EjB.

In order to address this problem, explant culture methods have been developed. I am able to isolate EjBs expressing a *UAS-GFP* reporter driven by the non-specific hs*Gal4* driver. EjBs can be dissected out of the host and cultured in Schneider's fly cell culture medium. I am able to keep explanted bulbs alive for up to 5 days at 30^{o}C, as assayed by bulb pumping and expression of GFP. The benefit of the explant culture is that cells can be disrupted and introduced into a host that does not express the reporter. Transplanted cells can then be monitored in the host for formation of tumors.

Alternatively, I was able to develop a method to transplant full intact EjBs from a fly expressing *UAS-GFP* driven by hs*Gal4* to a host without the GFP marker. A small incision was made on the side of the abdomen of the host fly. The transplant bulb was then slid carefully inside the abdomen, and allowed to grow. I was able to keep the bulb alive in the host carcass for up to three days, as assayed by expression of GFP in the transplant bulb (Fig 3.10). This method could be used to express Ras and a reporter simultaneously with *scribbled* or *bazooka* RNAi in an EjB that has been transplanted into a wild type host. This would allow us to determine if expression of Ras combined with knockdown of the apical-basal polarity genes can lead to metastasis in adult tissue.

Conclusion

Understanding the basic mechanisms that lead to the onset of a disease is important for determining how best to treat and prevent it. Unfortunately, it is often difficult to study the initiation of a disease in human patients. Illnesses such as cancer frequently cannot be seen or diagnosed until after the disease has already established itself. Healthy patients are also not very eager to subject themselves to exploratory testing in search of early factors that are significant to the onset of disease. Elements that impact the initiation and progression of human prostate cancer can be particularly problematic. As an age dependent carcinoma, the extended amount of time that factors have to accumulate and influence initiation of prostate cancer can make their identification difficult. Due to these issues, model systems are required to accurately and fully examine the onset of this disease.



Ras^{V12} induced transplant EjB

Figure 3.10. EjBs can be transplanted into host flies. EjB that was removed from a host *yw* carcass 3 days after transplantation into the abdomen. The bulb was originally harvested from a hs*GAL4;UASRas*^{V12} */ UAS GFP* fly. Overgrowth and GFP expression localized to the nucleus can be seen.

Several models for human prostate cancer have been developed. Cell culture lines can be useful for examining the pathways and interactions present during the specific point in disease progression they represent. They also allow for biochemical and genetic manipulations that can be useful in understanding the basic mechanisms of disease. Whole organism model systems allow for the investigation of more complex interactions that occur between cells and tissues in their normal biological context. Rat and mouse models of prostate cancer have been developed in which tumor formation and metastasis can be studied (Pollard, 1998; Roy-Burman et al., 2004). However, age influences prostate cancer (Bostwick et al., 2004), and these systems can be exceptionally time consuming (Pugh et al, 1994). The establishment of a short-lived prostate cancer model that allows for genetic manipulation while still maintaining biological context of cells would greatly improve the study of factors influencing the initiation of the disease. In this chapter, I have described a *Drosophila* model for prostate cancer.

The *Drosophila* ejaculatory bulb is similar in anatomy and function to the human prostate. Three prostate cancer markers, *acph-1, bagpipe,* and *shaven,* and two prostate cancer markers, *sprouty* and *AMACR*, are simultaneously expressed exclusively in the ejaculatory bulb of the fly. The normal EjB also expresses signaling pathway components and heparan sulfate proteoglycans known to be present in the normal prostate as well as in prostate cancer. The fly bulb is able to mimic neoplasia through two different methods: induction of a constitutively active form of the oncogene Ras (Ras^{V12}) causes an increase in the epithelial thickness, and in a separate line in which the

EjB experiences overgrowth spontaneously. Through incorporation and staining for the S-phase marker BrdU, I was able to show that overgrowth is caused by overproliferation of cells in the epithelial layer during Ras induction in the bulb.

