The Pennsylvania State University

The Graduate School

Department of Biology

THE ROLE OF THE WINGLESS SIGNALING PATHWAY IN CELL FATE SPECIFICATION

A Thesis in

Biology

by

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Submitted in Partial Fulfillment
of the Requirements
for the Degree of

Doctor of Philosophy

August 2004
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ABSTRACT

Cell-cell communication is essential for the proper development of any multicellular organism. Signal transduction pathways are crucial in the regulation of many cellular interactions during the growth and patterning of such organisms. The Wingless (Wg) signal transduction pathway is essential for many developmental processes during *Drosophila* embryogenesis. Wg is a member of the Wnt family of conserved signaling glycoproteins, and it is required for the segmentation of the embryonic epidermis, specification of cardiac and somatic muscle precursors, and patterning of the embryonic midgut. In addition, during the larval stages, Wg is involved in wing, leg, and eye imaginal disc growth and patterning.

The ultimate objective of the Wg signal transduction pathway is the cytoplasmic stabilization and subsequent nuclear translocation of the Armadillo (Arm) protein. Inside the nucleus, Arm functions as a transcriptional activator to activate Wg target genes. In the absence of Wg, Arm is rapidly phosphorylated and targeted for degradation by a collection of proteins that comprise the “destruction complex”. The serine/threonine kinase Zeste-White 3 (Zw3), a negative regulator of the Wg signaling pathway, is a component of this destruction complex.

The work presented in this thesis expands our understanding of the role that Wg signaling plays in the specification of the somatic musculature. An additional aim of this thesis is to add insight into the role of the N-terminus of Glycogen Synthase Kinase (GSK), the vertebrate homologue of Zw3.

Wg is required for the specification of the muscle progenitor cells expressing the muscle identity genes *S59* and *nautilus* (*nau*). To address whether this specification is
mediated by the canonical Wg pathway, reciprocal gain-of-function and loss-of-function experiments were performed. In addition, an examination into the relationship between the Wg and Notch (N) signaling pathways in the specification of muscle founder and fusion-competent cells was conducted. The results demonstrate that Wg and N act reciprocally in mediating a binary decision between muscle founder and fusion-competent cell fates in the somatic mesoderm.

Zw3 and GSK-3β are very closely related serine/threonine kinases that serve as essential negative regulators of Wg/Wnt signaling. In addition to their structural similarity, they share functional similarity as well since GSK-3β, but not the related GSK-3α, can rescue a zw3 mutant embryo. One hypothesis that is tested is that the difference in regulation between GSK-3β/α is due to differences found in their amino terminal region, which is the region that is required for pseudosubstrate inhibition. Various isoforms of Zw3, which have either the entire unique amino terminus deleted, the N-terminus replaced with that of GSK-3β or replaced with that of GSK-3α, were created and tested, utilizing in vivo assays, to determine differences in activity. The results suggest that unlike GSK regulation, Wg-mediated Zw3 inactivation does not depend on pseudosubstrate binding in vivo. Surprisingly, the N-terminus of GSK-3β was less active than the N-terminus of GSK-3α in Drosophila.
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ACKNOWLEDGEMENTS

Professional Acknowledgements:

This thesis is more than a set of experiments designed to address a biological question. It is the culmination of six years of professional and intellectual growth bolstered by the unbelievable support of my adviser Dr. Esther Siegfried. I thank her for giving me the opportunity to be a part of her laboratory. The analytical skills that I have acquired in the course of earning this Ph.D. are in large part due to the numerous conversations and discussions Esther and I shared. Her open-door policy allowed me to delve deeper into the data and not be afraid to ask a stupid question once in a while. Finally, I would like to thank Esther for generously awarding me research assistantships that enabled me to dedicate more time to research.

Several members of the Siegfried lab have helped me over the years and I would like to offer them my gratitude. I could always count on Dr. Maria Horvat-Gordon, Maria Steitz, and Rasika Kalamegham to virgin flies, collect embryos, or change a solution when I couldn’t. Thanks for being there.

I would also like to thank Dr. Susan Abmayr and members of her laboratory, especially the late Malabika Chakravarti, for their invaluable assistance with the SNS experiments. Malabika was the embodiment of gentleness and I will forever miss her genuineness. I would also like to thank the Thomas, Ordway, Schaeffer, Gilmour, and Lai Labs for providing various “crunch-time” reagents or fly strains. Finally, I would like to thank Manolis Dermitzakis for some illuminating discussions during the embryonic stages of this work.

Personal Acknowledgements:

There are so many wonderful people I need to thank for their love, support, and friendship during my graduate education. My parents Drs. Waheeb and Laila Rizkalla and my sisters Heba and Heidi and their families for their unflinching and neverending support and encouragement during the research phase of this thesis. My amazing wife Gina for her love and uplifting spirit that pushed me to finish during the writing phase and my fantastic daughter Jolene for her heavenly smiles that made preparations for the defense tolerable. Finally, I would like to thank my Lord and Savior Jesus Christ for blessing me with many riches, chief among them my wonderful family. His Love has kept me constant during variable times.

During my time at Penn State, I have been fortunate enough to meet and befriend a great many interesting people. I will forever remember the good times shared with Mike & Kim, Ben, Maria, Erin, Gioia, Alicia, Brian & Kim, Ian, Miro, Stephane, Manolis, and John. Finally, I would like to give a special thanks to Erin, Gioia, Sue, and Breea for giving me shelter when I was leaseless in State College.
Cell-cell communication is essential for the proper development of any multicellular organism. Signal transduction pathways play key roles in the regulation of many cellular interactions throughout the growth and patterning of such organisms. One important class of signaling molecules that is involved in the development of both vertebrate and invertebrate embryos is the Wnt family of secreted growth factors. Wnt genes encode secreted glycoproteins, approximately 350-400 amino acids in length, that are characterized by a conserved pattern of 23-24 cysteine residues. In addition to the 19 Wnts identified in human and mouse, multiple Wnts have also been discovered in *Xenopus* (16), *Danio rerio* (zebrafish) (12), *C. elegans* (5), and *Drosophila* (7) (Cadigan and Nusse, 1997; Siegfried, 1999; The Wnt gene Homepage at http://www.stanford.edu/~rnusse/wntwindow.html). In mice, mutations in Wnts lead to abnormal brain and kidney development (McMahon and Bradley, 1990; Stark *et al.*, 1994; Thomas and Capecchi, 1990), somite and tailbud irregularities (Takada *et al.*, 1994), defects in the female reproductive system (Vainio *et al.*, 1999), as well as many other patterning defects. In *Xenopus* and *C. elegans*, Wnts are involved in axis formation and embryo polarization, respectively (Cadigan and Nusse, 1997; Herman *et al.*, 1995; Rocheleau *et al.*, 1997; Thorpe *et al.*, 1997), while in zebrafish, Wnts are required for precise patterning of the mesoderm and neurectoderm (Lekven *et al.*, 2001) and for convergent extension movements during gastrulation (Heisenberg *et al.*, 2000). Of the seven Wnt genes identified in *Drosophila*, Wingless (Wg/DWnt-1) is the best
characterized (Cadigan and Nusse, 1997; Siegfried, 1999). Wg functions in many developmental processes during *Drosophila* embryogenesis including segmentation of the embryonic epidermis (Bejsovec and Martinez-Arias, 1991; DiNardo *et al*., 1988; Martinez-Arias *et al*., 1988), specification of cardiac and somatic muscle precursors (Baylies *et al*., 1995; Lawrence *et al*., 1995; Park *et al*., 1996; Ranganayakulu *et al*., 1996; Wu *et al*., 1995), and patterning of the embryonic midgut (Immergluck *et al*., 1990; Panganiban *et al*., 1990; Yu *et al*., 1996). Moreover, in the larval stages, Wg is involved in wing, leg, and eye imaginal disc growth and patterning (Siegfried, 1999; Lee and Treisman, 2001; Treisman and Rubin, 1995).

**Wingless/Wnt Signaling**

Research in several model systems has advanced our understanding of Wnt proteins and their roles in development. The integrative use of genetic, biochemical, and cell biological studies has led to the identification of a conserved pathway for the transduction of the Wg/Wnt signal. Homologues of the components of the Wg signaling pathway are found in many species including mouse, *Xenopus*, and *C. elegans*. Moreover, some of these components share functional similarities as well (Cadigan and Nusse, 1997; Siegfried, 1999; The Wnt gene Homepage at [http://www.stanford.edu/~rnusse/wntwindow.html](http://www.stanford.edu/~rnusse/wntwindow.html)).

The canonical Wg/Wnt signaling model that follows is rooted in data obtained from work on various systems. The ultimate objective of this signaling pathway is the cytoplasmic stabilization and subsequent nuclear translocation of Armadillo (the *Drosophila* homologue of β-catenin) (Peifer and Polakis, 2000; Wodarz
and Nusse, 1998). Inside the nucleus, Arm/β-cat functions as a co-transcriptional activator together with members of the TCF/LEF-1 (T-cell factor/Lymphocyte enhancer factor) family of transcription factors where they initiate the activation of Wg/Wnt target genes (Behrens et al., 1996; Brunner et al., 1997; van de Wetering et al., 1997). For that reason, a cell’s ability to regulate the cytoplasmic/nuclear levels of Arm/β-cat is of the utmost importance and is at the core of the Wg/Wnt signal transduction pathway.

In the absence of a positive Wnt signal, Arm/β-cat is rapidly phosphorylated and subsequently targeted for degradation by an intricate collection of proteins that comprise the “destruction complex” (Siegfried, 1999; Peifer, 1994b). Included in the destruction complex are two kinases, the serine/threonine kinase Zeste-White 3 (Zw3, the Drosophila homologue of the vertebrate Glycogen Synthase Kinase-3β) and Casein Kinase 1α (CK1α) (Amit et al., 2002; Bourouis et al., 1990; Liu et al., 2002; Ruel et al., 1993a; Siegfried et al., 1992; Siegfried et al., 1990; Yanagawa et al., 2002). In addition to these two kinases, several proteins play key roles in destabilizing the cytoplasmic pool of Arm/β-cat. Axin (the product of mouse fused) and APC (the gene product of the tumor suppressor gene adenomatous polyposis coli) serve as scaffolds bringing Arm/β-cat into close proximity to Zw3/GSK-3β (Hamada et al., 1999; Hart et al., 1998; Ikeda et al., 1998; Itoh et al., 1998; Rubinfeld et al., 1996; Sakanaka et al., 1998; Zeng et al., 1997) and CK1α. Lastly, the F-box/WD40-repeat protein Slimb (the Drosophila homologue of β-transducin repeat-containing protein, β-TrCP) is involved in targeting Arm/β-cat for degradation via the ubiquitin-proteosome pathway (Aberle et al., 1997; Jiang and Struhl, 1998; Maniatis, 1999).
The structural backbone of the destruction complex is Axin, which not only has binding sites for Zw3/GSK-3β and CK1α but also Arm/β-cat. In addition, the RGS domain of Axin binds APC (Hart et al., 1998; Ikeda et al., 1998; Itoh et al., 1998; Sakanaka et al., 1998). Free Arm/β-cat is also tethered into place by binding to APC (Rubinfeld et al., 1996; Rubinfeld et al., 1993). Once Arm/β-cat is sequestered, CK1 N-terminal phosphorylation primes it for subsequent phosphorylation by Zw3/GSK-3β (Amit et al., 2002; Liu et al., 2002; Yanagawa et al., 2002). As a result, phosphorylated Arm/β-cat interacts with Slimb/β-TrCP, an integral part of the SCF ubiquitin ligase complex. This interaction leads to the ubiquitination of Arm/β-cat which marks it for destruction via the proteosome (Maniatis, 1999).

Degradation of Arm/β-cat is not the only cellular response in the absence of a Wg/Wnt signal. In the absence of nuclear Arm/β-cat, TCF/LEF-1 can act as a transcriptional repressor by interacting with several co-repressors such as Groucho (Gro, a neurogenic gene and member of the Enhancer of Split Complex) and CtBP (C-terminal Binding Protein) (Cavallo et al., 1998; Roose et al., 1998). Furthermore, dTCF has been shown to interact with the histone acetyltransferase, CBP (Creb Binding Protein) to repress gene transcription when Wg/Wnt signaling is inactive (Waltzer and Bienz, 1998). Finally, a recently identified nuclear antagonist to the Wnt/Wg pathway has been identified. Chibby (Cby) was isolated in a screen for proteins interacting with the carboxy domain of Arm/β-cat. cby dsRNA interference mimics Wg hyperactivation and the authors suggest that in the nucleus, Cby competes with TCF/LEF-1 for binding to Arm/β-cat (Takemaru et al., 2003).
As previously stated, Wg/Wnt signaling stabilizes Arm/β-cat in the cytoplasm allowing it to enter the nucleus where it elicits a cellular response (Cadigan and Nusse, 1997). Wg/Wnt-1 is a secreted ligand that binds to an assembly of receptors on the cell membrane of a responsive cell (Bradley and Brown, 1990). This receptor complex is comprised of members of the Frizzled family of seven pass transmembrane proteins (Bhanot et al., 1996; Bhanot et al., 1999; Cadigan et al., 1998; Chen and Struhl, 1999; Zhang and Carthew, 1998) and to Arrow, a single pass transmembrane protein belonging to the LDL-receptor-related protein family (Pinson et al., 2000; Tamai et al., 2000; Wehrli et al., 2000). This ligand-receptor interaction is also bolstered by proteoglycans on the cell surface (Reichsman et al., 1996). Although their function is not fully understood, proteoglycans, like the protein Dally in Drosophila, can increase the stability of the ligand-receptor complex or aid in the presentation of Wg/Wnt to its receptors (Lin and Perrimon, 1999; Tsuda et al., 1999).

Binding of Wg/Wnt to its receptors results in the phosphorylation and activation of the cytoplasmic phosphoprotein Dishevelled (Dsh), a necessary transducer of the Wg/Wnt signal (Noordermeer et al., 1994; Yanagawa et al., 1995). In response to this phosphorylation, Dsh indirectly inactivates Zw3/GSK-3β (Siegfried et al., 1992; Siegfried and Perrimon, 1994). While it remains unclear exactly how Wg/Wnt signaling downregulates Zw3/GSK-3β, Dsh binding to Axin might compete with and limit Axin/Zw3/GSK-3β interactions. As a consequence of the interaction between Dsh and Axin, formation of the destruction complex collapses (Farr et al., 2000; Li et al., 1999; Smalley et al., 1999). As a result, Arm/β-cat escapes phosphorylation and its cytoplasmic levels stabilize (Orsulic and Peifer, 1996; Peifer et al., 1994a; Peifer, 1994b; Siegfried et al.,
1994; van Leeuwen et al., 1994). Hypophosphorylated Arm/β-cat enters the nucleus where it interacts with TCF/LEF-1 to activate target genes (Behrens et al., 1996; Brunner et al., 1997; Molenaar et al., 1996; Riese et al., 1997; van de Wetering et al., 1997) (Fig. 1.1).

**Signaling Components: a closer look outside the cell membrane**

Until 1996, researchers in the Wnt field were assembling a signaling pathway without an identifiable receptor. It was at that time that Fz proteins emerged as possible receptors for the Wg signal. In cell culture assays, DFz2 was able to bind Wg on the cell surface and bring about an increase in cytoplasmic levels of Arm (Bhanot et al., 1996). It was surprising then that flies mutant for *Dfz2* did not mimic the *wg* mutant phenotype (Chen and Struhl, 1999). Further studies revealed that removal of both *Dfz2* and *Fz*, which is required to mediate a Wg-independent planar polarity signal, abolishes the transduction of the Wg signal and that both proteins function as redundant receptors for Wg (Bhanot et al., 1999; Cadigan et al., 1998; Chen and Struhl, 1999; Zhang and Carthew, 1998).

Fz proteins are a family of serpentine transmembrane receptors that are characterized by an extracellular cysteine-rich domain (CRD) at the amino terminus (Cadigan and Nusse, 1997). The CRD is required for Wg/Wnt binding, and its crystal structure has recently been determined revealing novel protein folds that serve as putative Wnt-binding sites (Dann et al., 2001).

In Drosophila, five *frizzleds* have been identified (The Wnt gene Homepage at [http://www.stanford.edu/~rnusse/wntwindow.html](http://www.stanford.edu/~rnusse/wntwindow.html)). While the function of Dfz4 remains
Figure 1.1. The Canonical Wg Signaling Pathway in a Wg Responsive Cell.
The schematic on the left depicts a responsive cell in the absence of a signal, while
the one on the right is in the presence of a Wingless (Wg) signal. In the absence of
a Wg signal, Armadillo (Arm) is phosphorylated and targeted for degradation by a
collection of proteins that comprise the destruction complex. These proteins
include Zeste-White 3 (Zw3), Casein Kinase 1α (CK1α), Drosophila Axin (dAxin)
, and Adenomatous Polyposis Coli (APC). Once Arm is phosphorylated, Slimb
binds Arm leading to its degradation via the ubiquitin-proteosome pathway. Wg
binds to a receptor complex comprised of co-receptors DFrizzled2/Frizzled
(DFz2/Fz) and Arrow, and to the proteoglycan Dally. This results in the activation
and phosphorylation of the phosphoprotein Dishevelled (Dsh). Upon
phosphorylation, Dsh indirectly inactivates Zw3 resulting in the collapse of the
destruction complex. As a consequence of this collapse, Arm escapes
phosphorylation and subsequent degradation and its cytoplasmic levels stabilize and
accumulate. Hypophosphorylated Arm translocates into the nucleus where it
interacts with the transcriptional activator Drosophila T-Cell Factor (dTCF) to
activate Wg target genes. In addition to stabilizing Arm, Wg signaling also
upregulates the expression of naked (nkd). Nkd is an EF-hand containing protein
that binds to and inhibits Dsh to antagonize Wg signaling.
unknown, Dfz3 has been shown to bind Wg in cell culture assays and attenuate Wg signaling in some tissues (Sato et al., 1999), while *smoothened*, another member of the Fz receptor family, is required for Hedgehog signaling (Alcedo et al., 1996; van de Heuvel and Ingham, 1996). Additional Fz-Wnt interactions can also be found in other organisms. In *C. elegans*, the EMS cell fate decision is regulated by a *fz* homologue, the *mom-5* gene, and the gene coding for MOM-2, a Wnt protein (Rocheleau et al., 1997; Thorpe et al., 1997). Likewise, a Fz-Wnt receptor-ligand interaction is required for the migration of the QL neuroblast (Harris et al., 1996). In *Xenopus*, the coinjection of the rat *Rfz1* and *Xwnt-8* into frog embryos recruits XWnt-8 to the plasma membrane and increases expression of XWnt-8 target genes (Yang-Snyder et al., 1996). Additionally, axis duplication as a response to the injection of *Xwnt-5A* results only when embryos are coinjected with *Hfz5* (He et al., 1997).

In addition to Fz proteins, members of the low-density lipoprotein (LDL) receptor-related protein (LRP) family are thought to serve as Wg/Wnt receptors in various organisms. Failure to make these single transmembrane proteins, such as in *arrow* (the *Drosophila* LRP6 homologue) mutant embryos, results in a phenotype that duplicates a *wg* null mutation (Wehrli et al., 2000). Likewise, LRP-6 mutant mice similarly demonstrate mutant phenotypes reminiscent of Wnt loss of function (Pinson et al., 2000). While the genetic evidence strongly implicates a Wnt/LRP interaction, *in vitro* binding assays have failed to conclusively support a direct contact between these two proteins. However, LRPs provide the first link between a receptor and an intracellular pathway component. Recently, a yeast-two hybrid screen conducted by Mao et al., 2001 demonstrated that the intracellular C-domain of LRP-5 can bind and recruit Axin to the
cell membrane. Similarly, a yeast-two hybrid study carried out by Towlinski et al., 2003 verified that the cytoplasmic domain of Arrow interacts with Axin. This study further demonstrated that a Fz/Arrow chimera consisting of DFz2 and the Arrow cytoplasmic domain behaves as a ligand-independent activator of the Wg pathway (Tolwinski et al., 2003). Since the interactions presented in these two separate studies result in the destabilization of Axin, it is speculated that this is possibly one of the ways of transducing the canonical Wnt signal and preventing the degradation of β-catenin (Mao et al., 2001; Tolwinski et al., 2003).

The Wnt/receptor-complex interaction is further regulated by heparan sulfate proteoglycans (HSPGs) on the cell surface (Reichsman et al., 1996). These HSPGs make up part of the extracellular matrix and serve as positive regulators of Wg signaling. The synthesis and specification of these HSPGs requires the action of two gene classes: one class coding for the core protein (e.g. the glypicans dally and dally-like), and the other class coding for the formation and modification of the heparan sulfate glycosaminoglycan chains (e.g. kiwi/sugarless/suppenkasper which codes for the Drosophila homologue of uridine diphosphoglucose [UDP] dehydrogenase) attached to the core protein. Disruption of the expression of either gene class, via genetic mutation and/or dsRNA inhibition, results in segment polarity defects that resemble wg mutant embryos suggesting their requirement for proper Wg signaling (Binari et al., 1997; Hacker et al., 1997; Haerry et al., 1997; Lin and Perrimon, 1999; Tsuda et al., 1999). The requirement of HSPGs in Wnt signaling is also highlighted by the discovery of the evolutionarily conserved, extracellular sulfatase QSulf1. This cell-surface protein is related to heparan-specific N-acetyl glucosamine sulfatases that catalyze the hydrolysis of 6-O sulfates from N-acetyl
glucosamines of heparan during the degradation of HSPGs. In cell assays utilizing a catalytically inactive form of QSulf1, Wnt signaling activity was inhibited (Dhoot et al., 2001). HSPGs are thought to either act as Wnt ligand co-receptors or to aid ligand binding, presentation, distribution, and/or concentration. While an exact mechanism of HSPG action in Wnt signaling is yet to be determined, they clearly play an important role in the regulation of Wnt/receptor-complex interactions (Selleck, 2000).

**Signaling Components: a closer look inside the cell membrane**

In response to the binding of Wg/Wnt to its receptor-complex, the novel cytoplasmic protein Dsh is phosphorylated and recruited to the plasma membrane (Yanagawa et al., 1995). Although its function is not fully understood, Dsh is necessary for the intracellular transduction of the Wg signal (Klingensmith et al., 1994; Siegfried et al., 1994; Thiesen et al., 1994; Noordermeer et al., 1994). In addition, it is required in the Wg/β-cat-independent planar cell polarity (PCP) signaling mediated by the JNK pathway (Krasnow et al., 1995). This phosphoprotein contains three conserved domains: an amino-terminal DIX (Dishevelled, Axin) domain, a central PDZ (Postsynaptic density 95, Discs Large, Zonula occludens-1) domain, and a carboxy-terminal DEP (Dishevelled, Egl-10, Pleckstrin) domain (Cadigan and Nusse, 1997). The Dsh DIX domain, which is required in Wg signaling, is about 37% identical at the amino acid level to the carboxy-terminal DIX domain of Axin and may function in facilitating the binding of these two proteins (Smalley et al., 1999). PDZ domains are found in proteins that are involved in cytoskeletal organization and cell junctions. DEP domains are associated with proteins involved in mediating G protein signaling and are required for Dsh function in the PCP.
pathway (Axelrod et al., 1998). Recently, a genetic screen designed to identify additional dsh alleles has led to the discovery of a putative src homology 3 (SH3)-binding motif in Dsh. In addition to being involved in protein-protein interactions, SH3-containing-proteins are thought to localize proteins to the cell membrane (Penton et al., 2002).

Ongoing work in various model systems has identified a number of proteins that interact with Dsh. While the functional significance remains unclear, Drosophila Casein Kinase 2 (CK2) has been shown to phosphorylate Dsh both in vitro and in vivo (Willert et al., 1997). In addition, the unrelated Casein Kinase 1ε (CK1ε) binds to and increases the phosphorylation of XDsh at the PDZ domain (Peters et al., 1999). Another recently identified protein that associates with and phosphorylates Dsh is the serine/threonine kinase PAR-1. Interestingly, the interaction between PAR-1 and Dsh results in the stabilization of β-cat levels while suppressing the activation of the Dsh-mediated JNK pathway (Sun et al., 2001).

