IDENTIFYING THE DYNAMIC OLIGOMERIZATION OF WNTLESS

by

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ABSTRACT

Wnt proteins are a highly conserved family of secreted glycoproteins that act as ligands in receptor-mediated signaling pathways. These complex pathways are present, and tightly regulated, at various stages of development including early embryonic patterning, cell fate decisions, and proliferation of stem cells. The Wnt signal transduction pathway is one of the best characterized intercellular signaling networks. It is strictly regulated in signal-receiving cells by multiple cytoplasmic and nuclear factors, as well as receptor-ligand specificity. Regulation also occurs in the signal-producing cells at the level of Wnt production, transport, and secretion. Post-translational modifications are critical to proper intracellular trafficking and secretion of Wnt proteins. N-glycosylation and lipid modification mediated by the ER resident protein Porcupine is recognized by Wntless, the dedicated Wnt chaperone protein. Proper Wnt secretion and subsequent downstream activation is dependent upon Wntless (Wls) binding and its consequent escort of Wnts through the secretory pathway to the plasma membrane for release. Previous studies using co-immunoprecipitation assays revealed that Wls homo-oligomerization is required for interaction with Wingless (Wg), the prototypical *Drosophila* Wnt. In this study fluorescence correlation spectroscopy with photon counting histogram analysis was used to analyze the oligomeric state of Wls within *Drosophila* S2R+ cells. Results of these experiments indicate that Wls exists as a monomer within the plasma membrane in the absence of Wg; however, other intracellular compartments were not analyzed in this study.
Chapter 1

INTRODUCTION

The study of developmental biology has uncovered several families of signaling molecules crucial for controlling embryogenesis and also maintaining homeostasis in adult tissue. Embryogenesis and organogenesis is a complex process that requires specific cell-cell interactions to facilitate the formation of different cell types in the correct body regions at the correct times. Secreted short-range and long-range signaling polypeptides and their receptors are key players in these cell-cell interactions (Tanaka et al. 2000). Several families of signaling polypeptides have been identified and classified based on their amino acid sequences and structures. For example, the bone morphogenetic proteins (BMPs), the Hedgehogs, the fibroblast growth factors (FGFs), and the Wnts have all been identified, and their intracellular signal transduction networks have been elucidated to some extent (Logan and Nusse 2004). Aberrant regulation of these networks can lead to detrimental developmental outcomes such as birth defects. Additionally, considering the concept of cancer as inappropriate activation of development signaling in adults, it is not surprising that the above mentioned signaling pathways are often involved in this disease, as well as others.

1.1 Discovery of the Wnt Signal Transduction Pathway

In 1973 the physiological importance of the wg gene in Drosophila melanogaster was discovered, and confirmed in 1976, when loss-of-function
mutations caused impaired haltere development and wing deficient flies (Sharma and Chopra 1976). Then in 1982, the proto-oncogene \textit{integrase-1 (int-1)} was identified as an important gene in mouse mammary tumor virus (MMTV)-induced breast cancers in mice (reviewed by Baarsma Königshoff, and Gosens 2013). It wasn’t until five years later, in 1987, that scientists learned the \textit{Drosophila} homolog of \textit{int-1} was identical to the \textit{wg} gene. Ultimately the year 1991 brought about a change in nomenclature for \textit{wg/int-1} and all related genes: the Wnt ligand family (reviewed by Baarsma Königshoff, and Gosens 2013). The name Wnt is a union of \textit{wingless} and its homologous vertebrate oncogene \textit{integrase-1}.

According to The Wnt Homepage provided by Roel Nusse and Xinhong Lim at Stanford University, 19 different \textit{wnt} genes have been identified in mammalian genomes, including humans. There are also 7 \textit{wnt} genes in \textit{Drosophila} and 12 in zebrafish. Wg is still the most widely used protein for exploration of Wnt signaling, but the family is growing (Nusse and Lim 2016).

The Wnt proteins are a highly conserved family of secreted glycoproteins that act as ligands in receptor-mediated signaling pathways. These complex pathways are present, and tightly regulated, at various stages of development including early embryonic patterning, cell fate decisions, and proliferation and maintenance of stem cells (Tanaka, Kitagawa, and Kadowaki 2002; Willert \textit{et al.} 2003; Bänziger \textit{et al.} 2006). The overall purpose of this project was further characterization of the Wnt signaling pathway with a focus on events in the signal-sending cells.

### 1.2 Wnt Ligands

Wnt ligands are characterized as a type of morphogen in that they are secreted signaling molecules that inform cells about their appropriate response relative to the
cells position within a tissue. Morphogens form gradients in which immediate neighboring cells of a signal-producing cell, which see the highest concentration of ligand, undergo the greatest activity while those further away are exposed to lower levels of ligand and show a lesser response. This leads to differential target-gene expression across the tissue (Bartscherer et al. 2006). The use of genetic model systems such as *Drosophila melanogaster* is an effective way to elucidate the complexities of Wnt signaling. Wingless (Wg), the prototypical *Drosophila* Wnt (also known as Wnt-1), is functional in the embryo as a short-range inducer to pattern the epidermis; and at later stages Wg acts at longer distances in a concentration-dependent manner to help coordinate imaginal disc development (Bänziger et al. 2006; Bartscherer et al. 2006).

Wnt proteins are characterized by their amino acid sequence instead of their functional properties because these signaling molecules have been shown to participate in both canonical and non-canonical Wnt signaling pathways (reviewed by McNeill and Woodgett 2010). All Wnts contain a signal sequence, many highly charged amino acids, several potential glycosylation sites, and their trademark 22-25 cysteine residues at conserved positions in the protein molecules (Tanaka, Kitagawa, and Kadowaki 2002; reviewed by Logan and Nusse 2004; Zhai, Chaturvedi, and Cumberledge 2004). Additionally, Wnts have been shown to exist as monomers and achieve proper folds through intramolecular disulfide bonds (Tanaka, Kitagawa, and Kadowaki 2002). They are about 350-400 amino acids in length with a corresponding molecular mass of approximately 40 kDa (Tanaka et al. 2000).

Based on the amino acid sequences Wnt proteins should be soluble. No long stretches of hydrophobic residues have been found in the primary amino acid
sequences; however, their behavior does not align with the properties expected of hydrophilic proteins (Zhai, Chaturvedi, and Cumberledge 2004). A 2003 study by Willert and colleagues succeeded in creating a protocol for purification of Wnt proteins, including *Drosophila* Wingless. They were able to confirm at multiple steps in the purification procedure that Wnt proteins are hydrophobic. Utilization of mass spectrometry revealed that Wnt proteins are lipid modified on a conserved residue, which explains their high degree of insolubility (Willert et al. 2003).

Originally, it was thought that the conserved residue was Cys\(^{77}\) in murine Wnt3a; however, studies since have revealed serine residue 209 in Wnt3a as the lipid addition site (Takada et al. 2006; Janda et al. 2012). Most convincingly was the 2012 study by Janda and colleagues in which crystallization of *Xenopus* Wnt8 (XWnt8) and the cysteine-rich domain of its receptor Frizzled 8 (Fz8-CRD) was accomplished. Through Wnt crystallization, they were able to determine that the originally hypothesized Cys residue is engaged in a disulfide bond and, therefore, cannot accept a lipid addition. Consequently, the conserved serine (Ser\(^{209}\) in Wnt3a and Ser\(^{187}\) in XWnt8) was identified as the consensus lipidation site (Janda et al. 2012). The lipid modification is clearly important for signaling because loss of Wg activity was seen upon removal of the lipid and mutations in the serine residue (Willert et al. 2003; Takada et al. 2006). It is now understood that multiple accessory proteins are required for Wnt processing and secretion, such as Porcupine, the acyltransferase responsible for Wnt lipid modification (see section 1.4).

As mentioned previously, Wnt proteins are known primarily for their role in directing growth and cell fates in diverse processes such as embryonic segmentation, limb development, and CNS patterning. Wnt signaling is also implicated in cancer as
well as the postembryonic regulation of stem cell number and stem cell differentiation suggesting a potential use in medical studies (Tanaka, Kitagawa, and Kadowaki 2002; Willert et al. 2003; Bänziger et al. 2006).

1.3 Wnt Signaling: An Overview

The Wnt signal transduction pathway is one of the best characterized intercellular signaling networks. There are three intracellular signaling pathways by which Wnts elicit cellular responses; two of which are β-catenin independent, and one – known as the canonical pathway – that is dependent on β-catenin to produce its effects. In all three pathways activation of signal transduction occurs upon Wnt ligand binding to receptors on the cell surface – specifically members of Frizzled protein family.

The non-canonical, β-catenin independent, systems for Wnt signaling are the Planar Cell Polarity (PCP) pathway and the Ca$^{2+}$-dependent pathway. The PCP pathway regulates tissue morphogenesis and generally requires cell-cell interaction (reviewed by Baarsma, Königshoff, and Gosens 2013). Upon Wnt binding to cell surface receptors, actin cytoskeleton remodeling is controlled by small G-proteins such as Rho/Rac (reviewed by Mayor and Theveneau 2014).

