STUDY OF THE ROLE OF PAX TRANSCRIPTION FACTORS AND SP-RELATED FACTORS IN C. ELEGANS ORGAN DEVELOPMENT

DISSERTATION

Presented in Partial Fulfillment of the Requirements for
the Degree Doctor of Philosophy in the Graduate
School of The Ohio State University

by

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2008

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ABSTRACT

Organogenesis requires cells to coordinate their development. The similarities and differences between cells can be explained by looking at their gene expression profiles. At the center of these profiles, there are often transcription factors such as Pax and Sp-related factors that are critical for organ development.

To understand the specific role of Pax factors in organogenesis, it is important to determine how they function with other transcription factors to regulate target genes.

To better understand the different roles of EGL-38, I used an overexpression approach. I tested the effect of ectopic expression of EGL-38 and its mutants in animals. My results indicate that ectopic expression of EGL-38 and its male tail specific alleles (sy287 and gu22) in embryos induce embryonic lethality, whereas ectopic expression of its egg-laying defective allele (n578) does not have any effect. I hypothesized that these defects could be due to misexpression of different EGL-38 targets in response to the different alleles. Using gfp reporter transgenes, I have shown that indeed ectopic expression of EGL-38, induces ectopic expression of lin-48 (a known direct target).

Pax factors have different targets in different cells because they are known to act in a combinatorial manner with other transcription factors. For example, lin-48 is a direct target of EGL-38 in the hindgut, but it is not expressed in other organs where EGL-38 is known to function. To identify EGL-38 cofactors important for lin-48
hindgut expression, I conducted a genetic screen. I have isolated two mutations (\textit{gu84} and \textit{gu85}). I have shown that gu85 is a mutation in \textit{sptf-3}, a Sp-related factor.

I also characterized the role of \textit{sptf-3} in organogenesis. \textit{sptf-3(gu85)} animals show developmental defects that are similar to those seen in Wnt mutants. Specifically, mutants exhibit the Bivulva phenotype similar to \textit{lin-17/frizzled} and \textit{lin-18/Ryk} mutants. I show that \textit{sptf-3(gu85)} mutants are sensitive to the dose of \textit{lin-17}. In addition, \textit{sptf-3(gu85)} animals exhibit P12 to P11 cell fate transformations, and defects in B cell asymmetric division. These results provide the first description for the role of \textit{sptf-3} in \textit{C. elegans} development. They also suggest that SPTF-3 functions in Wnt-dependent decisions.
DEDICATION

To my parents, Fawwak and Randa Sleiman, for their unconditional love and support,
their guidance and their good example

To my brother, Kamal Sleiman, for supporting me in everything I do
ACKNOWLEDGMENTS

I am deeply grateful to my advisor, Dr. Helen Chamberlin for her guidance, support and patience throughout my graduate career. She has challenged and guided me to become a better scientist and has supported me through all my triumphs and failures.

I would like to thank my Ph.D. dissertation committee members, Dr. Helen Chamberlin, Dr. Stephen Osmani, Dr. Venkat Gopalan, and Dr. Heithem El-Hodiri for their advice, suggestions and their generosity with their time.

I have also been very lucky to have benefited from the advice of many Molecular Genetics faculty members. Specifically, I would like to thank Dr. Russell Hill and Dr. Paul Herman for the stimulating discussions and their wonderful suggestions. I would also like to extend my deep gratitude to Dr. David Bisaro for his guidance and support whenever I needed it.

This work was supported by grants from the National Science Foundation and National Institute of Health.

My gratefulness extends to all current and previous members of the Chamberlin lab. In addition to being wonderful and helpful colleagues, they have become my family away from my family. Dr. Xiaodong Wang, Dr. Vandana Rajakumar, Dr. Donha Park, Jennifer Zhang, Ryan Johnson, Kristin Armstrong, Hong Tao Jia, Xin Li, Jesse Sloan,
Jennifer Greenberg, and Stephen Sewell, I can’t thank you enough. In addition, I would also like to thank all the residents of the ninth floor in the Biological Sciences building throughout the years for making the floor a fun working place.

I have also been blessed to have a wonderful group of classmates and friends, who have laughed with me in the good days and who have been my shoulder to cry on in the tougher days. Dr. Nersine Affara, Nadine Bakkar, Dr. Danielle Dani, Dr. Ihab Younes, Dr. Hala Zahreddine, Mike Crowley, Zak Chehab, Mirna and Fadi Jaber, Mohammed Kabbout, Rami Doueri, and Mohammed Hotait, I am a better person for having known you.

I would like to acknowledge my family. I am grateful to my parents Fawwak and Randa Sleiman for making me believe that I can achieve anything I set my mind to. I am here today because of their sacrifices, unconditional support and love. My gratefulness also extends to my siblings, Kamal, Amir and Nehma Sleiman. Their smiles and encouragement have brightened my days.