Gene expression patterns in Ras induced and spontaneous overgrowth bulbs

To further examine the engineered and spontaneous *Drosophila* models of prostate cancer, and to study changes in the systems during aging, I used quantitative Real Time PCR to create gene expression profiles for the ejaculatory bulbs. I looked at expression of four signaling pathways ligands, *wg, dpp, hh,* and *bnl*, as well as readouts of signaling in their pathways. In the Ras^{V12} overgrowth EjBs, expression of *wg* and its response gene *slp* both increased compared to uninduced control bulbs. The only other gene upregulated in Ras induced bulbs is *pnt*. *pnt* is a response gene for the Bnl pathway, which works through the Ras-MAPK cascade. Overgrowth is achieved through overexpression of the oncogene Ras, so *pnt* upregulation is expected. In the spontaneous overgrowth model, upregulation of *slp* in overgrown bulbs is seen, similar to the engineered model. However, expression of the signaling ligand for that pathway, *wg*, is decreased. Similarly, expression of *ptc* and *salm* are increased, while *hh* and *dpp* expression is decreased. It is possible that some other factor increases *slp, ptc,* and *salm* in these EjBs, or that changes in heparan sulfate proteoglycan expression or structure allow for greater sensitivity of EjB cells to the ligands. Although expression levels of *slp* and *ptc* did not increase in the spontaneous model over time, they do show an increase when compared to aged *yw* control EjBs. This suggests that normal expression

of these genes declines with age, but expression in overgrown bulbs remains constant over time. The net result is a higher expression of the genes in overgrown bulbs than in normal controls, which may contribute to overgrowth.

HSPGs can modulate Wg, Dpp, and Hh signaling (Lindner et al., 2007), and differences in expression or modifications to these glycoproteins may allow cells to better respond to signals even with less ligand available. Previous work in our lab showed expression of HSPGs decreases with age in the normal EjB (Hernandez, unpublished data). My examination of HSPG expression levels showed Ras induced overgrowth bulbs upregulated one HSPG, *trol,* whose mammalian homolog Perlecan is known to be upregulated in advanced human prostate cancers (Datta et al., 2006). *dally* and *sdc* were both upregulated over time in the spontaneous overgrowth bulbs. This upregulation of HSPG expression may account for the increased signaling activity despite lower ligand expression in the spontaneous model. Furthermore, the large increase in *sdc* at all timepoints suggests it may be important in the spontaneous model overgrowth. Interestingly, Syndecan-2 was recently shown to be upregulated in advanced prostate cancers (Popovic et al., 2010), and Syndecan-1 is required for mammary tumor formation induced by Wnt (Alexander et al., 2000). This Syndecan-Wnt interaction in cancer cells may explain the increase in Wg signaling seen in the spontaneous overgrowth bulbs. This indicates that the spontaneous overgrowth model may imitate gene expression changes seen in human prostate cancer. Although genetic analysis suggests the overgrowth phenotype is multigenic, the spontaneous overgrowth line was originally derived from an *EP(Sdc)/CyO* fly stock, which may impact

expression levels of *sdc*. However, *sdc* expression is upregulated at all timepoints in the spontaneous model while overgrowth frequency increases with age, indicating that any impact genetic background has on *sdc* levels is not solely responsible for overgrowth.

The expression level of prostate and prostate cancer markers was assayed by qPCR. In spontaneous overgrowth bulbs, a large increase was seen in the prostate cancer marker *AMACR*, as well as a general increase in *sprouty*, whose upregulation in the Ras induced model was most likely due to Ras-MAPK pathway activation. I also assayed expression levels of the fly homolog of the ERG transcription factor, *ETS65A*, which is highly expressed in prostate cancer. ERG is upregulated in response to Ras-MAPK signaling, although its expression becomes independent of Ras in prostate cancer after a chromosomal rearrangement. The level of *ETS65A* increases dramatically over time in the spontaneous overgrowth model. Interestingly, this upregulation is independent of Bnl signaling. Both *bnl* and *pnt* are downregulated at all time points in the spontaneous overgrowth EjBs, suggesting the increase in *ETS65A* expression is independent of Ras signaling in this model, similar to prostate cancer. The *ETS65A* and *AMACR/sprouty* expression data is very exciting. Expression patterns in the spontaneous model closely resemble those in human prostate cancer. Upregulation and Ras-independence of ERG is important in human prostate cancer (Carver et al., 2009). The spontaneous model appears to upregulate the fly homolog of ERG in a Ras-independent manner. This suggests the spontaneous overgrowth of EjBs may be triggered by the same mechanisms as prostate cancer, which would make it an invaluable model for the initiation of the disease. The possibility remains that *ETS54A* is

being overexpressed in response to another growth factor signaling through the Ras-MAPK pathway, such as VEGF.