Besides kinases, Dsh also interacts with a number of proteins within the cell to mediate the Wg/Wnt signal. As mentioned above, the shared DIX domain between Axin and Dsh mediate their binding (Smalley et al., 1999). Additionally, the PDZ domain of Dsh binds to the amino-terminal portion of Axin (Li et al., 1999). This binding suggests a mechanism for Dsh-mediated disruption of Axin function in the destruction complex. Another recently identified protein that binds to the Dsh PDZ domain is Naked Cuticle (or Naked, Nkd) (Rousset et al., 2001; Yan et al., 2001). This EF-hand containing protein binds to and inhibits Dsh to antagonize Wg/Wnt signaling. Interestingly, nkd is upregulated in response to Wg/Wnt signaling suggesting a negative feedback loop to attenuate Wg/Wnt signaling. Moreover, this inducible antagonist to the canonical
Wg/Wnt signal has been shown to positively influence the PCP signaling pathway mediated by JNK. This has led to the hypothesis that Nkd acts as regulator of Dsh by limiting its role in the canonical Wg/Wnt pathway and increasing the role of Dsh in the PCP/JNK pathway (Yan et al., 2001).

In addition to negative regulators, Dsh also binds to positive regulators of Wnt signaling. The PDZ domain again serves as the region of interaction between Dsh and the amino-terminal portion of the vertebrate protooncogene Frat-1 (Frequently rearranged in advanced T-cell lymphoma) which has a positive role in Wnt signaling. Frat-1 is the homologue of *Xenopus* GBP (GSK binding protein) which also binds to XDsh (Farr et al., 2000; Li et al., 1999). The role of Frat-1/GBP is to inhibit GSK function (Yost et al., 1998). This interaction could be mediated by Dsh, since it can form a complex with both Axin and GSK and Frat-1/GBP can be recruited to this complex, to inhibit GSK kinase activity (Li et al., 1999; Smalley et al., 1999). So far, no homologues of GBP/Frat-1 have been found in *Drosophila*.

Recently, two yeast two-hybrid screens, conducted to identify Dsh-interacting proteins, yielded two proteins that interact differently with Dsh. The first, Dapper, identified in *Xenopus*, contains an amino-terminal putative leucine zipper domain and a PDZ binding motif in the carboxy end. The PDZ domain of XDsh binds Dapper at this carboxy-terminal motif. This interaction leads to the inhibition of XDsh-mediated Wnt signaling, as well as JNK signaling, and identifies Dapper as an antagonist of Dsh-mediated signaling pathways (Cheyette et al., 2002). The other screen identified a novel Dsh-interacting protein, Frodo (Functional regulator of Dsh in ontogenesis). Also identified in Xenopus, Frodo is an essential and positive regulator of Dsh in the canonical
Wnt signaling pathway. This regulation is mediated by binding of the region in the carboxy-terminal of Frodo to the XDsh DIX domain. Interestingly, Frodo was found to only bind the phosphorylated form of XDsh (Gloy et al., 2002). Like GBP/Frat-1, invertebrate homologues of either Frodo or Dapper have yet to be identified.

Two proteins that play a critical role in assembling the destruction complex that leads to the destabilization of Arm/β-cat are Axin and APC. Mutations in either d-axin or d-apc/apc2, the Drosophila homologues of axin and apc, results in the accumulation of Arm/β-cat in the cytoplasm as well as in the nucleus, suggesting that the normal function of these two proteins leads to the destruction of Arm/β-cat (Ahmed et al., 2002; Hamada et al., 1999; McCartney et al., 1999; Willert et al., 1999). Acting as scaffolds, Axin and APC not only bind to each other, but they also bring together components that are required in the destabilization of Arm/β-cat. Axin is able to bind Dsh, Arm/β-cat, Zw3/GSK-3β, and CK1α while APC binds Arm/β-cat (Behrens et al., 1998; Ikeda et al., 1998; Itoh et al., 1998). GSK-3β phosphorylation of Axin and APC enhances their binding to Arm/β-cat (Fagotto et al., 1999; Hart et al., 1998). Furthermore, it has been demonstrated that Axin can be dephosphorylated in response to Wnt signaling and that the serine/threonine Protein Phosphatase 2A (PP2A) is able to dephosphorylate Axin in vitro (Hsu et al., 1999). In addition, the B56 regulatory subunit of PP2A can bind APC (Seeling et al., 1999). The role of this phosphatase might be to counteract the kinase activity of GSK-3β in response to a Wg/Wnt signal. But acting together in the absence of a signal, Axin and APC negatively regulate Wg/Wnt signaling by down-regulating the level of Arm/β-cat via Zw3/GSK-3β phosphorylation (Behrens et al., 1998; Hamada et al., 1999; Itoh et al., 1998).
The stabilization and subsequent nuclear translocation of intracellular Arm/β-cat is the key step in Wg/Wnt signaling. In the absence of a positive signal, Arm/β-cat is degraded (Cadigan and Nusse, 1997; Siegfried, 1999). This is an extremely crucial step since the finding that many cancers are initiated as a result of mutations in either Arm/β-cat itself or in signaling components, such as APC, that lead to a cell’s inability to regulate Arm/β-cat levels (Polakis, 2000). In addition to its role in Wg/Wnt signaling, Arm/β-cat is also a component of cellular adhesion complexes (Cox et al., 1996; Peifer, 1993; Peifer et al., 1992). Arm/β-cat is an interesting protein comprised of an amino-terminal domain, that is important for regulating its stability as well as potentially serving as a transactivation domain, 12 imperfect repeats of 42 amino acids termed “arm repeats” that are centrally located, and a potent transcriptional activation domain in the carboxy terminus (Orsulic and Peifer, 1996; Peifer and Wieschaus, 1990). The Arm/β-cat crystal structure revealed that the 12 arm repeats form a superhelix comprised of α-helices that makes a positively charged shallow groove which may serve as potential binding sites for Arm/β-cat-interacting proteins (Huber et al., 1997).

N-terminal phosphorylation targets Arm/β-cat for degradation via the ubiquitin-proteosome pathway (Aberle et al., 1997; Yost et al., 1996). Four amino-terminal serine/threonine residues play a critical role in the downregulation of Arm/β-cat. CK1α phosphorylates S45 (on β-cat, S56 in Arm) which primes Arm/β-cat for Zw3/GSK-3β mediated phosphorylation on T41, S37, and S33 (T52, S48, and S44 in Arm) (Amit et al., 2002; Liu et al., 2002; Yanagawa et al., 2002). Removal of these phosphorylation sites results in an activated form of Arm/β-cat that is independent of Wg/Wnt signaling and can escape downregulation (Pai et al., 1997). Phosphorylated residues 33 and 37
comprise a site that can be recognized and bound by β-TrCP/Slimb, a subunit of the E3 ubiquitin ligase. This starts a chain of events whereby hyperphosphorylated Arm/β-cat is ubiquinated and eventually degraded via the proteosome pathway. Interestingly, activation of Arm/β-cat/TCF signaling results in the upregulation and accumulation of β-TrCP which suggests a negative regulatory feedback loop to control levels of Arm/β-cat (Ding and Dale, 2002; Jiang and Struhl, 1998).

In response to Wg/Wnt signaling, Arm/β-cat dissociates from the destruction complex. As its intracellular levels stabilize and accumulate, Arm/β-cat translocates to the nucleus where it associates with TCF/LEF, a high mobility group (HMG) domain-containing transcription factor (Cadigan and Nusse, 1997; Wodarz and Nusse, 1998). Once this bipartite transcriptional complex is formed in the nucleus, TCF is able to bind DNA while Arm/β-cat’s transactivation domain can activate the transcription of Wg/Wnt target genes (Brunner et al., 1997; van de Wetering et al., 1997). A recently identified nuclear protein is thought to take part in this TCF-mediated transcription. pygopus (pygo) encodes a nuclear protein containing a PHD (plant homology domain) finger, which is found in proteins that are involved in chromatin remodeling and transcription co-activation (Belenkaya et al., 2002; Parker et al., 2002; Thompson et al., 2002). Pygo forms a complex with Arm/β-cat/TCF in combination with the newly identified adapter protein, Legless (Lgs, homologue of BCL9) to aide in transcription of target genes (Kramps et al., 2002). Another nuclear protein that is believed to facilitate chromatin remodeling prior to Arm/β-cat transcriptional activation is the Brahma-related gene-1 (Brg-1, homologue of Drosophila Brahma) (Barker et al., 2001), a component of the mammalian SWI/SNF and Rsc chromatin-remodeling complex. Barker et al., 2001
propose that Arm/β-cat recruits Brahma/Brg-1 to TCF/LEF-1 target gene promoters to advance transactivation of Wnt/Wg target genes.

**Zw3/GSK-3β: a kinase in focus**

The downregulation of Zw3/GSK-3β is a fundamental step in the transduction of the Wg/Wnt pathway during animal development (Siegfried, 1999; Siegfried et al., 1994). Given such a prominent role in development, homologues of this serine/threonine kinase can be found not only in vertebrates and invertebrates but in yeast, plants and in the slime mold *Dictyostelium* as well (Dominguez et al., 1995; Harwood et al., 1995; Li and Nam, 2002; Plyte et al., 1999). There are two closely related GSK-3 genes in vertebrates, β and α. These two proteins share 98% amino acid identity in the kinase domain but differ considerably in the amino and carboxy terminals where identity falls to 36% at the C-terminus. In addition, GSK-3α contains a glycine-rich amino-terminal extension which accounts for the size difference between the two isoforms (Woodgett, 1990). As stated earlier, Zw3 is the *Drosophila* homologue of GSK-3β (Siegfried et al., 1992; Siegfried et al., 1990; Woodgett, 1990). These two proteins share 75% amino acid identity overall and 85% identity in the catalytic domain (Siegfried et al., 1992). In addition to this structural identity, GSK-3β, but not GSK-3α, can rescue *Drosophila* embryos lacking maternal and zygotic zw3 suggesting that they potentially share substrate specificity (Ruel et al., 1993a; Siegfried et al., 1992). The difference between the two GSK-3 genes is also highlighted by the phenotype observed in GSK-3β knockout mice. GSK-3β mutants exhibit embryonic lethality due to severe liver degeneration as a consequence of hepatocyte apoptosis, which is caused by excessive TNF toxicity brought
on by reduced NF-κB function (Hoeflich et al., 2000). While demonstrating that it is an essential gene, this lethality also points to a unique requirement for GSK-3β. However, the lack of any phenotype(s) resembling those observed in Wnt signaling perturbations, suggests that GSK-3α/β can potentially substitute for each other during early development. That finding notwithstanding, a recent study demonstrated a potentially unique role for GSK-3α in regulating the production of the amyloid-β peptides associated with Alzheimer’s disease (Phiel et al., 2003).

GSK-3 plays key roles in two very fundamental signaling pathways that affect growth and development. As previously stated, GSK-3 is involved in repressing β-cat in the absence of a Wnt signal. In addition, GSK-3 earned its name as a kinase that phosphorylates and inactivates glycogen synthase in glycogen metabolism (Embi et al., 1980). In the absence of Insulin stimulation, GSK-3 actively phosphorylates glycogen synthase to limit glycogen production. However, as a result of insulin signaling, GSK-3 activity is reduced via phosphatidylinositol 3-kinase (PI3K)-dependent Akt/Protein Kinase B phosphorylation at Ser-9/Ser-21 (GSK-3β/α, respectively) (Cross et al., 1995; Stambolic and Woodgett, 1994).

Aside from being an active inhibitory enzyme whose activity becomes suppressed in response to extracellular signaling, GSK-3 possesses several fascinating characteristics. Chief among them is GSK-3’s requirements for substrate recognition. Many GSK-3 substrates require prior phosphorylation by an earlier “priming” kinase such that the substrate consensus S/T-X-X-X-S/Tp is formed before phosphorylation by GSK-3 can take place (Fiol et al., 1987). At position p+4, the priming kinase would phosphorylate either a serine or threonine residue followed by GSK-3 phosphorylation at
position p. In Insulin signaling, Casein Kinase II is the priming kinase that phosphorylates glycogen synthase while Casein Kinase Iα has recently been shown to prime β-catenin prior to GSK-3 phosphorylation (Woodgett and Cohen, 1984; Amit et al., 2002; Liu et al., 2002; Yanagawa et al., 2002). In addition, the recent crystal structure of GSK-3β suggests a mechanism that addresses this peculiar substrate requirement (Dajani et al., 2001; ter Haar et al., 2001). A resolved pocket consisting of Arg-96, Arg-180, and Lys-205 was found positioned within the substrate binding cleft. These residues are thought to bind the substrate’s primed residue and potentially stabilize GSK-3 in its active conformation. Moreover, mutation of residue Arg-96 reduces GSK-3 phosphorylation of primed substrates (Frame et al., 2001). The presence of this phosphate binding cleft suggested a pseudo-substrate mechanism for GSK-3 regulation.

In Insulin signaling for example, GSK-3β would be phosphorylated on Ser-9 by Akt/PKB. The newly phosphorylated N-terminus would then act as a pseudo-substrate inhibitor by binding the phosphate binding cleft and effectively blocking the active site; thereby, competing with GSK-3 primed substrates and inhibiting its enzymatic activity.

While this pseudo-substrate activity helps clarify GSK-3 regulation in response to Insulin signaling, our understanding of GSK-3 regulation as a result of Wg/Wnt signaling is not as clear. Inactivation via Ser-9 phosphorylation, however, does not seem to be a mechanism utilized by Wnt signaling in regulating GSK-3 activity. Wortmannin is a PI3K inhibitor and thus can limit GSK-3 inactivation via Insulin signaling and Akt/PKB-mediated Ser-9 phosphorylation. While restoring GSK-3 activity in the presence of Insulin, use of wortmannin failed to suppress the Wg-mediated suppression of GSK-3 activity suggesting that Insulin and Wnt signaling regulate GSK-3 differently (Cook et
Moreover, in cell lines that are able to respond to both Insulin and Wnt signaling, addition of Insulin did not stabilize β-catenin levels and addition of Wnt-conditioned media did not increase glycogen synthase activity nor did it lead to the phosphorylation (and activation) of Akt/PKB (Ding et al., 2000). Finally, GSK-3β Ser-9 phosphorylation was not observed following Wnt stimulation (Ding et al., 2000; Ruel et al., 1999). Interestingly, GSK-3β associated with Conductin (an Axin-related protein) is not significantly phosphorylated on Ser-9 even after Insulin stimulation suggesting that GSK-3β complexed with Axin protects against Insulin signaling’s inhibitory Ser-9 phosphorylation (Ding et al., 2000).

So how does Wnt signaling inhibit GSK-3β activity? Although it still remains a relatively incomplete picture, recent insights have shed light onto the Wnt-mediated regulation of GSK-3β. In the absence of a Wnt signal, GSK-3β, along with Axin and APC, participate in the destruction complex that targets β-catenin for degradation (Behrens et al., 1998; Fagotto et al., 1999; Ikeda et al., 1998; Itoh et al., 1998). Both Axin and APC are phosphorylated by GSK-3β and this phosphorylation allows both of these proteins to more effectively bind β-catenin. In addition, GSK-3β phosphorylation of β-catenin is dramatically improved in the presence of Axin (Fagotto et al., 1999; Hart et al., 1998; Ikeda et al., 1998). Upon Wnt stimulation, the destruction complex collapses due to the intervention of two proteins, Dsh and GBP (Yanagawa et al., 1995; Yost et al., 1998). Initiation of the Wnt signaling pathway leads to the phosphorylation and activation of Dsh. As a result, Dsh is able to bind Axin and may either displace GSK-3β from the destruction complex or alter the conformation of Axin thereby limiting GSK-3β’s ability to phosphorylate β-catenin (Kishida et al., 1998; Li et al., 1999). Moreover, GBP and its
mammalian homologue Frat-1 can bind GSK-3β leading to a reduction in β-cat phosphorylation (Li et al., 1999; Smalley et al., 1999; Yost et al., 1998). Given the fact that GSK-3β cannot bind Axin and GBP/Frat-1 at the same time and that GBP/Frat-1 interacts with Dsh (Fraser et al., 2002), it has been speculated that Dsh could potentially recruit these GSK-3β inhibitors to the Axin-anchored destruction complex. Interestingly, a FRATtide (a peptide derived from Frat-1) can inhibit GSK-3β phosphorylation of β-cat and Axin while not affecting the phosphorylation of other primed substrates such as glycogen synthase leaving one to wonder that maybe not all primed substrates are the same (Thomas et al., 1999). Thus, Wnt stimulation leads to the eventual separation of the destruction complex participants. As a result, GBP/Frat-1 binds to and inhibits the activity of GSK-3β leading to the stabilization and hypophosphorylation of β-cat. It should be noted that the above mentioned Wnt-mediated downregulation holds true only for vertebrate GSK-3β since invertebrate homologues of GBP/Frat-1, or a similarly acting molecule, have not been identified.

**Wg/Wnt Signaling in Drosophila**

Of the seven Wnts in Drosophila, Wg, or DWnt-1 has been studied in the greatest detail (Siegfried, 1999). Proper growth and development of the fruit fly heavily depends on Wg signaling. Specification of the somatic mesoderm, embryonic midgut patterning, and patterning of adult appendages like the wing and leg all require Wg (Baylies et al., 1995; Immergluck et al., 1990; Panganiban et al., 1990; Ranganayakulu et al., 1996; Siegfried and Perrimon, 1994; Yu et al., 1996). That of segment polarity gene is perhaps the most investigated function of wg. Early in Drosophila development, maternal and
zygotic acting genes control segmentation, initially patterning the embryo along the anterior-posterior axis (DiNardo et al., 1988; Martinez-Arias et al., 1988). Upon formation of embryonic segmental units, intercellular communication (via the Wg and Hedgehog (Hh) signaling pathways) next determines cell fate and the polarity of each unit (Perrimon, 1994). Wg transcription, in single cell wide stripes, borders engrailed(en)-expressing cells at their anterior edge. This boundary between w g- and en-expressing cells marks the transient parasegmental border that will direct subsequent patterning of the embryo. The posterior edge of the en-expressing cells demarcates the permanent segmental border. En-expression in these cells is required for them to adopt a posterior segmental fate. Wg influence, on its posterior neighboring cells, stabilizes and maintains en expression so that these cells adopt this posterior cell fate (Martinez-Arias, 1993). Just as maintenance of en-expression is dependent on Wg signaling, maintenance of wg-expression is also dependent on Hh expressed in the adjacent En-expressing cells, which signals to maintain wg-expression. The establishment of A/P polarity within each segmental unit is therefore brought about by the combinatorial action of Wg and Hh (DiNardo et al., 1988; Heemskerk et al., 1991; Martinez-Arias et al., 1988).

The effect of this segmental patterning is evident in the differentiation of ventral cuticle pattern on the larval epidermis. Six rows of secreted small hair-like projections called denticles, that are precisely and uniquely arranged, decorate the anterior region of each segment. The posterior region of each segment, on the other hand, is devoid of such structures and instead secrete naked cuticle. The resulting pattern of alternating naked and denticle-secreting cuticle adorn the ventral surface of the embryo (Martinez-Arias, 1993). Wg is required for naked cuticle secretion in the posterior cells of each segment.
(DiNardo et al., 1988; Heemskerk et al., 1991; Martinez-Arias et al., 1988). The two en-expressing cells that neighbor the wg-expressing cell give rise to two different cell fates with the one closest secreting naked cuticle and the one most posterior secreting a denticle. In addition, cells located away from the range of Wg, in the anterior region, adopt a denticle fate (Bejsovec and Martinez-Arias, 1991; Dougan and DiNardo, 1992; Martinez-Arias et al., 1988). Cuticle (and denticle) identity is established through the opposing interplay of the Wg and Epidermal Growth Factor Receptor (EGFR) pathways. EGFR activation specifies denticle production while Wg signaling leads to the secretion of naked cuticle (O'Keefe et al., 1997; Szuts et al., 1997). Wg-mediated downregulation of shavenbaby (svb) is required for specification of naked cuticle while the EGFR pathway activates svb expression to specify denticle formation (Payre et al., 1999).

In addition to wg, mutations in three other Drosophila Wnt homologues, DWnt-2, DWnt-3/5, and DWnt-4, have been characterized (Cohen et al., 2002; Graba et al., 1995; Kozopas et al., 1998; Yoshikawa et al., 2003). Removal of DWnt-2 results in male sterility resulting from abnormalities in the size and morphology of the testes and the muscle layer of the testes sheath. Further characterization of this mutation revealed the lack of an outer pigment cell layer of the reproductive tract sheath. Additionally, misexpression of DWnt-2 in females results in the formation of ectopic pigment cells (Kozopas et al., 1998). DWnt-2 is also required during pupation for the correct patterning of the direct flight muscles of Drosophila and for their proper attachment to the epidermis (Kozopas and Nusse, 2002). Like DWnt-2, DWnt-4 mutants also exhibit a sex-specific phenotype. The highly divergent DWnt-4 has recently been shown to be required for the appropriate migration of the apical cells in the epithelial sheets that cover
the female ovariole (Cohen et al., 2002). Furthermore, these mutants exhibit reduced levels of focal adhesion kinase that is required for cell motility. An examination of other Wg-signaling components for a similar migration defect revealed that DFz-2 mutants phenocopy the DWnt-4 mutants, suggesting that DFz-2 could serve as a DWnt-4 receptor. In addition, a mutant allele of dsh, that is defective in PCP signaling but is capable of transducing the Wg signal, revealed a less severe DWnt-4 phenotype. While potentially utilizing DFz-2 and Dsh, the pathway that transduces the DWnt-4 signal is independent of many of the Wg- and PCP-signaling component and may represent a unique way of transducing a Wnt signal (Cohen et al., 2002). Finally, DWnt-5 (also known as DWnt-3) has recently been shown to be required for axon guidance in the Drosophila embryonic nervous system (Yoshikawa et al., 2003). A screen for regulators of the WIF domain-containing Derailed (Drl), a member of the RYK subfamily of atypical receptor tyrosine kinases, revealed that DWnt-5 can bind to this extracellular receptor. DWnt-5 mutants, much like drl mutants, are viable but uncoordinated with disorganized commissures suggesting that DWnt-5 is the ligand for Drl in guiding axons into anterior commissures (Yoshikawa et al., 2003).

**Wnts in other organisms**

Mouse int-1, the first Wnt gene discovered, was recognized as a protooncogene that was activated by the incorporation of a mouse mammary tumor virus (Nusse and Varmus, 1982). The ectopic Int-1 expression that followed resulted in mammary tumors. Int-1 was renamed Wnt-1 soon after the discovery that it was the mouse homologue of Drosophila Wg (Cabrera et al., 1987; Rijsewijk et al., 1987). wnt-1 knockout mutants
show significant loss of the midbrain, as well as apoptosis in the metencephalon (McMahon and Bradley, 1990; Thomas and Capecchi, 1990). The classical mouse mutant *swaying*, a mutant allele of *wnt-1*, results in defects in the anterior regions of the cerebellum (Thomas et al., 1991). Moreover, the simultaneous removal of both *wnt-1* and *wnt-3a* leads to a reduction in the number of neural crest progenitors (Ikeya et al., 1997). Additional mouse knockouts demonstrated the requirement of Wnt function in many developmental processes. Absence of *wnt-3* leads to defects in mesoderm formation, axis formation, as well as a missing primitive streak (Liu et al., 1999). Mutations in *wnt-3a*, the classical mouse mutant *vestigial tail*, exhibit abnormalities in somite and tailbud formation, vertebral abnormalities and a deformed neural tube (Greco et al., 1996; Takada et al., 1994). In addition to its requirement for the proper development of the kidneys and renal tubules, Wnt-4 is required during female sexual development (Stark et al., 1994; Vainio et al., 1999). *wnt-4* mutants appear male-like with the absence of “female” Mullerian ducts and the failure of “male” Wolffian ducts to undergo their normal regression. While *wnt-4* expression is downregulated in the male gonad, Wnt-4 is required in the ovary to suppress Leydig cell differentiation and thus may be required for the development of the female germ line (Vainio et al., 1999). Like *wnt-4*, *wnt-7a* is also important for sex organogenesis. Knockout of *wnt-7a* leads to the persistence of the Mullerian ducts in males suggesting that normally Wnt-7a acts to regulate Mullerian duct regression in males. *wnt-7a* females are sterile due to abnormally developed oviduct and uterus (Parr and McMahon, 1998). Also suggested by the observation that *wnt-7a* mutants display ventralized limbs, is the requirement for Wnt-7a as a dorsalizing signal...
for the establishment of the dorsal-ventral axis during normal limb development (Parr and McMahon, 1995).