The Wnt/Ca$^{2+}$ signaling pathway activation of phospholipase C (PLC) leads to the formation of inositol 1,4,5-triphosphate (IP3) and 1,2 diacylglycerol (DAG) from phosphatidylinositol 4,5-bisphosphate (PIP2), a membrane-bound phospholipid (reviewed by Baarsma, Königshoff, and Gosens 2013). The presence of IP3 and DAG causes an increase in intracellular Ca$^{2+}$ levels which results in the activation of calmodulin-dependent protein kinase II (CAMKII), protein kinase C (PKC), and the transcription factor nuclear factor of activated T cells (NFAT), as well as other
transcription factors (reviewed by McNeill and Woodgett 2010; reviewed by Baarsma, Königshoff, and Gosens 2013). The main focus of this laboratory, however, is on canonical Wnt signaling.

Recent literature has greatly increased the knowledge of events and participating components in the canonical Wnt signaling pathway (Figure 1). Regulation stems from multiple cytoplasmic and nuclear factors, as well as receptor-ligand specificity. Wnt ligands act on target cells by binding to the co-receptors Frizzled (Fz) and low-density-lipoprotein receptor-related proteins 5/6 (LPR5/6) which associate with one another only in the presence of Wnts. The lack of co-receptor association in the absence of Wnts, however, leaves the protein Dishevelled (Dsh) inactive allowing for formation of the β-catenin destruction complex. The complex is comprised of Glycogen Synthase Kinase-3β (GSK-3β), Casein Kinase-1 (CK-1), Axin, and Adenomatous Polyposis Coli (APC), and it works to phosphorylate β-catenin, targeting it for degradation. This is how cells normally keep cytoplasmic β-catenin (or Armadillo in Drosophila) at low levels (Logan and Nusse 2004).
Figure 1: Representation of the canonical Wnt signaling pathway in a signal-receiving cell when Wnt ligands are absent (left) and present (right). In the absence of Wnts, β-catenin is recognized by the destruction complex, composed of glycogen synthase kinase-3β (GSK-3β), casein kinase-1 (CK-1), axin, and adenomatous polyposis coli (APC), and subsequently targeted for degradation. In the presence of extracellular Wnt ligands, signaling occurs when a Wnt binds both the seven-transmembrane receptor Frizzled (Fz) and the co-receptor low-density lipoprotein receptor related proteins (LRP) 5 and 6. Activation of the receptors results in inhibition of the destruction complex and consequent accumulation of β-catenin, which enables its nuclear translocation to activate target genes. Reproduced from Baarsma, Königshoff, and Gosens, 2013.
When Wnts are present the co-receptors bind one another and the Wnt; this activates Dsh and recruits Axin to the plasma membrane inhibiting the destruction complex and allowing β-catenin to accumulate in the cell. Due to this effect, evaluating the amount and stability of cytoplasmic β-catenin has been used to assay the functionality of purified Wnts (Willert et al. 2003). The key effector of canonical Wnt signaling will eventually translocate to the nucleus where it interacts with the T-cell factor/lymphoid enhancer factor-1 (TCF/LEF) family of transcription factors to induce transcription of the canonical-targeted genes (Tanaka, Kitagawa, and Kadowaki 2002; Logan and Nusse 2004; reviewed by Baarsma, Königshoff, and Gosens 2013). Many Wnt target genes have been identified. These include some members of the Wnt transduction pathway itself indicating the presence of a feedback loop (Logan and Nusse 2004).

Much less is understood about the processes of Wnt secretion from signal-sending cells (Figure 2) and their movement from one cell to another (Bänziger et al. 2006). It is known that Wnt proteins are synthesized in the ER and then post-translationally modified via N-glycosylation, as well as lipidation. The attached lipid makes an otherwise soluble protein insoluble, and converts it to a membrane-anchored protein. The Wnt is subsequently escorted by Wntless (Wls) through the secretory pathway to the plasma membrane where it is secreted. However, the mechanism for Wnt release is still widely debated and may or may not be dependent on environmental conditions (reviewed by Logan and Nusse 2004).
Figure 2: Representation of Wnt processing, intracellular transport and secretion in signal-expressing cells. In the endoplasmic reticulum (ER) Wnt proteins are folded and post-translationally modified through $N$-glycosylation by the oligosaccharyltransferase complex and lipidation by the acyltransferase Porcupine. Lipid-modified Wnts are recognized by Wntless (Wls) and binding of the two allows for secretory pathway transit from the ER to the Golgi and on to the plasma membrane. Due to the lipid presence on Wnt ligands, it is clear that some morphological alteration or carrier protein is involved in their release; however, this mechanism has not yet been completely elucidated (see section 1.4 for details). Although Wnt activities in the extracellular space are still a bit of a mystery, it has been demonstrated that heparan sulfate proteoglycans (HSPG) aid in regulating Wnt ligand concentration, distribution, and stability. Reproduced from the Doctoral dissertation of Senel Sencer Tektas, 2015.
1.4 Wnt Proteins are Post-translationally Modified

As mentioned above, a Wnt must be glycosylated and lipid modified before it can be secreted. While the necessity for N-glycosylation was understood early on, it wasn’t until the 1990s that the protein’s lipid modification was realized (Papkoff, Brown, and Varmus 1987; Van den Heuvel et al. 1993). Early studies in Wnt signaling found that most of the Wnt protein in transfected cells accumulated in the ER and associated with BiP (immunoglobulin binding protein), indicating it was improperly folded and/or not fully processed (Van den Heuvel et al. 1993; Gething 1999; Tanaka et al. 2000). In 1993 Van den Heuvel and colleagues hypothesized that Wnt processing and secretion is dependent on an accessory protein and absence of said protein in cell culture was the reason for impaired secretion. The group then identified the resident ER protein Porcupine (Porc) as the accessory protein necessary for Wnt secretion when they noticed similar secretion defects between mutated porc and mutated wg. This issue was rectified when Drosophila Porc was expressed (Tanaka et al. 2000).

Porc is a multipass transmembrane ER protein encoded by porcupine (porc), a Drosophila segment polarity gene, and it is essential for normal Wg function (Tanaka et al. 2000; Tanaka, Kitagawa, and Kadowaki 2002; Willert et al. 2003; Reviewed by Logan and Nusse 2004; Zhai, Chaturvedi, and Cumberledge 2004). A study by Tanaka and colleagues in 2000 worked to identify and characterize the structure and patterns of expression of various porc homologs in fetal and adult stages of development. The group showed a lack of sequence similarities between vertebrate Porc homologs and other proteins which suggests a unique protein family with specific functionality. All Porc family proteins analyzed displayed the same overall structure with transmembrane domains at fixed positions and no signal sequence, indicating the first
transmembrane domain most-likely acts as an ER targeting signal (Tanaka et al. 2000).

As the existence and importance of this protein was confirmed, the question then became, what exactly is the function of Porc? What responsibility does it have in enabling proper Wnt secretion? The sequestration of Wnts in the absence of Porc was explained by highlighting Porc’s role in N-linked glycosylation of Drosophila Wg and demonstrating that said glycosylation is impaired when Porc is absent (Tanaka, Kitagawa, and Kadowaki 2002). The oligosaccharyltransferase (OST) complex is localized at the ER membrane and closely associates with a translocon in order to transfer an oligosaccharide chain to the asparagine within an NXS/T target site (asparagine, X = any amino acid except proline, serine/threonine) of a growing polypeptide chain (Silberstein and Gilmore 1996; Lodish et al. 2000). Due to the complex’s fixed localization on the ER membrane, N-glycosylation of proteins occurs before their translations are complete; however Wg seems to be an exception. According to Tanaka, Kitagawa, and Kadowaki (2002), Wg is post-translationally N-glycosylated instead of co-translationally due to the 23 cysteine residues that work to form disulfide bonds co-translationally (Shrimal, Cherepanova, and Gilmore 2015). This competition between the two modifications is the basis for Porcupine’s necessity – Porc aids in glycosylation by forcing Wg anchorage to the ER membrane, thus granting the OST complex the access it needs.

Porcupine is a putative membrane-bound acyltransferase that palmitoylates Wg at a conserved serine residue (Ser209 in murine Wnt-3a) tethering it to the ER membrane (Hofmann 2000; Zhai, Chaturvedi, and Cumberledge 2004; Takada et al. 2006; reviewed by Diaz et al. 2013; Ridgway and McLeod 2015). Many types of
proteins (cytosolic, transmembrane, and secreted) undergo palmitoylation; including Wnts. Palmitoylation is the uniquely reversible covalent attachment of fatty acids to a polypeptide chain and is thought to play a role in regulating both protein localization and function (Zhai, Chaturvedi, and Cumberledge 2004; Munday and López 2007). An unmodified Wnt protein does not associate with membranes. Nonetheless, lipidation facilitated by Porc allows for proper N-glycosylation and increases hydrophobicity, converting Wg from a soluble protein to a membrane-anchored protein which, in turn, affects intracellular trafficking.

Palmitate groups partition with cholesterol and sphingolipids. Lipid rafts are rich in cholesterol and glycosphingolipids and often form signal transduction centers (Simons and Ikonen 1997; Qanbar and Bouvier 2003). Therefore, it is believed that modified Wnts are directed to lipid raft microdomains on the plasma membrane where they are packaged for secretion (Zhai, Chaturvedi, and Cumberledge 2004; Goodman et al. 2006).