Overexpression of the ETS transcription factors have been implicated in the formation of prostate tumors. I was able to induce overgrowth in EjBs by overexpressing two different isoforms of the ETS factor Pnt, showing that overgrowth can be achieved in the fly bulb independent of the oncogene Ras.

Aging flies for several weeks, followed by induction and overexpression of Ras^{v12} for 3 or 7 days, results in unequal epithelial layer overgrowth between the two sides of the bulb. This uneven growth is not seen in Ras induced overgrowth samples that were not aged before induction. This asymmetric overgrowth is not unique. As many as 70% of all human prostate cancers arise from one specific zone of the prostate, the peripheral zone (Abel, 2001). It is possible that increased age of the flies results in changes that make Ras induced overgrowth more closely resemble tumor formation in human prostate cancer. I examined the expression of several genes in flies aged prior to induction to determine if increased age results in changes in expression levels, which could account for the asymmetric overgrowth. Of the genes assayed, most have the same general pattern whether they were not aged, aged 7, 14, 21, or 28 days prior to induction. *wg* expression and its signaling activity remained upregulated in aged samples compared to uninduced EjBs, although it was decreased when compared to un-aged induced bulbs. *sdc* expression decreased in flies that were aged before Ras induction, which is similar to expression changes seen in the normal EjB. This suggests that *sdc* does not participate in the overgrowth of Ras^{V12} induced bulbs at any age. This

is in stark contrast to the significant upregulation of *sdc* seen in the spontaneous overgrowth model with increasing age. The expression of the prostate cancer marker *AMACR*, already low in un-aged Ras induced overgrowth, is further decreased by aging samples before induction. These data indicate that changes to the assayed gene's expression levels are not likely the cause of the uneven overgrowth. Prostate cancer is known to be a multigenic disease, so failing to find a single gene that causes changes in overgrowth is not surprising. Further examination is needed to identify the age related differences that result in differential bulb overgrowth.

An early method for ejaculatory bulb transplantation was also described. In the absence of an EjB specific Gal4 driver, it is impossible to express markers solely in the bulb. Therefore, if potential metastasis from the bulb was to be studied, EjBs expressing a reporter must be removed from the original fly, which will have non-specific expression of the reporter. The bulb and its marked cells can then be used to investigate potential metastasis in a host fly. This can be achieved by disrupting the bulb and injecting the cells into new hosts, similar to xenograft studies in which human prostate cancer cells are injected into a mouse host. The advantage to this method is that cells from a single bulb could be injected into hosts of different ages, which would allow the effects of aging on tumor formation and metastasis to be studied. I described a method in which Ras ^{V12} induced overgrown EjBs marked by GFP were transplanted to the abdomen of wild type hosts. These bulbs were still expressing GFP after three days, indicating this method can also be useful in investigating metastasis in adult tissue.

The misregulation of growth factor signaling and heparan sulfate proteoglycans in prostate cancer has been well documented (Datta and Datta, 2006; Datta et al., 2006; Popovic et al., 2010). In this chapter, I described gene expression patterns for two separate *Drosophila* prostate cancer models. Both the engineered and spontaneous ejaculatory bulb overgrowth models resemble human prostate cancer in several ways, and each has all the benefits of a short-lived whole organism model system. This work will help to create a system in which age related changes leading to the onset of prostate cancer can be more thoroughly and easily studied.

Materials and Methods

Drosophila stocks

Fly stocks, including *UAS-GFP LacZ.nls, UAS-Pnt P1, UAS Pnt P2, UAS-Ras^{v12}, yw,* the *hsGal4* and *tubGal^{80}* stocks used to generate the line that drives overexpression, and the *EP(sdc)/CyO* stock the spontaneous overgrowth line was originally derived from are available through the Bloomington Stock Center. *dpp-LacZ* and *wg-LacZ* were obtained from Dr. Ginger Carney and Dr. Keith Maggert. Flies were grown in standard medium at 25°C and induced at 30°C unless otherwise stated.