The Wnt signaling pathway is required for the establishment of the dorsal-ventral axis in *Xenopus* embryos, although, there is no evidence for an endogenous Wnt signal (Moon and Kimelman, 1998). Overexpression of several *Wnts* (and *XWnts*) can induce a duplicated body axis when injected into ventral blastoderms of early *Xenopus* embryos (Wodarz and Nusse, 1998). Similarly, overexpression of other canonical pathway components such as β-cat and Dsh can also produce a duplicated axis (Guger and Gumbiner, 1995; Sokol *et al.*, 1995). However, overexpression of GSK-3β on the dorsal side, as well as a depletion of GBP, results in a completely ventralized embryo (He *et al.*, 1995; Yost *et al.*, 1998); where as a dominant negative GSK-3, can also lead to ectopic axis formation when injected ventrally into an embryo (Dominguez *et al.*, 1995). In addition, during normal development, Dsh and β-cat are both enriched in the future dorsal side of the embryo, while GSK-3β is depleted in an event mediated by GBP, solidifying the role of the canonical pathway components in axis formation (Dominguez and Green, 2000; Larabell *et al.*, 1997; Miller *et al.*, 1999; Yost *et al.*, 1998). Although still lacking, recent evidence may implicate a Wnt ligand since reduction in *XFzd7* (*via* antisense oligonucleotides) shows defects in dorsal development (Sumanas *et al.*, 2000). One role in *Xenopus* development where a Wnt is directly involved is in the formation of the pronephric tubules. Injection of antisense Wnt-4 morpholinos into *Xenopus* embryos has recently demonstrated a requirement for XWnt-4 for the tubulogenesis in the pronephric kidney (Saulnier *et al.*, 2002).
One well understood role of Wnts in *C. elegans* is in establishing polarity and affecting cell fate decisions in the embryo (Bowerman and Shelton, 1999). The asymmetric cell division that occurs in EMS blastomere in the 4 cell stage embryo, is directed by a signal emanating from P2, the sister blastomere of EMS. This signal induces A/P polarity in EMS resulting in two daughter cells, E and MS, with vastly different cell fates. The posterior E cell (and its descendents), which is closest to its sister P2, makes endodermal cells that comprise all of the gut while the anterior MS (and its descendents) form mesodermal structures like body wall muscle and pharyngeal tissue. If an EMS blastomere is allowed to develop in the absence of its P2 sister, both E and MS daughter cells adopt the MS cell fate and no longer make endodermal tissue. This polarizing signal has been identified as MOM-2, a Wnt-like ligand (Rocheleau *et al.*, 1997; Thorpe *et al.*, 1997). Subsequent genetic screens revealed other components of the canonical Wnt signaling pathway. These components include homologues of Fz (MOM-5), β-cat (WRM-1), APC (APR-1), GSK-3 and TCF (POP-1) (Lin *et al.*, 1995; Rocheleau *et al.*, 1997; Schlesinger *et al.*, 1999; Thorpe *et al.*, 1997). Although these proteins are all part of the canonical signaling pathway, their function in the P2-EMS signaling is quite different from their counterparts in other genetic systems. For instance, while APC and GSK-3 are normally required in the downregulation of β-cat, APR-1 and SGG-1 are required positively for endoderm induction (Rocheleau *et al.*, 1997; Schlesinger *et al.*, 1999). Another example of role reversal is found in the TCF homologue POP-1 which is downregulated by MOM-2 signaling in the E cell to permit endoderm fate. In the absence of the MOM-2 signal, as is the case of the MS blastomere, POP-1 represses endoderm formation (Lin *et al.*, 1995; Thorpe *et al.*, 1997).
In addition to polarizing EMS to induce endoderm formation, the Wnt signal from P_2 is also required to properly orient the mitotic spindle in EMS, a process that occurs before asymmetric cell division can occur (Schlesinger et al., 1999). After replication but before mitosis, the centrosomes, which were oriented along the left/right axis, rotate 90° so that the mitotic spindle forms along the A/P axis of division. While many components of the Wnt pathway are involved in the endoderm/mesoderm cell fate decision, only mom-2, mom-5, and gsk-3 are required for spindle orientation since disrupting the function of downstream components did not affect EMS spindle orientation. This suggested that gsk-3 might be a branch point between the two processes that control endoderm induction and spindle orientation (Schlesinger et al., 1999).

Recently, a role for Wnt signalling in haematopoietic stem cells (HSCs) has been described. HSCs are capable of both self-renewal and the ability to give rise to all lineages of the blood. Overexpression of activated β-cat expanded the pool of HSCs resulting in long-term growth while significantly reducing their differentiation. Conversely, overexpression of inhibitors of the Wnt signalling pathway lead to the inhibition of HSC growth in vitro. Furthermore, activation of a LEF-1/TCF reporter suggested that HSCs respond to Wnt signalling in vivo (Reya et al., 2003).

**Non-canonical Wnt signaling pathways**

The canonical model of Wnt signal transduction utilizes β-cat/TCF as the most downstream effectors (Cadigan and Nusse, 1997; Siegfried, 1999). However, non-canonical Wnt signal transduction does not lead to the activation of β-cat to bring about a cellular response. One example is the pathway that regulates planar cell polarity in
Drosophila, where both Fz and Dsh play key roles (Adler and Lee, 2001). Although Wg itself is not involved, the fact that it can bind Fz, suggests that a Wnt ligand functions as the polarity signal (Bhanot et al., 1996). This pathway, as expected in non-canonical transduction, does not employ β-cat but rather activates the JNK pathway to properly orient the hairs of the wing (Boutros and Mlodzik, 1999; Boutros et al., 1998). An additional non-canonical transduction of a Wnt signal, employing only Fz and Dsh homologues, is thought to regulate convergent extension during gastrulation in zebrafish and Xenopus in a similar manner (reviewed in Tada, 2002).

In addition to utilizing β-cat or the JNK cascade, several Wnts employ intracellular Ca\(^{2+}\) release to bring about a cellular response (Kuhl et al., 2000). Induction of a secondary axis in early Xenopus embryos, as a result of ectopic expression, is an assay used to categorize vertebrate Wnts into two functional groups. The Wnt-1 group, which includes Wnt-1, Wnt-3A, Wnt-8, and Wnt-8B, all function through β-cat resulting in a duplicated axis. The other group, which includes Wnt-4, Wnt-5A, and Wnt-11, do not induce a secondary axis but rather alter cell movement and reduce cell adhesion (Kuhl et al., 2000; Miller et al., 1999). Further characterization of this Wnt-5A group has shown that, acting through specific Fzs, they can stimulate intracellular Ca\(^{2+}\) release in a G-protein dependent manner (Sheldahl et al., 1999; Slusarski et al., 1997). Just as there are two classes of Wnts, Fzs as well have been divided into two functional groups. Those that can activate Wnt/β-cat target genes such as Rat Fz1, mouse Fz-7 and –8, Xenopus Fz-1, and DFz/DFz2, and those that cannot like RFz2, MFz-3, -4, and -6. Signaling via these Fzs, in the Wnt/ Ca\(^{2+}\) pathway, leads to the activation of Ca\(^{2+}\)-calmodulin-
dependent protein kinase II (CamKII) and PKC (Miller et al., 1999; Sheldahl et al., 1999).

**Antagonists to Wnt signaling**

Wnt activity must be tightly regulated since they are secreted growth factors that can lead to fundamental changes affecting cell fate during development. A set of secreted but unrelated vertebrate proteins that bind Wnts and aide in modulating their activity has been identified. The Frizzled-related protein (FRP) family was one of the first secreted Wnt antagonists to be discovered (Leyns et al., 1997; Rattner et al., 1997; Wang et al., 1997). So far, FRP homologues have been found in many vertebrate organisms including humans, mice, frogs, and chick but not in invertebrates. Members of this family all share homology with the extracellular amino-terminal cysteine-rich domain of Fz receptors but do not contain the serpentine transmembrane domain. These secreted antagonists inhibit Wnt signaling by binding Wnts in the extracellular space; thereby, limiting Wnt/receptor-complex interactions (Brown and Moon, 1998; Moon et al., 1997). Another secreted protein that acts to limit Wnt/receptor-complex interactions is Wnt-inhibitory factor-1 (WIF-1) (Hsieh et al., 1999). wif-1 encodes a novel protein that contains a signal sequence, a novel “WIF” domain, and five epidermal growth factor (EGF) repeats followed by a hydrophilic C terminus. Identified thus far only in vertebrates, WIF-1 also binds Wnts in the extracellular space. Finally, in addition to antagonizing Bone Morphogenetic Proteins (BMPs) in Xenopus, Cerberus also binds to and blocks XWnt-8 to regulate head induction (Piccolo et al., 1999).

While the antagonistic mechanism exercised by the above secreted proteins involved binding to and inhibiting the Wnt ligand, members of the cysteine-rich
Dickkopf (Dkk) protein family, chiefly Dkk-1, inhibit vertebrate Wnt signaling by a different mechanism (Fedi et al., 1999; Glinka et al., 1998). Dkk-1 binds to the extracellular domain of LRP6, one of the Wnt co-receptors, with a high-affinity. This binding essentially prevents formation of the Wnt/Fz/LRP receptor complex thereby inhibiting canonical Wnt signaling (Bafico et al., 2001; Mao et al., 2001; Semenov et al., 2001). A recently identified partner to Dkk, Kremen (Krm) is believed to aid in this inhibition. Krm is a transmembrane, high-affinity Dkk receptor (Mao et al., 2002). Acting in concert, Dkk and Krm form a complex with LRP-6 to block Wnt/β-cat signaling. This is achieved by inducing rapid endocytosis and removal of the LRP-6 receptor from the plasma membrane. Although homologues have yet to be identified in Drosophila, surprisingly, the ectopic co-expression of krm and dkk can inhibit Wg signaling in flies presumably by interacting with Arrow (Mao et al., 2002).

While the secreted antagonists discussed above are currently identified only in vertebrates, a recent screen designed to isolate Wg-target genes identified the first extracellular antagonist to Wg signaling. wingful (wf) encodes a novel protein that contains an N-terminal signal sequence with structural homology to a subfamily of hydrolases related to plant pectin acetylesterase (Gerlitz and Basler, 2002). Wf interferes with Wg signaling during larval development but it does not interact directly with either Wg or its co-receptors, DFz/DFz2 and Arrow. Instead, Gerlitz and Basler have suggested, that since it is presumed to be an active esterase, Wf inhibits Wg signaling by interfering with a component of the receptor-complex, the proteoglycan Dally.
Present Studies: The Wg signaling pathway and the specification of cell fates

Specification of cell fate, throughout the growth and development of *Drosophila*, is regulated by the Wg signaling pathway. The work presented in this thesis expands our understanding of the role that Wg signaling plays in the specification of the somatic musculature. An additional aim of this thesis is to add insight into the role of the N-terminus of GSK-3, a key regulator of the Wg/Wnt signaling pathway.

Wg plays many roles throughout development, one of which is its requirement in the specification of muscle progenitor cells expressing the muscle identity genes *S59* and *nautilus (nau)* (Baylies *et al.*, 1995; Ranganayakulu *et al.*, 1996). To address whether this specification is mediated by the canonical Wg pathway, reciprocal gain-of-function and loss-of-function experiments were performed. In addition, the relationship between Wg and Notch (N), another signaling molecule involved in somatic mesoderm specification (Corbin *et al.*, 1991; Rusconi and Corbin, 1998), in mediating a binary decision between muscle founder and fusion-competent cell fates in the somatic mesoderm, was examined.

Zw3 and GSK-3β are very closely related serine/threonine kinases that serve as essential negative-regulators of Wg/Wnt signaling. Their similarity does not end at the structural level, however, since GSK-3β, but not the related GSK-3α, can rescue a zw3 mutant embryo (Ruel *et al.*, 1993a; Siegfried *et al.*, 1992). This suggests that not only Zw3 and GSK-3β share substrate specificity but that they are also regulated by a potentially common mechanism. One hypothesis that is tested in this thesis is that the differences in regulation between GSK-3α/β is due to differences found in the amino terminal region, which is the region that is required for pseudosubstrate inhibition. Various isoforms of Zw3, containing no N-terminus, a β-N-terminus, and an α-N-
terminus, were created and tested, utilizing *in vivo* assays, to determine differences in activity.
Chapter 2

WINGLESS SIGNALING AND THE SPECIFICATION OF THE SOMATIC MESODERM

INTRODUCTION

Signaling molecules play an essential role in the development of any multicellular organism. Specification of cell fate, mediated by these molecules, allows for the formation of unique tissues. An example of this is the formation of the somatic muscle pattern in the developing *Drosophila* embryo. The *Drosophila* mesoderm arises from the ventral most cells in the cellular blastoderm (Bate, 1993). The nuclear accumulation of the maternal transcription factor Dorsal in these cells activates transcription of the zygotic mesoderm-specific transcription factors, Twist (Twi) and Snail (Sna), which in turn commits these cells to a mesodermal fate (Bate, 1993). These mesodermal cells then invaginate along the ventral furrow and migrate dorsally where they form a monolayer that becomes juxtaposed to the ectoderm (Bate, 1993). Under the inductive influence of Decapentaplegic (Dpp) and Wingless (Wg), and the regulatory influence of Even-Skipped (Eve) and Sloppy-Paired (Slp), the mesoderm subdivides into segmentally repeated domains that give rise to the progenitors of at least four different mesodermal cell types (Azpiazu *et al.*, 1996; Bate, 1993; Dunin Borkowski *et al.*, 1995; Maggert *et al.*, 1995; Riechmann *et al.*, 1997; Riechmann *et al.*, 1998). The cells in the anterior Eve domain give rise to the visceral mesoderm and the fat body while those cells in the posterior Slp domain go on to form the dorsal vessel and the somatic mesoderm (Baylies
Dpp from the ectoderm regulates the specification of the dorsal mesoderm which includes the cardiac cells of the dorsal vessel and the visceral muscles that surround the gut (Frasch, 1995; Staehling-Hampton et al., 1994). Wg from the ectoderm is required in the Slp domain for the formation of both cardiac and somatic muscles (Baylies et al., 1995; Baylies et al., 1998; Frasch, 1995; Park et al., 1996; Staehling-Hampton et al., 1994; Wu et al., 1995). In addition, the precise differentiation of somatic muscles in each hemisegment within the Slp/Wg domain requires high levels of Twi expression (Baylies and Bate, 1996; Dunin Borkowski et al., 1995).

After the subdivision of the mesoderm, the cells within each embryonic segment of the somatic mesoderm are specified to give rise to somatic muscle. In abdominal segments A2-A7, each hemisegment contains 30 unique somatic muscle fibers. Each one is a synctium formed by the fusion of anywhere from 3 to 25 myoblasts. Moreover, each muscle fiber is unique in terms of its size, shape, innervation pattern, and attachment point to the epidermis (Bate, 1993). Formation of such a complex pattern of muscle fibers is achieved through the specification of unique muscle founder cells (Bate, 1993; Rushton et al., 1995). Founder cells are unique myoblasts that possess all the necessary information required to make a unique muscle fiber (Bate, 1990; Bate, 1992). These cells, one founder for each muscle fiber, fuse to neighboring “naïve” fusion-competent myoblasts imparting the intrinsic determinants that are necessary for the formation of the syncytial fiber (Abmayr et al., 1995; Bate, 1993; Rushton et al., 1995). The concept for the “founder cell-model” of muscle patterning is based on the identification of muscle pioneers in grasshopper (Ho et al., 1983). These muscle pioneers are elongated cells that
extend processes spanning their territory and seed the process of fusion in grasshopper embryos (Ball et al., 1985). Evidence in support of the founder cell hypothesis in *Drosophila* is supplied by the presence of similar elongated muscle precursors in *myoblast city* (*mbc*) mutants (Rushton et al., 1995). The absence of myoblast fusion in these mutants allowed the morphological identification of unique cells that attempted to span their territories via cytoplasmic extensions. While these unique cells are identified as the muscle founder cells, the remaining population of unspecified mesodermal cells, in *mbc* mutants, are believed to be the fusion-competent cells. Additional evidence in support of the existence of muscle founder cells is provided by the identification of “muscle identity” genes such as *S59*, *apterous* (*ap*), *eve*, and *nautilus* (*nau*), that are expressed in distinct subsets of muscle precursors. The gene products of these genes are believed to be involved in specifying the characteristics required to form unique muscle fibers. Mutations in some of these genes can lead to alteration and disruption of the muscle pattern (Abmayr et al., 1992; Bourgouin et al., 1992; Dohrmann et al., 1990; Frasch et al., 1987; Keller et al., 1997; Keller et al., 1998; Knirr et al., 1999).

Although the process of how these unique founder cells are specified is not fully understood, recent studies have shed some light on how this fundamental step is accomplished. Founder cells arise from populations of mesodermal cells expressing high levels of Twi that appear to function as “equivalence groups” (Baylies and Bate, 1996; Carmena et al., 1995; Dunin Borkowski et al., 1995). An equivalence group is distinguished by cells that have equivalent potential to give rise to two cell types, either muscle progenitors or fusion-competent myoblasts. The presence of these “equivalent” cell clusters is thought to be marked by the expression of the proneural gene, *lethal of
scute (l’sc) (Carmena et al., 1995). Restriction of l’sc to single cells that go on to become muscle progenitors is achieved through a process of lateral inhibition mediated by the neurogenic gene Notch (N) (Baker and Schubiger, 1996; Bate et al., 1993; Baylies et al., 1998; Corbin et al., 1991). Thus, a single cell is selected from within each equivalence group to become a muscle progenitor, which will give rise to the muscle founder. In at least some cases, this progenitor divides asymmetrically to yield either two founder cells, or a founder cell and an adult muscle precursor (Carmena et al., 1998; Ruiz Gomez and Bate, 1997). This asymmetric division of the muscle progenitor involves the differential distribution of Numb. The selected founder cell will go on to seed the formation of a specific muscle fiber. The remaining cells within the equivalence group are assumed to adopt the fusion-competent cell fate. Identification of distinct founder cells can be achieved by examining the pattern of expression of muscle identity genes (Fig. 2.1).

The role of N signaling in the specification of the somatic mesoderm is to restrict muscle founder cell fate through two distinct mechanisms. Loss of zygotic N abolishes lateral inhibition, and results in an expansion of the number of Nau and S59 expressing muscle founders (Corbin et al., 1991; Rusconi and Corbin, 1998). This occurs with a concomitant reduction in the number of fusion-competent cells (Bour et al., 2000). A similar effect on muscle founder fate is observed in embryos mutant for zygotic Delta (Dl), the N ligand, or Suppressor of Hairless (Su(H)), a downstream component of the N signaling pathway. However, the removal of both maternal and zygotic gene product in embryos derived from females with homozygous N mutant germ line clones (N GLC mutant embryos) results in an even greater expansion of the number of S59 and Nau expressing muscle founders (Rusconi and Corbin, 1998). This indicates that maternal
Figure 2.1. Simplified Mechanism for the Selection of Founder Cells.

Two types of myoblasts are derived from a domain of cells within the mesoderm that express high levels of Twist. These cells are selected into equivalence groups where they will ultimately give rise to either the precursors of the muscle founder cells or the fusion-competent myoblasts. A single progenitor is selected within each equivalence group and, through a process of lateral inhibition, the neurogenic gene Notch [stop bars within High/Low Lethal-of-Scute (L’sc) expressing cells] restricts the surrounding cells from assuming this fate. The equivalence groups are thought to be marked by broad expression of the pro-neural gene L’sc, which becomes restricted to the single muscle founder progenitor as lateral inhibition occurs. The remaining cells within the equivalence group are assumed to adopt the fusion-competent cell fate.
protein participates in an early activity of N signaling to restrict muscle founder cell fates. This early function of N is independent of lateral inhibition and is mediated through a distinct signaling pathway. Absence of both maternal and zygotic (Dl) or (Su(H)), in the respective GLC mutant embryos, fails to increase the number of muscle founders over that observed in embryos solely lacking the zygotic gene product. This finding suggests that the early repressive role for N, in limiting the number of mesodermal cells that can become somatic muscle cells, is independent of lateral inhibition.

While the N signaling pathway acts to limit the founder cell fate, there are pathways that do the opposite by specifying the muscle progenitor cell fate. A requirement for Wg signaling, for example, has been demonstrated for the specification of several different muscle founder cells. In wg mutant embryos, S59 expressing muscle founders are absent, indicating that Wg is required to specify this subset of muscle founders (Bate and Rushton, 1993; Baylies et al., 1995). Moreover, Wg is required for the specification of some Nau-expressing muscle founders. In wg mutant embryos, the medial clusters of Nau-expressing muscle founders are completely absent (Ranganayakulu et al., 1996). Additionally, the activation of two receptor tyrosine kinase (RTK) pathways, the Drosophila Epidermal Growth Factor Receptor (DER) and the Heartless (Htl) Growth Factor Receptor pathways, are required for the specification of a group of muscle progenitors and founders in the dorsal mesoderm (Carmena et al., 1998b).

As illustrated above, the specification of the somatic mesoderm involves the coordinated activity of a number of signaling pathways. The focus of our laboratory is on the study of the Wg signaling pathway in the specification of cell fate. Consequently, the
role of this pathway, in the specification of pattern and cell fate in the mesoderm through interactions with other signals that can alter mesodermal cell fate, was investigated. The expression of S59 and Nau, two muscle identity genes that mark subsets of founder cells, and Sticks and Stones (SNS), a marker for the fusion-competent myoblasts, was monitored in various genetic backgrounds. It is demonstrated that Wg and N signaling influence the specification of both the founder and fusion-competent myoblasts within an equivalence group. Furthermore, it is shown that Wg and/or N signaling perturb muscle founders and fusion-competent cells in an inverse proportion, such that the number of fusion-competent myoblasts is reduced when the number of muscle founders is increased and vice versa. It is proposed that Wg and N signaling act differentially, but coordinately, to mediate a binary decision between the muscle founder and fusion-competent cell fates. Moreover, the requirement of L’sc expression as a marker for equivalence groups was examined. It is presented that some cells retain the ability to become either a muscle founder or fusion-competent myoblast in the absence of L’sc expression. These results suggest that, in contrast to previous models, L’sc expression does not determine the equivalence group, but, rather, is a product of the formation of the equivalence group. Finally, the potential for other DWnts to regulate the expression of muscle identity genes and to affect the specification of muscle founders was tested.
MATERIALS AND METHODS

Drosophila stocks

All stocks were maintained on standard cornmeal agar media at 25°C. \(wg^{CX4}\) is described in (Baker, 1987). \(dsh^{v26} FRT^{l01}\) is described in Siegfried et al. (1994). \(zw3^{M11-1} FRT^{l01}\) is described in Siegfried et al. (1992). \(arm^{258} FRT^{l01}\) is described in Riggleman et al. (1989). \(N^{55ell} FRT^{d84}\) and \(N^{alk1} FRT^{d101}\) are described in Rulifson and Blair, (1995) and Brennan et al. (1997), respectively. \(N^{5419} dsh^{v26} FRT^{l01}\) is described in Rulifson et al. (1996). \(DWnt4^{EMS23}\) and \(DWnt2^{o}\) are described in Cohen et al. (2002) and Kozopas et al. (1998), respectively. twist-GAL4 and Dmef2-GAL4 are both mesoderm-specific drivers driving GAL4 expression at approximately embryonic stage 6 and 7, and are described in Greig and Akam, (1993) and Ranganayakulu et al. (1996), respectively. UAS-\(zw3^{10A12A}\) is described in Steitz et al. (1998). UAS-wg (kindly provided by A. Bejsovec) is described in Hays et al. (1997), and UAS-\(arm^{SI0J}\) (kindly provided by M. Peifer) is described in Pai et al. (1997). UAS-\(N^{intra}\) (kindly provided by M. Young) is described in Kidd et al. (1998). UAS-\(DWnt4^{4-6}\) (kindly provided by E.L. Wilder) is described in Gieseler et al. (1999) and UAS-\(DWnt2\) (kindly provided by K.M. Kozopas) is described in Kozopas et al. (1998).

Loss and Gain of Function

Germ line clone (GLC) embryos that lack both maternal and zygotic gene products were generated using the FLP-DFS technique (Chou and Perrimon, 1992) as described in Siegfried et al. (1992). For example, to make a \(zw3^{M11-1}\) GLC, females of the genotype
were mated to ovo^{D1}FRT^{101}/Y; FLP^{38}/FLP^{38} males. The progeny of this cross were subjected to heat shock at 37°C for two hours per day for two days during the third instar larval stage to induce FLP-directed recombination. The resulting virgin females contain mosaic zw3 germlines (GLC). Mating of these zw3 GLC females to wild-type (Oregon-R) gave rise to two classes of embryos: null mutant embryos (zw3/Y) which lack both maternal and zygotic gene product, and paternally rescued mutant embryos which lack maternal product but express zw3 zygotically from the paternally inherited gene (zw3/+). For ectopic expression of a given gene product, we utilized the GAL4/UAS system (Brand and Perrimon, 1993). In brief, the appropriate UAS line was crossed to either twi-GAL4 or Dmef2-GAL4 at either 25°C or 29°C and embryos collected every 12 hours.