Finally, I would like to extend my thanks and love to Joseph Stephan. He has been there for me in every step of the way. He has been a source of joy, encouragement and support and the force that has kept me going in the tougher times.
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FIELDS OF STUDY

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TABLE OF CONTENTS

Abstract ............................................................................................................................. ii

Dedication ....................................................................................................................... iv

Acknowledgments ........................................................................................................ v

Vita ................................................................................................................................ vii

List of Tables ................................................................................................................ xi

List of Figures ................................................................................................................. xii

Chapters:

1. Introduction ................................................................................................................ 1

2. Alteration of the DNA binding domain disrupts distinct functions of the C. elegans Pax protein EGL-38 ....................................................................................... 24
   Introduction ................................................................................................................... 24
   Materials and Methods ............................................................................................. 26
   Results ......................................................................................................................... 29
   Discussion ................................................................................................................... 31

3. A screen for factors that regulate lin-48::gfp expression in the hindgut identifies SPTF-3 and other possible modulators ............................................................... 36
   Introduction ................................................................................................................... 36
   Materials and Methods ............................................................................................. 38
   Results ......................................................................................................................... 51
   Discussion ................................................................................................................... 57

4. The C. elegans Sp-related transcription factor SPTF-3 functions in Wnt-mediated developmental decisions ............................................................... 72
## LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1</td>
<td>Ectopic expression of EGL-38 induces ectopic lin-48 expression</td>
</tr>
<tr>
<td>3.1</td>
<td>Genetic mapping of gu84</td>
</tr>
<tr>
<td>3.2</td>
<td>Genetic mapping of gu84</td>
</tr>
<tr>
<td>3.3</td>
<td>Genetic mapping of gu85</td>
</tr>
<tr>
<td>4.1</td>
<td>Expression pattern of sptrf-3::gfp in animals</td>
</tr>
<tr>
<td>4.2</td>
<td>sptrf-3(gu85) exhibit an ectopic lumen posterior to the main vulval lumen</td>
</tr>
<tr>
<td>4.3</td>
<td>sptrf-3(gu85) animals exhibiting an ectopic lumen have normal vulval induction</td>
</tr>
<tr>
<td>4.4</td>
<td>Reducing the dose of lin-17 by half enhances the bivulva phenotype</td>
</tr>
<tr>
<td>4.5</td>
<td>sptrf-3(gu85) mutants exhibit reversals in P7.p lineage</td>
</tr>
<tr>
<td>4.6</td>
<td>sptrf-3(gu85) mutants exhibit defects in asymmetric B cell division</td>
</tr>
<tr>
<td>4.7</td>
<td>sptrf-3(gu85) mutants exhibit defects in P12.pa development and reduced expression of egl-5::gfp in that cell</td>
</tr>
</tbody>
</table>
### LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>Protein sequence alignment of the Paired domain of Pax proteins from different species</td>
</tr>
<tr>
<td>1.2</td>
<td>lin-48 is a hindgut specific target of EGL-38</td>
</tr>
<tr>
<td>1.3</td>
<td>Wnt pathway in signaling animals</td>
</tr>
<tr>
<td>1.4</td>
<td>Canonical and non Canonical Wnt signaling in animals</td>
</tr>
<tr>
<td>1.5</td>
<td>The EGF, Delta/Notch and Wnt signaling pathways mediate vulval development</td>
</tr>
<tr>
<td>1.6</td>
<td>Wnt signaling controls P7.p orientation</td>
</tr>
<tr>
<td>1.7</td>
<td>Model of Wnt signaling in the male B blast cell</td>
</tr>
<tr>
<td>1.8</td>
<td>P11/P12 development</td>
</tr>
<tr>
<td>2.1</td>
<td>Ectopic expression of EGL-38 induces ectopic lin-48 expression</td>
</tr>
<tr>
<td>2.2</td>
<td>Ectopic expression of EGL-38 affects embryonic viability</td>
</tr>
<tr>
<td>3.1</td>
<td>Sequence alignment between lre1 of C. elegans and its closely related species C. brigssae</td>
</tr>
<tr>
<td>3.2</td>
<td>Genetic map of chromosome X</td>
</tr>
</tbody>
</table>
3.3 Genetic map of chromosome I.......................................................................................... 63
3.4 Ire1 mutational analysis .................................................................................................. 64
3.5 lin-48::gfp expression in animals.................................................................................... 65
3.6 Comparison of the expression of the different lin-48::gfp transgenes in wild-type and gu85 animals ............................................................................................................. 66
3.7 EMSA gel showing that the EGL-38 DBD can bind to the Ire1 area.............................. 67
3.8 EGL-38 and SPTF-3 coimmunoprecipitation................................................................... 68
4.1 Induced expression of SPTF-3::FLAG rescues defects observed in sptf-3(gu85) animals .......................................................... 101
4.2 sptf-3 allele isolated in a genetic screen........................................................................ 102
4.3 Wild-type SPTF-3 binds to a mammalian consensus site, whereas the mutant protein does not ......................................................................................................................... 103
4.4 sptf-3 expression in wild-type animals ......................................................................... 104
4.5 sptf-3(gu85) and sptf-3 RNAi animals exhibit an ectopic lumen posterior to the main vulval lumen.................................................................................................................. 105
4.6 Both P12.pa cell fate and B cell asymmetric cell division are affected in sptf-3(gu85) animals ......................................................................................................................... 106
4.7 Models for the mechanism by which SPTF-3 functions to affect Wnt-dependent processes......................................................................................................................... 107
CHAPTER 1

INTRODUCTION

Organogenesis is a process that requires specific cells within an organism to perfectly coordinate their development. It involves cell fate specification, signaling events as well as organized spatial distribution of cells. The similarities as well as the differences between cells within an organ can be rationalized by examining expression profiles especially in the context of genes that are necessary for specific organ formation. Because many human diseases and a variety of birth defects result from either the malformation or the malfunction of an organ, deciphering the genes and genetic networks necessary for organogenesis can provide insights into human disease.