1.5 Wnt Secretion

The knowledge of Wnt lipid modifications raises questions as to how the proteins are released and how they travel between cells. Are they actively transported? Are Wnts always tethered to membranes, even in the extracellular space? Do carrier molecules bind to the palmitoyl group? There are many mechanisms thought to be involved in Wg/Wnt release but it is still unclear as to which mechanism is used when and why. Although Wnt activities in the extracellular space remain somewhat mysterious, it has been demonstrated that heparan sulfate proteoglycans (HSPG) aid in regulating Wnt ligand concentration, distribution, and stability particularly in the immediate area surrounding a signal-secreting cell (Baeg et al. 2001; reviewed by
Baarsma, Königshoff, and Gosens 2013). In *Drosophila*, phenotypes similar to *wingless* mutants are seen when Dally, an HSPG, is absent (Lin and Perrimon 1999).

Nevertheless, upon Wnt secretion there are a number of potential binding partners. For example, some data suggests that secreted Wnts can also bind Secreted Frizzled-Related Proteins (SFRP), which resemble the ligand-binding domain of the Frizzled family of Wnt receptors (reviewed by Logan and Nusse 2004). These are actually believed to function as extracellular Wnt inhibitors; but the possibility that they protect Wnts from degradation or aid in Wnt secretion and transport still exists (reviewed by Logan and Nusse 2004).

In terms of Wnt long-range signaling, there are a multitude of theories to explain the phenomenon of a hydrophobic protein traveling up to 30 cell diameters away in the extracellular space. Obviously some morphological alteration or carrier protein is required. One school of thought is that a number of secreted Wnt proteins may be assembled into lipoprotein aggregates to insulate the lipid chains and increase solubility (Chen et al. 2004; reviewed by Bartscherer and Boutros 2008). Another idea first introduced by Greco et al. in 2001 is that ligands are packaged into secretory vesicles, termed argosomes, which then carry Wnts as cargo through the extracellular space (reviewed by Logan and Nusse 2004).

1.6 Wntless Escorts Wnts through the Secretory Pathway in Signal-Producing Cells

For some time Porcupine (described previously) was the only accessory protein of Wnt signaling known to function exclusively in the signal-producing cell. Then, in 2006 three separate studies were published describing a gene that encoded a second protein of this sort. *wntless* (*wls*), *evenness interrupted* (*evi*), and *sprinter* (*srt*)
(to be referred to as \textit{wntless} or \textit{wls} from this point forward) are aliases for the same gene that encodes an evolutionarily and functionally conserved, eight-pass membrane protein (Bänziger \textit{et al.} 2006; Bartscherer \textit{et al.} 2006; Goodman \textit{et al.} 2006). The discovery of \textit{wntless} and its critical importance in Wnt signaling can be extrapolated to its importance in embryogenesis, development, and lifelong health of an animal. 

\textit{wls} is a segment-polarity gene found in \textit{Drosophila}. In \textit{wls} mutant cells there as an accumulation and retention of Wg in the signal-producing cells and a severe decrease in Wg levels in the medium and signal-receiving cells surrounding them (Bänziger \textit{et al.} 2006; Bartscherer \textit{et al.} 2006; Goodman \textit{et al.} 2006). These results indicated that Wls must be present in order for Wg to be secreted at the level necessary to produce a physiological effect (Bänziger \textit{et al.} 2006; Bartscherer \textit{et al.} 2006; Goodman \textit{et al.} 2006). Furthermore, these studies found that neither the presence nor absence of Wls had an effect on any other signaling pathway, secreted proteins, or cellular processes and was not required in the signal-receiving cells (Bänziger \textit{et al.} 2006; Bartscherer \textit{et al.} 2006; Goodman \textit{et al.} 2006). Yet it was observed that Wls functions similarly for all members of the Wnt family leading to the inference that Wls is an ancient partner for Wnts and the two have coevolved (Bänziger \textit{et al.} 2006; Bartscherer \textit{et al.} 2006).

While it is unclear if Wls is involved in releasing Wg from the cell surface, its necessity for proper maturation and intracellular transport of Wnt proteins is clear. Most likely Wls recognizes the porcupine-mediated lipidation on a Wnt ligand and is required to escort the ligand through the secretory pathway to promote proper secretion (Bänziger \textit{et al.} 2006; Bartscherer \textit{et al.} 2006; Goodman \textit{et al.} 2006; reviewed by Baarsma, Königshoff, and Gosens 2013).
Further studies revolving around Wls led to the realization that the retromer complex is also involved in Wnt signaling (Belenkaya et al. 2008). Retromer is known to mediate retrograde trafficking of proteins from endosomes to the trans-Golgi network (TGN). The core of the retromer complex is comprised of Vps35, Vps26, and Vps29 (vacuolar protein sorting-associated) subunits with Vps35 acting as the platform on which the complex assembles (Belenkaya et al. 2008; reviewed by Collins 2008). By generating a mutant vps35, Belenkaya and colleagues were able to determine that retromer’s main function in Wnt signaling is to maintain appropriate levels of Wls. In the model proposed, Wls acts as a Wnt cargo receptor and upon delivery of the cargo to the cell surface Wls is internalized. Two possibilities then exist for endocytosed Wls; in the presence of retromer it will be recycled back to the TGN, or in the absence of retromer Wls will be sent to lysosomes for degradation.

Yet there was a gap in the model. If Wnts are folded and lipid modified in the ER then they cannot travel to the Golgi without either aggregating or binding to a carrier protein. Obviously Wls would be the ideal candidate, however numerous studies found its localization limited to the Golgi and plasma membrane (Bänziger et al. 2006; Bartscherer et al. 2006; Goodman et al. 2006; Belenkaya et al. 2008). In spite of this, two recent and separate studies showed Wls is in fact trafficked from the Golgi to the ER via an ER-targeting sequence at the carboxyl terminus of Wls and a vesicular-tubular cluster called ERGIC2 (ER-Golgi intermediate compartment 2 – Yu et al. 2014; Ratchasanmuang, Tektas, and Selva, in preparation). Therefore, Wls appears to be the lone vital escort for Wnt proteins that loads its cargo in the ER and cycles from there to cell surface and back (Yu et al. 2014; Ratchasanmuang, Tektas, and Selva, in preparation).
While the pathways of Wls in Wnt signal-sending cells have been illuminated, the mechanism by which binding occurs between Wls and Wnts remains to be fully elucidated. Previous studies in the lab provided novel insights into the components and events necessary for Wls-Wg binding. In co-immunoprecipitation experiments performed by Tektas et al. (in preparation) the Wg binding region in Wls was discovered to be roughly within amino acids 113 to 137. Additionally, the study found that homo-oligomerization of Wls is required for Wg binding; and both the first transmembrane domain and residues between amino acids 137 to 223 are involved in this oligomerization (Figure 3).
Figure 3: Predicted topology of Wls. Due to alternative splicing Wls exists in two isoforms within the cell. The *Drosophila* gene encodes isoform A which contains three exons and results in a 594 amino acid (aa) protein, and isoform B – two exons and a 562 aa protein. The first luminal loop is also the largest non-membrane region. It includes an N-glycosylation site at Asn58; and the hypothesized Wg-binding region (aa113 – aa137) and Wls oligomerization region (aa137 – aa223). The first transmembrane domain has been shown to be involved in Wls oligomerization. Amino acid 223 is approximately the start of the second transmembrane domain of Wls. Image from the Doctoral dissertation of Senel Sencer Tektas, 2015.

This information is the basis for the project described here; with the hypothesis that the oligomeric state of Wntless directs intracellular trafficking of Wingless. The main goal of this research was to further investigate the oligomerization of Wls within cells.
To achieve this goal, cell culture was used to optimize a methodology for intracellular visualization of Wls to evaluate its oligomeric state and functionality.

1.7 Fluorescence Correlation Spectroscopy and Photon Counting Histograms

Fluorescence correlation spectroscopy (FCS) and photon counting histogram (PCH) analysis performed using laser scanning confocal microscopes are techniques with single molecule sensitivity and are appreciated for their use in live cells. FCS can be applied for examination of biophysical properties of protein complexes such as determining diffusion coefficients and oligomeric size (Herrick-Davis et al. 2012; Herrick-Davis and Mazurkiewicz 2013). FCS records the fluctuations in fluorescence of a fluorescently-tagged protein as it moves into and out of the laser-illuminated observation volume. A PCH can then be used to estimate the average molecular brightness of the sample (Herrick-Davis et al. 2012; Herrick-Davis and Mazurkiewicz 2013).

FCS was introduced over 40 years ago when Magde, Elson, and Webb (1972) wanted to analyze the diffusion and chemical reaction kinetics of the interaction of ethidium bromide with DNA in solution (reviewed by Briddon and Hill 2007; reviewed by Haustein and Schwille 2007; Herrick-Davis and Mazurkiewicz 2013). Studies thereafter ascertained that not only could FCS measure diffusion coefficients but also chemical rate constants, aggregation, and rotational dynamics (reviewed by Hess et al. 2002). Yet it wasn’t until the 1990s that FCS was paired with confocal microscopy to provide a method sensitive enough to monitor protein dynamics in living cells (reviewed by Briddon and Hill 2007; reviewed by Haustein and Schwille 2007; Herrick-Davis and Mazurkiewicz 2013). FCS has some key advantages over other techniques used to study these interactions in that it provides real time...
information and requires very low amounts of protein. This allows for both temporal and spatial resolution of protein properties at physiological expression levels (reviewed by Briddon and Hill 2007; Herrick-Davis et al. 2012; Herrick-Davis and Mazurkiewicz 2013).