**Immunohistochemical Staining of Whole-Mount Embryos**

Embryos were collected on either apple or grape juice-agar plates at either 25°C or 29°C for approximately 12 hours. All embryos were dechorionated in 50% bleach and then fixed in a 1:1 solution of 4% formaldehyde in PBS:heptane for 10-15 minutes. After removing the vitelline membrane in a 1:1 mixture of methanol:heptane, the embryos were thoroughly rinsed and stored in methanol at -20°C until used. Stainings were performed as described in Steitz *et al.* (1998). Colorimetric immunohistochemistry utilized rabbit polyclonal antiserum against S59 (1:500) (Baylies *et al.*, 1995) and rat polyclonal antisera directed against L’sc (1:400) (Carmena *et al.*, 1995) and SNS* (1:50) (Bour *et al.*, 2000). For the detection of Nau, both rat-αNau (1:50 or 1:30) and rabbit-αNau (1:500) polyclonal antisera were used (C. Keller, personnel communication). Goat-αrat or goat-
α-rabbit secondary antibodies were used at 1:500 and were detected using the Vectastain ABC Elite Kit (Vector Laboratories, Burlingame, CA) and the signal amplified using the TSA™ Biotin System (NEN™ Life Science Products, Inc., Boston, MA) as necessary. Immunostained embryos were photographed under Nomarski optics with a Zeiss Axiophot microscope using Fujicolor Superia ASA 100 color-print film. Prints were scanned and digitized with an Epson Expression 626 scanner using Adobe Photoshop (v.4.0.1) software. Composites were constructed and annotated using Adobe Illustrator 6.0. *All SNS stainings presented in this thesis were performed by Malabika Chakravarti in the laboratory of Susan M. Abmayr.

RESULTS

Wg has been shown to be essential for the specification of some muscle founder cells (Baylies et al., 1995; Ranganayakulu et al., 1996). The Wg signal is transduced through a multi-protein complex that regulates the nuclear accumulation of Armadillo (Arm) (Peifer and Polakis, 2000; Wodarz and Nusse, 1998). In the absence of the Wg signal, the so called “destruction complex” binds and phosphorylates Arm, thereby targeting it for rapid degradation via the ubiquitin/proteosome pathway. When Wg binds to its co-receptor complex, DFrizzled2/Frizzled/Arrow (DFz2/Fz/Arr), it leads to the inactivation of Zeste-White 3 (Zw3) and the destruction complex via the protein Dishevelled (Dsh). This inactivation leads to the stabilization and cytoplasmic accumulation of Arm. Stable Arm translocates into the nucleus where it associates with
its transcriptional co-activator, dTCF. In the nucleus, the Arm/dTCF complex binds DNA and activates transcription of Wg responsive genes. To determine whether this canonical Wg signaling pathway mediates Wg-dependent specification of muscle founder cells, embryos mutant for \(dsh\), \(zw3\), and \(arm\) were examined for a subset of muscle founder cells. Muscle founder fates were monitored by expression of the muscle identity genes, S59 and Nau, in embryos that lack both maternal and zygotic gene product derived from females with homozygous mutant germline clones (GLC mutant embryos). In addition, muscle founders in embryos where Wg signaling is hyperactivated, by ectopic expression of \(wg\) or an activated form of Arm, were examined.

**The Role of Wg in Specification of Muscle Founders: Wg-Dependent and Wg-Responsive Muscle Founder Cells**

S59, a homeodomain containing transcription factor (Dohrmann \textit{et al.}, 1990), is expressed in two clusters of muscle founders per hemisegment and in cells of the CNS on either side of the ventral midline in stage 11 wild-type embryos (Fig. 2.2A; Bate and Rushton, (1993); Baylies \textit{et al.} (1995)). In \(wg\) mutant embryos, the S59-expressing founders in the somatic mesoderm are completely absent, and the only observed S59 expression is in cells of the CNS (Fig. 2.2B; Baylies \textit{et al.} (1995). However, two phenotypes, designated Class I and Class II, were observed in \(dsh\) and \(arm\) mutant GLC embryos. A loss of S59 muscle founders, similar to that observed in \(wg\) mutant embryos, is observed in Class I \(dsh\) or \(arm\) mutant GLC embryos (Figs. 2.2C, E). In \(zw3\) mutant GLC embryos, on the other hand, there is an increase in the number of cells per cluster that express S59, supporting the idea that the Wg-dependent specification of S59 is
mediated through the repression of Zw3 (Fig. 2.2D). While the loss of S59 muscle founders in Class I \(dsh\) or \(arm\) mutant GLC embryos suggests that the Wg-dependent specification of S59-expressing muscle founders is occurring through the canonical signaling pathway, persistent mesodermal S59 expression in Class II \(dsh\) or \(arm\) mutant GLC embryos would argue against an absolute dependency on Wg signaling for specification of these muscle founders (Figs. 2.3C, D). Class I and II mutant embryos come from the same cross. They both exhibit the same segmentation defect that is a hallmark of Wg signaling inactivation (data not shown) and are true GLC embryos; Class II embryos, which accounted for 10-25% of the total number of GLC mutant embryos, therefore, are not simply examples of the paternally rescued class. To examine whether ectopic Wg could expand the number of S59 muscle founders, the mesoderm specific \(Dmef2\)-\(GAL4\) driver was used to overexpress Wg in the mesoderm (Ranganayakulu et al., 1996). As shown in Fig. 2.4B, there is an increase in the size of the clusters of S59 muscle founders under these conditions. To activate Wg signaling in a cell-autonomous fashion, the \(Dmef2\)-\(GAL4\) was used to express activated Arm (Arm\(^{S10}\)), a constitutively active form of the protein that is independent of Wg regulation (Pai et al., 1997). In these embryos, there is an increase in the number of S59 muscle founders per cluster, similar to embryos ectopically expressing Wg (Fig. 2.4C).

To determine whether Wg functions in an analogous fashion in the specification of other founder cells, the pattern of the Nau-expressing founder cells was examined in embryos in which Wg signaling is either absent or hyperactivated. Nau, the \(Drosophila\) homologue of Myo-D, is expressed in medial and lateral subsets of
Figure 2.2. The Transduction of the Wg Signal for the Specification of S59 Expressing Clusters Is Carried Out Through Dsh, Zw3, and Arm.

Anti-S59 immunostaining in stage 11 (A) wild-type, (B) wg\textsuperscript{cx4}, (C) Class I dsh\textsuperscript{v26} mutant GLC, (D) zw3\textsuperscript{M11-1} mutant GLC, and (E) Class I arm\textsuperscript{25B} mutant GLC embryo. In wild-type embryos (A), S59 muscle founders are located in two clusters per hemisegment as well as in cells of the CNS along the ventral midline. In wg mutant embryos (B), S59 expression in the somatic mesoderm is completely absent while the cells of the CNS continue to express S59. Additionally, S59 expression, in embryos mutant for either Class I dsh (C) or Class I arm (E), is absent from the somatic mesoderm but retained in the CNS. However, in zw3 mutant embryos (D), the clusters of cells expressing S59 are expanded. The pattern of specification is not grossly altered but the number of cells within each cluster is increased (arrowhead in D). Embryos were reared at 25°C. Embryos are shown in a ventral view and are oriented anterior to the left. Embryos in this and all subsequent figures were staged according to (Campos-Ortega and Hartenstein, 1985).
Figure 2.3. Persistent Mesodermal S59 Expression in Class II dsh and arm Mutant Embryos.

Anti-S59 immunostaining in stage 11 (A) wild-type, (B) wg^{ex4}, (C) Class II dsh^{26} mutant GLC, (D) Class II arm^{258} mutant GLC embryos. In wild-type embryos (A), S59 muscle founders are located in two clusters per hemisegment as well as in cells of the CNS along the ventral midline. In wg mutant embryos (B), S59 expression in the somatic mesoderm is completely absent while the cells of the CNS continue to express S59. In Class II dsh (C) and arm (D) GLC mutant embryos, expression of S59 is maintained in a subset of clusters in the somatic mesoderm. These GLC mutant embryos exhibit the segmentation defect that results from inactivation of Wg signaling and are therefore not simply examples of paternally rescued embryos. Class II embryos represented 10-25% of the total number of GLC mutant embryos. Embryos were reared at 25°C. Embryos are shown in a ventral view and are oriented anterior to the left.
Figure 2.4. Hyperactivation of Wg Signaling Can Expand the Number of S59-Expressing Cells.

Anti-S59 immunostaining in stage 11 (A) wild-type (B) UAS-wg/+; Dmef2-GAL4/+ (C) UAS-arm<sup>SI10f</sup>/+; Dmef2-GAL4/+ embryos. In wild-type embryos (A), S59 muscle founders are located in two clusters per hemisegment as well as in cells of the CNS along the ventral midline. Hyperactivation of Wg signaling by overexpressing either the ligand (B) or a ligand-independent, constitutively active form of arm (C), can increase the number of muscle founder cells per cluster that express S59. The smaller panel to the right of each figure is a higher magnification view (63X) of two segments (indicated by the horizontal bar in panel A). Horizontal bars in the smaller panels mark the ventral midline. Embryos were reared at 25°C. Embryos are shown in a ventral view and are oriented anterior to the left.
putative muscle founder cells in stage 11 wild-type embryos (Fig. 2.5A; Michelson et al., 1990). In \( \text{wg} \) mutant embryos, the medial clusters of Nau founders are absent but the lateral clusters remain (Fig. 2.5B; Ranganayakulu et al., 1996). In a similar fashion, the medial but not the lateral clusters of Nau muscle founders are absent in \( \text{dsh} \) and \( \text{arm} \) mutant GLC embryos (Figs. 2.5C, E). Conversely, in \( \text{zw}3 \) mutant GLC embryos, the medial clusters are slightly expanded, which is consistent with its role in Wg signaling (Fig. 2.5D). Interestingly, the lateral Nau-expressing clusters in \( \text{zw}3 \) mutant GLC embryos appear to be marginally reduced. Embryos in which Wg, or the activated form of Arm, are uniformly expressed in the mesoderm exhibit an increase in the size of not only the medial but the lateral clusters of Nau muscle founders as well (Figs. 2.6B, C). The effect of Wg hyperactivation on the medial Nau founders is expected since the specification of these cells is Wg dependent; however, the effect on the lateral Nau founders is surprising since these cells do not normally require Wg signaling for specification. Based on these results, it was concluded that the canonical Wg signaling pathway is required for the specification of Wg-dependent muscle founders S59 and the medial Nau-expressing cells. In addition, the consequences of both the absence and hyperactivation of Wg signaling supports the presence of two distinct classes of founder cells, Wg-dependent and Wg-responsive, that correspond to the lateral Nau founder cells which do not require Wg but are affected by hyperactivation of Wg signaling.
Figure 2.5. Specification of Medial Nau-Expressing Cells Is Mediated Through the Canonical Wg Signaling Pathway.

Anti-Nau immunostaining in stage 11 (A) wild-type, (B) wg\textsuperscript{cx4}, (C) dsh\textsuperscript{v26} mutant GLC, (D) zw3\textsuperscript{M11-1} mutant GLC, and (E) arm\textsuperscript{25B} mutant GLC embryo. In wild-type embryos (A), there are two clusters of Nau-expressing muscle founders in medial and lateral positions in the somatic mesoderm. In wg mutant embryos (B), the medial clusters of Nau founders are absent while the lateral clusters remain. In a similar fashion, expression of Nau in the medial clusters, in embryos mutant for either dsh (C) or arm (F), is absent but persists in the lateral clusters. Conversely, in zw3 mutant embryos (D), there is a slight increase in the number of cells per medial cluster that express Nau, while surprisingly, the lateral clusters appear marginally reduced in size. Embryos were reared at 25°C. Embryos are shown in a ventral view and are oriented anterior to the left.
Figure 2.6. Hyperactivation of Wg Signaling Can Expand the Number of Medial and Lateral Nau-Expressing Cells.

Anti-Nau immunostaining in stage 11 (A) wild-type, (B) UAS-wg/+; Dme2-GAL4/+, and (C) UAS-arm^{S10J}/+; Dme2-GAL4/+ embryo. In wild-type embryos (A), there are two clusters of Nau-expressing muscle founders in medial and lateral positions in the somatic mesoderm. Hyperactivation of Wg signaling by overexpressing either the ligand (B) or a ligand-independent, constitutively active form of arm (C), can increase the number of Nau-expressing founder cell in both medial and lateral clusters. The smaller panel to the right of each figure is a higher magnification view (63X) of two segments (indicated by the horizontal bar in panel A). Horizontal bars in the smaller panels mark the ventral midline. Embryos were reared at 25°C. Embryos are shown in a ventral view and are oriented anterior to the left. Anti-Nau immunostaining of the embryo in B was enhanced using the TSA Biotin System.
Overexpression of Zw3 Does Not Block the Wg-Dependent Specification of S59- or Nau-Expressing Cells

Previous work in our laboratory focused on the repression of Zw3 in response to the transduction of the Wg signal. It was demonstrated that high levels of Zw3 block endogenous Wg signaling in the embryonic midgut (Steitz et al., 1998). Therefore, an examination into the role of ectopic zw3 in the somatic mesoderm was performed to gauge what effect, if any, overexpression of this signaling repressor would have on the two distinct classes of founder cells. Initial overexpression of Zw3 at 25°C yielded no discernible change in Nau expression (Fig. 2.7B). However, repeating this at 29°C resulted in a very slight decrease in the size of the medial clusters and, surprisingly, in an increase in the size of the lateral clusters (Fig. 2.7C). This suggested that Zw3 has potentially dual functions: one, as the “normal” repressor in the medial clusters, and two, as a positive regulator, acting in an unknown pathway, in the specification of the lateral Nau founder cells. The overexpression of Zw3 at both 25°C and 29°C did not significantly reduce the number of S59-expressing founders per cluster (Figs. 2.7E, F).

The inability of ectopic Zw3 to block the specification of Wg-dependent muscle founders, unlike the ability of ectopic Zw3 to block endogenous Wg signaling in the embryonic midgut, could possibly be due to inherently different threshold levels in the different tissues.
Figure 2.7. Overexpression of Zw3 Does Not Block the Wg-Dependent Specification of Nau- or S59-Expressing Cells.

Anti-Nau (A-C) and Anti-S59 (D-F) immunostaining in stage 11 (A, D) wild-type, (B, E) UAS-zw3^{10A12A}/+; Dmef2-GAL4/+ at 25°C, and (C, F) UAS-zw3^{10A12A}/+; Dmef2-GAL4/+ embryos at 29°C. In wild-type embryos (A), there are two clusters of Nau-expressing muscle founders in medial and lateral positions. The overexpression of Zw3 at 25°C (B) yielded no discernible change in Nau expression. However, overexpressing Zw3 at 29°C (C) resulted in a minimal decrease in the size of the medial clusters and, surprisingly, led to an increase in the size of the lateral Nau-expressing clusters. In wild-type embryos (D), S59 muscle founders are located in two clusters per hemisegment as well as in cells of the CNS along the ventral midline. The overexpression of Zw3 at both 25°C (E) and 29°C (F) did not significantly reduce the number of S59-expressing cells. Embryos were reared at 25°C except where noted. Control wild type embryos reared at 29°C had wild type S59 and Nau expression patterns. Embryos are shown in a ventral view and are oriented anterior to the left.
**DWnts and the Specification of Muscle Founders**

The experiments above suggested that the specification of S59 and Nau muscle founders may include specification by other signaling molecules. Therefore, other signaling molecules that could potentially interact with Wg in the specification of founder cells were tested. Initial candidates were other Wnts since there are several Wnts in *Drosophila*, chiefly DWnt-2 and DWnt-4, that are expressed in the ectoderm at the time of muscle precursor specification (Eisenberg *et al.*, 1992; Graba *et al.*, 1995). To address the possibility that these other signaling pathways might involve other DWnts, the GAL4/UAS system was once again used to drive expression of DWnt-4 and DWnt-2 using mesoderm specific GAL4 drivers. We subsequently examined the expression patterns of S59 and Nau in these embryos. Ectopic expression of either DWnt-2 or DWnt-4 using either *twist-* or *Dmef2*-GAL4 drivers resulted in an increase in the number of Nau-expressing cells in the lateral clusters while not significantly altering the number of cells in the medial clusters (Figs. 2.8C-F). Similarly, overexpression of either DWnt-2 or DWnt-4 using either GAL4 driver also resulted in an increase in subsets of S59-expressing cells (Figs. 2.9C-F). The greatest expansion was seen in embryos overexpressing *Dwnt-2* using *Dmef2*-GAL4 (Fig. 2.9C) and *Dwnt-4* using *twist*-GAL4 (Fig. 2.9F).

The results obtained from ectopically expressing DWnts suggested that they may be able to regulate muscle founder specification. To further test the requirement of DWnts in the specification of founder cell fate, the reciprocal experiments to the overexpression studies were conducted. *Dwnt-2* and *Dwnt-4* mutant embryos were examined for the expression of S59 and Nau. In embryos mutant for either *Dwnt-2* or
Figure 2.8. Ectopic Expression of DWnt-2 or DWnt-4 Can Expand the Number of Lateral Nau-Expressing Cells.

Anti-Nau immunostaining in stage 11 (A) wild-type, (B) UAS-wg/+; Dmef2-GAL4/+ (C) UAS-DWnt-2/+; Dmef2-GAL4/+, (D) twist-GAL4/+; UAS-DWnt-2/+, (E) UAS-DWnt-4/+; Dmef2-GAL4/+; and (F) twist-GAL4/+; UAS-DWnt-4/+ embryo. In wild-type embryos (A), there are two clusters of Nau-expressing muscle founders in medial and lateral positions in the somatic mesoderm. As previously stated, hyperactivation of Wg signaling (B) can increase the number of Nau-expressing founder cell in both medial and lateral clusters. Surprisingly, overexpressing either Dwnt-2 (C, D) or Dwnt-4 (E, F), can expand the number of lateral Nau-expressing cells. The medial expression of Nau in these embryos remains largely unchanged. Experiments were performed at 25°C except in C, D, and F where crosses were carried out at 29°C. Control wild type embryos reared at 29°C did not exhibit a temperature effect. Embryos are shown in a ventral view and are oriented anterior to the left.
Figure 2.9. Ectopic Expression of DWnt-2 or DWnt-4 Can Expand Subsets of S59-Expressing Cells.

Anti-S59 immunostaining in stage 11 (A) wild-type, (B) UAS-wg/+; Dmef2-GAL4/+ (C) UAS-DWnt-2/+; Dmef2-GAL4/+, (D) twist-GAL4/+; UAS-DWnt-2/+; (E) UAS-DWnt-4/+; Dmef2-GAL4/+; and (F) twist-GAL4/+; UAS-DWnt-4/+ embryo. In wild-type embryos (A), S59 muscle founders are located in two clusters per hemisegment as well as in cells of the CNS along the ventral midline. As previously stated, hyperactivation of Wg signaling (B) can increase the number of muscle founder cells per cluster that express S59. While not resulting in a similar drastic increase in the number of cells that express S59, overexpression of either DWnt-2 (C, D) or DWnt-4 (E, F) can influence S59 expression such that expression expands in a subset of muscle precursor cells. The greatest expansion can be seen in embryos that overexpress DWnt-2 using the Dmef2-GAL4 driver (C) and DWnt-4 using the twist-GAL4 driver (F). Experiments were performed at 25°C except in C, D, and F where crosses were carried out at 29°C. Control wild type embryos reared at 29°C did not exhibit a temperature effect. Embryos are shown in a ventral view and are oriented anterior to the left.
Dwnt-4, expression of Nau in medial and lateral clusters remain at wild-type levels with no significant change to the expression pattern (Figs. 2.10 C, E). Furthermore, the wg and Dwnt2 double mutants phenocopy wg mutants with the lateral Nau founders still being present (Fig. 2.10D). Similarly, there is no sizable change to the number of S59 muscle founders in Dwnt2 and Dwnt4 mutant embryos (Figs. 2.11C, E). Similar to the Nau expression in wgDwnt2 double mutants, S59 expression in these embryos resembles the wg phenotype (Fig. 2.11D). So while the results obtained from the ectopic expression experiments suggested a potential role for DWnts in the specification of Wg-dependent and Wg-responsive muscle founders, the analysis of DWnt mutants greatly diminished the size of that role.

Wg-Dependent Muscle Founders: Specification of Cell Fate Directly by Wg Signaling or Indirectly by Alleviating N Restriction of Muscle Founder Fates

In addition to the instructive role of Wg signaling in the specification of founder cells, N signaling mediates the restriction of muscle founder cell fate. To investigate the interplay of Wg and N signaling in specification of the Wg-dependent and Wg-responsive cells described above, embryos in which both pathways are disrupted were examined. As expected, embryos lacking both the maternal and zygotic N gene product exhibit an increase in the number of both S59 and Nau expressing founder cells per cluster (Figs. 2.12B, 2.13B, respectively; (Rusconi and Corbin, 1998)). The Wg-dependent S59-expressing founders persist and are similarly expanded in embryos in which both N and Wg signaling is disrupted (Ndsh double mutant GLC embryos; Fig. 2.12D). This
Figure 2.10. Expression of Nau in Dwnt-2 and Dwnt-4 Mutant Embryos.
Anti-Nau immunostaining in stage 11 (A) wild-type, (B) wg<sup>ex4</sup>, (C) Dwnt2<sup>o</sup>, (D) wg<sup>ex4</sup>Dwnt2<sup>o</sup>, and (E) Dwnt4<sup>EMS23</sup> mutant embryo. In wild-type embryos (A), there are two clusters of Nau-expressing muscle founders in medial and lateral positions in the somatic mesoderm. In wg mutant embryos (B), the medial clusters of Nau founders are absent while the lateral clusters remain. In Dwnt2 mutant embryos (C), Nau expression in the somatic mesoderm is largely wild-type. Similarly, in Dwnt4 mutant embryos (E), the Nau expression pattern is not significantly altered from the wild-type pattern. Moreover, embryos doubly mutant for wg and Dwnt2 (D) phenocopy wg mutant embryos (B).
Embryos were reared at 25°C. Embryos are shown in a ventral view and are oriented to the left.
Figure 2.11. S59 Expression in Dwnt-2 and Dwnt-4 Mutant Embryos.
Anti-S59 immunostaining in stage 11 (A) wild-type, (B) wg<sup>cx4</sup>, (C) Dwnt2<sup>o</sup>, (D) wg<sup>cx4</sup>Dwnt2<sup>o</sup>, and (E) Dwnt4<sup>EMS23</sup> mutant embryo. In wild-type embryos (A), S59 muscle founders are located in two clusters per hemisegment as well as in cells of the CNS along the ventral midline. In wg mutant embryos (B), S59 expression in the somatic mesoderm is completely absent while the cells of the CNS continue to express S59. In Dwnt2 mutant embryos (C), S59 expression in the somatic mesoderm is largely wild-type. Similarly, in Dwnt4 mutant embryos (E), the S59 expression pattern is not significantly altered from the wild-type pattern. Moreover, embryos doubly mutant for wg and Dwnt2 (D) phenocopy wg mutant embryos (B). Embryos were reared at 25°C. Embryos are shown in a ventral view and are oriented to the left.
observation suggests that Wg signaling does not directly regulate the number of S59-expressing muscle founders but, rather, acts to alleviate the early N repression of this cell fate. This finding was also confirmed by others (Brennan et al., 1999). In contrast, the Wg-dependent medial Nau-expressing clusters are not observed in Ndsh double mutant GLC embryos, implying that Wg is the primary signal for specification of this subgroup of muscle founders (Fig. 2.13D). The Wg-responsive lateral Nau-expressing cell clusters are also expanded in Ndsh double mutant GLC embryos, reflecting the absence of the early N mediated restriction of this cell fate.