In this study, I used *Caenorhabditis elegans* (C. elegans) as a model organism to study how a single Pax transcription factor mediates the development of different organs through regulating different target genes in different cells. I also used *C. elegans* as a model organism to study the interaction between a Sp1-related transcription factor and the Wnt signaling pathway in cell fate specification and organ development. In this Chapter, I will first introduce Pax transcription factors and their role in *C. elegans*
hindgut development. Next, I will discuss the Sp-related transcription factors, the Wnt pathway and the interaction between them to mediate organ and animal development.

1.1. Pax genes and development

*Pax* genes play an important role in the development of a range of different cell types, and in promoting cell proliferation and cell survival (reviewed by Chi and Epstein, 2002). *Pax* genes encode transcription factors that are identified based on the presence of a conserved DNA binding domain called the paired domain. In addition to the paired domain, some Pax genes also have other conserved protein domains, the homeodomain and octapeptide sequence. All Pax proteins share several features common to the paired domain. The paired domain can interact with DNA as a monomer across a relatively large stretch of DNA of more than 20 base pairs (bp), but it also has a modular structure of two helix-turn helix domains which can bind to DNA independent of each other (Xu *et al.*, 1995; Jun and Desplan, 1996; Kozmik *et al.*, 1997; Jun *et al.*, 1998; Xu *et al.*, 1999a). Based on sequence, structure and function of the different Pax factors, the mammalian family of Pax proteins is subdivided in to four classes. For example, the Pax2/5/8 class of proteins shares the paired DNA binding domain, the octapeptide sequence and a partial homeodomain (reviewed by Chi and Epstein, 2002).

Genetic studies have shown that each Pax protein acts in the development of several different cell and organ types (reviewed by Chi and Epstein, 2002). Different functions are mediated through the regulation of different target genes, suggesting that
cellular context plays an important role in Pax protein function, and that cell-specific factors may allow the same Pax protein to regulate different target genes in different cell types. Pax proteins may function in a combinatorial manner with other proteins that act through separate enhancers (e.g., Brophy et al., 2003). In addition, examples in which Pax proteins form complexes or bind DNA synergistically with other transcription factors have also been described (Fitzsimmons et al., 1996; Roberts et al., 2001; Jin et al., 2002; Miranda et al., 2002; Sigvardsson et al., 2002; Di Palma et al., 2003). While these studies suggest possible mechanisms for combinatorial control, dissecting how a given Pax factor participates in target gene expression in different cell types in vivo remains an important question.

1.1.2. Pax 2/5/8 genes in C. elegans

There are two Pax2/5/8 genes in C. elegans: pax-2 and egl-38. They encode proteins that share a high sequence similarity in their paired domains and the two genes are proposed to arise from a recent gene duplication event (Wang et al., 2004). Both egl-38 and pax-2 play a role in C. elegans germ-line apoptosis (Park et al., 2006). They promote cell survival by regulating the expression of the Bcl-2 homolog, ced-9 (Park et al., 2006). Other potential roles for pax-2 are not defined. On the other hand, genetic studies have shown that egl-38 functions in the development of different cells and organ types including the egg-laying system, the hindgut, and the development of male mating spicules (Chamberlin et al., 1997). egl-38 also affects the expression of different genes in the different cell types. For example, expression of the epidermal growth factor gene
lin-3 in the hermaphrodite vulval cells is dependent on egl-38, whereas the Ovo zinc finger transcription factor gene lin-48 is a target for EGL-38 in hindgut cells (Chang et al., 1999; Johnson et al., 2001). lin-48 is not expressed in the egg-laying system, indicating it is a target in one cell type, but not the other (Johnson et al., 2001). Although egl-38 is an essential gene, its different functions have been characterized using non-null, reduction-of-function alleles that allow homozygotes to be viable. These mutant alleles affect the in vivo function of egl-38 in different ways, and each allele corresponds to a missense mutation that alters an amino acid in the EGL-38 DNA binding domain (DBD; Chamberlin et al., 1997; Chamberlin et al., 1999; Zhang et al., 2005) (Figure 1.1). For example, the n578 allele preferentially affects the egg laying ability of hermaphrodites, whereas the sy294 and sy287 alleles preferentially affect male tail development. To understand the tissue specific functions of Pax factors, I used lin-48 as a model target gene to further our understanding of how Pax factors act in combination with other proteins to mediate tissue specific gene expression.

1.1.3. Relation between egl-38 and lin-48

lin-48 encodes a C2H2 zinc finger protein similar to the Drosophila ovo gene (Johnson et al., 2001). A transcriptional reporter, lin-48::gfp, is expressed in multiple tissues in the animal, including the head neurons, the excretory duct cell, the hindgut, and the phasmid (Johnson et al., 2001) (Figure 1.2). lin-48 is expressed and is important for the cell fate specification of the same subset of hindgut cells as one of the C. elegans Pax2/5/8 genes, egl-38 (Johnson et al., 2001). Specifically, lin-48 is a direct target of
EGL-38 in the hindgut, but not in other tissues, such as the egg-laying system (Johnson et al., 2001). Previously, two redundant cis regulatory elements important for lin-48 hindgut expression (lre1 and lre2) were shown to be sensitive to EGL-38 activity in vivo (Johnson et al., 2001). However, EGL-38 only binds to lre2 in vitro (Johnson et al., 2001). As a result, I hypothesized that EGL-38 mediates hindgut lin-48::gfp expression through the lre1 element by forming a complex with unidentified factors. To identify potential EGL-38 cofactors, I conducted a genetic screen (refer to chapter 3). This screen yielded a mutant allele in sptf-3, a Sp1 family member homolog in *C. elegans*,

