THE EFFECTS OF THE O-XYLOSYLTRANSFERASE (OXT) MUTATION
ON WG, HH, AND DPP/TGF-β SIGNALING

by

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A thesis submitted to the Faculty of the University of Delaware in partial fulfillment of the requirements for the degree of Bachelor of Science in Quantitative Biology with Distinction.

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ON WG, HH, AND DPP/TGF-β SIGNALING

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ABSTRACT

The *o-xylosyltransferase (oxt)* encodes a glycosyltransferase needed for the first step of proteoglycan glycosaminoglycan (GAG) biosynthesis. Disruptions in heparan sulfate proteoglycan (HSPG) biosynthesis change the extracellular environment, which has been shown to block Wingless (Wg)/Wnt, Hedgehog (Hh), and Decapentaplegic (Dpp)/TGF-β intercellular signaling. Therefore, mutations in *oxt* are predicted to disrupt normal Wg, Hh, and Dpp signaling due to the absence of heparan sulfate (HS) and chondroitan sulfate (CS) modified extracellular proteoglycans. Characterization of the *oxt* mutant in *Drosophila* wing discs shows this mutation caused unexpectedly mild signaling defects as compared with other mutants acting further down in the HS biosynthetic pathway, such as *sulfateless (sfl)*, which is responsible for specific downstream sulfation and activation of only HS chains. The *oxt-sfl* double mutant was made to address why the *oxt* phenotype is less severe. Clonal analysis shows that the double mutant looks more like *sfl*, suggesting the *oxt* null mutation does not yield a fully penetrant phenotype, allowing some xylose addition to the core proteoglycan upon which Sfl can act. Because the *oxt* mutation is a nonsense mutation that eliminates the Oxt active site, the lack of penetrance can be attributed to either perdurance of residual Oxt protein or mRNA in mutant cells.
Chapter 1

INTRODUCTION

Intercellular signaling is part of a complex system of communication that is vital for multicellular life. Cells must be able to perceive and correctly respond to their environment. Cellular signaling forms the basis of development and this allows for growth and differentiation. Unique cell signals give rise to the regulation of many different mammalian cell types. Errors in cellular signaling can result in deformities, disease, and lethality. Signaling disruptions are the cause of many human diseases, countless numbers of which are currently undergoing research. For example, Parkinson’s disease is a degenerative disorder of the central nervous system caused by loss of function of dopamine signaling [Dopamine]. This disorder is characterized by tremors, rigidity, difficulty with gait, and cognitive problems such as late-stage dementia. Cancer is caused by unregulated cell growth and the over-expression of certain signals, leading to cancerous cells and tumors. An intricate balance of positive cellular signals is required for the maintenance of healthy tissues. By gaining a better understanding of cellular signaling and how it leads to phenotypic defects, diseases may be treated more effectively and efficiently.

Cell pattern formation during early developmental stages is controlled by signal transduction pathways that are activated by extracellular ligands. These pathways involve an extracellular signaling molecule binding to and activating a cell surface receptor, generating a response. This project focuses on heparan sulfate proteoglycans (HSPGs), which have been identified as important regulators of the
distribution of extracellular ligands such as Wingless (Wg), Hedgehog (Hh), and Decapentaplegic (Dpp) [Bornemann 2004]. Disruptions or mutations that compromise the function of HSPGs have been shown to disrupt the activity of these signaling pathways. Mutations affecting the biosynthesis of HSPGs are the cause of several human diseases such as Simpson-Golabi-Behmel syndrome (SGBS), multiple hereditary exostosis, and Ehlers-Danlos syndrome [Hacker 2005]. As this project demonstrates, mutations in HSPG biosynthesis result in changes in the extracellular environment and as a consequence, alter intercellular signaling. Using Drosophila melanogaster as a model organism, this project aims to look at proteoglycans that have been implicated in patterning events and analyze the factors that affect cell signals, including the physical distance between cells and the extracellular environment through which the cell signals travel. The goal of this project is to examine the role of HSPGs as modulators in several signaling pathways, gain a better understanding of the interactions between proteoglycans and extracellular signaling ligands, and to examine how changes in the extracellular environment influence signaling.

1.1 Proteoglycans

Proteoglycans consist of a core protein and one or more covalently attached glycosaminoglycan (GAG) sugar chains. GAGs are linear polysaccharides consisting of amino sugars (N-acetylglucosamine or N-acetylgalactosamine) and uronic acid (glucuronic acid or iduronic acid). Nearly all mammalian cells produce proteoglycans. These macromolecules can be secreted into the extracellular matrix (ECM), inserted into the plasma membrane, or stored in secretory granules [Varki 2009]. The negative charge carried on GAG chains allows proteoglycans to regulate the availability of
positively charged components. Proteoglycans are diverse in structure, allowing for many roles in a wide variety of biological processes including regulation of cell proliferation and differentiation [Varki 2009].

Heparan sulfate proteoglycans (HSPG) are cell surface and ECM macromolecules that consist of heparan sulfate (HS) GAG chains attached to serine residues in core proteins via xylose. Studies show that HSPGs may be required in a variety of biological phenomena such as embryonic development [Baeg 2001], and they play a crucial role in modulating growth factor signaling [Bornemann 2004]. In regards to signal transduction, HSPGs play a vital role in several signaling pathways, including Wnt, Hedgehog (Hh), and Bone Morphogenetic Protein (BMP)/Transforming Growth Factor-β (TGF-β). There are three classifications of HSPG: glypicans, syndecans, and perlecans [Baeg 2001].

Glypicans are cell surface HSPGs that consist of a core protein, HS chains, and a glycosylphosphatidyl inositol (GPI) anchor which links the macromolecule to the plasma membrane (Figure 1B). The Drosophila genome has two glypicans: division abnormally delayed (Dally) and Dally-like (Dlp). Recent evidence suggests that Dally and Dlp play major roles in regulating gradient formation of morphogens, including Wingless (Wg), Hh, and Decapentaplegic (Dpp) [Yan and Lin 2009].

Syndecans are cell surface HSPGs that are linked to the plasma membrane by a transmembrane domain on the core protein (Figure 1A). Syndecans are decorated with both HS and chondroitin sulfate (CS) chains. The Drosophila genome has a single syndecan: Sdc [Yan and Lin 2009].
Perlecans are secreted HSPGs that are mainly distributed in the ECM. Like glypicans, perlecans exclusively bear HS GAG chains (Figure 1C). The *Drosophila* genome has a single perlecans: terribly reduced optic lobes (Trol) [Yan and Lin 2009].

![Three classifications of HSPGs](image)

**Figure 1: Three classifications of HSPGs.** A) Syndecans are attached to the plasma membrane by a transmembrane domain and contain both HS and CS chains. B) Glypicans are attached to the plasma membrane by a GPI anchor and contain HS chains and a large extracellular domain. C) Perlecans are released into the ECM and contain only HS chains. From Yan and Lin 2009.