Confocal microscopy-based FCS experiments are performed using a small detection volume created by focusing a laser beam to a diffraction-limited spot (~0.3 μm) using an objective lens with a high numerical aperture (Figure 4). Positioning of a pinhole in the confocal plane creates a detection volume on the order of 10⁻¹⁵ L (reviewed by Briddon and Hill 2007; Herrick-Davis et al. 2012; Herrick-Davis and Mazurkiewicz 2013).
Figure 4: Representation of the experimental set up and light path for an FCS experiment. The laser and high numerical aperture objective are used to generate a small observation volume. When fluorescent molecules pass through the volume they are excited by the laser. The resulting emitted fluorescence is captured by the objective, passes through the dichroic mirror and any necessary emission filters and is focused through a pinhole onto a photon detector. This detector is what records the fluctuations in fluorescence over time. Adapted from Hess et al. 2002; Haustein and Schwille 2007; and Herrick-Davis and Mazurkiewicz 2013.

This technique takes advantage of the natural, spontaneous fluctuations in fluorescence emission of the molecules in thermodynamic equilibrium (reviewed by Haustein and Schwille 2007). So, as fluorescent molecules pass through this volume
they are excited and the emitted photons are recorded in real time by a photon-counting detector.

Autocorrelation analysis of the fluorescence signal depicts the fluctuations as a function of particle number and diffusion time (Herrick-Davis et al. 2012; Herrick-Davis and Mazurkiewicz 2013). Specifically, it compares the size of the fluctuation (δI) from the average intensity (⟨I⟩) at time t with a subsequent fluctuation at a time t + τ later (reviewed by Hess et al. 2002; reviewed by Briddon and Hill 2007; reviewed by Haustein and Schwille 2007; Herrick-Davis and Mazurkiewicz 2013). The autocorrelation function, G(τ), results from using a range of values for τ and can yield information about the average dwell time (τ₀) and the average number of molecules (N) of the fluorescent species within the volume during measurement (Figure 5) (reviewed by Hess et al. 2002; reviewed by Briddon and Hill 2007; reviewed by Haustein and Schwille 2007; Herrick-Davis and Mazurkiewicz 2013). Further, with knowledge of the size of the detection volume, the diffusion coefficient of the species can be calculated; however, that was not completed as part of this project.
Figure 5: Example of a fluorescence intensity trace and an autocorrelation curve. (A) Fluorescence intensity traces are generated for each observation period and show the variations in fluorescent intensity as they are recorded in real time by the photon detector. Generally 5 – 10 consecutive 10-second observation intervals are taken per FCS recording. Some photobleaching usually occurs in the first 10 second interval and, therefore, may not be incorporated into the data. (B) Autocorrelation analysis of the fluorescence intensity trace. From the midpoint of an autocorrelation curve the dwell time ($\tau_D$) can be calculated, as depicted on the graph. Not shown is that there is an inverse relationship between the autocorrelation function at time zero [$G(0)$] and the number of diffusing particles (N). Image reproduced from Briddon and Hill, 2007.

\[
G(\tau) = 1 + \frac{\langle \delta I(t) \cdot \delta I(t + \tau) \rangle}{\langle I \rangle^2}
\]

Analysis of the amplitude of the fluctuations in fluorescence intensity can be used to generate a photon counting histogram (PCH) and determine the molecular brightness of a fluorescent species (Herrick-Davis et al. 2012). Molecular brightness is
a term used to describe the number of photon counts per molecule (Herrick-Davis et al. 2012; Herrick-Davis and Mazurkiewicz 2013). Since the molecular brightness of a protein oligomer is directly proportional to the number of fluorescent molecules present in the complex, it can be used to determine the oligomeric size of that complex (Herrick-Davis et al. 2012; Herrick-Davis and Mazurkiewicz 2013).
Chapter 2
MATERIALS AND METHODS

2.1 Cloning Techniques and Plasmids

Numerous plasmids were generated using various cloning techniques. The original plan for this project was to create plasmids capable of insertion into the Drosophila melanogaster genome to generate transgenic lines, one with the fusion WlsB-mEGFP and another fusion WlsB-mCherry (WlsB is the Wls protein isoform B, Figure 3). However, it was decided to first test the hypothesis of Wls oligomerization in cell culture using FCS. Therefore, only plasmids with the mEGFP fluorescent protein were utilized and are described below. For information regarding plasmid constructs generated with the mCherry fluorescent protein please see Appendix A. All constructs described in this section and in the Appendix were verified by restriction digest and Sanger sequencing (Genewiz).

2.1.1 Traditional Cloning Methods

The empty backbone plasmid pmEGFP-1 was a gift from Benjamin Glick (Unpublished – Addgene plasmid # 36409). This plasmid was chosen because the encoded GFP protein (mEGFP) was changed to be both fluorescently enhanced and exist as a monomer – alanine at amino acid position 206 was mutated to a lysine (A206K mutation). First, primers listed in Table 2.1 were used to obtain mEGFP with the indicated restriction sites (all primers were from Integrated DNA Technologies [IDT]). The NotI site was added for the purpose of fusing mEGFP in frame to WlsB,
whereas the EcoRI site was added to make a soluble mEGFP control in the same vector. PCR reactions were carried out with the Q5 High-Fidelity polymerase kit purchased from New England Biolabs (NEB) according to manufacturer specifications including addition of the optional enhancer. To enable propagation, the cloneJET PCR cloning kit (for blunt-ended cloning – Thermo Scientific) was used, but not according to manufacturer specifications. pJETmEGFP-control plasmid ligations were set up with a 1:4 vector/insert molar ratio and the reactions incubated at 16°C for 14 hours. For the pJETmEGFP-fusion plasmid NEB’s T4 DNA ligase was used under the same conditions.

Table 2.1: Primers used for traditional cloning methods for mEGFP

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Restriction Site Added</th>
<th>Primer Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>pJETmEGFP</td>
<td>NotI</td>
<td>5’ – GTGGTGGGCGGCGGAGAATGCAATGGTGAGGCAAGGGCGAGGAG – 3’</td>
</tr>
<tr>
<td></td>
<td>Xhol</td>
<td>5’ – GTGGTGGCTCGAGTCAGTACAGCTCGTCCATGCGAG – 3’</td>
</tr>
<tr>
<td>pJETmEGFP</td>
<td>EcoRI</td>
<td>5’ – GTGGTGGGATTCATGGTAGCAAGGGGCGAGGAG – 3’</td>
</tr>
<tr>
<td></td>
<td>Xhol</td>
<td>5’ – GTGGTGGCTCGAGTCAGTACAGCTCGTCCATGCGAG – 3’</td>
</tr>
</tbody>
</table>

To generate a plasmid capable of integration into the *Drosophila* genome, a pUASTwlsB-HA vector previously generated in the lab was digested (enzymes from NEB) at the restriction sites listed in Table 2.1 to remove the HA tag (for creation of
the WlsB-mEGFP fusion) and the WlsB-HA fusion (to make the soluble mEGFP). Unfortunately, difficulties with traditional ligations arose and methods to create the desired plasmids shifted to Gibson Assembly techniques. For further information regarding troubleshooting for all cloning techniques please refer to Appendix A.

2.1.2 Gibson Cloning Methods

The NEBuilder HiFi DNA Assembly Cloning Kit (NEB) was used, and the primers listed in Table 2.2 were designed, according to manufacturer recommendations. The same Q5 High-Fidelity polymerase (NEB) was used for PCR reactions to generate the inserts; and the vector, pUASTwlsB-HA, was digested as describe above. After some failed attempts with the Gibson reactions, manufacturer suggestions included using a 1:3 vector/insert molar ratio starting with 200 ng of vector and the total amount of DNA in the reaction was 0.12 pmols. Also, 20% DMSO was added to the reaction and the incubation time was extended from 15 minutes to 1 hour (due to a high GC content in the insert and vector). The reaction was followed by transformation in NEB 10-beta chemically competent *E. coli*. Still, just the pUASTwlsB-mEGFP plasmid was made in this manner; creation of the pUAST-mEGFP control plasmid remained elusive.
Table 2.2 Primers used in Gibson cloning techniques

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>pUASTwlsB-mEGFP</td>
<td>5’ – AAGATCCTCTAGAGGTACCCCTCGAGTCAGTACAGCTC GTCC – 3’</td>
</tr>
<tr>
<td></td>
<td>5’ – TCGTTCACACGCAAGGTGGCCTTCGATGGCGTGGTGG CGGCCG – 3’</td>
</tr>
<tr>
<td>pUAST-mEGFP</td>
<td>5’ – TTCCTTCAAAAGATCCTCTAGAGGTACCCGTGGTGGCT CGAGTCAGTAC – 3’</td>
</tr>
<tr>
<td></td>
<td>5’ – CAAGAAGAGAGAAGACTCTGAATAGGGAATTGGGGTGGTGG GAATTCAATGGTGAGC–3’</td>
</tr>
</tbody>
</table>