1.2. **Sp1 family of transcription factors and animals development**

The mammalian Sp1 family of transcription factors is comprised of nine members (Sp1-Sp9) (reviewed by Suske, 1999; Bouwman et al., 2002; Kaczynski et al., 2003). These all have three highly conserved C2H2 zinc fingers that make up the DNA binding domain and that are located at the C-terminus of the protein (reviewed by Suske, 1999; Bouwman et al., 2002 Kaczynski et al., 2003). These factors bind to GC or GT boxes in the proximal promoter of genes, and they regulate the transcription of a wide array of target genes (Kadonaga et al., 1986; Kadonaga et al., 1988; Bucher, 1990; Mastrangelo et al. 1991, Su et al., 1991). The zinc finger domain also mediates protein-protein interactions, thus modulating DNA binding specificity. In addition, to the DNA binding domain, these factors all have a Buttonhead (Btd) box that may be required for transactivation (Courey and Tjian, 1988; Athanikar et al., 1997). The N-terminus of these proteins is more variable. Based on the domains present in the N-terminus, Sp1,
Sp2, Sp3 and Sp4 form a subgroup. These proteins either contain one or two transactivation domains (domains A and B) as well as an inhibitory domain (ID) in the case of Sp3 (reviewed by Safe and Abdelrahim, 2005). Sp5, Sp6, Sp7 and Sp8 form a second subgroup. These proteins appear to be truncated versions of Sp1-Sp4 (reviewed by Safe and Abdelrahim, 2005). In this dissertation, I will refer to all proteins in this class as Sp-related transcription factors.

Sp-related transcription factors function as activators or repressors depending on the promoter they bind to and on the cofactors with which they interact (Hagen et al., 1994; Dennig et al., 1995; Kwon et al., 1999). For example, Sp1 activates transcription by binding to DNA and interacting with general transcriptional machinery proteins, such as the TATA binding protein (TBP) and TATA binding protein associated factors (TAFs) or with other transcription factors, such as p107, YY1, E2F and p300 (Gill et al., 1992; Hoey et al., 1993; Seto et al., 1993; Emili et al., 1994; Chiang and Roeder, 1995; Datta et al., 1995; Lin et al., 1996; Billon et al., 1999; Doetzlhofer et al., 1999; Suzuki et al., 2000; Lee et al., 2002). Repression by Sp-related transcription factors occurs either through the competition of the repressors for the GC boxes or through recruitment of chromatin modifying enzymes such as HDAC (Birnbaum et al., 1995; Hagen et al. 1995; Majello et al. 1997; Won et al. 2002; Yu et al. 2003; Moorefield et al. 2004; Phan et al. 2004). Because of their diverse mechanisms of action, the Sp-related transcription factors play critical roles in animal development.

Sp-related transcription factors play important roles in vertebrate embryonic development (reviewed by Zhao and Meng, 2005). Murine Buttonhead and Drosophila Buttonhead, both Sp1 homologs, are required for murine limb development and for the
generation and specification of *Drosophila* head segments, respectively (Treichel *et al.*, 2003 and Wimmer *et al.*, 1993; Schock *et al.*, 1999; Estella *et al.*, 2003). Moreover, Sp-related transcription factors mediate the functions of signaling pathways to promote animal development and disease progression, including cancer (Reviewed by Safe and Abdelrahim, 2005). For example, zebrafish Sp5 mediates the anteroposterior patterning activity of FGF signaling in the neuroectoderm (Sun *et al.*, 2006). In addition, Sp5 is a direct target of the Wnt pathway and is upregulated in human colorectal carcinoma (Takahashi *et al.*, 2005). Even though there is evidence for an interaction between the Wnt pathway and Sp-related transcription factors in vertebrates, it is not clear if Sp-related transcription factors mediate all Wnt dependant decisions, promote the functions of all types of Wnt signaling pathways or only function in Wnt responsive cells. To explore how Sp-related transcription factors interact with the Wnt signaling pathway, we used *C. elegans* to study the role of and the interaction between the Wnt signaling pathway and a Sp-related transcription factors. In *C. elegans*, three genes have been annotated as Sp-related transcription factors: *sptf-1*, *sptf-2*, and *sptf-3*. However, prior to this work, none of these genes has been characterized for their role in animal development.

**1.3. Wnt signaling in animals**

In this dissertation I present evidence that the *C. elegans* Sp1-related protein SPTF-3 functions with the Wnt signaling pathway. The Wnts form a large family of growth factors that are grouped together based on amino acid sequence (reviewed by
Gordon and Nusse, 2006). Wnt signaling involves multiple different downstream signaling effectors. The most commonly studied type of Wnt signaling is the canonical β-catenin dependent Wnt signaling. However, there is an increased interest in the non-canonical or β-catenin independent Wnt signaling.