BrdU incorporation and staining

Newly eclosed males were anesthetized and a 1mg/mL BrdU solution in DMSO was administered to the abdomen of the fly once a day for up to three days. EjBs were then dissected

and fixed as described (Park et al., 2003). BrdU incorporation was visualized with a mouse primary antibody (BD Biosciences) and a peroxidase conjugated secondary antibody (Jackson Immunoresearch). Diaminobenzidine (DAB) was used to develop the secondary antibody. EjBs were mounted on slides for visualizing with a compound microscope.

β-galactosidase activity staining

Dissection of ejaculatory bulbs was performed at 24 days post hatching and *β*-galactosidase visualization was achieved by fixing tissue with ET fix (1 X buffer B: formaldehyde) for 10 min at RT, washing three times with 1X PBST, and incubating in X-gal stain for 3 hours at 37°C.

DAPI staining

Ejaculatory bulbs were dissected and mounted immediately onto a slide containing Vectashield mounting medium with DAP1 (Vector Labs). DAPI staining in the bulbs was then visualized with a compound microscope

Quantitative RealTime PCR

Whole ejaculatory bulbs were dissected and used for RNA isolation. RNA was isolated using Trizol (Invitrogen) following manufacturer's directions, and further purified using the RNeasy kit (Invitrogen). Samples were DNAsed and reverse transcribed using oligo dT and random hexamer primers. The resulting cDNA was used to perform quantitative Real Time PCR with SYBR Green dye (Applied Biosystems). All qRT-PCR reactions were carried out in triplicate at two different template concentrations. Primer sequences are available upon request. β-actin expression was used as an internal control. Data were analyzed using the delta-delta calculation method to yield fold change compared to controls.

CHAPTER IV

CONCLUSIONS AND FUTURE DIRECTIONS

My research is focused on understanding how developmental signaling pathways are differentially regulated in various biological contexts. The vast number of developmental and biological processes that are influenced by growth factor signaling during the lifetime of an organism dwarfs the number of signaling pathways present that elicit those responses. Consequently, the same signaling components and pathways must be recycled many times over in order to impact so many different processes. I am interested in examining how the regulation of these pathways helps to create the enormous diversity in function during an organism's life. I use *Drosophila melanogaster* as a model to study the ways in which the same growth factor signaling pathways can be used in different biological contexts. Specifically, I investigate the ability of a heparan sulfate proteoglycan to regulate signaling in both similar and contrasting biological events during *Drosophila* development. I also examine how the pathways and regulators that control development in the fly can influence the initiation and progression of aberrant tissue growth in a *Drosophila* model for prostate cancer. By utilizing the vast knowledge base and biological tools available in the fruit fly, I hope to gain insight into the differential regulation of signaling during development and disease. Through my work here, I hope to not only improve our understanding of the basic mechanisms by which signaling impacts an organism throughout its life, but also

contribute to the field of cancer research through the description of a powerful model in which to study the initiation of prostate cancer.

Trol Regulates Growth Factor Signaling to Activate Neuroblast Proliferation and Other Developmental Processes in *Drosophila*

Heparan sulfate proteoglycans are essential for proper signaling by many different growth factors (Hacker et al., 2005). Numerous studies have shown that improper regulation of signaling pathways will lead to developmental defects or death. Therefore, the study of how heparan sulfate proteoglycans function in different developmental contexts, and how they modulate signaling by growth factors during these processes, is critical. In the second chapter, I demonstrated the requirement of fully functional Trol during biological development in the *Drosophila* larval stages. The ability of Trol to modulate signaling by multiple different growth factors during larval development was examined. Through the use of several developmental events including neuroblast reactivation, plasmatocyte proliferation, and larval disc development, I was able to demonstrate how Trol differentially regulates growth factor pathways in both similar and different developmental contexts. These studies help to expand the known roles of heparan sulfate proteoglycans, and Trol in particular, during development.