2.1.3 Gateway Cloning Methods

Finally, Gateway technology (BP Clonase product #1255-029 and LR Clonase product #12538-120 – Invitrogen) was utilized to make the remaining plasmids necessary for the FCS experiments. All primers used for the addition of \( attB1 \) and \( attB2 \) sites for subsequent homologous recombination are listed in Table 2.3 and were designed according to Invitrogen recommendations. Additionally, PCR reactions were performed using Q5 High-Fidelity DNA Polymerase (NEB) according to NEB protocol. All subsequent reactions for Gateway were carried out according to Invitrogen recommendations except the incubation times for the BP and LR reactions were extended from one hour to three hours.
Table 2.3: Primers required for Gateway cloning

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Addition</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>pJET-mEGFP-Control</td>
<td>attB1</td>
<td>5’ – GGGGACAAGTTTGTACAAAAAAGCAGGCTTTCTAGAAAGATGTGGTGGGAATTTC – 3’</td>
</tr>
<tr>
<td></td>
<td>attB2</td>
<td>5’ – GGGGACCACTTTGTACAAGAAGGCTGGGTTCAAGCAGGCTGGTGGAGTCGAGTCA – 3’</td>
</tr>
<tr>
<td>pCMV-CD86-mEGFP</td>
<td>attB1</td>
<td>5’ – GGGGACAAGTTTGTACAAGAAGGCTGCTGCCTAGAAAGATGTGGTGGGAATTTC – 3’</td>
</tr>
<tr>
<td></td>
<td>attB2</td>
<td>5’ – GGGGACCACTTTGTACAAGAAGGCTGGGTTCAAGCAGGCTGGCCTGCAGTCA – 3’</td>
</tr>
<tr>
<td>pCMV-CD86-mEGFP-mEGFP</td>
<td>attB1</td>
<td>5’ – GGGGACAAGTTTGTACAAGAAGGCTGCTGCCTAGAAAGATGTGGTGGGAATTTC – 3’</td>
</tr>
<tr>
<td></td>
<td>attB2</td>
<td>5’ – GGGGACCACTTTGTACAAGAAGGCTGGGTTCAAGCAGGCTGGCCTGCAGTCA – 3’</td>
</tr>
</tbody>
</table>

Original plasmids coding for CD86 (a known monomeric single-pass transmembrane protein) tagged with either mEGFP or tandem mEGFP-mEGFP were a generous gift from the laboratory of Donna Woulfe (Herrick-Davis et al. 2013). The original constructs were under control of the CMV promoter; however, after transfection into Drosophila S2R+ cells (see next section for details) no expression was detected (data not shown). It was therefore determined the constructs would need to be moved to plasmids where the promoters are known to function in Drosophila. Since the GAL4-UAS system was already in place for plasmids previously described, it was utilized again here. Gateway destination vectors with this promoter, specifically the Gateway 1 Collection (Murphy, unpublished), were obtained from the Drosophila Genomics Resource Center (DGRC – supported by NIH grant 2P40OD010949-10A1).
While the destination vector used here has the ability to add a C-terminal EGFP tag, this function was not used for two reasons. First, the constructs gifted to us by the Woulfe lab already contained these tags. Second, the EGFP in the destination vector does not have the A206K mutation to render the protein monomeric; making it unsuitable for FCS purposes.

Thus, the expression clones created and utilized in FCS experiments were CD86-mEGFP and CD86-mEGFP-mEGFP both under control of the GAL4-UAS system. For all fusion proteins generated for this project, the mEGFP tag was added to the C-terminal end of the protein of interest. Both CD86 and WlsB are membrane proteins in which the C-terminus is cytosolic, indicating that mEGFP remained cytosolic for all fusion proteins. For this project, the soluble mEGFP was used primarily as a transfection control, but could be used as an additional control for FCS measurements if desired. For additional information on Gateway cloning methods used please see Appendix A.3.

2.2 Cell Culture and Transfections

_Drosophila_ S2R+ cells were cultured in Schneider’s cell media (Gibco) supplemented with 10% fetal bovine serum (FBS – Gemini) and 1% Penicillin-Streptomycin (HyClone) and grown at 25°C. S2R+ cells were plated at least 5 hours before transfection in Nunc Cell-Culture Treated Multidishes (4-well – Thermo Scientific) at a density of 5 x 10^5 cells/well and then transfected with the indicated plasmid DNA using Effectene Transfection Reagent (Qiagen) according to manufacturer specifications. At 20 hours post-transfection cells were scraped from their individual wells and transferred to LabTekII Chambered 1.5 German Coverglass System wells (4 wells) previously coated with 0.25g/L Concanavalin A (ConA – see
Appendix B for coating details). Cells were incubated in ConA wells for 30 minutes prior to FCS experiments. Just before loading the wells onto the microscope the Schneider’s media was replaced with 1x Live Cell Imaging Solution (Life Technologies).

2.3 Fluorescence Correlation Spectroscopy (FCS)

FCS measurements were made using a Zeiss LSM880 confocal microscope at the Delaware Biotechnology Institute (microscopy equipment was acquired with a shared instrumentation grant [S10 OD016361] and access was supported by the NIH-NIGMS [P20 GM103446], the NSF [IIA-1301765] and the State of Delaware). Photon excitation with a continuous argon ion laser was performed using a 40x (numerical aperture 1.2) C-apochromat water immersion objective. FCS measurements were made on the plasma membrane, away from the cell nucleus and endoplasmic reticulum, of *Drosophila* S2R+ cells previously transfected with the indicated plasmids. Measurements were recorded at 25°C for 50 seconds, as 5 consecutive 10-second intervals. As the mEGFP-tagged proteins moved through the observation volume they were excited by a 488 nm laser with an intensity of 0.2%. The emitted fluorescence was captured by the objective, passed through a 488 nm beam splitter and was focused onto the photon detector using a pinhole of 1 Airy unit (32 μm). The recordings were analyzed using the Zeiss Zen 2.1 (black) software with a correlation bin time of 0.2 μs and a PCH bin time of 10 μs.

For instrument alignment and calibration 1.6 nM and 0.8 nM dilutions of the calibration dye rhodamine 123 were prepared and placed in LabTekII Chambered 1.5 German Coverglass System wells. With the microscope focused in the 1.6 nM solution, pinhole adjustments for the X and Y planes were performed. Then FCS
measurements were recorded for both dilutions at 25°C for 100 seconds, as 10 consecutive 10-second intervals with excitation from a 488 nm laser, 5% intensity. The light pathway was otherwise the same as described previously; however the recordings were analyzed with a PCH bin time of 200 μs instead of 10 μs.

After pinhole alignment and calibration, FCS recordings were rotationally performed on approximately five each cells transfected with the control plasmids (expression clones CD86-mEGFP and CD86-mEGFP-mEGFP) and those transfected with the experimental plasmid (pUASTwlsB-mEGFP). Cells with an average plasma membrane photon count rate ranging from 50 to 150 kHz were selected and membrane regions containing ruffles or filopodia were avoided. Even with a low laser intensity of 0.2% some photobleaching was observed in most of the first of five 10-second intervals.
Chapter 3

DETERMINATION OF PROMOTER FOR APPROPRIATE EXPRESSION LEVELS IN FCS EXPERIMENTS

3.1 Introduction

As mentioned in the previous chapter the experimental plasmid pUASTwlsB-mEGFP was made originally for integration into the *Drosophila* genome. However, when it was decided to instead use FCS measurements to investigate the oligomeric state of WlsB in cell culture the suitability of the promoter came into question. The GAL4-UAS system is widely used for the ectopic expression of genes in *Drosophila*. In the system, expression of GAL4, a yeast transcriptional activator, is driven by the promoter (or enhancer) for ectopic expression (Phelps and Brand 1998). GAL4 then targets and activates only genes marked with the GAL4 binding site – UAS (Upstream Activation Sequence). Therefore, the activation of a target gene fused to UAS is indirectly controlled by the promoter fused to GAL4. The key feature of this system is that the *GAL4* gene and the UAS-target gene are separated in two individual transgenic lines (Phelps and Brand 1998). Crossing these two lines produces progeny in which the target gene is activated in a spatiotemporal pattern dependent upon the promoter controlling GAL4 (Phelps and Brand 1998 – Figure 6). Thus, cell cultures must be transfected with two plasmids, one containing the promoter and GAL4 construct and another with the UAS-target gene fusion.
Figure 6: Representation of the GAL4-UAS system for ectopic gene expression. In this system target gene expression is controlled by the activation of GAL4, a yeast transcriptional activator. GAL4 expression is driven by a tissue-specific promoter or enhancer. When expressed, GAL4 will bind to its target site, the upstream activation sequence (UAS) and promote expression of the target gene. The two components of the system are kept separate in two different transgenic lines. In the GAL4 line the protein is expressed but has no site to which to bind or gene to activate. In the UAS-target gene line the gene is silent without GAL4 for activation. Crossing these two lines produces progeny in which the target gene is turned on. Image reproduced from Phelps and Brand, 1998.