The central event that defines the canonical Wnt signaling is the nuclear translocation of β-catenin. In the absence of a Wnt signal, a β-catenin destruction complex comprised of Axin, adenomatous polyposis coli (APC) and glycogen synthase kinase 3 (GSK3) controls the cytoplasmic levels of β-catenin (reviewed by Gordon and Nusse, 2006). GSK3 phosphorylates β-catenin, which in turn leads to its ubiquitination and degradation by the proteosome. Upon binding of Wnt ligands to their receptors, a complex of Frizzled and LRP, the destruction complex is inhibited (Bhanot et al., 1996; Kroicher et al., 2001; Yoshikawa et al., 2003; Lu et al., 2004). This initiates an intracellular signaling cascade that leads to the stabilization of the β-catenin protein, its accumulation in the cytoplasm, followed by its nuclear translocation. In the nucleus, β-catenin interacts with TCF/LEF transcription factor to regulate target gene expression (Molenaar et al., 1996) (Figure 1.3).

Several non-canonical Wnt pathways have also been described. These pathways do not utilize β-catenin and differ in the signaling molecules downstream of the receptor. The most studied example of a non-canonical Wnt pathway is the planar cell polarity (PCP) pathway described in Drosophila melanogaster (Vinson et al., 1989 and reviewed in Strutt, 2003) (Figure 1.4). Recently, Wu and Herman (2006) reported a novel non-canonical Wnt pathway that is important for the asymmetric division of the C. elegans B blast cell. This pathway functions in a β-catenin, GSK3 and Axin
independent fashion and shares some effectors with the *Drosophila* PCP pathway, including LIN-17/frizzled, MIG-5/Dsh, RHO-1/RhoA and LET502/ROCK (Wu and Herman, 2006). However, this pathway was described as novel because unlike other non-canonical Wnt pathways, it utilizes the *C. elegans* TCF factor, POP-1 (Wu and Herman, 2006).

In addition to signaling via frizzled receptors, characteristic of the above described canonical and non-canonical Wnt signaling pathways, Wnts also utilize another class of receptors. This class includes atypical members of the receptor tyrosine kinase (RTK) family, such as Ror1, Ror2, and Ryk (Schmitt *et al.*, 2006). Wnt signaling through Ror2 was shown to antagonize canonical Wnt in mammals and the *C. elegans* vulva (Mikels and Nusse, 2006; Green *et al.*, 2007) and its signaling through Ryk mediates repulsion during axonal guidance in mammals and *Drosophila* (Yoshikawa *et al.*, 2003; Liu *et al.*, 2005)(Figure 1.4). In addition, Wnt signaling through the *C. elegans* Ryk homolog LIN-18 is important for polarization of the vulval lineage (Inoue *et al.*, 2004). In conclusion, the number and variety of the above described Wnt signaling pathways and effectors accounts for the wide range of Wnt functions during animal development.

The Wnt signaling pathway has been shown to play many important roles in animal development. For example, it is critical for early embryonic patterning, generation of cell polarity, cell fate specification and organ development (Logan and Nusse, 2004).
Hyper-activation of the Wnt pathway is associated with many diseases, such as human colon cancer and Wilms tumor (reviewed by Koesters et al., 1999; Nusse, 2005 and Nusse, 2007). Moreover, Wnt signaling plays an important role in stem cell and progenitor cell maintenance and renewal (Reya and Clevers, 2005).

1.3.1. Wnt and C. elegans development

In C. elegans, the Wnt signaling pathway controls many cell polarities, and asymmetric cell divisions. Some examples of Wnt-dependent processes that I have studied in my work include vulval mirror symmetry (Ferguson et al., 1997), P12.pa cell fate specification (Jiang and Sternberg, 1998) and B blast cell asymmetric cell division (Herman et al., 1995; Sawa et al., 1996). In the following sections, I will describe what is known about the role of the Wnt pathway in these developmental decisions.

1.3.2. Wnt signaling and vulval development

The C. elegans vulva is a mirror symmetrical opening through which eggs are extruded and males can mate. Vulval development is mediated by the EGF, Delta/Notch and Wnt signaling pathways. The vulva forms from ventral epidermal cells that develop in response to a gradient of the EGF signal, LIN-3, produced by the anchor cell (AC) in the gonad (Hill and Sternberg, 1992; Katz et al., 1995). The ventral epidermal cell closest to the AC (P6.p) is induced to form vulval tissue and adopts the primary cell fate. The two adjacent ventral epidermal cells (P5.p and P7.p) are also induced, but
adopt the secondary cell fate (Sulston and Horvitz, 1977; Sulston and White, 1980).

Delta/Notch signaling through LIN-12 mediates a lateral inhibition from the P6.p cell to promote secondary fates (Greenwald et al., 1983; Sternberg, 1988; Sternberg and Horvitz, 1989). Hyperactive EGF or Delta/Notch signaling can lead to additional cells being induced to form vulval tissue; as a result, animals exhibit a “Multivulva” phenotype (Ferguson and Horvitz, 1985 and Ferguson and Horvitz 1989). (FIGURE 1.5) This phenotype causes additional vulval lumens to form.