### 1.2 Biosynthesis of HS and CS Proteoglycans

The two classes of proteoglycans involved in this project are linked to serine residues in core proteins via the sugar xylose. HS and CS biosynthesis begin with a common pathway: in the Golgi, nucleotide sugar precursors form a tetrasaccharide linker that is attached to the core protein [Bornemann 2004]. First, an O-xylosyltransferase (Oxt) utilizes a UDP-xylose to link xylose to a serine on the protein. Then, a linkage tetrasaccharide forms by the addition of two galactose residues and one glucuronic acid [Varki 2009]. Following this, the two pathways diverge into HS and CS biosynthesis (Figure 2). HSPGs are composed of alternating
glucuronic acid-N-acetylglucosamine (GlcA-GlcNAc) repeating disaccharide units. Chondroitin sulfate proteoglycans (CSPGs) are composed of alternating glucuronic acid-N-acetylgalactosamine (GalNAc-GlcA) disaccharide units [Bornemann 2004].

Figure 2: Bifurcation of HS and CS biosynthesis. HS and CS biosynthesis initiates after the establishment of a tetrasaccharide linker on the serine residue of the core protein. These processes are similar but require different enzymes. From Hacker et al 2005.
HS chains are modified with GlcN N-sulfation on N-acetylglucosamine units, followed by 2-O-sulfation of iduronic acid units. Some glucuronic units also undergo 2-O-sulfation. Next, 6-O-sulfation occurs on select N-acetylglucosamine residues. Finally, sulfated sugar residues and uronic epimers form the targets of 3-O-sulfotransferase. Posttranslational modification of HS chains tends to occur in clusters along the core protein, resulting in regions lacking sulfate addition. CS chains, on the other hand, commonly have long links of fully modified disaccharides. The precise arrangement of sulfated residues and uronic acid epimers on HS chains forms specific binding sequences for ligands. HS chains are highly conserved from D. melanogaster to C. elegans [Varki 2009].

Sulfation of chondroitin chains is a similar process to heparan chain sulfation, however, different enzymes are utilized. Enzymes are first used for the epimerization of glucuronic acid to iduronic acid, followed by 2-O-sulfation, 4-O-sulfation, and 6-O-sulfation. CS chains are highly conserved and are the most abundant GAG in human tissues, such as articular cartilage [Varki 2009].

Proteoglycan have many diverse functions. An important feature of proteoglycans is their ability to bind water and form stable matrices. In cartilage, the proteoglycans provide a steady matrix capable of absorbing high compressive loads by water retention. This ability aids in the structural organization of many tissues. Many of these biological functions are dependent on the interaction of GAG chains with select protein ligands [Varki 2009].

Many HSPG studies have demonstrated the significance of HS chains. As seen in figures 2 and 3, various glycosyltransferases and modification enzymes are involved in the polymerization and modification processes of HSPG biosynthesis.
Figure 3: Mutations identified in the HSPG biosynthetic pathway in *Drosophila* (red). From Lin 2004.
These enzymes are conserved in *Drosophila* and vertebrates [Lin 2004]. HS chains have been widely studied and are of major interest in developmental biology. Intensive exploration of HS biosynthetic mutants in *Drosophila* have revealed much about the function of HSPGs. Figure 3 shows several of the mutations that disrupt enzymes in the HS biosynthetic pathway: Brother of tout-velu (Botv), Tout-velu (Ttv), Sister of tout-velu (Sotv), and Sulfateless (Sfl). These four *Drosophila* enzymes are homologs of vertebrate EXTL3, EXT1, EXT2, and N-deacetylase/N-sulfotransferase.

Botv is a HS polymerase that adds the first GlcNAc to the linkage tetrasaccharide before chain elongation begins. *botv* encodes the *Drosophila* homologue of human EXTL3. Ttv and Sotv are copolymerases that add alternating GlcA-GlcNAc to polymerize HS chains. *ttv* and *sotv* encode the *Drosophila* homologues of human EXT1 and EXT2, respectively. All three homologues are members of the EXT tumor suppressor family. Studies have shown that Wg, Hh, and Dpp signaling are defective in cells mutant for *ttv, sotv,* or *botv* [Izumikawa 2005]. However, Wg signaling is defective in *botv* mutants or *ttv-sotv* double mutants, but is not defective in tissues that harbor *ttv* or *sotv* mutations alone. This suggests that Ttv and Sotv are redundant in Wg signaling [Han 2004]. Sfl is responsible for specific downstream sulfation and activation of HS chains.

Numerous studies of mutants in the HS biosynthetic pathway have demonstrated the crucial role of HSPGs in regulating cellular signaling events. However, due to the lack of discovered mutants in CS, a more abundant and widely expressed GAG in mammalian tissues, investigators have found it difficult to determine the role CSPGs play in development. A mutation identified by Erica M. Selva in *oxt,* which encodes the O-xylosyltransferase responsible for adding the first
xylose to the core protein in the biosynthesis of both HS and CS chains, was found to be a premature stop resulting in a complete loss of the enzyme’s active site (Figure 4). Hence, oxt should result in the complete absence of proteoglycan HS and CS modified chains. This novel mutation now allows for the indirect investigation of CS. By comparing the effects of $oxt^{7H24}$, a mutant resulting in abrogation of both HS and CS chains, to a mutation in downstream HS biosynthesis only, we hope to better understand the role of CS in glycoprotein function in cellular signaling events. For this project, a mutation in $sfl$ was selected to compare to $oxt^{7H24}$ (Figure 5).

Figure 4: **The $oxt^{7H24}$ mutation.** 7H24 in oxt is a C to A transversion at nucleotide 251 of the coding sequence converting a cysteine codon (TGC) at amino acid 83 to a stop codon (TGA). Thus, 7H24 is a nonsense allele of oxt, and if translated, would yield an 82 amino acid protein fragment that lacks all functional domains of the protein.
1.3 Morphogens

Cell signaling and communication occur via messenger molecules. Some of these molecules, called morphogens, are secreted signaling molecules that govern the pattern of tissue development. During development, morphogens are secreted from their producing cells into the ECM, where they bind to their receptors, triggering changes in gene expression through the activation of signal transduction cascades in a direct concentration-dependent manner [Yan and Lin 2009].

Commonly studied morphogens include members of the Wnt, Hh, and BMP/TGF-β families. These secreted signaling proteins have been shown to affect cell identities within tissues. Numerous studies have shown that HSPGs act as regulators...
of morphogen gradient formation. HSPGs can directly influence morphogen gradient formation through changes in morphogen movement, signaling, and trafficking [Yan and Lin 2009].

_Drosophila_ wing discs provide a good model for analyzing the effects of mutations on the Wg, Hh, and Dpp morphogens (Figure 6). Wing discs (mesothorax)

Figure 6: The _Drosophila_ wing disc is a good model for studying cellular signaling. Wing discs allow for the visualization of the activation of short-, mid-, and long-range targets of Wg, Hh, and Dpp signaling. From Held, L. I. Jr. 2005.
are the largest discs in the developing *Drosophila* larvae and allow for the visualization of morphogen gradient formation after immunofluorescent staining.