In previous experiments in this lab, the constitutive actin promoter was used to drive expression of the GAL4 gene leading to high expression levels of the protein of interest, WlsB. While this system worked well for co-immunoprecipitation experiments that lead to this laboratory’s first discovery of the oligomerization of Wls, its use in FCS experiments was questionable. Fluorescence correlation spectroscopy requires low levels of protein expression in order to properly measure the fluctuations in fluorescence.
3.2 Results

To determine the appropriate promoter to use for FCS experiments *Drosophila S2R+* cells were transfected with the indicated plasmids and monitored for 48 hours. Visible differences in the expression levels were seen in terms of the brightness of cells. As seen in Figure 7, cells transfected with genes under control of hsp70 promoter appear to have the lowest expression levels compared to those transfected with the actin 5C promoter or the GAL4-UAS system. Nevertheless, based on these experiments alone it was difficult to be certain if a change of promoter was necessary. FCS measurements were performed on cells transfected using the GAL4-UAS system and because the expression varied enough from cell to cell it was still possible to identify plenty with appropriate levels of expression (those with an average photon count rate on the plasma membrane ranging from 50 to 150 kHz).
Figure 7: S2R+ cells transfected with CD86-mEGFP-mEGFP under control of the hsp70 promoter show the lowest expression levels, but there is variation in the cells transfected using the GAL4-UAS system. All images were taken at approximately 40 hours post-transfection using a Zeiss Axio Observer D1 microscope and a 40x objective. To see the difference in expression levels the exposure time was kept constant – 56.4 ms. The slight green background is due to some autofluorescence in the cell media.
3.3 Discussion

Due to the fact that there were enough cells expressing at suitable levels under the control of the GAL4-UAS system it was determined that this method could still be used for FCS experiments. However, if a lower-expressing promoter was used and the cells were allowed to incubate for longer after transfection but before FCS experiments, perhaps the state of the cells would be more consistent. While there were plenty of cells expressing adequate protein levels (determined by the photon count rate during an FCS experiment [50 – 150 kHz]), less variability amongst the cells would be helpful. A more steady level of expression would allow for quicker scanning and focusing on the cells meaning they would be exposed to less laser light overall. This could help to decrease the amount of photobleaching that can occur before FCS measurements even begin.

For future experiments, the constructs should be moved to the control of the hsp70 promoter and should be evaluated at a series of times post-transfection to ascertain the optimal time frame for executing FCS experiments. This change in promoter will also hopefully increase the transfection efficiency as it will avoid the binary GAL4-UAS system.
Chapter 4

FLUORESCENCE CORRELATION SPECTROSCOPY

4.1 FCS measurements in the plasma membrane show Wntless to be monomeric

Previous studies in the lab have shown that Wntless (Wls) not only exists as a dimer, but that its homo-oligomerization is required for functionality. For this project, FCS and PCH were used to investigate the oligomeric state of Wls specifically in the plasma membrane. Prior to measurements of cells transfected with the wlsB construct the molecular brightness of mEGFP monomer and dimer mimic were determined using membrane protein controls, CD86-mEGFP and CD86-mEGFP-mEGFP respectively. Even under the same conditions, the brightness of these controls can vary from day to day and must be measured multiple times throughout each experiment.

Photon counting histogram (PCH) analysis estimates the average molecular brightness, in the units of counts per second per molecule (CPSM), of a fluorescent species in an observation volume based on the measurements recorded during an FCS experiment. Drosophila S2R+ cells were transfected with the indicated plasmids and proteins were allowed to express for 22 hrs before performing FCS and PCH techniques. The average molecular brightness of 37 cells expressing the monomeric CD86-mEGFP construct was found to be 35,384 CPSM while it was determined that 36 cells expressing the dimer mimic had an average brightness of 51,291 CPSM. Though the brightness of the dimer was predicted to be closer to double that of the monomer, the difference between the two are still statistically significant (Figure 8).
Reasons for the decreased brightness may include disturbances in protein folding, or some photobleaching of the second mEGFP could have occurred (Monillas et al. 2015).

Figure 8: Molecular brightness of CD86-mEGFP and CD86-mEGFP-mEGFP controls. Error bars show the standard deviation and the asterisk indicates statistically significant difference between monomer and dimer brightness (student’s t-test, p < 0.0001).

The same methods were applied to cells transfected with pUASTwlsB-mEGFP in effort to determine the oligomeric state of WlsB in the plasma membrane. The average molecular brightness of 25 cells expressing WlsB-mEGFP was 36,474 CPSM. The brightness of WlsB-mEGFP fusion at the plasma membrane was more
comparable to the monomeric control and was statistically different from the dimer mimic control (Figure 9). Figure 9B shows the overall spread of the data; and while there is considerable spread in each data set the brightness of WlsB-mEGFP is the most consistent of the three. Moreover, all of the data points collected for the brightness of WlsB-mEGFP fall within the spread of data points for the brightness of monomeric control CD86-mEGFP.

Figure 9: Molecular brightness of mEGFP controls and WlsB-mEGFP. A: Error bars show standard deviation and the asterisks indicate statistically significant difference between brightness of the CD86-mEGFP-mEGFP dimer mimic and WlsB-mEGFP (student’s t-test, p < 0.0001). There was no significant difference between CD86-mEGFP brightness and WlsB-mEGFP brightness (student’s t-test, p = 0.699). B: Box plot shows the spread of the data for brightness. There is less overall variation in the brightness data for WlsB-mEGFP showing consistency.
To test for the presence of a mixture of monomers and dimers in the observation volume of cells expressing WlsB-mEGFP, the data was fit to a two-component PCH analysis using the Zeiss Zen 2.1 (black) software. The average molecular brightness values for the monomer and dimer controls acquired from the original one-component fit of the data were used to fix the molecular brightness here. For comparison, the PCH data for both the CD86-mEGFP monomer control and WlsB-mEGFP were subjected to this two-component analysis. However, results of this only further confirmed WlsB-mEGFP as a monomer in the plasma membrane as the component concentrations (monomer vs. dimer) were nearly identical to those of CD86-mEGFP. Additionally, $\chi^2$ analysis determined that the data is better fit to a one-component model.

4.2 Discussion

The results of these studies were unexpected. Co-immunoprecipitation studies previously done in the lab indicate that Wls exists as an oligomer, and most likely as a dimer. So it was very much a surprise that the results of this project indicate otherwise. Upon further research into the S2R+ cell line using the FlyBase Consortium (Attrill et al. 2016), it was discovered that this cell line has a “moderately high” endogenous expression level of the Wls protein. This information was provided to the Drosophila community as part of the modencode project (Celniker et al. 2009).

When performing the next set of FCS experiments at least one additional control is recommended. CD28 is a single-pass transmembrane protein that is known to dimerize and would prove useful in order to confirm the occurrence of dimerization. Additionally, detection of this dimer control in comparison with the dimer mimic
control used in the above experiments would provide further assurance that the
FCS/PCH system is working properly.
Chapter 5

DISCUSSION AND FUTURE DIRECTIONS

Unfortunately, the potential effects of endogenous Wls expression on FCS/PCH experiments were not realized until after the results were obtained. One explanation for the monomer-like brightness of cells transfected with pUASTwlsB-mEGFP may be that oligomers actually are forming, but the proteins in the complex are a mix of products from transfected and endogenous DNA. The proteins encoded by the transfected DNA will have a mEGFP tag while endogenous proteins will not; the binding of the two will therefore appear monomeric instead of dimeric. Alternatively, a mouse or even human cell line could be used for these experiments as the lines would not contain Wls at all. However, another issue arises in that there is no literature to say whether or not oligomerization between the mouse or human homolog of Wls and Wls can occur. Therefore, the potential for endogenously and exogenously coded proteins to bind and interfere with the FCS data still exists. Additional information gleaned from the modENCODE project (Celniker et al. 2009) and FlyBase Consortium (Attrill et al. 2016) is that the Drosophila embryonic S1 cell line expresses “low” levels of Wls. In fact the S1 cell line was reported to express almost seven times less Wls than S2R+ cells (Celniker et al. 2009; Attrill et al. 2016) making it much more suitable for these FCS/PCH experiments.

With that being said, it should be noted that monomeric structures were also seen in the co-immunoprecipitation experiments which were done exclusively on whole-cell lysates. Therefore, neither set of experiments, coIP or FCS/PCH take into
account the possibility that the oligomeric state of Wls may change depending on its subcellular localization. As described in the introduction, Wls functions to escort Wnts through the secretory pathway to the plasma membrane where they are released to the extracellular space. It is possible then that Wls oligomerization is required to bind Wnts for travel through the secretory pathway, but upon reaching the membrane the oligomer dissociates. Perhaps this aids in Wnt secretion, or maybe this dissociation is necessary for Wls recycling via the retromer complex.

Previously in the laboratory an amino acid sequence comparison of Wls residues 1 - 238 across various organisms from Drosophila to humans discovered four fully conserved residues (Tektas 2015). Two of which, glutamine and phenylalanine, were found in the first transmembrane domain (TMD1) as well as two cysteine residues in the first luminal loop at amino acid positions 50 and 72 (Tektas 2015). Both glutamine and phenylalanine are rarely found in transmembrane domains, particularly glutamine due to its polar qualities, indicating it is most likely involved in protein oligomerization (Senes, Gerstein, & Engelman, 2000; Tektas 2015). As part of the Doctoral Dissertation of S.S. Tektas, immunoprecipitation experiments were conducted on a truncated Wls protein; Wls137 – Wls protein of amino acids 1 – 137 where the first oligomerization region, TMD1, and the Wg binding region, aa113 – 137, were retained but the second oligomerization region, aa137 – 223 was removed (see Figure 3). Versions of this truncation were made with and without mutations on the four conserved amino acids in order to shed light on their involvement in Wls oligomerization (Tektas 2015).