The Wnt pathway utilizes both LIN-17/Frizzled and LIN-18/Ryk receptors to mediate vulval mirror symmetry through its control of P7.p orientation. These receptors function in parallel to each other, but converge at the level of the TCF/LEF transcription factor, POP-1 (Inoue et al., 2004). Normally, the P5.p and P7.p descendents have identical cell lineages, but are organized in opposite and mirror symmetrical orientations along the anterior-posterior axis (Figure 1.6). To achieve the mirror symmetry in the vulva, the Wnt signaling pathway reorients the cell division pattern of the P7.p lineage and the fate of its progeny. Mutations in Wnt receptors, \textit{lin-17} and \textit{lin-18}, cause a reversal in the P7.p lineage, leading the descendents of the P7.p cell to adopt the P5.p orientation (Ferguson et al., 1987; Deshpande et al., 2005). This reversal in the P7.p lineage is morphologically observed as an ectopic lumen posterior to the main vulval lumen, a phenotype referred to as Bivulva (Biv).
1.3.3. Wnt signaling and asymmetric B cell division

The Wnt pathway also mediates asymmetric cell divisions in *C. elegans*. The first division of the B cell is an example of an asymmetric cell division that is dependent on the Wnt pathway. The B cell is a male-specific blast cell located in the hindgut. In hermaphrodites, this cell does not divide during postembryonic development. In contrast, in males, this cell undergoes several rounds of post-embryonic cell divisions, and its progeny differentiate to form the male copulatory spicules (Sulston, 1980). In late L1 males, the B cell divides asymmetrically to yield a larger anterior daughter cell (B.a) and smaller posterior cell (B.p). In *lin-17*/Frizzled mutants, the B cell polarity is lost (Sternberg and Horvitz, 1988). The cell divides symmetrically, and the daughters adopt similar B.a-like fates. In *lin-44*/Wnt mutants, the B cell polarity is reversed. A model that involves a novel Wnt pathway was proposed to explain the role of Wnt in asymmetric B cell division (Wu and Herman, 2006). The LIN-44 signal is expressed in the tail hypodermal cells that are located posterior to the B cell. The binding of the LIN-44 ligand to the LIN-17 receptor localized on the posterior membrane of the B cell leads to the formation of a LIN-17-MIG-5 complex. This complex is internalized and possibly relocated to the anterior cortex. This causes a redistribution of cytoplasmic determinants and cytoskeletal elements leading to a displacement of the mitotic spindle and thus to asymmetric cell division. In the absence of LIN-44, LIN-17 receptors, expressed in neighboring cells, such as the F cell, dimerize with LIN-17 receptors present on the anterior surface of the B cell. This activates signaling independent of LIN-44. In this case, it is possible that the presence of LIN-17 or the LIN-17/MIG-5
complex at the posterior also leads to redistribution of cytoplasmic determinants and cytoskeletal elements leading to a displacement of the spindle. This time, however, the polarity of the cell division occurs in the reverse orientation (Figure 1.7). In the absence of LIN-17, no signaling is activated and no asymmetric localization of determinants or cytoskeletal elements occurs. This leads to symmetric cell division (Wu and Herman, 2006).

1.3.4. Wnt signaling and P12 development

The P11 and P12 cells are the most posterior ventral epithelial cells in *C. elegans*. P11 and P12 are initially equivalent, but *lin-3/EGF* and *lin-44/Wnt* promote the P12 fate (Jiang and Sternberg, 1998) (Figure 1.8). These signals also promote the expression of the HOM-C gene, *egl-5* in the more posterior cell, which confers P12 fate on that cell (Jiang and Sternberg, 1998).

1.4. Sp-related transcription factors and Wnt signaling in animal development

There is evidence in vertebrates for an interaction between Sp-related transcription factors and the Wnt pathway. This interaction is critical for both normal animal development as well as disease progression. Several recent papers have reported that Sp5 is a direct target of the Wnt pathway. For example, Zebrafish Sp-5l and Sp5 function downstream of Wnt8, and are important for mesoderm and neuroectoderm patterning (Weidinger *et al.*, 2005). In addition, Sp5 levels are elevated in response to
overexpression of β-catenin in the mouse CNS. Moreover, Sp5 represses Sp1 targets in response to Wnt. (Fujimura et al., 2007). Human Sp5 is a direct target of Wnt signaling and is overexpressed in colorectal cancer and has been shown to deregulate negative inhibitors of the Wnt signaling pathway, such as p21WAF1 in these cells (Takahashi et al., 2005). These results in vertebrates show that the Wnt pathway mediates a wide variety of its functions in development and disease progression through transcriptional regulation of the Sp-related transcription factors. Moreover, comparative studies show that mammalian FZD7 and FZD5 both have highly conserved Sp1 binding sites in their upstream regulatory sequences (Katoh and Katoh, 2007a and Katoh and Katoh, 2007b). In addition, there is also evidence for the presence of an Sp1 binding site in the Wnt5a proximal promoter (Danielson et al., 1995) and human LEF-1 promoter (Hovanes et al., 2000). These studies raise the possibility that a Sp1-related transcription factor could be involved in regulation of Wnt pathway member expression. As a result, it remains unclear whether Sp-related transcription factors only function as targets of the Wnt pathway or whether they can also regulate the gene expression of Wnt pathway members. It also remains unclear whether any interaction between the Wnt signaling pathway and Sp-related transcription factors occurs in C. elegans.

1.5. Goals of this study

The main purpose of my dissertation is to understand how transcription factors and signaling pathways coordinate to mediate animal development. Specifically, I am interested in understanding how a single transcription factor, EGL-38, participates in
the transcription of different genes in different organs. I hypothesized that this is can be achieved through its recruitment of and interaction with different binding partners in different tissues. To understand this postulate, I conducted a genetic screen to identify cofactors that specifically function with EGL-38 in the hindgut. I was also interested in understanding how another transcription factor, SPTF-3, functions in animal development and in understanding its interaction with the Wnt signaling pathway to mediate the polarization of P7.p lineage, asymmetric cell division and cell fate specification.