### 1.4 Wnt Signaling and the Wg Morphogen

The Wnt family of secreted proteins are signaling molecules that affect cell fate and proliferation in many different biological processes [Zoltewicz 2009]. These molecules are involved in the regulation of many biological phenomena during development and have increasingly been implicated in tissue homeostasis in adult organisms [Nusse 2005]. The Wnt family is highly conserved and evidence indicates that Wnts act as morphogens in the developing embryo, forming concentration gradients across many tissues. In early development, Wnts generate changes in cell proliferation, fate, and movement in a direct concentration-dependent manner. In adults, defects in Wnt signaling and Wnt malfunction have been shown to result in various forms of disease, including cancer and neurodegenerative diseases [Moon 2004]. Recently, Tetra-amelia syndrome, a rare human disease characterized by the absence of limbs, has been proposed to result from WNT3 loss of function mutations [Nusse 2005]. Studies suggest that Wnts may activate more than one type of signaling pathway [Moon 2004]. Wingless (Wg) is one of the 7 Wnts in *Drosophila* and is the founding member of the family.

*Drosophila* provides a simple model for Wnt signaling. In the *Drosophila* wing imaginal disc, Wg is secreted from the dorsal-ventral (D-V) boundary and forms a distinct band 2 – 3 cells wide (Figure 7, top). Wg acts as a long-range morphogen by inducing the expression of Wg target genes, including short-range *achaete* (*ac*) and *senseless* (*sen*), and long-range *distilless* (*dll*) and *vestigial* (*vg*) (Figure 6) [Yan and Lin 2009]. A proposed model for Wg signaling supports a restricted diffusion
mechanism in which secreted Wg morphogen molecules interact with their receptors and extracellular matrix (ECM) proteins, in particular HSPGs. The Wg morphogen

Figure 7: Morphogenic gradient formation patterns of Wg, Hh, and Dpp. In the Drosophila wing disc, Wg-expressing cells form a band along the dorsal-ventral boundary and signals dorsally and ventrally. Hh is expressed in the posterior compartment only and signals to the anterior compartment. Dpp is expressed in a stripe along the anterior-posterior boundary and signals to both the anterior and posterior compartments.
moves through this restricted diffusion mechanism by attaching to GAG chains of HSPGs at the cell surface. Evidence suggests that Wg associates with the two *Drosophila* HSPG glypicans, Dally and Dlp, also bound to cell surfaces. Dally and Dlp are the major core proteins providing effective proteoglycan GAG chains. Dally and Dlp play important roles in modulating Wg signaling and distribution [Yan and Lin 2009]. It has been suggested that Dally encodes the protein core of the HSPGs involved in Wg signaling. Loss of Dally is associated with defects comparable to loss of Wg signaling activity. Loss of Dlp results in a deficiency of Wg reception, while overexpression of Dlp leads to an accumulation of extracellular Wg [Baeg 2001].

Altogether, this suggests that both glypicans play important roles in the distribution and reception of the Wg signal. This project aims to visualize the effect of mutations in *oxt* and *sfl* in the hopes of gaining a better understanding of the role of CS chains on the Wg morphogen gradient.

1.5 **Hedgehog Signaling**

Hh is another morphogen shown to play a key role in development. The Hh signaling pathway is required for many fundamental features of cell and tissue development such as the patterning of cells and the regulation of cell differentiation and proliferation [King 2008]. The human homolog is Sonic Hedgehog (Shh). Hh binds to receptor in the ECM and releases an intercellular signal with many critical functions in the regulation of embryonic development, the control of stem cell behavior, and management of homeostasis in the adult [King 2008]. Disruption of the Hh pathway in the form of inactivation or overactivation can result in serious diseases, such as Pallister-Hall syndrome and Gorlin syndrome [King 2008]. Evidence suggests that HSPGs are involved in the movement and transport of Hh proteins through tissues.
Studies have shown that Hh protein accumulates in front of HSPG-defective cells, suggesting that Hh fails to move across HSPG mutant cells. HSPGs may also play a role in controlling the stability of Hh. Similar results are observed in clones mutant for both \textit{dally} and \textit{dlp}, suggesting that Hh movement is mediated by a restricted diffusion model involving Dally and Dlp [Yan and Lin 2009].

In the \textit{Drosophila} wing disc, Hh is produced and expressed by cells in the posterior compartment and signals into the anterior compartment (Figure 7, middle). Hh acts as a short range morphogen and travels a distance of 8 – 10 cell diameters from its site of production to induce expression of target genes, including \textit{engrailed (en)}, \textit{patched (ptc)}, and \textit{decapentaplegic (dpp)} along the anterior-posterior (A-P) boundary (Figure 6) [Baeg 2001]. Hh also initiates transcriptional regulation of numerous targets, including cyclins and growth factors, and intracellular signaling molecules such as Cubitus interruptus (Ci) [Bornemann 2004]. This project aims to visualize the effect of mutations in \textit{oxt} and \textit{sfl} in Hh expression and signaling.

1.6 TGF-β Signaling and the Dpp Morphogen

A third morphogen widely studied in \textit{Drosophila} is Decapentaplegic (Dpp), which is a member of the TGF-β family. TGF-β is a family of dimeric polypeptide growth factors. An important human homolog is Bone Morphogenic Protein (BMP). Nearly all mammalian cells produce TGF-β and have receptors for it. TGF-β regulates the propagation and differentiation of cells, and plays a role in embryonic development and angiogenesis [Blobe 2000]. Mutations in the TGF-β pathway are linked to the pathogenesis of disease, in particular, cancer and hereditary hemorrhagic telangiectasia [Blobe 2000].
In the *Drosophila* wing disc, Dpp is induced in a stripe of cells along the dorsal-ventral (D-V) boundary and signals into both the anterior and posterior compartments (Figure 7, bottom). Dpp acts as a long-range morphogen to induce expression of its target genes including *spalt (sal)*, *optomotor-blind (omb)*, and *vestigial (vg)* (Figure 6). *Drosophila* Dpp is also a target of Hh signaling. Studies have shown that Dpp is a heparin-binding protein. In mutant clones of HS GAG biosynthetic enzymes, both Dpp signaling activity and extracellular levels of Dpp are significantly reduced, suggesting that Dpp signaling is compromised during loss of HS polymerase activity [Yan and Lin 2009]. In addition to the direct binding of Dpp, HSPGs may regulate Dpp signaling and gradient formation through interactions with these proteins. It has been suggested that Dpp moves through the epithelial sheet in the wing disc through a restricted diffusion mechanism [Yan and Lin 2009]. This project aims to visualize the effect of mutations in *oxt* and *sfl* in Dpp expression and signaling.