It was discovered that mutations in the amino acids of TMD1 were not enough to completely prevent Wls dimers from forming, however the amount of dimers
formed was decreased (Tektas 2015). With this information, subsequent immunoprecipitation experiments were conducted to further elucidate the possible contribution of the conserved cysteine residues on Wls-Wls binding. It was then determined that intermolecular disulfide bonds are formed between cysteines at the same position on each Wls monomer to form Wls homo-dimers (Tektas 2015). These results led to the theory that covalently bound Wls dimers form a binding pocket for Wg, and the complex as a whole is then trafficked through the secretory pathway to the plasma membrane (Tektas 2015).

The possible dissociation of Wls and its effect on Wg release was not incorporated into that theory until the results of this study showed Wls to exist as a monomer at the plasma membrane. This sparked further research into the literature on disulfide bond rearrangement. It was discovered that there is a rapidly expanding field of research on the concept of disulfide bonds acting as a “dynamic scaffold to present mature proteins in different conformational and functional states on the cell surface,” (reviewed by Jordan and Gibbins 2006). Another review by Yi and Khosla (2016) lists many examples of proteins in which cleavage of disulfide bonds can alter protein activity. Additionally, both reviews cite that a number of studies have shown thiol isomerase proteins (chaperones that enable the formation, reduction, and rearrangement of disulfide bonds), including PDIs (protein disulfide isomerases), to function at the cell surface (reviewed by Jordan and Gibbins 2006; reviewed by Yi and Khosla 2016). This information supports the theory that Wls must dimerize to form a Wg binding pocket; the complex of two Wls proteins and one Wg protein then travels through the secretory pathway to the plasma membrane. Once at the membrane, dissociation of the Wls dimer may occur with the help of a PDI or another thiol
isomomerase promoting the release of Wg. To address this theory, additional FCS measurements can be conducted using constructs in which a C-terminal mEGFP tag has been added to the previously made Wls137 truncations with and without cysteine residue mutations that were described above.

To address the idea that Wls oligomerization is dependent on localization, additional FCS studies need to be completed targeting distinct intracellular compartments including the endoplasmic reticulum and the Golgi. ThermoFisher Scientific has a variety of dyes for live cells that can be used to ensure observations of the desired intracellular compartments. For example, the ER-Tracker™ Red (glibenclamide BODIPY® TR) is a cell-permeant, live-cell stain. Glibenclamide binds to the sulphonylurea receptors of ATP-sensitive K⁺ channels that are found on the ER making this dye highly selective for the organelle (Molecular Probes 2005). The red version of the ER-Tracker dye undergoes peak excitation/emission at 587/615 nm compared to GFP which has peak excitation/emission at 488/508 nm (ThermoFisher Scientific 2016). The differences in these two fluorescence spectra along with use of emission filters in the confocal setup should minimize potential cross-talk, allowing these fluorescent molecules to be used simultaneously. There are similar live-cell dyes for targeting the Golgi.

Alternatively, plasmids encoding fluorescently tagged ER and Golgi resident proteins could be co-transfected with the plasmids for WlsB-mEGFP. Many such constructs exist, such as the pDsRed-Monomer-Golgi vector from Clontech; however, most were designed to function in mammalian cells and it is unclear if functionality would remain if transfected into insect cells. Assuming a suitable intracellular marker is found, a two channel setup for the confocal microscope can be used to scan the cell
field. Since the intensity levels of the two fluorophores will most likely be very different, adjusting the contrast should help to identify the useful cells. When a cell is found with the appropriate expression levels of WlsB-mEGFP (photon counts of 50 – 150 kHz), FCS measurements only need to be taken on the channel assigned to that protein.

Once use of the appropriate cell line is established, and all of the desired controls are generated, FCS can be used not only to resolve the oligomeric state of Wls in multiple intracellular compartments but also to determine its diffusion coefficient. Additionally, this research can be taken a step further by utilizing fluorescence cross-correlation spectroscopy (FCCS) to determine the stoichiometry of the Wls-Wg complex. Co-immunoprecipitation results have led to the inference that the complex for Wg movement through the secretory pathway is comprised of two Wls proteins for every one Wg. Dual-color FCCS is a technique in which two spectrally distinct chromophores, such as GFP and RFP, can be measured simultaneously using different channels on a confocal microscope (reviewed by Haustein and Schwille 2007). This method has already been used to study protein-protein interactions in signaling processes where Ca$^{2+}$-dependent binding of calmodulin (CaM) was monitored by separate labeling of calmodulin and the Ca$^{2+}$/CaM-dependent kinase II (reviewed by Bacia, Kim, and Schwille 2006). Also dual-colored FCCS has been used to observe dynamic colocalization in intracellular trafficking (reviewed by Bacia, Kim, and Schwille 2006). Similar methods applied to Wls and Wg can provide further insight to the mechanisms of Wg secretion.
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product porcupine stimulates the posttranslational N-glycosylation of wingless in the

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van den Heuvel M, Harryman-Samos C, Klingensmith J, Perrimon N, Nusse R.,
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3rd, Nusse R., (2003). Wnt proteins are lipid-modified and can act as stem cell


Appendix A

PLASMIDS AND CLONING TROUBLESHOOTING

A.1 mCherry Plasmids and Traditional Cloning Troubleshooting

As mentioned in the materials and methods section, some plasmids were created but not used for FCS experiments. The empty backbone plasmid pcDNA3 mCherry LIC cloning vector (6B) was a gift from Scott Gradia (Unpublished – Addgene plasmid # 30125). Using traditional cloning methods, primers listed in Table A.1 were used to obtain mCherry with the indicated restriction sites. PCR reactions were carried out using the Q5 High-Fidelity polymerase kit purchased from New England Biolabs (NEB) according to manufacturer specifications including addition of the optional enhancer. PCR products were gel purified and extracted using the NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel).
Table A.1: Primers used for traditional cloning methods for mCherry

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Restriction Site Added</th>
<th>Primer Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>pJETmCherry-Fusion</td>
<td>NotI</td>
<td>5’ – GTGGTGGGCGGCCGCGGAAGTGCAATGGTGAGCAAGGGCGAGGAG - 3’</td>
</tr>
<tr>
<td></td>
<td>Xhol</td>
<td>5’ – CCACCACCTCGAGTCATTTATACAGCTCGTCCATGCC - 3’</td>
</tr>
<tr>
<td>pJETmCherry-Control</td>
<td>EcoRI</td>
<td>5’ – GTGGTGGAATTTCATGGTGAGCAAGGCAGGAGGAG - 3’</td>
</tr>
<tr>
<td></td>
<td>Xhol</td>
<td>5’ – CCACCACCTCGAGTCATTTATACAGCTCGTCCATGCC - 3’</td>
</tr>
</tbody>
</table>

To enable propagation, the cloneJET PCR cloning kit (for blunt-ended cloning – Thermo Scientific) was used, but not according to manufacturer specifications. pJETmCherry-control plasmid ligations were set up with a 1:4 vector/insert molar ratio and the reactions incubated at 16°C for 14 hours. For the pJETmCherry-fusion plasmid NEB’s T4 DNA ligase was used under the same conditions. The change in ligase was because there were difficulties in ligating the fusion PCR products into the pJET vector, but it is still unclear why. Troubleshooting the issue included: altering the concentrations of vector and insert; altering the molar ratio of vector to insert; altering the incubation time and temperature; and either ethanol precipitating the ligations or not before transformation (chemically competent DH5α bacterial cells were used for all transformations). Positive control transformations were always efficient. Eventually the NEB T4 DNA ligase was used because it contained
approximately 80-fold more enzyme per μL than the ligase in the cloneJET kit. While this trick worked to produce pJETmEGFP-fusion and pJETmCherry plasmids, it did not work when applied to other ligations.

To generate a plasmid capable of integration into the *Drosophila* genome, a pUASTwlsB-HA vector previously generated in the lab was digested (enzymes from NEB) at the restriction sites listed in Table A.1 to remove the HA tag and the WlsB-HA fusion, respectively, for insertion of *mCherry*. Unfortunately, difficulties with these traditional ligations arose even using the same T4 DNA ligase (NEB) as before. Again I varied the concentration of vector and insert as well as the molar ratio between the two. At first it seemed as though a 1:5 vector/insert ratio was sufficient as it produced many colonies after transformation using ElectroMAX DH5α-E Competent Cells (Invitrogen); however these colonies did not possess the desired final construct.