The requirement of normal Perlecan function during development has been demonstrated in multiple systems. In C. elegans, mutations in the Perlecan homolog unc-52 lead to muscle cell defects that can cause paralysis of the worm (Rogalski et al., 2001). Studies in the mouse show that Perlecan is required for normal maintenance of

cartilage and the basement membrane, proper vascular development, and normal cerebral cortex development (Costell et al., 1999; Gonzalez-Iriarte et al., 2003). Furthermore, Perlecan mutations can cause Silver-Handmaker syndrome, a disease with skeletal abnormalities due to defects in chondrogenesis (Arikawa-Hirasawa et al., 2001), and Schwartz-Jampel syndrome in humans, which is characterized by muscle tone abnormalities (Nicole et al., 2000). Thus, a significant homology exists between organisms in their requirement of Perlecan/Trol for normal development,

In *Drosophila*, mutations in *trol* lead to decreased larval neuroblast activation and lethality (Datta and Kankel, 1992; Datta 1995). It first appears defects in central nervous system development inhibit survival of the animals. However, this is not the case. Combining *trol* lethal mutants with *anachronism*, a mutant that enhances neuroblast proliferation (Ebens et al., 1993), rescues larval neuroblast proliferation, but not lethality (Datta, 1995). This leads to the conclusion that *trol* must be essential for other developmental processes in the developing fly.

I was able to use *Drosophila* central nervous system development to help assess the role of *trol* in different developmental events. Mutations in *trol* lead to a distinct neuroblast proliferation phenotype allelic series in the first instar optic lobe and central brain neuroblasts (Park et al, 2003). However, lethal phase analysis showed the most severe mutant for first instar neuroblast reactivation progressed farther in development than mutants with less severe neuroblast phenotypes. The ability of *trol* mutants to influence these two developmental events in a dissimilar fashion suggests Trol is able to

function differently in these processes. One way in which Trol can have different impacts on developmental events is through modulation of growth factor signaling.

Regulation by heparan sulfate proteoglycans is required for normal signaling by several growth factors (Hacker et al., 2005). Tissues and organs use different HSPGs to regulate these signaling pathways. Differential regulation of signaling by proteoglycans may be accomplished in several ways. Signaling molecules may have varying affinities for the type of modifications heparan sulfate chains exhibit. Various sulfotransferases can differently modify heparan sulfate chains, leading to a high level of GAG chain diversity (Nakato and Kimata, 2002). This variability gives each heparan sulfate chain the potential of containing numerous growth factor binding sites. The regions of the heparan sulfate chains may be modified to target certain growth factors at specific times and locations during development (Hacker et al., 2005). For example, Perlecan from different epithelial sources can have varied affinity for FGF binding (Knox et al., 2002). The same signaling pathway can utilize different heparan sulfate proteoglycans in different tissues, and multiple HSPGs can also modulate the same pathway. For example, Perlecan, Glypicans, and Syndecan-3 can all modulate Hh signaling (Park et al., 2003; Shimo et al., 2004; Hacker et al., 2005), and Perlecan and Syndecan-4 can both regulate FGF2 signaling (Aviezer et al., 1997; Tkachenko et al., 2005). The protein core of HSPGs can also interact with growth factors. Along with interactions modulated by its heparan sulfate chains, the protein core region of Syndecan is also required for FGF2 signaling (Volk et al., 1999). Therefore, the great diversity among HSPGs may make specific proteoglycans uniquely suited for a given tissue or developmental event.