To further examine the role of N repression of muscle founder cell fate, we expressed a ligand-independent activated form of N (N\text{intra}) specifically in the mesoderm using the twist-GAL4 driver (Kidd et al., 1998). In these embryos, the specification of most S59- and Nau-expressing muscle founders is abolished (Figs. 2.12C and 2.13C, respectively). This effect is opposite to that observed in embryos that lack maternal and zygotic N (Figs. 2.12B and 2.13B). A distinction cannot be made that would separate the effects of N hyperactivation on the early function of N or lateral inhibition, since it is predicted that the outcome of N hyperactivation in either pathway would result in identical phenotypes. Nonetheless, hyperactivation of N signaling affects both Wg-dependent and Wg-independent muscle founders in a similar fashion.

**S59-Expressing Muscle Founders Are Specified in the Absence of L’sc Expression**

After demonstrating that Wg and N signaling alternately promote and repress specification of muscle founders, it was questioned at what stage in the the restriction of
Figure 2.12. Wg Specifies S59 Expression by Alleviating an Early N Repression.
Anti-S59 immunostaining in stage 11 (A) wild-type, (B) \( N^{35e11} \) mutant GLC, (C) \( \text{twist-GAL4/}^{+};\text{UAS-N}_{\text{intra}/}^{+} \), and (D) \( N^{3419}dsh^{526} \) double mutant GLC embryo. In wild-type embryos (A), S59 muscle founders are located in two clusters per hemisegment as well as in cells of the CNS along the ventral midline. There is an increase in the number of S59-expressing founders in \( N \) GLC mutant embryos (B); whereas, expression of an activated form of \( N \) (C) significantly reduces the number of S59-expressing muscle founders. The lack of both Wg and N signaling (D) result in an increase of Wg dependent S59-expressing muscle founders. The smaller panel to the right of each figure is a higher magnification view (63X) of two segments (indicated by the horizontal bar in panel A). Horizontal bars in the smaller panels mark the ventral midline. Embryos were reared at 25°C. Embryos are shown in a ventral view and are oriented anterior to the left.
Figure 2.13. Wg Directly Specifies the Medial Clusters of Nau-Expressing Muscle Founder Cells.

Anti-Nau immunostaining in stage 11 (A) wild-type, (B) N55e11 mutant GLC, (C) twist-GAL4/+;UAS-N\textsuperscript{intra}/+ and (D) N3419 dsh\textsuperscript{v26} double mutant GLC embryo. In wild-type embryos (A), Nau is expressed in a subset of muscle founder cell clusters in medial and lateral positions in the somatic mesoderm. In N mutant GLC embryos (B), both medial and lateral clusters of Nau-expressing muscle founders are expanded. Conversely, these clusters are completely absent in embryos overexpressing an activated form of N (C). In N\textit{dsh} double mutant GLC embryos (D), the medial clusters of Nau-expressing founders are absent while the lateral clusters are expanded. The smaller panel to the right of each figure is a higher magnification view (63X) of two segments (indicated by the horizontal bar in panel A). Horizontal bars in the smaller panels mark the ventral midline. Embryos were reared at 25°C. Embryos are shown in a ventral view and are oriented anterior to the left.
mesodermal cell fate do these pathways act. Within the domain of mesodermal cells destined to give rise to the somatic muscle, equivalence groups are specified that have the potential to give rise to both muscle founder and fusion-competent cells. The proneural gene *l’sc* has been implicated in the determination of the equivalence groups and in selection of founder cells from within these groups (Carmena et al., 1995). It has been suggested that clusters of cells expressing L'sc define and/or mark the equivalence group and that, within this cluster, the cell that expresses the highest levels of L'sc will be selected as the muscle founder. L'sc expression was examined in the same genetic backgrounds used to monitor muscle founders in order to distinguish whether Wg and N signaling restrict the equivalence group or act to select founder cells within the equivalence group. L'sc is normally expressed in roughly 18 clusters per abdominal hemisegment (Fig. 2.14A). In *wg* mutant embryos, L'sc expression is lost except in the median neuroblasts along the ventral midline (Fig. 2.14B; Carmena et al., 1998b). A similar loss of L'sc is observed in *dsh* mutant GLC embryos, suggesting that Wg acts through the canonical signaling pathway to regulate L'sc expression (Fig. 2.14C). Conversely, expression of L'sc is expanded in these clusters in *N* mutant GLC embryos (Fig. 2.14D). Since the Wg and N pathways appeared to have opposite effects on L'sc expression, we examined the interplay between these two signals. In *Ndsh* double mutant GLC embryos, expression of L'sc is absent (Fig. 2.14E), which is unexpected since S59 muscle founders are present and expanded in number in the same genetic background (Fig. 2.12D). This observation
indicates that specification of muscle founders can occur in the absence of L'sc expression.

**Wg and N Signaling Have Opposite Effects on the Specification of SNS-Expressing Fusion-Competent Cells**

Given the appearance of muscle founders in embryos that lack L'sc expression, the correlation between equivalence groups and L'sc expression in embryos mutant for Wg and N signaling was questioned. The existence of the equivalence groups can be determined independently by examining the two cell types that define the equivalence group: the muscle founders and fusion-competent myoblasts. Having already monitored the specification of the muscle founders, the specification of the fusion-competent myoblasts in embryos mutant for Wg and N signaling was examined. SNS expression in the somatic mesoderm first appears in wild-type embryos at stage 12 (Bour *et al.*, 2000). At this stage, expression in the ventral-most region of the embryo is restricted to mesodermal cross bridges that are 1-2 cells in width (Fig. 2.15A). In both *wg* mutant and *dsh* mutant GLC embryos, one observes a significant expansion in the number of SNS expressing cells along the ventral midline and within the cross bridges (Figs. 2.15B, C), concurrent with a loss of founder cells (Figs. 2.2B, C and 2.5B, C). Compared to the expanded populations of founder cells observed in *N* mutant GLC embryos (Figs. 2.12B, 2.13B), the SNS-expressing fusion-competent cells are completely absent in embryos lacking *N* (Fig. 2.16B; Bour *et al.*, 2000). Thus N signaling, like Wg signaling, appears to influence both founder and fusion-competent cell fates in a reciprocal manner. However, Wg clearly has an opposite effect on the specification of the fusion-competent cells from
**Figure 2.14. Wg and N Signaling Differentially Regulate L’sc Expression.**

Anti-L’sc immunostaining in stage 11 (A) *dsh^{v26}/+*, (B) *wg^{cx4}*, (C) *dsh^{v26}* mutant GLC, (D) *N^{k11}* mutant GLC, and (E) *N^{5419}dsh^{v26}* double mutant GLC embryos. In wild-type paternally rescued *dsh^{v26}* embryos (A), L’sc is expressed in ~18 clusters per hemisegment. In embryos mutant for Wg signaling (B, C), L’sc expression in the somatic mesoderm is largely absent with expression persisting in the median neuroblasts that are positioned along the ventral midline. Conversely, in N mutant embryos (D), L’sc expression is dramatically increased. However, when Wg and N signaling are simultaneously perturbed (E), L’sc expression is completely absent. The panel to the right is a higher magnification view (63X) of four segments (indicated by the horizontal bar in A). Embryos were reared at 25°C. Embryos are shown in a ventral view and are oriented anterior to the left.
that of N, in that N is essential for the specification of the SNS-expressing fusion-
competent cells whereas Wg signaling appears to repress their specification.

The opposing effects of Wg and N signaling on SNS expression are also
seen when either signal is hyperactivated. When Wg or activated Arm is
overexpressed in the mesoderm using twi-GAL4, the level of SNS expression is
reduced (Figs. 2.15D, E). On the other hand, expression of activated N in the
mesoderm results in a dramatic increase in SNS expressing cells, reminiscent of
the pattern seen in wg mutant embryos (Figs. 2.16C, 2.15B). Intriguingly, only a
small number of SNS expressing cells in the somatic mesoderm are detected
along the ventral midline in Ndsh double GLC mutants (Fig. 2.16D). Based on the
position of these fusion-competent cells, it is speculated that they are derived
from the equivalence groups that would normally give rise to the Wg-dependent
medial clusters of Nau-expressing founder cells. In the absence of both Wg and
N signaling, these muscle founders are absent but the other muscle founders
examined (S59 and lateral Nau founders) are present and greatly increased in
number (Figs. 2.12D, 2.13D). Therefore in the absence of Wg and/or N signaling,
there is a reciprocal relationship between the numbers of founder cells and fusion-
competent cells within each equivalence group. Where founder cell fates are
expanded, fusion-competent cell fates are reduced. These observations suggest
that 1) both the N and Wg pathways mediate a binary decision between the
founder and fusion-competent cell fates and 2) the Wg and N signaling pathways
have opposite effects on the specification of each of these myoblast populations.
Figure 2.15. Wg Restricts the Fusion-Competent Cell Fate.
Anti-SNS immunostaining in stage 12 (A) wild-type, (B) $\text{wg}^{254}$, (C) $\text{dsh}^{26}$ mutant GLC, (D) $\text{twist-GAL4; UAS-wg}$, (E) $\text{UAS-arm}^{S10J}/\text{twist-GAL4}$ embryos. In wild-type embryos (A), the ventral most SNS-expressing fusion-competent myoblasts are detected in the mesodermal cross bridges that are approximately 1-2 cells wide and cross the ventral midline. In embryos mutant for Wg signaling (B, C), there are ectopic SNS fusion-competent myoblasts and the cross bridges are wider than in wild-type. Conversely, the number of SNS fusion-competent myoblasts significantly decrease when Wg signaling is hyperactivated (D, E). Bracket in A-C marks the mesodermal cross bridges that span the ventral midline. Embryos were reared at 25°C. Embryos are shown in a ventral view and are oriented anterior to the left.
Figure 2.16. N and Wg Differentially Regulate the Fusion-Competent Cell Fate.

Anti-SNS immunostaining in stage 12 (A) wild-type, (B) $N^{55e11}$ mutant GLC, (C) twist-GAL4; $UAS-N^{\text{intra}}$, and (D) $N^{5419} dsh^{v26}$ double mutant GLC embryos. In wild-type embryos (A), the ventral most SNS-expressing fusion-competent myoblasts are detected in the mesodermal cross bridges, spanning the ventral midline, that are approximately 1-2 cells wide. In $N$ mutant embryos (B), SNS-expressing fusion-competent cells are absent. However, some SNS expression persists in the visceral mesoderm of these embryos (arrowheads in B). In embryos expressing an activated form of N (C), there is a dramatic increase in the number of SNS fusion-competent cells in the somatic mesoderm, including the ventral cross bridges. Expression of SNS in the visceral mesoderm is also expanded in these embryos. When both Wg and N signaling are absent (D), SNS fusion-competent cells can be found across the midline. In addition, visceral expression of SNS persists (arrowheads in D). Bracket in A, C, and D marks the mesodermal cross bridges that span the ventral midline. Embryos were reared at 25°C. Embryos are shown in a ventral view and are oriented anterior to the left.
**DISCUSSION**

Wg is a very versatile signaling protein that specifies pattern and cell fate in a number of embryonic and adult tissues in *Drosophila*. For example, Wg influences posterior cell fates in the embryonic ectoderm, cardiac and somatic muscle cell fates in the embryonic mesoderm, and long range patterning of the imaginal wing and leg discs (Siegfried, 1999). In all cases examined to date, Wg signaling is mediated through the canonical pathway and results in the accumulation of Arm in the nucleus where it controls patterns of gene expression that alter cell fates. In myogenesis, Wg has been implicated in the specification of distinct muscle founders. Previous studies have demonstrated that Wg is required to specify distinct classes of muscle founders that include the S59-, Eve-, Vestigal- and a subset of the Nau-expressing myoblasts (Bate and Rushton, 1993; Baylies et al., 1995; Ranganayakulu et al., 1996; Carmena et al. 1998b).

In this work, the role of Wg in the specification of myogenic cell fates, including those of several different founder cells as well as the fusion-competent cells was investigated.

**Wg Signaling Specifies Muscle Founders and Restricts Fusion-Competent Cell Fates**

Wg signaling has a reciprocal effect on the regulation of muscle founder cells and fusion-competent myoblasts. In embryos that are deficient for Wg signaling, there is a reduction of S59 and medial Nau-expressing muscle founders. Concurrently, as revealed by the expression of SNS, the number of fusion-competent cells is expanded. These results indicate that, in addition to specification of muscle founders, Wg signaling restricts fusion-competent cell fates. The ability of Wg signaling to affect both myogenic cell fates is corroborated by the shift in cell fates observed in embryos in which Wg
signaling is hyperactivated. Overexpression of Wg itself, or an activated form of Arm, results in an increase in S59- and Nau-expressing founders and a simultaneous reduction in the number of fusion-competent cells. The ability of Wg signaling to influence cell fate is cell autonomous, since ectopic Wg and activated Arm result in a similar reduction in fusion-competent cells and simultaneous increase in muscle founders. These data suggest that Wg signaling regulates a binary fate choice by promoting expression of muscle identity genes and differentiation of founder cells and, at the same time, repressing a genetic program necessary for the differentiation of fusion-competent cells.

Wg Specification of Muscle Founder Cells Can Be Direct or Indirect

Wg signaling is essential for the specification of different founder cell populations; however, the mechanism by which Wg specifies these muscle founders is not universal. Wg signaling specifies distinct Wg-dependent muscle founders either directly or indirectly. The S59-expressing muscle founders are specified indirectly by Wg since, in these cells, Wg signaling acts to alleviate an early N mediated restriction of this cell fate, that is independent of lateral inhibition. The number of S59 founders is expanded in N mutant GLC embryos and, conversely, these cells are absent in wg mutant embryos. Embryos in which both signaling pathways are absent exhibit a large number of S59 founders, similar to that observed by the loss of N signaling alone. These results indicate that the S59-expressing muscle founders require Wg to lift the N-imposed restriction on cell fate, and do not require Wg for specification in the absence of N signaling. In addition, the limited but persistent expression of S59 in a small number of Class II dsh and arm mutant GLC embryos provides further support against a direct and
absolute requirement for Wg signaling for the specification of this cell type. This mechanism is in contrast to that acting on the medial Nau-expressing muscle founders, which require Wg signaling directly for specification. These muscle founders are absent in embryos lacking just Wg signaling or both Wg and N signaling, suggesting that Wg signaling determines these cell fates directly, independent of any restriction imposed by N.

Interestingly, the lateral Nau-expressing founders that do not require Wg for specification are, nonetheless, expanded when Wg signaling is hyperactivated. The expanded Nau expression in these dorsally located cells suggests that they are capable of receiving and transducing the Wg signal, but that Wg is normally restricted from affecting their fate. One plausible explanation for the difference in response to Wg between the medial and lateral Nau cells may be their position relative to the stripe of ectodermal cells expressing Wg. The medial Wg-dependent cells are adjacent to the ventral stripe of Wg in the ectoderm (Ranganayakulu et al., 1996). However, the lateral cells lay in a position that corresponds to a gap in the circumferential stripe of Wg expression. Perhaps at this position, lateral Nau cells experience an insufficient level of Wg to activate the pathway and specification of cell fate is established through some other inductive signal. Thus, the founder cells can be classified into at least two distinct groups: the Wg-dependent founders, which are more ventrally located, and the Wg-independent but responsive founders, which are located more dorsally.

The expansion of the Wg-independent lateral Nau-expressing clusters upon Zw3 overexpression was a surprising result. Moreover, in zw3 mutant GLC embryos, the lateral clusters appeared to be marginally reduced in size. The combination of these two
results suggested, apart from the repressive role it plays in restricting the fate of the medial Nau-expressing clusters, that Zw3 potentially plays a positive role in the specification of the lateral Nau founder cells. Support for this could possibly be found in a recent report that demonstrated GSK-3β can phosphorylate and inactivate N activity (Espinosa et al., 2003). Therefore, a tempting explanation to the increase in the size of lateral Nau-expressing clusters in response to Zw3 overexpression is that the exogenous Zw3 is downregulating the N repression of this cell fate. Thus, the overexpression of Zw3 would result in an increase in the size of the lateral clusters while in the absence of zw3, N repression is no longer challenged resulting in a decrease in size of these lateral Nau-expressing founders.

**DWnts Do Not Specify nor Restrict S59- and Nau-Expressing Founders**

From the above observation of Wg-dependent and independent specification, two potentially related questions emerged. What if Wg is prevented from affecting the dorsal Nau founders not by positional limitations but by an antagonistic molecule? And, are there molecules that could potentially interact with Wg in the specification of founder cells? Two candidate molecules were other Wnts. In the embryonic ectoderm, ectopic DWnt-4 has been demonstrated to antagonize the activity of endogenous Wg upstream of Arm regulation (Giesler et al., 1999). Therefore, if DWnts function to antagonize Wg signaling in the mesoderm in an analogous manner, then ectopic expression of DWnts would presumably lead to a reduction in the number of Wg-dependent founder cells, such as the medial Nau founders. If, however, DWnts have overlapping function with Wg in regulating muscle founder specification, then the overexpression of DWnts would alter
the fate of Wg-responsive muscle founders, such as the lateral Nau founder cells, in a manner similar to Wg hyperactivation.

The ectopic expression of DWnt-2 and DWnt-4, while not completely resulting in the same expansion in the numbers of S59 and Nau-expressing founders as Wg hyperactivation, did alter the fate of Wg-responsive founders. The number of lateral Nau-expressing founders were expanded in response to overexpressed DWnt-2 and DWnt-4. However, the Wg-dependent medial Nau-expressing founders were not significantly altered. Likewise, only subsets of S59-expressing muscle founders were expanded in embryos where the DWnts were ectopically expressed. However, the potential for DWnts to alter cell fate in the somatic mesoderm was reduced upon examination of S59 and Nau expression in Dwnt mutants. Both S59- and Nau-expressing founders were present, in near wild-type levels, in Dwnt-2 and Dwnt-4 mutant embryos suggesting that while hyperactivation may alter the fate of these muscle founders, DWnt-2 and DWnt-4 are not required for their specification.

**Notch Functions to Restrict Muscle Founders and Specify Fusion-Competent Cell Fates**

In N mutant embryos, the number of muscle founders is expanded and the fusion-competent myoblasts are absent. Furthermore, ectopic activation of N signaling reduces the number of S59- and Nau-expressing founders while increasing the number of fusion-competent cells. As described above, N also has an early role in the repression of founder cell fates, independent of lateral inhibition. Consequently, in the absence of both N and Wg signaling, S59-expressing muscle founders are not only present but are increased in
number compared to wild type. By comparison, the lateral clusters of Nau founders are also expanded in *Ndsh* mutant embryos, while the medial clusters are absent.

Interestingly, SNS expression is severely reduced in the somatic mesoderm of embryos mutant for both Wg and N signaling, with the exception of the cells along the ventral midline. In this region of the embryo, in which there is a loss of muscle founders (medial Nau cells), there is a simultaneous increase in fusion-competent cells. Conversely, in regions of the embryos where there is an increase in the number of muscle founders (lateral Nau and S59) we observe a loss of fusion-competent cells. Even in the absence of both Wg and N signaling, one observes an inverse proportion of muscle founders to fusion-competent cells, suggesting that Wg and N signaling act independently and differentially to regulate both muscle founder and fusion-competent cell fates. Furthermore, this inverse effect on muscle founders and fusion-competent cells indicates that Wg and N signaling regulate a binary cell fate decision within a fixed equivalence group: a defined number of cells capable of assuming either the muscle founder or fusion-competent cell fates. This is in contrast to a previous model suggesting that the sequential action of Wg and other signals define a pre-cluster of L'sc expressing cells from which the equivalence group is derived (Brennan *et al.*, 1999; Carmena *et al.*, 1998b).

**L'sc Expression Does Not Define the Equivalence Group**

Current models have suggested that Wg specification, and N restriction, of muscle founders occurs through the specification of the equivalence group. These conclusions have been made, in part, from observations that Wg and N signaling effect the expression
of L'sc, which was assumed to mark the cells of the equivalence group as well as the muscle founder that was selected from amongst the cells of this group.

L'sc was initially identified as a "proneural gene" (Cabrera et al., 1987). It is expressed in a group of equivalent neuroectodermal cells, one of which will be selected to become a neuroblast. L'sc expression becomes restricted to this one neuroblast as it delaminates from the remaining epidermoblasts, and this restriction does not occur in mutants such as \( N \) in which the neurogenic fate is expanded at the expense of the ectodermal cell fate. In a similar fashion, L'sc has been implicated in restriction of the founder cell fate in the somatic mesoderm (Carmena et al., 1995). L'sc expression, within the high-Twist domain, is believed to mark the group of equivalent somatic muscle cells. In one case, the restriction of L'sc expression coincided with expression of the muscle identity gene, S59 (Carmena et al., 1995). In addition, ectopic expression of L'sc resulted in a duplication of 1-2 muscles per segment. These data suggested that L'sc expression is a prerequisite for muscle founder specification. However, there is a minimal disruption of the somatic muscle pattern in \( l'sc \) mutant embryos, undermining the necessity for L'sc in muscle founder specification.

The conclusion reached on the role of L'sc in the specification of these myoblasts are slightly different from those of Carmena et al. (1995), which suggest that the L'sc-expressing clusters are sufficient to account for the selection of all muscle progenitors and, consequently, all founder cells. It has been demonstrated that in embryos lacking both Wg and N signaling, in which expression of L'sc is absent, both S59 muscle founders and some fusion-competent cells are specified. Furthermore, the numbers of muscle founder and fusion-competent cells are coordinately and inversely regulated.
Even in the absence of L'sc, muscle founder and fusion-competent cells are both still found, suggesting that L'sc is not essential for myogenic cell fate. This conclusion does not preclude the possibility that L'sc acts in a combinatorial fashion with other myogenic factors to determine the equivalence group and specify muscle founders. It is only concluded that it is not essential for either of these processes and that both can occur in its absence.

**Wg and N Signaling Act Coordinately to Regulate Myogenic Cell Fates**

Wg signaling specifies the founder cell fate and restricts the fusion-competent cell fate. Conversely, N signaling restricts the founder cell fate and promotes the fusion-competent cell fate. The coordinate action of these two signaling pathways mediates a binary decision between muscle founder and fusion-competent cell fates. The following model is proposed for the mechanism of Wg and N signaling in the specification of both founder and fusion-competent myogenic cell fates within a pre-determined equivalence group (Fig. 2.17). The role of Wg signaling can be described in the specification of three distinct muscle founder cells: S59-expressing, medial Nau-expressing and lateral Nau-expressing founders. Wg promotes the muscle founder cell fate by alleviating an early N repression (S59 founders) or independent of this activity of N (medial Nau founders). Other founders (lateral Nau) are not normally subject to Wg induction but can respond to Wg hyperactivation. Once a founder is selected, N lateral inhibition is activated in the neighboring cells preventing them from assuming the founder fate by restricting the genetic program for founder cell fate. Conversely, activation of N signaling promotes the fusion-competent fate. This model proposes that Wg signaling and N lateral inhibition
regulate common myogenic factors that determine alternatively muscle founder and fusion-competent cell fates.

The proposal that Wg and N signaling act to regulate a binary switch between two distinct cell fates is supported by a similar mechanism that determines cuticle specification. A precedent for two antagonistic signaling pathways regulating a binary cell choice has been set with the interaction between DER and Wg signaling pathways in the embryonic ectoderm (O'Keefe et al., 1997; Szuts et al., 1997). The binary cell fate decision in the ectoderm is a choice between cells that secrete naked cuticle or denticles. Wg signaling is required for the differentiation of naked cuticle, whereas DER signaling is required for denticle differentiation. The differentiation of these cell types is regulated by the expression of the zinc-finger transcription factor Ovo/shavenbaby (Ovo/svb) (Payre et al., 1999). ovo/svb expression is necessary and sufficient for denticle differentiation. Its expression is, alternately, activated and repressed by DER and Wg signaling, suggesting that the regulation of Ovo/svb expression is the point of integration of these two distinct pathways. The results presented here suggest that Wg and N signaling may specify myogenic cell fates in an analogous fashion by regulating the expression of factors necessary for muscle founder and fusion-competent cell fates. One possible target for both the Wg and N signaling pathways is L'sc. L'sc expression is not essential for specification of myogenic cell fates, however, it may have redundant function with other basic helix-loop-helix proteins that act in combination to regulate the expression of muscle identity genes. Wg and N signaling may act coordinately to regulate the expression of L'sc and its partners, which in turn modulate muscle founder and
fusion-competent-specific gene expression. The test of this molecular model awaits the identification of other myogenic regulatory proteins and their targets.
Figure 2.17. Wg Signaling Specifies Different Muscle Founders via Distinct Mechanisms.