### A.2 Gibson Assembly Troubleshooting

Due to frustration with failed attempts at traditional cloning, efforts to create the desired plasmids shifted to Gibson Assembly techniques; however these reactions did not work right away either. Table A.2 shows the primers used for Gibson assembly techniques for the plasmids containing the *mCherry* gene. After contacting NEB multiple times for recommendations; altering the concentration of DNA in the reaction; using different vector/insert ratios; transforming using NEB 5-alpha electrocompetent and chemically competent *E. coli* and NEB 10-beta chemically competent *E.coli*; and adding DMSO to the reaction finally I was able to make the pUASTwlsB-mCherry plasmid coding for a fusion protein. What finally worked was a 1:3 vector/insert molar ratio where I used 200ng of vector and the total amount of DNA in the reaction was 0.12 pmols. Also, 20% DMSO was added and the incubation
time was one hour at 50ºC followed by transformation in NEB 10-beta chemically competent *E. coli*.

Table A.2 Primers used for Gibson Assembly for plasmids containing mCherry gene

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>pUASTwlsB-mCherry</td>
<td>5’ – TCGTTCACACGCAAGGTGGCCTCGATGGCGTGGTGCTGGGC GGCCG – 3’</td>
</tr>
<tr>
<td></td>
<td>5’ – TTCCTTCACAAAGATCCTCTAGAGGTACCCCCACCACCT CGAGTCATTTTATACAG – 3’</td>
</tr>
<tr>
<td>pUAST-mCherry</td>
<td>5’ – CAAGAAGAGAACTCTGAATAGGGAATTGGGGTGCTGGG AATTCATGTTGAGC – 3’</td>
</tr>
<tr>
<td></td>
<td>5’ – TTCCTTCACAAAGATCCTCTAGAGGTACCCCCACCACCT CGAGTCATTTTATACAG – 3’</td>
</tr>
</tbody>
</table>

These conditions were applied to generate pUASTwlsB-mEGFP, pUAST-mEGFP, and pUAST-mCherry plasmids; and while they worked well for the mEGFP fusion construct, they did not work for either of the soluble control constructs – the fluorescent genes alone. I went about troubleshooting these issues in the same manner as described above. I also tried digesting the pUASTwlsB-mEGFP and pUASTwlsB-mCherry plasmids previously generated to use as the vector, but to no avail.

Due to these difficulties I decided to apply the knowledge I gained from Gibson assembly and retry traditional cloning to make the control plasmids. Troubleshooting involved much of the same adjustments of concentrations and molar ratios; however this time when I still was unable to get the ligations to work I narrowed my focus to identify the issue. The most informative results were when I
digested the pUASTwlsB-HA plasmid with a single-site restriction enzyme. I then gel purified and extracted half of that digest to simulate the other ligations and left the other half as it was. This was followed by a ligation reaction and transformation of both halves; colonies grew on the plate with DNA not run on a gel, but no colonies grew from the purified and extracted DNA. Clearly these results indicated that something in the gel purification and/or extraction process was making the DNA not suitable for either ligation or transformation.

Some ideas to rectify this were to purify digested DNA using phenol chloroform or a different gel extraction kit. However, due to time constraints the decision was made to instead use Gateway cloning to make the control plasmids and I was unable to confirm my theories. Nevertheless, for future members of the lab attempting traditional ligations I would recommend the following: first digest the vector at one site and see if it will re-ligate with or without gel purification. I would try a few gel extraction techniques including making all necessary buffers in the lab.

Lastly, about a month after abandoning traditional cloning methods, it was discovered that the lab’s miniprep kit was not producing high quality DNA. It is unclear if this did or did not contribute to the cloning difficulties I experienced, but it should be considered.

A.3 More information on plasmids made using Gateway cloning methods

There are three essential steps to Gateway cloning with gel purifications, transformations, and minipreps in between. First the attB1 and attB2 sites must be added on to the 5’ and 3’ ends of your gene of interest, respectively, through PCR. PCR products are then purified and used to create entry clones via the BP recombination reaction in which an appropriate attB substrate and a donor vector
(pDONR221 – Invitrogen) homologously recombine to produce a plasmid with the desired gene of interest. The entry clones created as part of this project were: EntryGFP-MonCon (Soluble Monomeric EGFP), EntryCD86-mEGFP, and EntryCD86-mEGFP-mEGFP. Their associated primers are shown in Table 2.3.

Next an LR recombination reaction must be performed to create an expression clone using the entry clone previously made and a destination vector with the appropriate homologous sites. As mentioned in the materials and methods section, the destination vectors used were part of Gateway Collection 1 from DGRC; specifically I used the vectors listed in Table A.3 to create the expression clones listed in the same table. Each of these vectors are able to generate a C-terminal EGFP tag; however, the EGFP does not have the A206K mutation to render the protein monomeric. The constructs were placed under the control of different promoters in order to test the expression levels of each. As described previously, one advantage to FCS experiments is the necessity for low expression levels and therefore the ability to view proteins at a more physiological level. While it did seem as though genes under control of the hsp70 promoter had the lowest expression levels of the three (Figure 7), I continued to use the GAL4-UAS system for two reasons. One was because the expression varied enough from cell to cell that I was still able to identify those with suitable levels of expression; and the second was because I had already made the WlsB-mEGFP fusion protein in a UAS plasmid ensuring it could later be used to create a transgenic line.
Table A.3: Gateway *Drosophila* destination vectors and resulting expression clones

<table>
<thead>
<tr>
<th>Destination Vector</th>
<th>Promoter</th>
<th>Expression Clones Generated</th>
</tr>
</thead>
<tbody>
<tr>
<td>pAWG (DGRC #1072)</td>
<td>Actin 5C</td>
<td>mEGFP-MonCon in pAWG</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CD86-mEGFP in pAWG</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CD86-mEGFP-mEGFP in pAWG</td>
</tr>
<tr>
<td>pHWG (DGRC #1074)</td>
<td>Hsp70</td>
<td>CD86-mEGFP-mEGFP in pHWG</td>
</tr>
<tr>
<td>pTWG (DGRC #1076)</td>
<td>UAST</td>
<td>mEGFP-MonCon in pTWG</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CD86-mEGFP in pTWG</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CD86-mEGFP-mEGFP in pTWG</td>
</tr>
</tbody>
</table>

Finally, Table A.4 gives more information about the proteins encoded by the plasmids made for this project. A construct encoding CD28 tagged with Venus was not used in this project, but was also a gift from the Woulfe laboratory (Herrick-Davis *et al.* 2013). I would have liked to use it as an additional control for FCS experiments, but unfortunately time constraints kept me from accomplishing that goal. A DNA fusion construct of *CD28-mEGFP* must be generated and aside from specific primers for the addition of *attB1* and *attB2* sites, the laboratory is equipped with all of the necessary materials to achieve this using Gateway cloning. However, as mentioned earlier, the *EGFP* gene in the destination vectors obtained from DGRC do not have the A206K mutation. Thus, this mutation should first be made in the desired destination vector before continuing with Gateway techniques.
Table A.4: Basic qualities of the proteins used in FCS experiments and encoded by the plasmids described in this appendix

<table>
<thead>
<tr>
<th>Protein</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>WlsB</td>
<td>Described in detail in the introduction</td>
</tr>
<tr>
<td>mEGFP</td>
<td>mEGFP stands for monoclonal enhanced green fluorescent protein. The original GFP was identified in the jellyfish <em>Aequorea Victoria</em> and has since been genetically altered to emit higher-intensity fluorescence. When concentrated enough the wildtype protein forms aggregates and thus the necessity for a monoclonal version.</td>
</tr>
<tr>
<td>CD86</td>
<td>A known monomeric receptor. This protein is a single pass, type I membrane protein, meaning that its N-terminus is luminal/extracellular while its C-terminus is cytoplasmic.</td>
</tr>
<tr>
<td>CD28</td>
<td>A known dimeric receptor. This protein is also a single pass, type I membrane protein.</td>
</tr>
</tbody>
</table>
Appendix B

CONCANAVALIN A WELL COATING PROTOCOL

_Drosophila_ S2R+ cells are only mildly adherent and have a very spherical shape with many filapodia projections. Therefore, it is extremely difficult to target the membrane of a cell without some sort of dish coating. First I tried coating wells with laminin; then poly-D-lysine; and then the two substrates together. However, ConA is what finally made the cells spread out. Below is the protocol used for coating LabTekII chambered German coverglass system wells (adapted from a protocol by the Sabatini Lab [http://sabatinilab.wi.mit.edu/sabatini_public/fly_array/ConA.htm]).

**B.1 Concanavalin A Solution**

1. Measure 0.0625 g of Concanavalin A (ConA).

2. In a graduated cylinder, add ConA to 200 mL nuclease free water. Mix by inversion.

3. After ConA is dissolved, bring volume to 250 mL.

4. Pour ConA into a 250 mL jar. Add a sterilized stir bar to jar, seal top of jar and stir ConA on a stir plate for 4 hours at a speed that creates a sufficient vortex in the solution.

5. After 4 hours of stirring, filter solution into a new, sterilized container with 0.22 um vacuum filter.

**B.2 Slide Coating**

The following is performed in a tissue culture hood:
1. Pipette up and down 1 mL of ConA solution 5 times. Do this for all wells and then leave the ConA in the wells for two minutes.

2. Remove ConA from wells and lean them vertically against a pipette tip box in the tissue culture hood.

3. Allow wells to dry like this for 1 hour.

4. Pipette up and down 1 mL of nuclease free water 5 times. Then rinse with 1 mL of fresh nuclease free water. Do this for all wells.

5. Dry wells for 1 hour as before.

Store wells in sterile conditions. The coating quality should be good for 1 week.