To examine differences in Trol's modulation of signaling during different developmental events, I analyzed proliferation in a second spatially and temporally distinct set of neuroblasts in the second instar *Drosophila* brain. Thoracic neuroblast proliferation closely resembles the allelic series seen in first instar optic lobe/central brain neuroblasts, suggesting Trol works in a similar way during reactivation of both sets of cells. Previous studies in our lab have shown that the Bnl and Hh growth factors interact with Trol to prompt first instar neuroblasts to exit quiescence and resume proliferation (Park et al., 2003). Bnl and Hh were also shown to operate in a positive feedback loop to reactivate first instar optic lobe and central brain neuroblasts (Barrett et al., 2008). I wanted to investigate whether these same pathways were initiating neural stem cell proliferation in the second instar thoracic ganglion. I showed both the Bnl and Hh ligands are expressed in the thoracic ganglion, and signaling by their pathways is active at the appropriate time for neuroblast reactivation. I revealed through overexpression and mutant analysis that both the Hedgehog and Branchless pathways directly influence thoracic neuroblast proliferation. Furthermore, I showed using genetic interaction studies that Trol is regulating Hh and Bnl signals in the ventral ganglion. This data correlates with what is known about the regulation of the first instar optic lobe population of neural stem cells. Therefore, the heparan sulfate proteoglycan Trol regulates the Hh and Bnl signaling pathways in a similar, yet distinct, developmental context.

Other studies suggested that Trol would not be able to modulate Hh and Bnl signaling during the reactivation of quiescent neuroblasts because it was not present near

the cells (Voigt et al., 2002). Previous studies from our lab have shown both Hh and Bnl are able to interact with Trol (Park et al., 2003). To regulate growth factor signaling in this system, our model implies Trol must be near the cells that are receiving the signals. Voigt et al showed *trol* mRNA expression was present only in a few cells that were not near the neural stem cells. However, they performed *in situ* hybridizations during third instar, well after the optic lobe, central brain, and thoracic neuroblasts resume proliferation. Furthermore, Trol is a secreted HSPG with a long half-life, and mRNA expression may not accurately reflect protein localization. Using a *trol-GFP* fly line, I was able to show that Trol protein was present near the proliferating neuroblasts at the appropriate times. This is further evidence that Trol directly interacts with the two ligands to help reactivate quiescent cells.

I wanted to examine how Trol might differentially modulate signaling during different developmental contexts. To accomplish this, I looked at the production of hemocytes during larval life. Mature circulating hemocytes remain proliferative, although at a slow rate. Activation of the Ras-MAPK pathway with Ras^{V12} increases the number of proliferating hemocytes (Asha et al., 2003). The Ras-MAPK cascade can be activated by Vascular Endothelial Growth Factor (VEGF) and Platelet Derived Growth Factor (PDGF), and signaling by mammalian homologs of both growth factors has been linked to mammalian Perlecan (Iozzo, 2005). Furthermore, decreasing PDGF/VEGF receptor function resulted in an increase in hemocyte cell death (Evans et al., 2003). I wanted to test whether Trol could modulate PDGF and VEGF in this developmental event. I showed that Trol-GFP is expressed on the plasmatocytes, and mutations in *trol*

decreased the number of circulating cells. Furthermore, the mutants resulted in decreased VEGF/PDGF signaling. This data shows that Trol is able to modulate the Ras-MAPK signaling pathway in another developmental context.

I wanted to examine how Trol may affect other signaling pathways. To accomplish this, I looked at the Dpp, Wg, Hh, and Bnl signaling pathways in the second instar ventral ganglion, as well as in another developmental context, the third instar eye discs/brain lobes. Gene expression analysis of *dpp*, *wg*, *hh*, *bnl*, and their pathway response genes in *trol* mutants indicates Trol is able to regulate signaling by all four growth factors in the second instar ventral ganglion. However, in the third instar brain lobes/eye discs, Hh, Wg, and Bnl signaling efficiency was not affected by decreased Trol function.

Taken together, this data is indicates that the *Drosophila* Perlecan homolog Trol is able to differentially modulate multiple signaling pathways in different developmental contexts. Trol similarly modulates signaling by all four pathways tested in the ventral ganglion, yet only Bnl signaling is dependent on Trol in a different developmental context (the third instar eye disc and brain lobe). These data show that Trol's regulation of growth factor signaling is variable based on the developmental circumstance, and is tissue and context specific. My research highlights the high degree of variability that regulation by heparan sulfate proteoglycans can provide. I have shown that the presence of both HSPGs and active signaling pathways together is not sufficient for proteoglycans to regulate signaling, and that HSPG modulation of growth factor pathways is a highly specific and regulated event.