1.7 Specific Aims

The focus of this research is on the role of HSPGs in Wg, Hh, and Dpp intercellular signaling. The *oxt*\(^{7H24}\) mutation, which disrupts both HS and CS GAG biosynthesis, is predicted to alter HSPG structure and expression in *Drosophila* and consequently alter morphogen formation in the wing imaginal disc. The *oxt*\(^{7H24}\) mutation has been shown to be a premature stop, resulting in a complete loss of the enzyme’s active site. Initial characterization of the *oxt* mutation causes an unexpectedly mild phenotype as compared with another mutant acting further down the HS biosynthetic pathway, *sulfateless (sfl)*, responsible for specific downstream sulfation and activation of HS chains only.
The observation that sfl displays a more severe phenotype than oxt on Wg, Hh, and Dpp signaling leads to two possible hypotheses: first, the core protein of proteoglycans has an intrinsic function in cell signaling that is masked by unsulfated sugar chains, or second, that oxt is not completely null and xylose is added to at least some HS target sites in core proteoglycans. In response to this, the double mutant oxt-sfl was made to address why the oxt phenotype is less severe. If the double mutant looks similar to oxt, then this suggests that the core proteoglycan has intrinsic function in cell signaling that is blocked by sugar addition. If the double mutant looks similar to sfl, then this suggests that the oxt null mutation does not yield a fully penetrant phenotype, allowing some xylose addition to the core proteoglycan upon which sfl can act further down the pathway.

There are three specific aims of this project. The first is to complete comparative clonal analysis of oxt, sfl, and oxt-sfl in Wg, Hh, and Dpp signaling to determine if the oxt mutant has a less severe phenotype in all these signaling contexts, as well as a quantitative analysis of extracellular Wg gradient formation. The second aim is to determine the mechanism of the long half-life of Oxt, or the perduring factor. It is thought to be either RNA or protein. The third aim is to characterize the role of oxt in proteoglycan modification, signal transduction, and development.

The study of the novel oxt mutation will lead to a better understanding of HSPG implication in Wg, Hh, and Dpp developmental signaling and homeostasis. It is hoped that these and future studies will help define its role in situations when signaling is aberrant, such as cancer and disease. Due to the unique phenotype of this mutation, in comparison to HS-specific mutations, this study may also provide insights into the role of CS in cellular signaling.
Chapter 2

MATERIALS AND METHODS

A fundamental feature of *Drosophila* that is utilized in this project is the ability to generate mosaics. Mosaic tissue denotes the presence of multiple populations of cells with different genotypes. *Drosophila* larval wing discs are created in which wild-type and mutant clonal tissue lay side by side in the same wing disc. The mechanism by which mosaic tissue is created is mitotic recombination. Genetic mosaics allow for the study of genes important for early events in development. The intentional generation of mosaic tissue allows for the direct comparison of phenotypes after antibody staining. Differences in phenotype are directly related to differences in genotype and not external factors. This provides a dramatic visualization of the effects of a mutation on ligand expression.

2.1 Use of FLP/FRT System to Perform Mosaic Analysis

The use of the FLP/FRT system to generate somatic clone patches, adapted from Theodosiou and Xu, 1998, is used to manipulate *Drosophila* DNA under controlled conditions to directly compare mutant and wild-type tissue *in vivo*. Strains are established containing the yeast site-specific Flip Recombinase (FLP), a gene which recognizes Flip Recombinase Target (FRT) sites, and chromosome wild-type target sites possessing visible markers. To use the FLP/FRT system to generate homozygous mutant clone patches, the mutation of interest must first be recombined onto a FRT-containing chromosome. Heat shock during early larval stages induces
FLP, which allows for high levels of recombinase to be expressed at specific developmental stages. The heat shock induces mitotic recombination at the FRT sites on homologous chromosomes. *Drosophila* used in this study are heterozygous for a gene encoding the visible marker, green fluorescent protein (GFP), and an allele of the gene to be studied. After induction of recombination by FLP expression and segregation of the homologous non-sister chromatids, cells that have undergone recombination will have some progeny that receive two mutant chromosomes and two wild-type chromosomes. These daughter cells are homozygous for either the allele being studied or GFP. Cells that do not express GFP are identified as those carrying the homozygous mutation of interest. Table 1 shows a list of specific fly stocks used during the course of this project.
### Table 1. Fly Stocks Used

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Experiment</th>
</tr>
</thead>
<tbody>
<tr>
<td>$w^{118}$</td>
<td>wild-type</td>
</tr>
<tr>
<td>$y w hs$-flp/ $y w hs$-flp; $M(3)_{105}$ hs-GFP FRT$^{2A}$/TM6B, $Tb$</td>
<td>Clone Generating Stock (w/ HS)</td>
</tr>
<tr>
<td>$e^{22} c$-Gal4 UAS flp/ CyO; ubn GFP FRT$^{2A}$/TM6B, $Tb$</td>
<td>Clone Generating Stock (w/o HS)</td>
</tr>
<tr>
<td>$w/w; oxt^{H24}$ FRT$^{-2A}$/TM6B, $Tb$</td>
<td>$oxt$ mutant clones</td>
</tr>
<tr>
<td>$w/w; sfl^{9B4}$ FRT$^{2A}$/TM6B, $Tb$</td>
<td>$sfl$ mutant clones</td>
</tr>
<tr>
<td>$w/w; oxt^{H24} sfl^{9B4}$ FRT$^{2A}$/TM6B, $Tb$</td>
<td>$oxt$-$sfl$ mutant clones</td>
</tr>
<tr>
<td>$w/w; Vg$-Q-lacZ/Vg$-Q$-lacZ; $oxt^{H24}$ FRT$^{2A}$/TM6C</td>
<td>$oxt$ mutant clones (Vg-Q-lac Z)</td>
</tr>
<tr>
<td>$w/w; Vg$-Q-lacZ/CyO; $sfl^{9B4}$ FRT$^{2A}$/TM6C</td>
<td>$sfl$ mutant clones (Vg-Q-lac Z)</td>
</tr>
<tr>
<td>$w/w; Vg$-Q-lacZ/CyO; $oxt^{H24} sfl^{9B4}$ FRT$^{2A}$/TM6C</td>
<td>$oxt$-$sfl$ mutant clones (Vg-Q-lac Z)</td>
</tr>
<tr>
<td>$w$ Omb-lacZ/w Omb-lacZ; $oxt^{H24}$ FRT$^{2A}$/TM6C</td>
<td>$oxt$ mutant clones (omb-lac Z)</td>
</tr>
<tr>
<td>$w$ Omb-lacZ/w Omb-lacZ; $sfl^{9B4}$ FRT$^{2A}$/TM6C</td>
<td>$sfl$ mutant clones (omb-lac Z)</td>
</tr>
<tr>
<td>$w$ Omb-lacZ/w Omb-lacZ; $oxt^{H24} sfl^{9B4}$ FRT$^{2A}$/TM6C</td>
<td>$oxt$-$sfl$ mutant clones (omb-lac Z)</td>
</tr>
</tbody>
</table>
2.2 **Immunohistochemistry of *Drosophila* Wing Discs**

Before beginning either the conventional or extracellular staining protocol, the appropriate crosses must be set up and cultured at room temperature (RT), or 25°C. For the crosses that required heat shock a 90 minute heat shock at 37.5°C during the second and third days (first instar) induced mitotic recombination. Prior to dissection, a 60 minute heat shock at 37.5°C induced GFP expression, and is followed by a 60 minute incubation at RT to allow proteins to be translated and localized to the nucleus.

A second clone generating stock was made towards the end of the project without the need to heat shock during early larval stages or before dissection.