There exists at least three distinct categories of muscle founders based on their differing response to Wg signaling: S59-expressing founders (blue), medial (green) and lateral (red) Nau-expressing founders. For the specification of the S59 founders, Wg signalling alleviates the early N restriction on founder cell fate within the equivalence group. Wg signaling promotes the expression of myogenic factors within this cluster of myoblasts by removing N mediated repression, and facilitates the selection of one cell to express higher levels of these factors (e.g. L’sc). This cell will become the muscle progenitor (blue, S59) and will restrict the expression of the myogenic factors in the surrounding cells through lateral inhibition mediated by N (N lat); thereby, restricting them to the fusion-competent cell fate (gray). In the case of medial Nau founders that are also Wg dependent, the requirement for Wg signaling to specify muscle progenitors (green) is independent of the early N restriction of cell fate. The lateral Nau founders do not require Wg signaling to specify muscle progenitors (red); however they respond to high levels of Wg or hyperactivation of the pathway suggesting that normally the Wg signal is inaccessible to these cells.
Chapter 3

AN IN VIVO ANALYSIS OF THE GSK N-TERMINUS

INTRODUCTION

In any multi-cellular organism, cell-cell communication is necessary for its proper
growth and development. One important class of signaling molecules that are involved in
the regulation of cell growth and differentiation of both vertebrate and invertebrate
embryos is the Wnt family of secreted growth factors (Cadigan and Nusse, 1997;
Siegfried, 1999). Wingless (Wg/DWnt-1) is the best understood of all the *Drosophila*
Wnts. The integrative use of genetic, biochemical, and cell biological studies has led to
the identification of a conserved pathway for the transduction of the Wg/Wnt signal
(Siegfried, 1999; Siegfried *et al.*, 1994; Wodarz and Nusse, 1998). Homologs of the
components of the Wg signaling pathway are found in many species including human,
mouse, *Xenopus*, and *C. elegans* (The Wnt gene Homepage at
http://www.stanford.edu/~rnusse/wntwindow.html). Moreover, some of these
components share functional similarities as well (Cadigan and Nusse, 1997).

Wg is a secreted ligand that binds to a receptor complex comprised of members of
the Frizzled (Fz) family of seven pass transmembrane proteins (Bhanot *et al.*, 1996;
Bhanot *et al.*, 1999; Cadigan *et al.*, 1998; Chen and Struhl, 1999; Zhang and Carthew,
1998) and Arrow, a single pass transmembrane protein belonging to the LDL-receptor-
Binding of Wg to its receptors activates the cytoplasmic phosphoprotein Dishevelled
(Dsh), a necessary transducer to the Wg signal (Noordermeer et al., 1994; Yanagawa et al., 1995). As a response to the phosphorylation of Dsh, Zeste White-3 (Zw3) is indirectly inactivated (Siegfried et al., 1992; Siegfried and Perrimon, 1994). Zw3 is a serine/threonine kinase and is the homolog of vertebrate Glycogen Synthase Kinase-3β (Bourouis et al., 1990; Siegfried et al., 1992; Siegfried et al., 1990). Zw3 inactivation prevents the destruction of Armadillo (Arm), the key effector of Wg/Wnt signaling, and results in its stabilization and accumulation (Peifer et al., 1994b; Peifer et al., 1994a; Siegfried and Perrimon, 1994). Normally, in the absence of a Wg signal, cytoplasmic Arm is initially targeted for degradation by phosphorylation in a process mediated by a destruction complex that includes Zw3, the priming kinase Casein Kinase 1α (CK 1α), the scaffold protein Axin, and APC (the gene product of the tumor suppressor gene adenomatous polyposis coli) (Hamada et al., 1999; Hart et al., 1998; Ikeda et al., 1998; Itoh et al., 1998; Rubinfeld et al., 1996; Sakanaka et al., 1998; Zeng et al., 1997; Amit et al., 2002; Yanagawa et al., 2002). The F-box/WD40-repeat protein Slimb subsequently targets phosphorylated Arm for degradation via the ubiquitin-proteosome pathway (Aberle et al., 1997; Jiang and Struhl, 1998). However, as a result of a Wg signal, hypophosphorylated Arm escapes degradation and translocates into the nucleus where it interacts with the HMG box-containing transcriptional activator dTCF to activate transcription of Wg responsive genes (Behrens et al., 1996; Brunner et al., 1997; van de Wetering et al., 1997).
The Role of Zw3/GSK-3β in Wg/Wnt Signaling

An essential step in the transduction of the Wg/Wnt signaling pathway is the downregulation of Zw3/GSK-3β activity. Since loss of Zw3 parallels hyperactivation of the Wg signaling pathway (see Figs. 2.2D, and 2.5D), control of this key regulatory enzyme is therefore extremely important in establishing correct cell fate and pattern during animal development. So how is GSK-3β activity restricted in response to Wnt signaling? Although it still remains a relatively incomplete picture, recent insights have increased our understanding of the Wnt-mediated regulation of GSK-3β. In the absence of a Wnt signal, GSK-3β, along with Axin and APC, participate in the destruction complex that targets β-catenin for degradation (Behrens et al., 1998; Fagotto et al., 1999; Ikeda et al., 1998; Itoh et al., 1998). Both Axin and APC can be phosphorylated by GSK-3β and this phosphorylation is thought to allow both of these proteins to more effectively bind β-catenin. In addition, in the presence of Axin, GSK-3β phosphorylation of β-catenin is dramatically improved; most likely due to a stabilizing, bridging effect (Fagotto et al., 1999; Hart et al., 1998; Ikeda et al., 1998). However, upon Wnt stimulation, the intervention of two proteins, Dsh and GSK-3β Binding Protein (GBP) (Yanagawa et al., 1995; Yost et al., 1998) leads to the eventual collapse of the destruction complex.

Initiation of the Wnt signaling pathway results in the phosphorylation and activation of Dsh (Yanagawa et al., 1995). Upon activation, it is believed that Dsh binds to Axin and may either displace GSK-3β from the destruction complex or alter the conformation of Axin. The result of either scenario is a reduction of GSK-3β’s ability to effectively phosphorylate β-catenin (Kishida et al., 1998; Li et al., 1999). Moreover, the inhibitory interaction of GSK-3β with GBP (and its mammalian homologue Frat-1) can lead to a
reduction in β-cate phosphorylation (Li et al., 1999; Smalley et al., 1999; Yost et al., 1998). As a result of overlapping binding sites in its C-terminus, GSK-3β cannot bind Axin and GBP/Frat-1 at the same time (Dajani et al., 2001; Ferkey and Kimelman, 2002; Fraser et al., 2002). Therefore, given the facts that GBP/Frat-1 is able to interact with Dsh (Fraser et al., 2002), and that the binding of Axin and Frat-1 to GSK-3β is mutually competitive, it has been speculated that Dsh could potentially recruit these GSK-3β inhibitors (GBP/Frat-1) to the Axin-anchored destruction complex. Thus, upon Wnt stimulation, there is a subsequent separation of the destruction complex participants. The result of this would be GBP/Frat-1 binding to and inhibiting the activity of GSK-3β leading to the stabilization and hypophosphorylation of β-cate. It should be noted that the above mentioned Wnt-mediated downregulation holds true only for vertebrate GSK-3β since homologues of GBP/Frat-1, or a similarly acting molecule, have not been identified in the Drosophila genome.

GSK-3: Form and Function

Apart from GSK-3’s role in repressing β-cate in the absence of a Wnt signal, this regulatory kinase also plays a key role in another fundamental signaling pathway. GSK-3 earned its name as the kinase that phosphorylates and inactivates glycogen synthase in glycogen metabolism (Embi et al., 1980). The lack of Insulin stimulation results in GSK-3’s active phosphorylation of glycogen synthase. This brings about a reduction in glycogen production. However, as a result of insulin signaling, GSK-3 activity is reduced via phosphatidylinositol 3-kinase (PI3K)-dependent Akt/Protein Kinase B
phosphorylation at Ser-9/Ser-21 (GSK-3β/α, respectively) (Cross et al., 1995; Stambolic and Woodgett, 1994).

The interesting fact that GSK-3 is an active inhibitory enzyme, whose activity becomes suppressed in response to extracellular signaling, is just one of the fascinating properties possessed by GSK-3. Another peculiar characteristic is GSK-3’s requirements for substrate recognition. Many GSK-3 substrates require prior phosphorylation by an earlier “priming” kinase such that the substrate consensus S/T-X-X-X-S/Tp is formed before phosphorylation by GSK-3 can take place (Fiol et al., 1987). At position p+4 (located four residues carboxy-terminal to the site of GSK phosphorylation), the priming kinase would phosphorylate either a serine or threonine residue followed by GSK-3 phosphorylation at position p. In Insulin signaling for example, Casein Kinase II has been shown to be the priming kinase that phosphorylates glycogen synthase. Similarly, Casein Kinase Iα has recently been shown to prime β-cat prior to GSK-3 phosphorylation (Woodgett and Cohen, 1984) (Amit et al., 2002; Liu et al., 2002; Yanagawa et al., 2002). In addition, the recent crystal structure of GSK-3β suggests a mechanism that addresses this peculiar substrate requirement (Dajani et al., 2001; ter Haar et al., 2001). A resolved pocket consisting of Arg-96, Arg-180, and Lys-205 was found positioned within the substrate binding cleft. This pocket is thought to bind the substrate’s primed residue (either phospho-serines or threonines) and potentially stabilize GSK-3 in its active conformation to facilitate its kinase activity. Additional support is provided with the observation that mutation of residue Arg-96 reduces GSK-3 phosphorylation of primed substrates (Frame et al., 2001). Moreover, the presence of this phosphate binding cleft in the catalytic domain suggested a pseudo-substrate mechanism for GSK-3 regulation. In
response to Insulin signaling for example, GSK-3β would be phosphorylated on Ser-9 by Akt/PKB. The newly phosphorylated N-terminus would then act as a pseudo-substrate inhibitor by binding the phosphate binding cleft and effectively blocking the active site; in turn, it would compete with GSK-3 primed substrates and inhibit GSK-3 enzymatic activity.

While this pseudo-substrate activity begins to address GSK-3 regulation in response to Insulin signaling, our understanding of GSK-3 regulation as a result of Wg/Wnt signaling, as described above, is not as clear. Phosphorylation of Ser-9 as a means of inactivation, however, does not seem to be a mechanism utilized by Wnt signaling in regulating the activity of GSK-3. Use of wortmannin, a PI3K inhibitor, can reduce the inactivation of GSK-3 due to Insulin signaling and the associated Akt/PKB-mediated Ser-9 phosphorylation. However, use of wortmannin failed to suppress the Wg-mediated suppression of GSK-3 activity. The different effects on GSK-3 activity obtained with the use of wortmannin suggested that Insulin and Wnt signaling regulate GSK-3 differently (Cook et al., 1996; Cross et al., 1994). Additionally, in cell lines that are responsive to both Wnt and Insulin signaling, the addition of Insulin did not stabilize the cytoplasmic levels of β-cat; moreover, in the reciprocal experiment, the addition of Wnt-conditioned media did not increase glycogen synthase activity nor did it lead to the phosphorylation (and activation) of Akt/PKB (Ding et al., 2000). Another compelling observation, that supports the notion that Wnt signaling-mediated inactivation of GSK-3 is not mediated by Ser-9 phosphorylation, is simply that GSK-3β Ser-9 phosphorylation was not detected following Wnt stimulation (Ding et al., 2000; Ruel et al., 1999). Interestingly, GSK-3β that is associated with Conductin (an Axin-related protein) is not
significantly phosphorylated on Ser-9 even after Insulin stimulation. This suggests that one way of protecting GSK-3β activity from Insulin signaling’s inhibitory Ser-9 phosphorylation is to have GSK-3β in a complex with Axin (Ding et al., 2000).

The Two Isoforms of GSK-3

Homologues of this regulatory serine/threonine kinase can be found not only in vertebrates and invertebrates but also in plants, yeast, and Dictyostelium as well (Dominguez et al., 1995; Harwood et al., 1995; Li and Nam, 2002; Plyte et al., 1999). Vertebrates possess two closely related GSK-3 genes, α (51 kDa) and β (47 kDa) (Woodgett, 1990). The proteins encoded by these two genes share 98% amino acid identity in the kinase domain but differ considerably in the amino and carboxy terminals where identity falls to 36% at the C-terminus. A phylogenetic analysis of the vertebrate GSKs and Zw3 indicates that the vertebrate GSK-3α and GSK-3β are monophyletic (Fig. 3.1). Moreover, GSK-3α contains a glycine-rich amino-terminal extension that accounts for the size difference between the two isoforms (Woodgett, 1990) (Fig. 3.2A). The phenotype observed in GSK-3β knockout mice further demonstrates the difference between the two GSK-3 genes. Embryos mutant for GSK-3β die due to severe liver degeneration as a consequence of hepatocyte apoptosis. This is caused by excessive TNF toxicity brought on by reduced NF-κB function (Hoeflich et al., 2000). The embryonic lethality phenotype, while demonstrating that GSK-3β is an essential gene, also suggests a unique requirement for GSK-3β. However, the failure to observe phenotype(s) resembling those associated with defects in Wnt signaling suggests that GSK-3α/β can potentially substitute for each other during early development. As stated earlier, Zw3 is
Figure 3.1. Phylogenetic Analysis of the GSK Family of Proteins.
Neighbor-joining tree using amino acid sequence of vertebrate GSK, Zw3, and NGSK (D. me GSK homolog). All the vertebrate GSKαs and GSKβs are monophyletic. M. mu (mouse), H. sa (human), R. No (rat), Xla (frog), D. re (zebrafish), P. li (sea urchin), C. el (nematode), D. di (Dictyostileum).
the *Drosophila* homologue of GSK-3β (Siegfried et al., 1992; Siegfried et al., 1990; Woodgett, 1990). These two proteins share 75% amino acid identity overall and 85% identity in the catalytic domain (Siegfried et al., 1992). In addition to their shared structural identity, GSK-3β can rescue *Drosophila* embryos lacking maternal and zygotic zw3 suggesting that they potentially share substrate specificity. Unlike the β-isoform, GSK-3α, however, cannot rescue this mutant phenotype (Ruel et al., 1993a; Siegfried et al., 1992).

What can account for the different activities observed upon expression of the two vertebrate GSK-3s in *Drosophila*? A region in the amino terminus which differentiates the GSK-3βs from the GSK-3αs, and which is more similar to Zw3 has been identified (Fig. 3.2B). A hypothesis that is tested here is that this distinct amino terminal region could be responsible for the different activities of GSK-3α and -β in *Drosophila*. Various isoforms of Zw3, which have either the entire unique amino terminus deleted, the N-terminus replaced with that of GSK-3β or replaced with that of GSK-3α, were generated and used to address this question (Fig. 3.3). Transgenic lines were established that expressed these isoforms as HA epitope tagged transgenes under UAS control. The GAL4 driver c96-GAL4 was used to test the activity of the various transgenes and to compare the ability of these mutant isoforms of Zw3 to mimic Zw3 overexpression in the imaginal wing. In addition, these mutant isoforms were tested to see if they could rescue the zw3 mutant phenotype.
Figure 3.2A. Sequence Alignment of the Amino Terminal Regions of GSK3 Proteins. Residues in red indicate identity. Residues in yellow indicate identity mostly among GSK-3\(\alpha\)s.

Figure 3.2B. Sequence Alignment of the Unique Amino Terminal Regions of Zw3, GSK-3\(\beta\), and GSK-3\(\alpha\). Residues in red indicate amino acid identity. The Serines shown in bold are subject to phosphorylation and have been implicated as a pseudo-substrate competing for binding to the kinase.
Figure 3.3. Schematic Constructs of Representative Mutant Isoforms of Zw3.
The above constructs were used to generate transgenic lines that express an HA epitope tagged transgene under UAS control. Yellow bars indicate the two HA tags at the carboxy terminus. The red bar is the stop codon. Construct A was used to generate epitope tagged wildtype Zw3 ($UAS$-$zw3^{WDT-HA}$). Construct B was used to generate an isoform of Zw3 with a deleted amino terminus ($UAS$-$zw3^{\Delta NT-HA}$). Constructs C and D were used to generated chimeric genes where the N-terminus was replaced with either the amino terminus of GSK3α ($UAS$-$zw3^{\alpha NT-HA}$) or GSK3β ($UAS$-$zw3^{\beta NT-HA}$).
MATERIALS AND METHODS

Drosophila Stocks

All stocks were maintained on standard cornmeal agar media at 25°C. c96-GAL4 is described in Gustafson and Boulianne, (1996). en-GAL4 is described in Yoffe et al., (1995). zw3/FM7;arm-GAL4 was generated in the Siegfried lab (E.S., personal communication).

Construction of Zw3 Isoforms

An HA epitope tagged wildtype Zw3 under UAS control was constructed as a wild type control. For cloning purposes, a Not site was engineered into pbOV1 zw3 cDNA (Siegfried et al., 1992) at the 5’UTR. The primers used were Not 5’ –GCA TGC GGC CGC GAA TTC CTT GTA AAT and ES KpnI –CTC GGT ACC ATG AGC to PCR the 5’ UTR. The conditions for the PCR reaction, using Pfu polymerase (Stratagene), were: step 1- 95°C for 5 min.; step 2 - 95° for 45 sec.; step 3-50°C for 30 sec.; step 4- 72°C for 2 min.; step 5- cycle 30 times to step 2. The fragment was cut with NotI and KpnI, gel purified, cloned into pBSK, and sequence confirmed. After subsequent subclonings into pBSK, a ~2.9kb fragment was ligated into pUAST (Brand and Perrimon, 1993) and labeled pUAST-WDT (Whole Damn Thing), which contained the zw3 coding region along with the entire 5’ and 3’UTRs. The Not ~2.9kb fragment (from pUAST-WDT) was ligated into pGEM5f (Promega) to facilitate introduction of the HA epitope tag. Two HA tags (sequence TAC CCC TAC GAT GTG CCA GAT TAC GCC) separated by an SpeI site were introduced just upstream of the stop codon into the pGEM5f-WDT and relabeled pGEM5f-WDT-HA. The Not ~2.9kb fragment (from
pGEM5f-WDT-HA) was ligated into pUAST. This construct is the wildtype Zw3 under UAS control (pUAST-WDT-HA).

To construct GSK-3α and GSK-3β N-terminal chimeras, two primary PCR reactions were carried out. The first round of PCR reactions consisted of two separate steps. In the first step, a PCR reaction was done to PCR out the N terminus of human GSK-3α (~324bp) and GSK-3β (~135bp). Human GSK-3β is identical to rat GSK-3β. Human GSK-3α and rat GSK-3α only have one change. The primers for GSK-3α were GSKAF- GCG GTA CCA TGA GCG GCG GGC CT and GSKAR- TTG CAA CAA CTG TTG TGA TTT CCC GCT GTC ACG GCC CAG. The primers for GSK-3β were GSKBF- GCG GTA CCA TGT CAG GGC GGC CCA AGA and GSKBR- TTG CAA CAA CTG TTG TTA TTT GCT GCC GTC CTT GTC TCT. The 5’ primer incorporated a KpnI site for cloning while the 3’ primer included sequence at the end that are complementary to GSK common sequence. The conditions for these primary PCR reactions, using Pfu polymerase (Stratagene), were: step 1- 94°C for 5 min.; step 2- 94°C for 45 sec.; step 3- 58°C for 45 sec.; step 4- 72°C for 2 min.; step 5- cycle back 30 times to step 2; step 6- 72°C for 10 min. The second portion of the first step of the PCR was to amplify a ~250bp fragment from pbOV1 containing the “GSK common domain” which contained an internal Hind III (H3) site. Combining the products of these 2 PCR reactions, a second PCR reaction was performed using the 5’Kpn primer from human GSK-3 and the 3’ Zw3 H3 primer. The conditions for these secondary PCR reactions, using Pfu polymerase (Stratagene), were: step 1- 95°C for 5 min.; step 2 - 95°C for 45 sec.; step 3-50°C for 30 sec.; step 4- 72°C for 2 min.; step 5- cycle 30 times to step 2. After resolving and isolating the products using the Mermaid Kit (Bio 101, Inc.), the PCR
products were cut with KpnI and H3 and then cloned into KpnI-H3 cut pBSK. Sequence confirming the chimeric isoforms were performed by the Pennsylvania State University Biotechnology Institute’s Nucleic Acid Facility. After confirmation, the above KpnI-H3 fragment was cloned into pGEM5f-WDT-HA that was cut with KpnI and H3. From this, a Not fragment, containing chimeric N-terminus, was isolated and cloned into pUAST. Transgenic lines containing the chimeric constructs were generated using P-element mediated transformation using the stock yw; ∆2-3Sb/TM6, Ubx (Robertson et al., 1988) according to standard procedures (Spradling, 1986). For the GSK-3α N-terminal chimera, seven independent sites of insertion were established. UAS-zw3NT-HA-3-47.5 was chosen for further study; while for GSK-3β, of the multiple insertion sites established, UAS-zw3NT-HA-3-2A was chosen. Both insertions are on the second chromosome.

To construct the UAS-zw3NT-HA-11, which has the entire N-terminus deleted, a modified version of the GSKcom primer was used along with the Zw3 H3 primer in a PCR reaction. This primer, GSKcomAUG- GCG GTA CCA TGA AAA TCA CAA CAG TTG TTG CAA, included a KpnI site and an AUG for translational initiation. The KpnI/H3 fragment was cloned into pBSK. After sequence confirmation, the internal KpnI/H3 fragment of pGEM5f-WDT-HA was replaced with the N-terminal deleted fragment. From this, a Not fragment was isolated and cloned into pUAST. Transgenic lines containing this construct were generated as described above.

**Immunohistochemical Staining of Whole-Mount Embryos**

Embryos were collected on either apple or grape juice-agar plates at 25°C. All embryos were dechorionated in 50% bleach and then fixed in a 1:1 solution of 4%
formaldehyde in PBS:heptane for 10-15 minutes. After removing the vitelline membrane in a 1:1 mixture of methanol:heptane, the embryos were thoroughly rinsed and stored in methanol at -20°C until used. Stainings were performed as described in Steitz et al. (1998). Colorimetric immunohistochemistry utilized mouse monoclonal antiserum against HA (1:10,000) (Boehringer Manheim). Goat-α-mouse secondary antibody was used at 1:500 and detected using the Vectastain ABC Elite Kit (Vector Laboratories, Burlingame, CA). Immunostained embryos were photographed under Nomarski optics with a Zeiss Axiophot microscope using Fujicolor Superia ASA 100 color-print film. Prints were scanned and digitized with an Epson Expression 626 scanner using Adobe Photoshop (v.4.0.1) software. Composites were constructed and annotated using Adobe Illustrator 6.0.

**Rescue of zw3 mutant phenotype**

Germ line clone (GLC) embryos that lack both maternal and zygotic gene products were generated using the FLP-DFS technique (Chou and Perrimon, 1992) as described in Siegfried et al. (1992). Females of the genotype zw3<sup>M11-1</sup> FRT<sup>101</sup>/FM7; arm-GAL4/arm-GAL4 were mated to ovo<sup>D1</sup> FRT 101/ Y ; FLP<sup>38</sup>/FLP<sup>38</sup> males. The progeny resulting from this cross were subject to heat shock at 37°C for two hours during the third instar larval stage, for two consecutive days, to induce FLP-mediated recombination. Virgin GLC females containing one copy of arm-GAL4 were mated to either homozygous UAS-zw3<sup>αNT-HA-3-47.5</sup>, UAS-zw3<sup>βNT-HA-3-2A</sup>, UAS-zw3<sup>αNT-HA-11</sup>, or UAS-zw3<sup>WDT-HA-4A1</sup> males. The ability to rescue was gauged by the reemergence of denticle
identity. As a control, $zw^{3 M11-1}$ mutant GLC; $arm$-GAL4/+ females were mated to wildtype males. A restored cuticle pattern was not observed in the progeny of this cross.

**Cuticle Preparations**

Cuticles were prepared according to directions described in Siegfried et al. (1992). Crosses were carried out at 25°C. Cuticles were viewed and photographed under dark field using a Zeiss Axiophot microscope. Images were taken on Kodak T-MAX 100 black and white film. A Polaroid SprintScan 35 Slide Scanner was used to scan and digitize the negatives using Adobe Photoshop (v.4.0.1) software. Composites were constructed and annotated using Adobe Illustrator 6.0.