Future Directions

The research I have presented here gives insight into the variable function heparan sulfates have during development. Evidence that Trol is able to modulate signaling by Hh, Dpp, Wg, and Bnl in the second instar ventral ganglion, but only regulate Bnl signaling in the third instar brain lobes and eye discs, adds to the idea that pathway regulation by heparan sulfate proteoglycans is a highly specific and regulated process. I believe it is necessary to further examine the specific regulation of heparan sulfate modification in *Drosophila* central nervous system development. If the regulation of signaling pathways by HSPGs is as tissue and context specific as it appears to be, the regulation of heparan sulfate chain modification must also be tightly controlled. For example, the variable binding affinity of FGF for Perlecan from different epithelial sources (Knox et al., 2002) demonstrates how HSPG modification can tissue specific. The reactivation of two distinct populations of quiescent neuroblasts is regulated by very similar mechanisms in the fly brain. Bnl and Hh signaling is modulated by Trol, which results in neural stem cell proliferation. It is likely that Trol is specifically modified in this system, which allows the HSPG to enhance the proper pathways and initiate proliferation. If the specific modification enzymes and patterns that allow Trol to modulate signaling in this tissue can be identified, it is possible they could lead to treatments that may help with neurodegenerative diseases.

In certain biological contexts, more than one HSPG can modulate the same pathway in the same tissue. For example, Syndecan-4 and Perlecan are both able to regulate signaling by FGF2 (Tkachenko et al., 2005; Aviezer et al., 1997). This suggests

it may be possible, under the right circumstances, to modify a proteoglycan not normally involved with a specific pathway in a tissue in such a way to allow it to act as a modulator of signaling for that pathway. Examining how HSPGs can substitute for one another in certain cases may lead to insights on how to better treat human diseases caused by mutations to HSPGs, such as the Schwartz-Jampel and Silver-Handmaker syndromes.

The *Drosophila* Ejaculatory Bulb Can Model Initiation and Progression of Prostate Cancer

The importance of developmental signaling pathways to the progression of cancer has been well established (Datta and Datta, 2006; Cronauer et al., 2003; Wakefield and Roberts, 2002; Ruiz i Altaba et al., 2004). Misregulation of signaling pathways has been reported in many types of cancer. Therefore, proper regulation of these growth factor pathways is as important later in life as it is during development. In Chapter II, I showed that heparan sulfate proteoglycans can specifically regulate growth factor signaling in multiple developmental contexts. The ability of HSPGs to modulate signaling remains intact later in life. In fact, previous studies from our lab have shown that Perlecan can modulate growth factor signaling in prostate cancer (Datta et al., 2006). The signaling pathways and regulators important in cancer are the same pathways I showed were important to neural stem cell proliferation in Chapter II. However, these pathways and their modulators are not the only things cancer and stem cells have in common.

The ability of a single hematopoietic stem to proliferate and reconstitute the bone marrow in an X-ray irradiated mouse was the first discovered by Till and McCulloch in 1961 (reviewed, Huntly and Gilliland, 2005). Since that time, the idea of cancer stem cells has been generally accepted. Studies have shown that populations of cells in tumors can have stem cell characteristics. In an integral study, cancer cells expressing the specific cell surface marker CD34, but not the marker CD38, were shown to have the ability to proliferate and reconstitute a cancer cell population in mice (Bonnet and Dick, 1997). These cancer cells therefore display stem cell-like qualities, which allow them to form tumors from a single cell. It is possible that the same mechanisms involved in the reactivation of quiescent stem cells are involved in the activation and proliferation of stem cell-like cancer cells.

Prostate cancer is an age related disease, which makes examination of the onset and early progression difficult. To investigate initiation of prostate carcinomas, model systems must be used. The *Drosophila* ejaculatory bulb has the same anatomy and function as the human prostate. It also expresses prostate and prostate cancer specific markers. As in the activation of quiescent neuroblasts, growth factor signaling is important in prostate cancer. Normal prostates exhibit growth factor signaling, which is then misregulated in cancers. The fly ejaculatory bulb also expresses the signaling pathway components, as well as heparan sulfate proteoglycans. Similar anatomy, function and markers, along with the short life span and molecular tools available, make the *Drosophila* ejaculatory bulb a good model for the human prostate.