2.2.1 **Conventional Immunofluorescent Staining Protocol**

First, larvae were selected based on appropriate genetic markers. For the third chromosome mutations, non-tubby, or long and slender, morphology indicates the larvae harbor the two FRT chromosomes described above. Then, a primary larval dissection is performed in 1X phosphate buffered saline (PBS). The primary dissection consists of separating the anterior and posterior ends of the larva under a dissection microscope. The posterior end is discarded while the anterior end is inverted to expose the internal structures. The majority of the internal structures are removed, exposing the wing imaginal discs. Next, the dissected larvae are fixed in a solution of 1X PBS with 4% formaldehyde for 20 min at RT. After fixation, the tissue is rinsed and washed four times each in PBS with 0.1% TritonX-100 (PBT) at RT. Then, the tissue is blocked in PBT with 5% Normal Horse Serum (PBTN) for 30 minutes at RT. Afterwards, the primary antibody is added and incubated overnight on a rocker at 4°C.

The next day, the tissue is rinsed and washed four times each in PBT at RT before adding the secondary antibody in PBTN and incubating for one hour at RT.
After rinsing and washing four times in PBT again, a secondary dissection is performed, in which the wing discs are separated from the rest of the tissue under the microscope and mounted in 70% glycerol in PBS on a microscope slide for analysis.

In a few cases in which an anti-rabbit primary antibody is used, there is a third incubation for the rabbit GFP antibody prior to the secondary dissection. This incubation is for one hour on a rocker at RT after a 20 min fixation, and then followed by another series of rinses and washes. Tables 2a. and 2b. list the primary and secondary antibodies used during the course of this project.

### 2.2.2 Extracellular Staining Protocol

The process for extracellular staining is very similar to the process for conventional staining. However, to obtain more accurate results for the quantitative analysis, the entire process of oxt, sfl, and oxt-sfl stainings were done simultaneously. To differentiate between the three mutations, sfl was stained with Vg-Q-lac Z, oxt-sfl was stained with omb-lac Z, and oxt had no other specific staining.

First, the primary antibody is diluted in cold Schneider’s cell culture media and kept on ice until use. The primary dissection is performed in cold Schneider’s cell media on ice and dissected larvae are placed in a tube of cold cell media on ice. Afterwards, the cold cell media is removed and replaced with diluted antibody. Then, the tissue is incubated on ice for one hour, without agitation. Next, the antibody is removed and the tissue is washed in cold cell media three times on ice with no agitation. Next, the tissue is fixed in PBS with 4% formaldehyde for 2 minutes on ice and then 18 minutes at RT. Beyond this step, the protocol is identical to the conventional staining protocol.
Table 2a. Primary Antibodies Used

<table>
<thead>
<tr>
<th>Target</th>
<th>Ig</th>
<th>Concentration (diluted in PBTN)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wg – ligand</td>
<td>mouse</td>
<td>1:10</td>
</tr>
<tr>
<td>Wg – Vg-Q, β-gal (long)</td>
<td>chicken</td>
<td>1:100</td>
</tr>
<tr>
<td>Wg – Ac (short)</td>
<td>mouse</td>
<td>1:4</td>
</tr>
<tr>
<td>Wg – Sen (short)</td>
<td>guinea pig</td>
<td>1:1000</td>
</tr>
<tr>
<td>Hh - ligand</td>
<td>rabbit</td>
<td>1:500</td>
</tr>
<tr>
<td>Hh – Ci (long)</td>
<td>rat</td>
<td>1:20</td>
</tr>
<tr>
<td>Hh – Ptc (short)</td>
<td>mouse</td>
<td>1:10</td>
</tr>
<tr>
<td>Dpp – SalM (long)</td>
<td>rat</td>
<td>1:500</td>
</tr>
<tr>
<td>Dpp – Omb, β-gal (mid)</td>
<td>chicken</td>
<td>1:100</td>
</tr>
<tr>
<td>Dll</td>
<td>mouse</td>
<td>1:500</td>
</tr>
<tr>
<td>DLP</td>
<td>mouse</td>
<td>1:5</td>
</tr>
<tr>
<td>Rab 5</td>
<td>rabbit</td>
<td>1:500</td>
</tr>
<tr>
<td>HRS</td>
<td>guinea pig</td>
<td>1:500</td>
</tr>
<tr>
<td>Pmad</td>
<td>rabbit</td>
<td>1:1000</td>
</tr>
</tbody>
</table>
Table 2b. Secondary Antibodies Used

<table>
<thead>
<tr>
<th>Ig</th>
<th>Fluorophore</th>
<th>Concentration (diluted in PBTN)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Goat anti-guinea pig</td>
<td>A647</td>
<td>1:500</td>
</tr>
<tr>
<td>Goat anti-mouse</td>
<td>A488</td>
<td>1:500</td>
</tr>
<tr>
<td>Goat anti-mouse</td>
<td>A568</td>
<td>1:500</td>
</tr>
<tr>
<td>Goat anti-mouse</td>
<td>A647</td>
<td>1:500</td>
</tr>
<tr>
<td>Goat anti-rabbit</td>
<td>A488</td>
<td>1:500</td>
</tr>
<tr>
<td>Goat anti-rabbit</td>
<td>A568</td>
<td>1:500</td>
</tr>
<tr>
<td>Goat anti-rabbit</td>
<td>A647</td>
<td>1:500</td>
</tr>
<tr>
<td>Goat anti-rat</td>
<td>A488</td>
<td>1:500</td>
</tr>
<tr>
<td>Goat anti-rat</td>
<td>A647</td>
<td>1:500</td>
</tr>
<tr>
<td>Rabbit anti-GFP</td>
<td>A488</td>
<td>1:500</td>
</tr>
</tbody>
</table>

2.3 Data Analysis

Mounted, stained wing discs were analyzed with a Zeiss LSM510 and LSM780 confocal microscope using a 40X oil objective. Z-series projections were rendered as a maximum intensity projection using Zeiss LSM510 (version 3.2) or ImageJ software. Data are presented as projections of optical sections. Wing discs were rotated and oriented following convention: dorsal side on the top, ventral on the bottom, anterior to the left, and posterior to the right. ImageJ and Adobe Photoshop were used to adjust brightness, contrast, and color balance.
Chapter 3

RESULTS

3.1 Mutations in oxt have a mild effect on Wg signaling while sfl, and oxt-sfl strongly disrupt Wg signaling in the wing disc

The Wg ligand is packaged into vesicles by expressing cells in a band 1 - 2 cells wide along the dorsal-ventral (D-V) boundary of the wing disc, where it forms a concentration gradient that controls growth and patterning. Wg forms a morphogen gradient and directs cells in a concentration-dependent manner for both short- and long-range targets. Clones in oxt, sfl, as well as the double mutant oxt-sfl show a reduced spreading of the morphogen gradient in Wg signaling. However, mutants in sfl and oxt-sfl show a greater reduction.