The data presented in the following chapters will provide insight into the different roles of two families of transcription factors, the Pax and Sp1 families, in organ development. It will also provide the first report describing the roles of a Sp1-related factor in *C. elegans* development.
Figure 1.1: Protein sequence alignment of the Paired domain of Pax proteins from different species. The amino acid changes that result from the different egl-38 mutations are indicated. In the sy287 mutants, glycine 9 is mutated to a serine. In the gu22 mutants, methionine 29 is mutated to an isoleucine. In the sy294 mutants, glycine 33 is mutated to a valine. In n578 mutants, glycine 69 is mutated to a glutamic acid (Modified from Zhang et al., 2005)
Figure 1.2: *lin-48* is a hindgut specific target of EGL-38 (A) DIC micrograph of a wild-type animal. (B) *lin-48::gfp* expression in the animal in panel A. *lin-48::gfp* expression is observed in head cells, excretory duct, and hindgut cells. (C) DIC micrograph of an egl-38 mutant animal. (D) *lin-48::gfp* expression in the animal in panel D. *lin-48* expression is retained in the head cells and excretory duct cell, but is lost in the hindgut of egl-38 mutant animals (Modified from Zhang et al., 2005). The arrow indicates the position of the hindgut cells.
**Figure 1.3:** Wnt pathway signaling in animals. (A) In the absence of a Wnt ligand, the destruction complex composed of Axin, APC and GSK3 is functional. GSK3 phosphorylates β-catenin, which in turn leads to its ubiquitination and degradation by the proteosome. At the same time, TCF represses target gene transcription through its interaction with repressors such as Groucho. (B) Wnt ligands bind to a receptor complex composed of the Frizzled receptor and the LRP co-receptor. This in turn activates Dishevelled and inactivates the destruction complex. β-catenin is stabilized, accumulates in the cytoplasm and then translocates to the nucleus where it interacts with TCF to activate gene transcription (reviewed in Gordon and Nusse, 2006).
Figure 1.4: Canonical and non canonical Wnt signaling in animals. (A) In the canonical pathway, Wnt signaling through the Frizzled/LRP complex mediates nuclear translocation of β-catenin. In the nucleus, β-catenin interacts with TCF to activate gene expression. Wnt signaling through a different receptor of the receptor tyrosine kinase (RTK) family, the Ror2 receptor, inhibits this pathway. (B) In one non canonical Wnt pathway, Frizzled and Dishevelled mediate planar cell polarity in Drosophila or convergence extension in vertebrates through regulation of cytoskeletal elements. The signal for this process remains unknown. (C) Wnt signaling through another RTK receptor, Ryk, mediates repulsion during axonal guidance (reviewed in Gordon and Nusse, 2006).
**Figure 1.5:** The EGF, Delta/Notch and Wnt signaling pathways mediate vulval development. EGF is the inductive signal. Loss of function mutants in members of this pathway show a decreased number of induced cells and exhibit the vulvaless (no or absent vulva) phenotype. Gain of function mutants in members of this pathway show an increased number of induced cells and exhibit the multivulva phenotype. The Delta/Notch pathway mediates lateral inhibition from P6.p to its neighbors and contributes to secondary cell fate specification. Delta/Notch mutants are characterized by defects in secondary fate. The Wnt pathway mediates mirror symmetry in the vulva through its control of P7.p orientation. Mutations in the Wnt pathway cause reversal in the P7.p lineage which is observed by the presence of an ectopic vulval lumen posterior to the main vulva.
Figure 1.6: Wnt signaling controls P7.p orientation. (A) The Wnt pathway signals via both LIN-17/Frizzled and LIN-18/Ryk to mediate P7.p orientation. These pathways function in parallel to each other. The two pathways converge at the level of POP-1/TCF. (B) In wild-type animals, Wnts signal to P7.p and its progeny. This causes this lineage to adopt a mirror symmetrical orientation to the P5.p lineage orientation. In Wnt pathway receptor mutants, the P7.p adopts the default P5.p orientation. This defect is referred to as a reversal in the P7.p orientation (reviewed in Hie, 2005).
Figure 1.7: Model for Wnt signaling in the male B blast cell. (A) The LIN-44 signal, expressed in the tail hypodermis cells, binds to the LIN-17 receptor located on the posterior B cell membrane. This leads to its internalization along with MIG-5/Dsh. This complex may be displaced to the anterior cortex, which in turn leads to the redistribution of cytoplasmic determinants and cytoskeletal elements along with the mitotic spindle. As a result, the cell divides asymmetrically resulting in a large anterior daughter cell (B.a) and small posterior daughter cell (B.p). (B) In the absence of the Wnt signal, LIN-17 on the anterior surface of the B cell self-dimerizes with molecules present on other neighboring cells. This in turn leads to its internalization along with MIG-5/Dsh. This complex may be displaced to the posterior cortex. This leads to redistribution of cytoplasmic determinants and cytoskeletal elements along with the mitotic spindle. As a result, the cell divides asymmetrically resulting in a small anterior daughter cell and large posterior daughter cell (Wu and Herman, 2006).
Figure 1.8: P11/P12 development. (A) The P11 cell divides once to yield an anterior cell that develops into a neuroblast, and the posterior cell that adopts the P11.p fate. The P12 cell divides twice. After the first division, the anterior daughter cell develops into a neuroblast. The posterior cell divides to produce an anterior cell that adopts the P12.pa fate and a posterior cell that undergoes apoptosis. (B) The EGF and Wnt signaling pathways promote the P12.pa cell fate through the regulation of expression of the HOM-c gene, egl-5. The difference in the fates can be observed by following the lineage of these cells (Jiang and Sternberg, 1998).
CHAPTER 2

ALTERATION OF THE DNA BINDING DOMAIN DISRUPTS DISTINCT
FUNCTIONS OF THE C. ELEGANS PAX PROTEIN EGL-38

2.1. INTRODUCTION

This chapter has been reformatted and adapted from Zhang, G., Sleiman, S.F. et al., 2005 to discuss my contribution to the paper.