**Dissection and Mounting of Adult Wings**

Wings of the progeny of appropriate crosses were dissected in 95% ethanol under an Olympus dissecting scope. Wings were permanently mounted in Hoyer’s media (water, glycerol, gum arabic, choral hydrate), and were viewed and photographed using a Zeiss Axiophot microscope. Images were captured on Kodak T-MAX 100 black and white film. A Polaroid SprintScan 35 Slide Scanner was used to scan and digitize the negatives using Adobe Photoshop (v.4.0.1) software. Composites were constructed and annotated using Adobe Illustrator (v.6.0.) software.
RESULTS

It has been previously demonstrated that the expression of vertebrate GSK-3β, but not GSK-3α, could rescue a zw3 mutant (Ruel et al., 1993a; Siegfried et al., 1992). To address whether the distinct amino terminal regions of the two vertebrate genes could account for their different activities when expressed in Drosophila, various isoforms of Zw3 which have either the entire amino terminus deleted (ΔNT-Zw3HA), the N-terminus replaced with that of human GSK-3β (βNT-Zw3HA) or with human GSK-3α (αNT-Zw3HA) were generated and compared to wildtype Zw3 (Zw3HA). Transgenic lines were established that express these constructs as HA epitope tagged transgenes under UAS control.

Transgenic Lines Express Full-Length Chimeric Protein

Before investigating the ability of these chimeric constructs to mimic overexpression of wildtype Zw3 in either the wing margin, or in the ability to rescue a zw3 mutant phenotype, a test to demonstrate the expression of full-length HA-tagged transgenes was first conducted. The embryonic GAL4 driver, engrailed (en)-GAL4, was crossed to HA epitope tagged wild-type Zw3. The embryos derived from this cross were immunostained with anti-HA antibody (Fig. 3.4A). 14 stripes of protein expression was observed demonstrating that full-length HA-tagged Zw3 is being expressed. Similarly, en-GAL4 was crossed to ΔNT-Zw3HA, βNT-Zw3HA, and αNT-Zw3HA. In all resulting progeny, 14 stripes of protein expression were observed upon anti-HA immunostaining (Figs. 3.4B, C, and D). The results from these stainings confirmed that
the transgenic lines generated, containing chimeric N-termini, express full-length protein.

Ectopic Expression of Zw3 Isoforms Can Mimic Overexpression of Wildtype Zw3

The results from the HA immunostaining established that the transgenes were making full-length protein, it did not however, imply anything about the functionality of these proteins. The activity of the various transgenes, and their ability to mirror Zw3 overexpression, was tested using the GAL4/UAS system binary expression system (Brand and Perrimon, 1993). Overexpression of Zw3 in the embryonic midgut blocks Wg signaling (Steitz et al., 1998). Moreover, overexpression of Zw3 in the imaginal wing margin blocks the endogenous Wg signaling pathway through its interaction with dAxin (Steitz, 2000).

The chimeric transgenes were expressed in the presumptive wing margin using the GAL4 driver, c96-GAL4, which is expressed in a domain 10-12 cells wide along the Dorsal-Ventral compartment border (Gustafson and Boulianne, 1996). The Drosophila adult wing is normally decorated with sensory bristles on the anterior margin and non-innervated hairs on the posterior margin (Fig. 3.5A). Similar to UAS-zw3; c96-GAL4, individuals overexpressing Zw3HA using the c96-GAL4 driver exhibit notching of the wing and a decrease in the number of marginal bristles (data not shown, and Fig. 3.5B) implying that Wg signaling at the wing margin is being disrupted. This also suggests that the presence of the epitope tag does not alter the activity of the transgene since wildtype Zw3 with, or without, the HA tag perform similarly in vivo. Like ectopic Zw3HA expression, overexpression of ΔNT-Zw3HA in the wing margin results in a comparable phenotype with the corresponding loss of marginal bristles and notching (Fig. 3.5C).
Figure 3.4. All Isoforms of Zw3 Express Full-Length Protein.

Anti-HA immunostaining in stage 11 (A) en-GAL4/UAS-zw3\textsuperscript{WDT-HA}, (B) en-GAL4/UAS-zw3\textsuperscript{ANT-HA}, (C) en-GAL4/UAS-zw3\textsuperscript{\(\beta\)NT-HA}, (D) en-GAL4/UAS-zw3\textsuperscript{\(\alpha\)NT-HA} embryos. These embryos expressed the Zw3 transgene in the \textit{engrailed} pattern as evidenced by the HA immunostaining. Therefore, it is confirmed that all transgenes tested express full-length, tagged protein. As a negative control, UAS-transgene embryos, without the GAL4, were immunostained with anti-HA and no signal was detected (data not shown). Embryos were reared at 25°C. Embryos are shown in a ventral view and are oriented anterior to the left.
However, the effect of ΔNT-Zw3HA is almost always weaker than Zw3HA. This is a surprising result since the amino terminal region contains the Ser-9 that has been implicated as a pseudosubstrate (Dajani et al., 2001; Frame et al., 2001). It was anticipated that deletion of the entire amino terminal region would behave as a constitutively active since a mutant form of Zw3, in which Ser-9 is altered to Alanine, behaves as a constitutively active kinase (Hazelett et al., 1998).

Overexpression of βNT-Zw3HA and αNT-Zw3HA in the wing margin also produced unexpected results. An early assumption was that since GSK-3β and not -α can replace Zw3 in vivo, that βNT-Zw3HA would be more active in the overexpression assay than αNT-Zw3HA. Contrary to this expectation, expression of βNT-Zw3HA had very little effect on the wing margin (Fig. 3.5D). In fact, there was very little loss of marginal bristles when compared to Zw3HA overexpression (Fig. 3.5B). While the amino termini of Zw3 and GSK-3β are very similar, the few differences between them are apparently enough to alter the regulation of the kinase activity in vivo. Surprisingly, expression of αNT-Zw3HA resulted in wing margin phenotypes that closely resembled Zw3HA overexpression (compare Figs. 3.5E and B; see Table 1).

Expression of Chimeric Transgenes Can Rescue zw3 Mutant Embryos

To further test the activity of the chimeric transgenes, the ability of ΔNT-Zw3HA, βNT-Zw3HA, and αNT-Zw3HA to rescue a zw3 mutant embryo was examined. Normally, the ventral surface of wild-type embryos is decorated with an alternating array of naked cuticle and denticle-secreting cuticle (Fig. 3.6A). zw3 is expressed maternally and is required for this normal segmentation. zw3 mutant germ line clone (GLC) females
Figure 3.5. Overexpression of Zw3 Isoforms at the Wing Margin Disrupt Bristle Formation.

Wings dissected from adult (A) wild-type Oregon-R, and (B) UAS-zw3\textsuperscript{WDT-HA/+}; c96-GAL4, (C) UAS-zw3\textsuperscript{\Delta NT-HA}, (D) UAS-zw3\textsuperscript{βNT-HA}, (E) UAS-zw3\textsuperscript{αNT-HA}. The c96-GAL4 driver drives expression in B, C, D, and E along the presumptive wing-margin in the wing imaginal disc. The \textit{Drosophila} adult wing is decorated with sensory bristles on the anterior margin and non-innervated hairs on the posterior margin (A). Overexpression of \textit{zw3} results in a “notching” effect with the loss of margin tissue and bristles that decorate the wing margin (B). Likewise but to a lesser extent, overexpression of \textit{zw3}\textsuperscript{\Delta NT-HA} results in the loss of marginal bristles and notching (C). Overexpression of the two chimeric \textit{zw3} genes at the wing margin yields different results. Overexpression of \textit{zw3} with a GSK-3\textit{β} N-terminus results in little loss of marginal bristles (D). Conversely, overexpressing \textit{zw3} with a GSK-3\textit{α} N-terminus results in a wing margin phenotype that resembles the overexpression of wild-type \textit{zw3} (E). All flies were reared at 25°C.
Table 1. Overexpression of αNT-Zw3HA, and Not βNT-Zw3HA, Can Severely Disrupt Wing Margin Formation

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*Five different regions, defined as the margin between longitudinal veins LI-II, LII-LIII, LIII-LIV, LIV-LV, and LV-LVI, were scored for gaps or notches. For each genotype, four wings were analyzed.*
produce embryos that exhibit a distinctive naked cuticle phenotype that is lacking dentine-secreting cuticle (Fig. 3.6B and Siegfried et al., 1992). zw3 GLC females carrying a copy of arm-GAL4 were crossed to UAS-zw3HA males. Restoration of the segmental pattern of denticles was observed in a portion of embryos resulting from this cross (Fig. 3.6C), suggesting the Zw3HA transgene is producing normal protein with wildtype activity that is able to substitute for the missing endogenous protein. The zw3 naked cuticle phenotype was also rescued with the expression of ∆NT-Zw3HA (Fig. 3.6D).

Overexpression of βNT-Zw3HA and αNT-Zw3HA once again produced surprising results that were consistent with their effect on the wing margin. Crossing βNT-Zw3HA males with zw3 GLC; arm-GAL4 females yielded embryos that were only partially rescued (Fig. 3.6E). While these embryos were rescued to a greater extent than the paternally rescued embryos (data not shown), restoration of dentine-secreting cuticle in zw3; arm-GAL4/βNT-Zw3HA embryos was significantly less than the rescue observed with the expression of Zw3HA or ∆NT-Zw3HA (compare Figs. 3.6E to C and D). It is therefore very unexpected that expression of an isoform of Zw3 that contains the GSK-3β amino terminus is less active than Zw3HA even though the N-termini of both proteins are very similar. Surprisingly, expression of αNT-Zw3HA can restore segmentation to zw3 mutant embryos to levels that are comparable to wildtype Zw3HA (Fig. 3.6F). This result is somewhat unpredicted given that the N-termini of these two proteins are quite dissimilar (Fig. 3.2B) and that expression of GSK-3α does not rescue the zw3 mutant phenotype (Ruel et al., 1993a; Siegfried et al., 1992).
Figure 3.6. Ectopic Expression of the Zw3 Isoforms Can Rescue the zw3 Mutant Phenotype.

Dark field photomicrographs of cuticles of (A) wild-type, and (B) zw3/Y null mutant embryos. (C-F) zw3 mutant embryos expressing the particular transgene under the control of arm-GAL4, (C) zw3; arm-GAL4/UAS-zw3\textsuperscript{WDT-HA}, (D) zw3; arm-GAL4/UAS-zw3\textsuperscript{\Delta NT-HA}, (E) zw3; arm-GAL4/UAS-zw3\textsuperscript{\beta NT-HA}, (F) zw3; arm-GAL4/UAS-zw3\textsuperscript{\alpha NT-HA}. The ventral surface of wild-type embryos is decorated with an alternating array of naked cuticle and denticle-secreting cuticle (A). In zw3 null (GLC) mutant embryos, the ventral surface is completely naked (B). The alternating belts of naked and denticle cuticle can be restored in zw3 null mutants ectopically expressing zw3\textsuperscript{WDT-HA} (C). A similar phenotype is seen in mutant embryos ectopically expressing zw3\textsuperscript{\Delta NT-HA} (D). However, the two chimeric zw3 genes had different abilities in rescuing the zw3 mutant phenotype. The ectopic expression of zw3\textsuperscript{\beta NT-HA} in zw3 mutants resulted in a subtle improvement to the segmental pattern with a limited restoration to denticle secretion (E). Conversely, ectopically expressing zw3\textsuperscript{\alpha NT-HA} in zw3 mutants phenocopies the ectopic expression of zw3\textsuperscript{WDT-HA} such that there is a significant restoration to the segmental pattern of denticles (F). All embryos were reared at 25°C. Embryos are oriented anterior to the top and ventral to the left. Magnification in A is smaller than C-F.
DISCUSSION

Wg signaling is mediated by the inactivation of the serine/threonine kinase Zw3 (Siegfried *et al.*, 1992). While the precise mechanism behind the Wg-dependent modulation of Zw3 kinase activity remains unresolved, work on its vertebrate counterpart, GSK-3β, is providing us with insights into its regulation. Regulation of GSK-3, in response to Insulin signaling, occurs through phosphorylation of Ser-9 of GSK-3β and Ser-21 of GSK-3α via PI 3-Kinase-dependent PKB/Akt (Cross *et al.*, 1995; Stambolic and Woodgett, 1994). This phosphorylation creates a pseudosubstrate site that can potentially interact with the substrate-docking domain. Binding of the pseudosubstrate reduces “normal” substrate binding; thereby, inhibiting GSK-3 kinase activity (Frame *et al.*, 2001). Conversely, it has been revealed that in Wnt signaling, binding of GSK-3β to GBP/Frat-1 can inhibit its kinase activity (Yost *et al.*, 1998). It has been suggested that this process may involve competition between Axin and GBP/Frat-1 for binding to GSK-3β (Fraser *et al.*, 2002). As there are currently no GBP/Frat-1 homologues identified in *Drosophila*, it remains to be seen if Zw3 is regulated by a similar mechanism. Different as these two homologues may be, expression of vertebrate GSK-3β, but not GSK-3α, in zw3 mutant embryos can substitute for Zw3 in *vivo* (Ruel *et al.*, 1993a; Siegfried *et al.*, 1992). While behaving differently when expressed in *Drosophila*, GSK-3α and GSK-3β, however, appear to have overlapping function in the mouse (Hoeflich *et al.*, 2000). The identification of a unique amino terminal region, which distinguishes the GSK-3αs from the GSK-3βs, prompted an investigation into its role on the different activities of the two vertebrate genes when expressed in *Drosophila*. 106
Wg-mediated Zw3 Inactivation Does Not Depend on Pseudosubstrate Binding *In Vivo*  

Expression of an isoform of Zw3, that lacks the entire unique amino terminus, is able to mimic overexpression of full-length wildtype Zw3. The activity of ΔNT-Zw3HA was tested using the GAL4 driver, *c96-GAL4*, to drive expression in the imaginal wing disc. Like overexpression of wildtype Zw3HA, ectopically expressing ΔNT-Zw3HA in the wing margin results in notching and loss of marginal bristles (Figs. 3.5B and C). The effect of ΔNT-Zw3HA is considerably weaker than Zw3HA. This result is rather surprising since the deleted amino terminal region contained the Ser-9 residue that has been implicated as a pseudosubstrate. The ΔNT-Zw3HA isoform was initially expected to behave as a constitutively active kinase since a mutant form of Zw3 in which Ser-9 is altered to Ala behaves as such (Hazelett *et al.*, 1998). Supporting this, expression of a *UAS-zw3* transgene with this mutation (S9A) using the *c96-GAL4* at 25°C proves to be lethal (data not shown). However, when this cross is performed at 18°C, viable flies emerge harboring moderate wing margin defects (data not shown). The difference in the activity of this transgene, as a result of temperature modulation, is due to the characteristic temperature-sensitivity of GAL4 (Wilder and Perrimon, 1995). Wing margin defects could not be detected upon expression of the other *zw3* transgenes at this lower temperature (data not shown). It is therefore rather interesting that a mutation of a single residue results in a constitutively active kinase while the removal of the 33 amino acid N-terminus, which includes this residue, is less active. These results hint at the presence of other residues in the amino terminus that are involved in the regulation of
kinase activity. Additionally, these results suggest that Zw3 activity via Wg signaling is regulated by a mechanism that does not depend on pseudosubstrate binding in vivo.

Since one way of inhibiting GSK-3 kinase activity is through the binding of the pseudosubstrate to the catalytic domain (Frame et al., 2001), removal of this region should not reduce kinase activity. However, what was observed was less activity without the N-terminus than with it. The activity of the transgene with a deleted N-terminus, that is incapable of pseudosubstrate binding, was less than the activity of the transgene with an intact amino terminus (Figs. 3.5C and B). The reduced activity of ΔNT-Zw3HA as compared to Zw3HA was once again observed in the rescue attempt of the zw3 mutant phenotype. Expression of the ΔNT-Zw3HA transgene was able to restore denticle-secreting cuticle to an otherwise naked zw3 mutant embryo (Fig. 3.6D). However, this result was weaker than the rescue observed upon expression of Zw3HA (Fig. 3.6C). This again suggests that other residues within the amino terminal are involved in regulating Zw3 kinase activity and that phosphorylation of Ser-9, creating an inhibitory pseudosubstrate, is not the way Zw3 is inactivated following Wg stimulation.

Several studies have addressed the issue of Ser-9 phosphorylation and pseudosubstrate binding in response to Wnt signaling. Cell culture assays performed by Ruel et al. (1999) demonstrated that in response to Wg signaling, via induction of Dsh expression, Zw3 was phosphorylated on serine residues. However, they found that the phosphopeptides of Zw3 could not be equated with the phosphorylation of the site analogous to Ser-9 in GSK-3β, suggesting the presence of other Wg/Dsh-inducible serine residues on Zw3 (Ruel et al., 1999). Additionally, Ding et al. (2000) confirmed that, unlike Insulin signaling, Wnt signaling did not lead to Ser-9 phosphorylation of GSK-3β.
Furthermore, they found that GSK-3β complexed with Axin is protected from the inhibitory Ser-9 phosphorylation that follows Insulin stimulation (Ding et al., 2000). Finally, in an experiment conducted by Frame et al. (2001), they found that by using a truncated peptide of 8 residues that matches a phosphorylated N-terminus of GSK-3β, phosphorylation of Axin did not decrease but phosphorylation of glycogen synthase by GSK-3β was selectively inhibited. This implies that occupation of the phosphate-binding site (presumably via a pseudosubstrate) is not responsible for inactivation of GSK-3β in response to Wnt signaling, since the interaction of this short phosphopeptide with the phosphate binding-site did not inhibit phosphorylation of substrates of the Wnt signaling pathway (Frame et al., 2001).

The GSK-3β N-terminus Is Less Active Than the GSK-3α N-terminus in Drosophila

Overall, Zw3 and GSK-3β are 76% identical; and within the kinase domain, identity increases to 85% (Siegfried et al., 1992). Not surprisingly, expression of rat GSK-3β was able to rescue the zw3 mutant phenotype (Ruel et al., 1993a; Siegfried et al., 1992). Moreover, the unique N-terminal region of Zw3 and its vertebrate counterpart, GSK-3β, differ by only 3 residues (Fig. 3.2B). It is therefore quite surprising to find that replacing the endogenous Zw3 N-terminus with that of GSK-3β resulted in a significant reduction to the kinase activity. Expression of βNT-Zw3HA in the imaginal wing margin using the c96-GAL4 driver did not lead to a drastic reduction in marginal bristles as compared to expression of Zw3HA (Figs. 3.5B and D). In fact, the activity of an isoform of Zw3 with a deleted N-terminus was more active, resulting in stronger wing margin defects (Fig. 3.5C). Furthermore, arm-GAL4 driven expression of
βNT-Zw3HA in a zw3 mutant embryo resulted in a mild rescue of the mutant phenotype (Fig. 3.6E). The patchy and sparse restoration of denticle-secreting cuticle in response to βNT-Zw3HA paled in comparison to what was observed with Zw3HA expression (Figs. 3.6E and C). Once again, the βNT-Zw3HA transgene was less active than the transgene that did not contain an N-terminus. The above results suggest that although the unique amino termini of Zw3 and GSK-3β are very similar, the three differences between them appear to alter the regulation of the kinase activity in vivo. Moreover, this decrease in activity hints at the presence of a molecule(s) that is able to selectively inhibit the βNT-Zw3HA transgene.

Even more surprising was the activity of the chimeric Zw3 isoform with the N-terminus of GSK-3α, which is almost three times as large as the endogenous N-terminus, when expressed in the wing margin. Even though expression of GSK-3α could not rescue a zw3 mutant phenotype (Ruel et al., 1993a; Siegfried et al., 1992), expression of αNT-Zw3HA in the wing margin produced phenotypes similar to those observed with the expression of wildtype Zw3HA (Figs. 3.5E and B). The notching and loss of marginal bristles that resulted suggested that this transgene is as active as the wildtype transgene in blocking Wg signaling at the wing margin. Furthermore, expression of αNT-Zw3HA in zw3 mutants restored denticle identity to levels comparable to those observed with Zw3HA expression (Figs. 3.6F and C). The restoration of normal segmentation with the αNT-Zw3HA transgene was far greater than that seen with βNT-Zw3HA expression. So while it appears that the activity of βNT-Zw3HA is unexpectedly reduced in these assays, the activity of the αNT-Zw3HA transgene is, surprisingly, very similar to that of Zw3HA.
What Could Be Inhibiting the Activity of the βNT-Zw3HA Transgene in

*Drosophila*?

The only difference between the chimeric transgenes, βNT-Zw3HA and αNT-Zw3HA, is in their amino terminal region. So why is their activity so different when their catalytic domains are identical? To help address this question, an exploration into molecules that are able to regulate GSK-3 would be insightful. An initial assumption was that perhaps, PKB/Akt, the kinase that inactivates GSK-3β in response to Insulin signaling, was responsible for inactivating βNT-Zw3HA activity. But several findings would suggest against any PKB involvement in downregulating Zw3 activity in Wg signaling. First, unlike Insulin stimulation, Wnt signaling did not cause threonine-308 or serine-473 phosphorylation on PKB, which are required for its activation (Alessi *et al.*, 1996; Ding *et al.*, 2000). Secondly, activation of PKB alone could not activate transcription of Wg target genes (Yuan *et al.*, 1999). Thirdly, the phenotype of flies mutant for dakt1, a *Drosophila* PKB/Akt homolog, suggests a role that is more in line with cell survival rather than Wg signaling or Zw3 regulation for DAkt1 (Staveley *et al.*, 1998). Finally, activated PKB equally phosphorylates both GSK-3β and -α isoforms (Cross *et al.*, 1995). Therefore, if PKB phosphorylation was somehow responsible for suppressing the activity of the βNT-Zw3HA transgene, then the activity of αNT-Zw3HA should also have been equally suppressed, but this was not observed. While the reasons listed above present a strong argument against PKB’s role in inhibiting Zw3 activity, it does not preclude PKB from inactivating the exogenous transgene. One way to test any PKB involvement in downregulating βNT-Zw3HA transgene activity would be to express this transgene in a PKB mutant background. If in this background, the resulting
activity of βNT-Zw3HA, as measured by the ability to cause wing defects or rescue the
naked cuticle phenotype, is increased and is at least comparable to the activity of
Zw3HA, then that would imply a role for PKB-mediated inhibition of kinase activity.
Unfortunately however, there is precedent again against the role of PKB in Zw3
inactivation. No phenotypic changes were observed when Zw3 was overexpressed in a
PKB heterozygous mutant background suggesting that Drosophila PKB may not be
involved in Zw3 inactivation (Steitz, 2000).