The EjB is able to mimic neoplasia by overproliferation and overgrowth in the epithelial layer. Both a Ras induced and spontaneous overgrowth model have been isolated. The same pathways and regulators I studied during the reactivation of neural stem cells have roles during prostate cancer. I created gene expression profiles for the signaling ligands, pathway readouts, HSPGs, and prostate biomarkers for both the engineered and spontaneous fly models. The expression of the transcription factor ERG *(ETS65A* in *Drosophila)*, which is highly expressed in prostate carcinomas, is increased in the spontaneous overgrowth model, as was the prostate cancer marker *AMACR*. The expression pattern of these genes in the spontaneous model is very exciting. The similarity to the expression patterns in human prostate cancer suggests that valuable information can be obtained from the spontaneous model regarding initiation of the disease. Several differences in expression patterns exist between the Ras engineered and spontaneous overgrowth, which indicates the mechanisms in which they achieve overgrowth are different. This leaves us two distinct models, each of which may be useful. Cancer is a highly heterogeneous disease, and model systems that have different hallmarks can be used to examine this heterogeneity.

Taken together, this data describes the *Drosophila* ejaculatory bulb as a model for prostate cancer. My initial characterization of gene expression patterns will allow others to better study how the onset of this disease occurs. The fly models will also help simplify analysis of the contribution age makes to prostate cancer, and my work will help better understand the role aging plays in gene expression changes that lead up to the initiation of prostate cancer.

Future Directions

The ejaculatory bulb model for human prostate cancer is an intriguing system. The ability of this short-lived model to mimic neoplasia through overproliferation in the EjB epithelial layer may lead to many insights into prostate cancer initiation and progression. I have done an initial gene expression characterization of signaling pathway components and heparan sulfate proteoglycans in both an oncogenic Ras engineered overproliferation model, as well as in a model line that displays spontaneous overgrowth. I have also examined how signaling and expression of pathway regulators change over time in the overgrown bulbs. It is now important to investigate the spontaneous overgrowth model further, in an attempt to determine the exact causes of overproliferation in these bulbs. Determining the causes of spontaneous overgrowth of the EjB may lead to insights into the early initiation of prostate cancer.

I assayed expression of several genes in aged Ras induced overgrowth bulbs that exhibited uneven epithelial overgrowth. However, I was not able to find significant changes that may have accounted for the abnormal overgrowth. The fact that this only

occurs in bulbs aged before Ras induction drives overgrowth suggests that some age dependent component(s) are responsible for the irregular overgrowth. Prostate cancer is an age related carcinoma (Bostwick et al., 2004), and most prostate cancers arise from one particular zone of the gland (Abel, 2001). The asymmetric overgrowth observed may arise due to the aged bulb gaining features that make it more like a cancerous prostate. It is therefore important to examine why older ejaculatory bulbs behave differently than young EjBs in response to Ras induction.

Another important direction to take is the induction of metastasis in the fly prostate cancer model. Metastasis can be induced in larvae by combining overexpression of Ras with knockdown of apical-basal polarity genes (Brumby and Richardson, 2003; Pagliarini and Xu, 2003). Generating an ejaculatory bulb specific Gal4 driver would greatly simplify many of the experiments, as you could induce genes specifically in the tissue you want, and monitor the fly for metastasis and secondary tumor growth. Also, introducing a reporter and the apical-basal knockdown genes into the spontaneous overgrowth background would be interesting. The larval metastasis model requires Ras induced overproliferation, and determining if the spontaneous model can generate metastasis without engineered overexpression of an oncogene would be exciting.

Conclusions

I have used the *Drosophila* model system to demonstrate the ability of heparan sulfate proteoglycans to differentially regulate multiple growth factor pathways in different developmental contexts. I have shown that the fly Perlecan gene regulates signaling pathways in a tissue- and time-specific manner. I have described a *Drosophila* model for prostate cancer, and examined gene expression profiles in engineered and spontaneous overgrowth ejaculatory bulbs. These studies have provided insight into the specific regulation of growth factor signaling by heparan sulfate proteoglycans, and described a powerful model in which to study the initiation and progression of prostate cancer.

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