3.1.1 Distribution of Wg and Wg Short-Range Sen Expression

In the wild-type tissue Wg expressing cells form a band along the D-V boundary, and senseless (sen), a short-range target of Wg, forms two bands along the D-V boundary. In oxt clones, Wg distribution and Sen expression are subtly reduced. Thus, oxt has a mild effect on the distribution of Wg in receiving cells and downstream Sen target expression. Whereas, sfl and oxt-sfl show a greater reduction in the number of Sen expressing cells and in the distance Wg traveled from the D-V boundary in mutant tissues (Figure 8).
Figure 8: *oxt* has a mild effect on the distribution of Wg in receiving cells and downstream Senseless target expression, while *sfl* and the double mutant block both. Mutant tissue marked by the absence of green. Distribution of Wg in receiving cells is shown in red. Downstream Sen target expression is shown in blue.

### 3.1.2 Wg Short-Range Ac Expression

Another short-range target of Wg signaling is *achaete (ac)*. In the wild-type tissue, Ac is detected in the anterior compartment close to the D-V boundary on either side of the Wg domain. In mutant clones of *oxt*, *sfl*, and *oxt-sfl*, Ac staining is strongly reduced in all three signaling contexts suggesting that proper proteoglycan formation is more essential for expression of Ac than other short-range Wg target genes (Figure 9). *sfl* and *oxt-sfl* show a slightly more severe disruption than *oxt*, as low levels of Ac is expressed in *oxt* mutant tissues.
Figure 9: *oxt, sfl, and oxt sfl double mutant strong block short-range Achaete target expression*. Mutant tissue marked by the absence of green. Ac expression is shown in red.

3.1.3 Wg Long-Range Target Vg-Q Expression

In the wild-type tissue *vestigial* (*Vg*) expression throughout the wing pouch quadrant is dependent on Wg signaling at long-range. An enhancer trap that places the *lazZ* gene under the control of the *vestigial-quadrant* enhancer was added to the background of our mutant lines and therefore, expression of B-galactosidase is readout of Wg long-range signaling in these lines. In *oxt, sfl, and oxt-sfl* mutant clones, expression of Vg-Q is reduced in comparison to the wild-type tissue. However, the effect is more severe in *sfl* and *oxt-sfl* double mutant than in *oxt* (Figure 10).
Figure 10: *oxt* has a mild effect on the distribution of Vg-Q in receiving cells while *sfl* and the double mutant show a more severe signaling disruption. Mutant tissue marked by the absence of green. Vg-Q expression is shown in red.

### 3.1.4 Extracellular Wg Signaling

An extracellular staining protocol, in which the primary antibody is applied before fixation, was used to better visualize the extracellular Wg gradient. Clones in *oxt*, *sfl*, and the double mutant *oxt-sfl* all show reduced spreading of the morphogen gradient in terms of reduced intensity and distance traveled of Wg vesicles from the D-V boundary in comparison to the wild-type tissue. However, the reduction in *oxt* is subtle. *oxt-sfl* shows a greater reduction in both number and distance traveled than *oxt*. Mutation in *sfl* shows the greatest amount of disruption in signaling and expression (Figure 11).

A lower extracellular Wg concentration would be expected to reduce short- and long-range target activation and could be due to a decreased stability of the Wg ligand. Mutant cells could also cause a decreased ability of extracellular Wg to bind to target cells [Bornemann 2004]. The reduction in extracellular Wg and the ectopic Wg expression in mutant clones near the D-V boundary are indicative of compromised Wg signal transduction [Bornemann 2004].
Figure 11: **Extracellular Wg is mildly reduced in oxt clones.** The *oxt sfl* double mutant has a more pronounced effect, while *sfl* almost completely blocks Wg expressing cells. Mutant tissue marked by the absence of green. Extracellular Wg expression is shown in red.

Analysis of the mean intensity was used to compare the relative level of extracellular Wg in *oxt, sfl, oxt-sfl,* and wild-type tissue (Figure 12). An area of approximately 420 μm² was analyzed in each case. Results show that *oxt* had the smallest mean intensity of 82.53, and wild-type had the largest mean intensity of 1112.19. *sfl* and *oxt-sfl* had a similar mean intensity, however, it was unexpectedly higher than *oxt.*

![Mean Intensity Chart](chart.png)

Figure 12: **Analysis of extracellular Wg levels shows that the reduction is greatest in oxt.**
3.2 Mutations in oxt have a small effect on Hh signaling while sfl, and oxt-sfl strongly disrupt Hh signaling in the wing disc

In the wing disc, Hh is expressed in the posterior compartment and moves towards the anterior-posterior (A-P) boundary where it binds the Hh receptor to induce transcription of patched (ptc) and stabilizes the transcription factor Cubitus interruptus (Ci) in cells along the A-P boundary [Bornemann 2004]. The Hh signaling ligand is found in punctated vesicles within receiving cells in the anterior compartment and is present throughout the cell. Mutant clones in oxt, sfl, and oxt-sfl were analyzed for Hh ligand movement and activation of its targets. Clones of oxt, sfl, and oxt-sfl show a reduced spreading of the morphogen gradient in Hh signaling. However, mutants in sfl and oxt-sfl show a more severe reduction.

3.2.1 Distribution of Hh Ligand

In wild-type tissue the Hh ligand is expressed in the entire posterior compartment and moves towards the A-P boundary. Hh can be detected as punctated vesicles within receiving cells 3 – 4 cell diameters from Hh producing cells. Two observations were made in oxt, sfl, and oxt-sfl mutant tissues. First, there was a marked decrease of Hh in posterior mutant clones. When sfl and oxt-sfl clones were found at the A-P boundary there was an absence of punctated vesicles in receiving cells, suggesting Hh is not being taken up by receiving cells. Suitable A-P clones to evaluate this phenotype in the oxt mutant tissues were not observed (Figure 13).
Figure 13: *sfl* and *oxt-sfl* show disruptions in Hh distribution and a reduction in distance traveled in the posterior compartment. Mutant tissue marked by the absence of green. Distribution of Hh in producing and receiving cells is shown in red.

3.2.2 Hh Long- and Short-Range Targets Ci and Ptc Expression

In the wild-type tissue, Ci can be detected at low levels throughout the entire anterior compartment, with a more intense band of cells forming along the A-P boundary, where it is stabilized by Hh [Bornemann 2004]. The width of this band is reduced in mutant clones in *oxt, sfl*, and *oxt-sfl* at the A-P boundary and into the anterior compartment, with a significant reduction in *sfl* and *oxt-sfl*. The severe reduction in the number of cells that show Ci stabilization suggests that *sfl* and *oxt-sfl* reduce the effective range of Hh signaling in the anterior compartment more so than *oxt* (Figure 14).

Ptc is expressed in a band along the A-P boundary. This receptor is expressed in the anterior compartment in response to binding the Hh ligand. Ptc is expressed in a gradient from the A-P boundary into the anterior compartment. This gradient is reduced in clones of *oxt, sfl*, and *oxt-sfl*, with a significant reduction seen in *sfl* and *oxt-sfl* (Figure 14).
Figure 14: *oxt* has a small effect on the distribution of short-range Patched expression and long-range Cubitus interruptus, while *sfl* and the double mutant strongly block both Hh targets. Mutant tissue marked by the absence of green. Ptc expression is shown in red. Long-range Ci is shown in blue.