PKB is not the only kinase capable of inactivating GSK-3β. Overexpression of
the serine/threonine kinase, Integrin-linked Kinase (ILK), results in not only the nuclear
localization of β-catenin but the formation of the LEF-1/β-cat transcriptional activation
complex (Novak et al., 1998). Further examination revealed that ILK can directly
phosphorylate GSK-3 and inhibit its activity (Delcommenne et al., 1998). Since ILK can
also activate PKB via Ser-473 phosphorylation, it has not been established whether the
inhibition of GSK-3 activity is due to ILK phosphorylation directly or via PKB activity
indirectly. Interestingly, experiments attempting to detect GSK-3 Ser-9 phosphorylation
in response to ILK overexpression have been inconclusive (Delcommenne et al., 1998).
However, the mutant phenotype of the Drosophila homolog, dilk, like that of dakt, ruled
out a requirement in Wg/β-cat signaling. dilk mutant embryos, while dying at the end of
embryogenesis, have a completely normal cuticle pattern and show no defects associated
with perturbations of Wg signaling (Zervas et al., 2001). While this may rule out a role
for dILK in Wg signaling, it does not necessarily mean that it cannot on some level,
selectively phosphorylate, and potentially inactivate, the βNT-Zw3HA transgene.
As discussed above, one way to inactivate GSK-3β is via phosphorylation. But what if the activity of the kinase is not so much inhibited as the kinase is prevented from acting on the substrate? What if regulation is a product of targeting GSK-3 to different subcellular localizations such that it is unavailable to its substrates? The emergence of research into the subcellular localization of GSK-3 has offered explanations to interesting observations. For example, many of GSK-3’s putative substrates, such as Cyclin D1, C-Myc, and Heat shock factor-1, are transcription factors that are inhibited upon phosphorylation (Doble and Woodgett, 2003). While mainly thought of as a cytoplasmic kinase, recent studies have established that the localization of GSK-3 inside the cell is dynamically regulated. There does not appear to be any nuclear import or export signals in the GSK-3 amino acid sequence, raising the question of its localization within the cell. A report by Franca-Koh et al. (2002) has demonstrated that GBP/Frat-1 may play a role in regulating GSK-3 localization. A GSK-3 mutant isoform that is unable to bind Frat accumulates in the nucleus (Franca-Koh et al., 2002). Since there are no GBP/Frat-1 homologues in Drosophila, it’s unclear whether the mechanism of Zw3 localization is mediated by similarly acting proteins. One indication that it might be is Zw3 sensitivity towards LMB, an inhibitor of the nuclear export receptor Crm1 (Franca-Koh et al., 2002). So could the βNT-Zw3HA transgene be sequestered in the nucleus and therefore unable to block Wg signaling? One way to find out would be to determine the subcellular localization by confocal microscopy using antibodies directed against the HA tag. If this transgene is selectively confined to the nucleus, then that would imply it is unable to be exported from the nucleus by a presently unknown mechanism.
Zw3/GSK-3β Inhibition via Wg/Wnt Signaling

The formation of a destruction complex involving Zw3/GSK-3β, that results in β-cat destabilization, is at heart of the current model of Wg/Wnt-mediated inhibition of Zw3/GSK-3β kinase activity. A pool of GSK-3β participates in this complex by phosphorylating a number of substrates in addition to β-cat. Both APC and Axin are phosphorylated by GSK-3β resulting in their stabilization while increasing their affinity for β-cat and for each other (Behrens et al., 1998; Ikeda et al., 1998; Rubinfeld et al., 1996; Yamamoto et al., 1999). In fact, GSK-3β phosphorylation of β-cat is greatly improved in the presence of Axin, suggesting that Axin acts as a scaffold protein that brings together kinase and substrate (Ikeda et al., 1998). Therefore, the formation of this active complex results in the eventual destruction of β-cat. Consequently, if formation of this complex is prevented, β-cat is stabilized and able to activate transcription of Wnt responsive gene in combination with TCF/LEF-1 upon translocation to the nucleus (Hart et al., 1998; Hinoi et al., 2000). At least in the regulation of vertebrate Wnt signaling, the activity of GBP/Frat-1 is thought to be responsible for the disassembly of the destruction complex (Farr et al., 2000; Ferkey and Kimelman, 2002; Yost et al., 1998). GBP/Frat-1 is believed to competitively bind GSK-3 away from Axin, leading to the instability and subsequent break up of the destruction complex (Dajani et al., 2003; Ferkey and Kimelman, 2002; Fraser et al., 2002). However, it is still unclear whether the inhibition of Zw3 in response to Wg signaling is mediated by a similar mechanism since homologues of GBP/Frat-1 have not been identified in Drosophila. So in the absence of such a molecule, how can we explain the decreased activity of the βNT-Zw3HA transgene in Drosophila? One possibility is that the minimal change in the amino
terminal region is affecting the way the destruction complex players get together. That could be due to inefficient phosphorylation of dAPC and/or dAxin. The result of this would be a destruction complex that is not as stable as the one formed by the exogenous Zw3HA transgene. With such a gathering of loosely associated proteins, there would be minimal Arm phosphorylation and Wg signaling would not be blocked. It should be noted however, that all the transgenes tested should potentially bind Axin to equal levels since Axin binding has been mapped to the C-terminus of the protein and they all originated from the same construct prior to the changes in their N-termini. In any case, the effect of dAxin on Zw3 overexpression is well documented. The effect of ectopically expressing Zw3 can be suppressed in a dAxin mutant background. Conversely, the effect of Zw3 overexpression can be enhanced with the concomitant overexpression of dAxin (Steitz, 2000). One test to determine if dAxin is responsible for the inactivity of the βNT-Zw3HA transgene is to overexpress βNT-Zw3HA and dAxin simultaneously. If the effect of this resembles the overexpression of βNT-Zw3HA alone, then that would imply that the transgene is not properly interacting with dAxin and thus not forming an efficient destruction complex that is able to destabilize Arm.

**Summary**

The effect of the N-terminus on GSK-3 activity has been tested. It has been suggested that Wg signaling-mediated regulation of Zw3 does not depend on pseudosubstrate binding since an isoform with a deleted amino terminal region is as active as an isoform with an intact N-terminus. Furthermore, an isoform containing the GSK-3α N-terminus is more active than an isoform that contains the N-terminus from
GSK-3β. The reason behind the difference in activity between the two isoforms has yet to be elucidated. Even though, 2-3 independent insertions of each transgene were tested for each assay, one cannot rule out that the two isoforms express different levels of protein.
Signal transduction pathways direct the growth and patterning of any multicellular organism. The extracellular signals and the signaling cascades that ensue are essential for the proper development of invertebrate and vertebrate embryos. One of the most heavily studied classes of signaling molecules is the Wnt family of secreted growth factors (Cadigan and Nusse, 1997; Siegfried, 1999). Wnt genes encode secreted glycoproteins that are utilized in the regulation of cell growth and differentiation across species (Wodarz and Nusse, 1998). Wingless (Wg) is the best characterized Wnt identified in *Drosophila*. Wg directs cell fate specification and patterning throughout the embryonic and larval development of *Drosophila* (Siegfried, 1999).

The studies presented in this thesis examined the role of the Wg signaling pathway in the specification of cell fate in the somatic mesoderm. It is demonstrated that Wg and Notch (N) signaling act in concert to regulate the differentiation and patterning of the embryonic somatic musculature. Both loss-of-function and gain-of-function experiments were used to investigate the interaction of these two signaling pathways on the specification of S59- and Nautilus (Nau)-expressing muscle founders, Sticks and Stones (SNS)-expressing fusion-competent cells and Lethal of Scute (L’sc) expression. Wg signaling is required to selectively specify some muscle founders and to restrict the fusion-competent cells. Conversely, N signaling restricts muscle founders and is required for fusion-competent cell specification. It is shown that Wg and N signaling coordinately
but differentially specify distinct subsets of muscle founders and fusion-competent myoblasts within an equivalence group.

An additional aim of this thesis focused on the role of the N-terminus of Glycogen Synthase Kinase-3 (GSK-3), a key negative-regulator of the Wg/Wnt signaling pathway. This was achieved by expressing isoforms of Zeste White-3 (Zw3), the Drosophila homolog of vertebrate GSK-3β, that differ in their amino terminal region. These isoforms were tested in their ability to mimic Zw3 overexpression in various in vivo assays. It is demonstrated that Zw3 inactivation in response to Wg signaling does not depend on pseudosubstrate binding in vivo. Furthermore, it is presented that a chimeric transgene with the N-terminus of GSK-3β is less active than one with the N-terminus of GSK-3α when expressed in Drosophila.

The Different Roles of Wg Signaling in the Specification of Distinct Muscle Founder Cell Fate

The somatic mesoderm of a Drosophila embryo gives rise to an elaborate pattern of 30 distinct body wall muscles per abdominal hemisegment (Bate, 1990; Bate and Rushton, 1993). Each of these fibers is formed from a series of fusion events between a founder cell, which dictates the ultimate identity of the muscle fiber, and neighboring fusion-competent cells (Bate, 1992; Rushton et al., 1995). Both of these cell types arise from a population of myoblasts within a discrete equivalence group and selection of the founder cell occurs through a process mediated by lateral inhibition (Baylies et al., 1998). Moreover, Wg has been shown to be required for the specification of S59- and Nau-expressing muscle founder cells (Baylies et al., 1995; Ranganayakulu et al., 1996).
The mechanism by which Wg specifies muscle founders is not universal and specification of S59- and Nau-expressing muscle founders occurs through different Wg-dependent and –independent mechanisms. In the specification of S59-expressing muscle founders, Wg signaling acts to alleviate an early N mediated restriction of this cell fate that is independent of lateral inhibition. Brennan et al. (1999) also confirmed this finding. It was demonstrated that this Wg-dependent expression of S59 is mediated through the canonical Wg signaling pathway, which culminates in Arm-mediated regulation of gene expression. Therefore, the Wg derepression of N restriction on cell fate is likely to occur at the level of competition for regulation of common target genes. Integration of the Wg and N signaling pathways has been suggested to occur at the level of Wg binding to N, or some component of the N pathway (Brennan et al., 1999), or integration through Dishevelled (Dsh) which has been shown to bind N (Axelrod et al., 1996). The observation that founder cell fate is expanded by overexpression of cytoplasmically activated Arm is consistent with a competition of the Wg and N pathways for regulation of gene expression. This regulation can occur directly on S59 expression or indirectly, mediated through the regulation of common and/or competing myogenic activators and repressors which in turn control S59 expression. Conversely, the medial clusters of Nau founders are absent in embryos lacking Wg signaling as well as in embryos lacking both Wg and N signaling. These observations suggest that the Wg signaling-dependent specification of these cell fates is independent of any restriction imposed by N. The distinct mechanisms of Wg-dependent expression of the S59 and medial Nau cells may reflect differences in the promotor organization of these two genes, or differences in the requisite myogenic factors. Interestingly, a quick scan of the s59
and *nau* promotor regions did not reveal any consensus TCF binding sites (H. Rizkalla, and E. Dermitzakis, unpublished observation).

Unlike the medial cluster, the lateral clusters of Nau-expressing founders, surprisingly, do not require Wg for specification, but nonetheless, are expanded upon hyperactivation of the Wg signaling pathway. This response suggested that these cells are capable of receiving and transducing the Wg signal, but that Wg normally does not affect their fate. One plausible explanation for the difference in response to Wg between the medial and lateral Nau cells may be their position relative to the stripe of ectodermal cells expressing Wg. The Wg-dependent medial cells are adjacent to the ventral stripe of Wg in the ectoderm (Ranganayakulu *et al.*, 1996). However, the lateral cells lay in a position that corresponds to a gap in the circumferential stripe of Wg expression. It is conceivable that at this position, lateral Nau cells experience an insufficient level of Wg to activate the pathway and specification of cell fate is established through some other inductive signal. Finally, a recent finding regarding N inhibition may offer yet another explanation for the differences observed between the medial and lateral Nau-expressing cells. Espinosa *et al.* (2003) demonstrated that GSK-3β can phosphorylate and inactivate N activity. This finding, in combination with the observations that a) Zw3 overexpression can increase the size of the Wg-independent lateral Nau-expressing cells and b) the lateral clusters are slightly reduced in *zw3* mutant GLC embryos, raises the possibility that Zw3, in a Wg-independent role, could potentially play a positive role in the specification of the lateral Nau founder cells. Therefore, a tempting explanation to the increase in the size of lateral Nau-expressing clusters upon overexpression of Zw3 is that exogenous Zw3 is downregulating the N repression of this cell fate. Therefore,
overexpressing Zw3 would result in an increase in the size of the lateral clusters since
this would, in essence, repress a repressor. Conversely, in zw3 mutants, the lateral
clusters would decrease in size since the N repression of this cell fate would be
uncontested.

**DWnts Are Not Required for the Specification of S59 and Nau Founder Cells**

The presence of the lateral Nau-expressing founders, in embryos mutant for Wg
signaling, suggested that their specification is mediated by another inductive signal.
Two candidate molecules that were tested were DWnt-2 and DWnt-4. Their ectopic
expression, while not completely resulting in the same expansion in the numbers of S59
and Nau-expressing founders as Wg hyperactivation, did alter the fate of Wg-responsive
founders. Overexpressing DWnt-2 or DWnt-4 expanded the number of lateral Nau-
expressing founders, whereas the Wg-dependent medial Nau-expressing founders were
not significantly altered. Furthermore, only subsets of S59-expressing muscle founders
were expanded in response to DWnt overexpression. However, examination of S59 and
Nau expression in Dwnt mutants did not support a role for DWnts in specifying cell fate
in the somatic mesoderm. Both S59- and Nau-expressing founders were present, in near
wild-type levels, in Dwnt-2 and Dwnt-4 mutant embryos suggesting that while
hyperactivation may alter the fate of these muscle founders, DWnt-2 and DWnt-4 are not
required for their specification.
The Early N Repression of Muscle Founder Cell Fate Is Alleviated Differently in the Specification of S59 and Nau Founders

Rusconi and Corbin (1998) previously reported that S59 and Nau muscle founder cell fates are restricted by an early activity of N that is independent of lateral inhibition. Wg signaling alleviates this early N restriction on S59 muscle founder fates; however, this is not true for the Nau founder fate. The medial Nau founders are Wg-dependent but they are not restored in an embryo mutant for both Wg and N signaling. Conversely, the lateral Nau founders are Wg-independent and are not only present but are expanded as well in embryos mutant for both Wg and N signaling. Therefore, there must be an alternative means of N derepression for the Nau founders. One possibility is that the unidentified signaling pathway(s) which induce(s) lateral Nau founders may also function to alleviate the early N repression of this cell fate. The Zw3 loss-of-function/gain-of-function findings temptingly suggest that Zw3 may be involved in this unidentified signaling pathway.

The Selection of Founder and Fusion-Competent Cell Fates Within Equivalence Groups Is Regulated by Wg Signaling and N-Mediated Lateral Inhibition

In embryos where Wg signaling is perturbed, there is a reduction of some founder cells (S59 and medial Nau) and concurrently, as revealed by the expression of SNS, an expansion in the number of fusion-competent cells. Overexpression of the Wg ligand, or an activated form of Arm, results in an increase in all muscle founders examined and a corresponding reduction in fusion-competent cells. The opposite is observed in N mutant embryos, in which the number of muscle founders is expanded and the fusion-competent
myoblasts are absent. Additionally, ectopic activation of N signaling reduces the number of both S59- and Nau-expressing founders while increasing the number of fusion-competent cells. Interestingly, SNS expression is significantly reduced in the somatic mesoderm of embryos mutant for both Wg and N signaling, with the exception of the cells along the ventral midline. In this region of a Ndsh double mutant GLC embryo, in which there is a loss of muscle founders (medial Nau), there is a simultaneous increase in fusion-competent cells. Conversely, in regions of the embryo where there is an increase in the number of muscle founders (lateral Nau and S59) we observe a loss of fusion-competent cells. Consistent with the findings presented, in the absence of both Wg and N signaling, an inverse proportion of muscle founders to fusion-competent cells is observed. These results suggest that Wg signaling acts within pre-determined equivalence groups to select the muscle progenitors, rather than to restrict or position the equivalence groups. Furthermore, N signaling also activates lateral inhibition in the surrounding cells to restrict the muscle founder fate and promote the fusion-competent cell fate. This is consistent with the observations that hyperactivation of Wg, or loss of N signaling, results in an expansion of the number of cells per founder cluster, but the number and position of the clusters remain largely unchanged (see Table 2 for summary).

**All Equivalence Groups Do Not Express L’S C**

As summarized above, the results presented indicate that Wg and N signaling regulate a binary cell fate decision within a fixed equivalence group. The presence of an equivalence is thought to be marked by the expression of L’sc (Carmena et al., 1995). In embryos lacking both Wg and N signaling, some founders and fusion-competent cells are
### Table 2. Summary of S59 and Nau Expression in Various Genetic Backgrounds

<table>
<thead>
<tr>
<th>Genotype/Transgene Expressed</th>
<th>S59</th>
<th>Nau-Medial</th>
<th>Nau-Lateral</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>wg</em></td>
<td>-</td>
<td>-</td>
<td>NC</td>
</tr>
<tr>
<td><em>dsh</em></td>
<td>-</td>
<td>-</td>
<td>NC</td>
</tr>
<tr>
<td><em>zw3</em></td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td><em>arm</em></td>
<td>-</td>
<td>-</td>
<td>NC</td>
</tr>
<tr>
<td><em>Dwnt2</em></td>
<td>NC</td>
<td>NC</td>
<td>NC</td>
</tr>
<tr>
<td><em>Dwnt4</em></td>
<td>NC</td>
<td>NC</td>
<td>NC</td>
</tr>
<tr>
<td><em>N</em></td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td><em>Ndsh</em></td>
<td>+++</td>
<td>-</td>
<td>+++</td>
</tr>
<tr>
<td>UAS-<em>wg</em></td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>UAS-<em>arm</em>&lt;sup&gt;S1J&lt;/sup&gt;</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>UAS-<em>zw3</em> @ 29 °C</td>
<td>NC</td>
<td>-</td>
<td>+++</td>
</tr>
<tr>
<td>UAS-<em>Dwnt2</em></td>
<td>+</td>
<td>NC</td>
<td>+++</td>
</tr>
<tr>
<td>UAS-<em>Dwnt4</em></td>
<td>+</td>
<td>NC</td>
<td>+++</td>
</tr>
<tr>
<td>UAS-*N&lt;sup&gt;intra&lt;/sup&gt;</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

+++/- - - = greatly expanded/completely absent  
++/- - - = marginally expanded/reduced  
+/- = slightly expanded/reduced  
NC = no change
specified even though no L’sc expressing cells are observed under these same conditions. These results suggested that the equivalence group can be specified although the cells of this equivalence group do not express L’sc. Thus, expression of L’sc may not be a faithful and accurate marker for the presence of an equivalence group in all instances. The results presented are in general agreement with the conclusions made by Carmena et al. (1995) that state L’sc may potentially have redundant activity with other factors required to specify equivalence groups. In addition, the furnished data is consistent with two different interpretations for the role of L’Sc in specification of the equivalence groups. First, equivalence groups may exist that normally do not express L’sc. This possibility is unexpected for the S59-expressing founders, since the expression of S59 has been closely correlated with that of L’sc (Carmena et al., 1995). Secondly, although expression of both L’sc and S59 is induced by Wg and repressed by N, it is still possible that the integration of these signaling pathways in the regulation of expression of L’sc and S59 is distinct.

A Model for Wg Signaling in the Specification of the Somatic Mesoderm

The model in Chapter 2 presents a model for the mechanism of Wg and N signaling in the specification of both founder and fusion-competent myogenic cell fates within a pre-determined equivalence group. It describes the role of Wg signaling in the specification of three distinct muscle founder cells: S59-expressing, medial Nau-expressing and laternal Nau-expressing founders. Wg promotes the muscle founder cell fate by alleviating an early N repression (S59 founders) or independent of this activity of N (medial Nau founders). Other founders (lateral Nau) are not normally subject to Wg
induction but can respond to Wg hyperactivation. Once a founder cell is selected, N lateral inhibition is activated in the neighboring cells to restrict them from assuming the founder fate and to promote the fusion-competent fate. It is proposed that Wg signaling and N lateral inhibition regulate common myogenic factors that determine alternatively muscle founder and fusion-competent cell fates. The test of this model awaits the identification of the target genes regulated by Wg and N signaling in the mesoderm.

The Regulation of Zw3/ GSK-3β

Zw3 kinase activity is downregulated in response to Wg signaling. Upon Wg stimulation, mediated by a currently undefined process, Dsh indirectly regulates the activity of Zw3. The inactivation of Zw3 leads to the accumulation and stabilization of cytoplasmic Arm. Stable Arm protein moves into the nucleus where it associates with dTCF to activate transcription of Wg responsive genes (Siegfried, 1999). While the mechanism behind Zw3 inactivation in response to Wg signaling remains unclear, work on GSK-3β has increased our understanding of the Wnt-mediated regulation of this serine/threonine kinase. GSK-3β, along with Axin and APC, in the absence of Wnt stimulation, participate in the destruction complex that targets β-cat for degradation (Behrens et al., 1998; Fagotto et al., 1999; Ikeda et al., 1998; Itoh et al., 1998).

However, upon Wnt stimulation, the destruction complex collapses as a result of the interaction of two proteins, Dsh and GSK-3β Binding Protein (GBP) (Yanagawa et al., 1995; Yost et al., 1998). It has been demonstrated that since the C-terminus of GSK-3β contains overlapping Axin and GBP binding sites, and that these two proteins cannot bind GSK-3β at the same time (Dajani et al., 2001; Ferkey and Kimelman, 2002; Fraser et al.,
Furthermore, it has been speculated that Dsh could recruit these GSK-3β inhibitors (GBP/Frat-1) to the Axin-anchored complex to promote the disassociation of GSK-3β from this complex (Fraser et al., 2002). Therefore, upon Wnt stimulation, GBP/Frat-1 would bind to and inhibit the activity of GSK-3β, which would result in the stabilization of unphosphorylated β-cat. Since a GBP/Frat-1 like molecule has yet to be identified in *Drosophila*, it is unclear exactly how Zw3 activity is inactivated in response to Wg.

The mechanism behind Insulin signaling’s downregulation of GSK-3 is, on the other hand, better understood. Insulin stimulation downregulates GSK-3 and promotes glycogen biosynthesis (Embi et al., 1980). GSK-3 is subject to Ser-9 (β)/Ser-21(α) phosphorylation by PKB in response to Insulin signaling (Cross et al., 1995; Stambolic and Woodgett, 1994). The phosphorylation of that residue creates a pseudosubstrate site that can bind the kinase’s substrate docking domain. This inactivates the kinase by reducing substrate binding (Dajani et al., 2001; Frame et al., 2001).

**Wg Signaling Inactivation of Zw3 Does Not Utilize Pseudosubstrate Binding In Vivo**

The expression of an isoform of Zw3, with a deleted N-terminus, is able to mimic overexpression of full-length wildtype Zw3. However, the observation that the effect of ∆NT-Zw3HA expression was considerably weaker than Zw3HA was surprising since the deleted amino terminal region contained the Ser-9 residue that has been implicated as a pseudosubstrate. It was believed that the ∆NT-Zw3HA isoform would behave as a consitutively active kinase since a mutant form of Zw3 in which Ser-9 is altered to Ala behaves as such (Hazelett et al., 1998). Additionally, expression of a *UAS-zw3* transgene
with this mutation (S9A) using the c96-GAL4 at 25°C proves to be lethal while at 18°C viable flies emerge harboring moderate wing margin defects (data not shown). Interestingly, expression of the other zw3 transgenes at this lower temperature did not produce wing margin defects (data not shown). It is therefore rather intriguing that a mutation of a single residue would result in a constitutively active kinase while the deletion of the amino terminal region, which includes this residue, is less active. This finding suggests the presence of other residues in the amino terminus that are involved in the regulation of kinase activity. Additionally, these results suggest that Zw3 activity via Wg signaling is regulated by a mechanism that does not depend on pseudosubstrate binding in vivo.

The N-terminus of GSK-3β, but not GSK-3α, Imparts Reduced Activity When Expressed in Drosophila

Zw3 and its vertebrate counterpart, GSK-3β are 85% identical within the kinase domain (Siegfried et al., 1992). In fact, expression of GSK-3β, but not GSK-3α, can rescue the zw3 mutant phenotype (Ruel et al., 1993a; Siegfried et al., 1992). Furthermore, the unique N-terminal regions of Zw3 and GSK-3β differ by only 3 residues while differing considerably from the N-terminus of GSK-3α. It was therefore quite surprising to find that replacing the endogenous Zw3 N-terminus with that of GSK-3β resulted in a significant reduction to the kinase activity. An even greater surprise was the level of activity observed when the N-terminus was replaced with that of GSK-3α. In the two assays used, the activity of the βNT-Zw3HA transgene was consistently weaker than αNT-Zw3HA. The mild loss of wing margin tissue and poor rescue of the zw3...
phenotype observed with βNT-Zw3HA expression were significant in contrast to those observed with αNT-Zw3HA expression. These results imply that while the unique amino termini of Zw3 and GSK-3β are very similar, the three differences between them appear to alter the regulation of the kinase activity in vivo. In addition, the decreased activity of the βNT-Zw3HA transgene suggests the possible presence of a molecule(s) that is able to selectively inhibit it when expressed in Drosophila.

While experimental data elucidating the differences in activity between the βNT-Zw3HA and the αNT-Zw3HA transgenes were not presented, several possible explanations were brought forth. Inactivation by phosphorylation, either by PKB or ILK, could be responsible for the observed differences in activity of the two transgenes. Moreover, dissimilarity in the subcellular localization of the two isoforms could also explain the disparity in activity. Baring the discovery of a GBP like molecule in Drosophila, the most probable explanation is that the minimal change in the amino terminal region, between βNT-Zw3HA and Zw3HA, is interfering with the assembly of the destruction complex. Whether this alters the phosphorylation of dAPC and/or dAxin remains to be tested. In any event, the association of βNT-Zw3HA would result in a destruction complex that is not as stable as the one formed by the exogenous Zw3HA transgene. A less stable destruction complex would result in minimal Arm phosphorylation and Wg signaling would not be blocked as effectively. The precise mechanism behind the differences in activity of βNT-Zw3HA and αNT-Zw3HA awaits further testing.
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