3.3 Mutations in *oxt*, *sfl*, and *oxt-sfl* all strongly disrupt Dpp signaling in the wing disc

*Drosophila* Dpp is a target of Hh signaling. Dpp is induced in a stripe of cells along the A-P boundary in wing discs and signals into the anterior and posterior compartments to act as a long-range morphogen by inducing expression of its target genes *spalt-major (salm)* and *optomotot-blind (omb)*. Clones in *oxt*, *sfl*, and *oxt-sfl* all show a strong reduction in the expression of Dpp signaling targets.

3.3.1 Dpp Mid-Range Omb Expression

Omb is a mid-range transcriptional target of Dpp signaling. In wild-type tissue Omb is expressed in a wide band around the A-P boundary that does not reach the ends of the wing disc in response to Dpp. Omb expression is severely restricted in *oxt*, *sfl*, and *oxt-sfl* mutant clones (Figure 15).
Figure 15: **Optomotor-blind, a mid-range target of Dpp signaling, expression is reduced in oxt, sfl and oxt sfl mutants.** Mutant tissue marked by the absence of green. Omb expression is shown in red. oxt, sfl and the double mutant show a similar phenotype.

### 3.3.2 Dpp Long-Range Salm Expression

Salm is a long-range transcriptional target of the Dpp pathway in the wing pouch. In wild-type tissue, salm is expressed along the A-P boundary and into the entire anterior and posterior compartments, showing expression throughout the wing disc. Salm expression is severely restricted in oxt, sfl, and oxt-sfl mutant tissue reflecting diminished Dpp signal transduction. Unlike in the earlier Wg and Hh signaling contexts, it appears that oxt, sfl, and the double mutant all have an equally severe effect in Dpp signaling (Figure 16).
3.4 Dally-like Protein appears to accumulate on the apical surface of the mutant columnar epithelia

In addition to examining the effects of \textit{oxt, sfl}, and \textit{oxt-sfl} mutation on ligand movement and Wg, Hh, and Dpp signaling, \textit{Drosophila} wing discs were examined for overall expression of one of the \textit{Drosophila} proteoglycans, the glypican Dally-like protein (Dlp) in order to determine whether glypican modification caused any changes in Dlp. Dlp is a target of Oxt and Sfl, and is a component of the extracellular matrix required for signaling in the wing pouch. In wild-type discs, Dlp is evenly expressed throughout the extracellular space, except for two bands adjacent to the D-V boundary. When proteoglycan biosynthesis is disrupted, Dlp accumulates on the apical surface of the mutant columnar epithelia in the wing disc showing cellular relocalization of Dlp (Figure 17), the function of which at this time is not clear.
Figure 17: **Dlp accumulates on the apical surface of the mutant columnar epithelia in the wing disc showing cellular relocalization of Dlp.** Mutant tissue marked by the absence of green. Dlp apical and central slices are shown in red.
Chapter 4
DISCUSSION AND CONCLUSIONS

4.1 The Lack of Penetrance of the $oxt^{7H24}$ Mutation

The initial aim of this project was to determine the role, if any, of CSPGs by comparing the effects of mutations in $oxt$, which lack HS and CS biosynthesis, and $sfl$, which has loss of HS function only. Clonal analysis revealed that $sfl$ has a more severe phenotype and a greater reduction of Wg and Hh signaling than $oxt$ clones. This was unexpected because the $oxt$ mutation is theoretically more disruptive to proteoglycan biosynthesis. Clonal analysis of the double mutation $oxt$-$sfl$ shows a similar phenotype to $sfl$, indicating that the $oxt^{7H24}$ mutation is not acting as a functional null in this system, suggesting that a mutation in $oxt$ is not fully penetrant and some residual functional $oxt$ is present in mutant clones. The penetrance of a character is the frequency with which a genotype will manifest itself in a given phenotype. The $oxt$ mutation shows a phenotype intermediate between $sfl$ and the WT, therefore displaying characteristics of non-penetrance. Several possible reasons that could account for the lack of penetrance is perdurance of residual Oxt protein or RNA in mutant cells and initiation of tetrasaccharide linker biosynthesis by alternative sugar added or the addition of xylose to target sites through a different glycosyltransferase.

4.1.1 The Phenomenon of Perdurance

Perdurance occurs when a small amount of stable protein or RNA is sufficient to rescue a wild-type phenotype. $oxt$ was first discovered in an embryonic lethal screen in which both the chromosomal and maternally-loaded RNAs and proteins of normal $oxt$ was removed from Drosophila embryo [correspondence with E.M. Selva].
In this context, \textit{oxt} mutation is an embryonic lethal with a “lawn of denticles phenotype” indicative of loss of Hh and/or Wg signaling. Zygotic loss of \textit{oxt} in which the maternal-load is not removed yielded a late pupal lethal phenotype, which suggests the maternal component is sufficient to support development almost to adulthood. Hence, \textit{oxt} was found to be both stable and essential for early development processes. In the wing discs studied here, the mutation is induced and a single mutant cell grows into a clone. Since \textit{oxt} perdures from embryonic to pupal stages it is likely that traces of functional \textit{oxt} protein or mRNA would also perdure in mitotic clones. These small concentrations may be enough to initiate HS and CS biosynthesis on the core proteoglycans at low levels thus allowing some core proteoglycan to be HS and CS modified.

The issue of perdurance can be addressed in several ways. First, \textit{oxt} embryonic germline clones can be studied. Embryos are more limited in cellular signaling visualization in comparison to wing disc mosaics. A different protocol would be required. Second, small versus large \textit{oxt} clones could be compared. Because Oxt from the progenitor cell would be spread throughout the entire clone, it would be expected that a smaller clone would retain higher concentrations of Oxt than a clone with a larger surface area. Therefore, signaling would theoretically be more disrupted in larger clones. A quantitative analysis similar to the one used to analyze extracellular Wg concentration would be used to accurately compare signaling disruptions. Because wild-type and mutant clone boundaries are sometime difficult to distinguish, it may be difficult to accurately analyze small clones. Difficulties aside, if it is clear that there is no obvious differences in signaling disruptions between small and large clones, this experiment could prove an easy way to eliminate the issue of perdurance. If
perdurance is truly to account for the lack of penetrance, it would be interesting to explore whether the perdurance is due to protein or mRNA through RNA interference experiments.

4.1.2 Alternative Glycosylation

Another possibility, Oxt function could be rescued by alternative glycosylation. Vertebrates have two genes encoding xylosyltransferase I and xylosyltransferase II. However, oxt is the only xylosyltransferase in Drosophila predicted to participate in glucosaminoglycan biosynthesis. It is possible that the core protein is susceptible to the addition of a non-xylose sugar. This alternate sugar could functionally allow the assembly of the tetrasaccharide linker onto the core protein, thus allowing for HS and CS biosynthesis in the absence of Oxt.

To examine the possibility of alternative glycosylation, an experiment could be performed to analyze glycosylation patterns of proteoglycans in the presence and absence of Oxt. This could be done by comparing wild-type and mutant germline embryos. If alternate glycosylation of the core protein can occur, a similar molecular weight will be observed in both.

4.2 Role of HSPGs in Cellular Signaling

Despite reduced penetrance of the oxt mutation, these data support previous findings that HSPGs are essential for proper signaling of the Wg, Hh, and Dpp morphogens [reviewed in Yan and Lin 2009]. Clonal analysis results reveal that the oxt mutation has a strong effect on Dpp and Hh signaling, and a minor effect on Wg signaling (Dpp > Hh > Wg), implying that HSPGs are required in a higher concentration in the Dpp and Hh signaling pathways. These results suggesting that
HSPGs are required for proper signaling support previous findings that the EXT family proteins Tout velu (Ttv), Sister of tout velu (Sotv), and Brother of tout velu (Botv), required for elongation of HS sugar chains, disrupt Wg, Hh, and Dpp gradient formation and intercellular signaling [Bornemann 2004]. However, a complex problem still to be analyzed is how HSPGs and signaling ligands interact in vivo.

Many studies have implicated HSPGs in Wg signaling. Both Wg levels and signaling efficiency are reduced when HSPG concentration is decreased in the ECM [Bornemann 2004]. In tissue culture cells, Wg proteins have close interactions with cell membranes and the ECM, possibly through sulfated proteoglycans. Baeg et al. proposed two models regarding the function of HSPGs in Wg signaling: first, HSPGs may be required for stabilizing a complex between Wg and its receptor, frizzled, and second, HSPGs may restrict the extracellular diffusion of the Wg ligand. Wg binds to GAGs on proteoglycans in the ECM, and appears to interact with the glypican Dly, suggesting that the concentration of HSPGs and Dly may be involved in shaping the gradient of extracellular Wg formation. Clonal analysis reveals that the function of HSPGs in Wg signaling may be to limit Wg extracellular diffusion. An alternative possibility is that HSPGs prevent Wg from being degraded by extracellular proteases [Baeg 2001].

The Wg and Hh pathways are linked by a feedback loop during segment polarity determination [Perrimon 2004]. This linkage can make it difficult to determine which pathways are affected by specific mutations during HS and CS biosynthesis. Evidence shows that HS synthesis is essential for Hh signaling. This is demonstrated when Ci stabilization and Ptc expression are severely reduced in mutant clones in the wing disc (Figure 10). Ci and Ptc levels are maintained in only a thin row
of mutant cells along the A-P boundary, suggesting that cells lacking HSPGs are deficient and unable to spread the Hh signal. Previous studies have shown that other mutants in the HSPG pathway also limit the domain of Ci stabilization, indicating that the range of Hh signaling is impaired and properly modified proteoglycans are required for proper Hh transport [Bornemann 2004]. It is observed that Hh levels are reduced in the posterior compartment clones suggesting that HSPGs may also alter ligand stability [Bornemann 2004]. Altogether, these data show that GAGs are required to facilitate the spreading of the Hh morphogen through the anterior compartment of wing disc cells. It has been shown that GAGs are required to a lesser extent for Wg signaling than for Hh signaling in wing discs. This could be due to the overexpression of Wg compensating for the absence of GAGs that are normally present in the ECM [Perrimon 2004]. The severe phenotype associated with loss of GAGs may be the result of amplification of a reduction in signaling activity in either one or both of the Wg and Hh signaling pathways connected by a regulatory loop [Perrimon 2004].

Data has also shown that HS chains are required for Dpp signaling. Dpp signaling in the wing disc is reduced in mutant clones confirming that HS GAG chains are required for optimal activity of the Dpp pathway. Contrary to the results in this study, other data suggests that like Wg, Dpp can signal in the absence of HSPGs, and Hh is the most severely affected by loss of HS proteoglycans [Bornemann 2004].

4.2.1 Models of Morphogen Gradient Formation

There have been several proposed models of morphogen gradient formation. Recent evidence favors a model in which secreted morphogen molecules interact with their receptors and ECM proteins, including HSPGs in a restricted diffusion manner
(Figure 18a). This model fits well for the movement of Wg, Hh, and Dpp ligand molecules in the wing disc. The binding of these morphogens to GAG chains on HSPGs restricts their diffusion along the surface of receiving cells [Yan and Lin 2009]. This prevents their loss into the ECM. In the absence of properly synthesized HSPGs, signaling ligands may degrade rapidly, resulting in fewer punctates reaching their signaling targets.

Another model of diffusion suggests that signaling molecules undergo planar transcytosis in which morphogens move from their source by active transport through repeated endocytosis and resecretion (Figure 18b). Some studies have shown that endocytosis may play a role in the movement of the Wg ligand [Yan and Lin 2009].

Other proposed models include transfer by lipoprotein particles (Figure 18c). Lipoproteins play a role in mediating morphogen movement in early development and are able to interact with HSPGs. Therefore, it is likely that lipoproteins interact with HSPGs in the ECM through a restricted diffusion mechanism in order to contribute to morphogen movement [Yan and Lin 2004].

Lastly, another model suggests that morphogen movement can occur through direct cell contact through cytonemes (Figure 18d). Cytonemes are apical filopodia that extend from the Drosophila wing disc, allowing direct contact with morphogen producing cells [Yan and Lin 2009].
Figure 18: **Four models of morphogen gradient formation.** A) In the restricted diffusion model, morphogens diffuse by interaction with ECM proteins such as HSPGs. B) In the planar transcytosis model, morphogens are actively transported through repeated rounds of endocytosis and resecretion. C) In the lipoprotein transfer model, morphogens are packaged into lipoprotein particles and transported to receiving cells. D) In the cytoneme model, morphogen receiving cells extend actin-based filopodia from the apical surface toward the morphogen source. From Yan and Lin 2009.

It is very much possible that mechanisms from all these models, and possibly others, contribute to the overall distribution of morphogens from their source to their receiving cells.
4.3 Wg, Hh, and Dpp Signaling in Disease and Development

Many studies have shown that the Wg, Hh, and Dpp signaling pathways play crucial roles in regulating cellular signaling events in early development as well as adult homeostasis. Aberrant signaling of these pathways has been linked to a wide range of diseases. A better understanding of these pathways may lead to new therapies for clinical conditions. For example, aberrations in the Wnt pathway have been linked to cancer and degenerative diseases [Nusse 2005]. Inhibition of the Wnt signaling pathways may form the basis of an effective therapy for certain cancers and other diseases [Moon 2004].

Proper Hh signaling is important not just for early development, but also for tissue maintenance and differentiation in the adult [King 2008]. Inappropriate activation of Hh signaling has been linked to several cancers. The Hh signaling pathway may be a target for therapies to inhibit the growth of both prostate cell lines and tumors [King 2008].

Evidence also supports the importance of GAG chains of proteoglycans in developmental processes. A mutation of the xylosyltransferase gene in the worm sqv-6 has been associated with defective vulval morphogenesis [Wilson 2004]. As shown in this project, proper proteoglycan formation is essential for appropriate Wg, Hh, and Dpp signaling.

This project has demonstrated the complexity and importance of cellular signaling in development. Both the cellular signal and the mode of transportation are vital to gaining a better understanding of intercellular signaling. Hopefully, a better understanding of cell signaling and extracellular regulation will facilitate the development of treatments for diseases related to cell signaling defects.
REFERENCES