_Pax_ genes have been shown to play an important role in the development of a range of different cell types, and to promote cell proliferation and cell survival (reviewed by Chi and Epstein, 2002). _Pax_ genes encode transcription factors that are identified based on the presence of a conserved DNA binding domain called the paired domain. Although four main classes of Pax proteins have been identified, all Pax proteins share several features common to the paired domain. The paired domain can interact with DNA as a monomer across a relatively large stretch of DNA of more than 20 bp, but it also has a modular structure of two helix-turn helix domains which can bind to DNA independent of each other (Xu _et al._, 1995; Jun and Desplan, 1996; Kozmik _et al._, 1997; Jun _et al._, 1998; Xu _et al._, 1999). Many DNA sequences that bind Pax proteins have been identified. However, how Pax proteins interact with DNA _in_
vivo, and how they recognize appropriate targets from among all sequences that could act as PAX binding sites, remain open questions.

Genetic studies have shown that each Pax protein acts in the development of several different cell and organ types (reviewed by Chi and Epstein, 2002). Different functions are mediated through the regulation of different target genes, suggesting that cellular context plays an important role in Pax protein function. Thus other factors may allow the same Pax protein to regulate one set of target genes in one cell type, and a different set of targets in a different cell type. Pax proteins may function in a combinatorial manner with other proteins acting through separate enhancers (e.g., Brophy et al., 2003). In addition, examples in which Pax proteins form complexes or bind DNA synergistically with other transcription factors have also been described (Fitzsimmons et al., 1996; Roberts et al., 2001; Jin et al., 2002; Miranda et al., 2002; Sigvardsson et al., 2002; Di Palma et al., 2003). While these studies suggest possible mechanisms for combinatorial control, dissecting how a given Pax factor participates in target gene expression in different cell types in vivo remains an important question.

To better understand how a single Pax transcription factor can regulate different target genes in different cells, we have characterized the functions of the C. elegans Pax2/5/8 gene egl-38. Genetic studies have shown that egl-38 functions in the development of several different cells and organ types including the egg-laying system, the hindgut, and the development of male mating spicules (Chamberlin et al., 1997). egl-38 also affects the expression of different genes in the different cell types. For example, expression of the epidermal growth factor gene lin-3 in the hermaphrodite vulval cells is dependent on egl-38, whereas the Ovo zinc finger transcription factor
gene *lin-48* is a target for EGL-38 in hindgut cells (Chang et al., 1999; Johnson et al., 2001). *lin-48* is not expressed in the egg-laying system, indicating it is a target in one cell type, but not the other. Although *egl-38* is an essential gene, its different functions have been characterized using non-null, reduction-of-function alleles that allow homozygotes to be viable. These mutant alleles affect *egl-38 in vivo* function in different ways, and each allele corresponds to a missense mutation that alters an amino acid in the EGL-38 DNA binding domain (DBD; Chamberlin et al., 1997; Chamberlin et al., 1999; Zhang et al., 2005) (Figure 1.1). Consequently, to investigate the relationship between Pax proteins and their *in vivo* targets, we have tested the *in vivo* properties of these mutant EGL-38 variants.

### 2.2. MATERIALS AND METHODS

#### 2.2.1. *C. elegans* strains and transgenes:

*C. elegans* strains were cultured according to standard techniques (Sulston and Hodgkin, 1988). Mutations are described in the wormbase (http://www.wormbase.org/). Mutations used: *unc-119(e2498) III; egl-38(n578), egl-38(sy294), egl-38(sy287), egl-38(gu22), egl-38(s1775) IV; him-5(e1490) V*. Integrated transgene: *saIs14 (lin-48::gfp; Sewell et al., 2003)*. This transgene contains the pTJ1157 reporter gene with the upstream regulatory sequences from *lin-48* that include both identified EGL-38-responsive elements (lre1 and lre2; Fig. 3E).
In wild-type animals, these elements exhibit functional redundancy, but transgenes containing a single response element exhibit enhanced sensitivity in different egl-38 genetic backgrounds (Johnson et al., 2001). Thus, the most robust EGL-38 response is mediated through both elements.

2.2.2 Ectopic expression of EGL-38 proteins in worms

A cDNA coding for full-length wild-type EGL-38 protein was cloned downstream of the hsp16-41 promoter in the vector pPD49.83 (a gift from A. Fire). Sequences corresponding to the FLAG epitope were introduced in-frame at the 3’ end of the cDNA to allow detection of the expressed protein. This construct represents hsp::egl-38(+) and also served as the template to produce egl-38 mutant variants using the QuikChange mutagenesis method (Stratagene). Mutant clones were sequenced to confirm the clone and ensure that additional mutations were not incorporated during the mutagenesis process. Transgenes for the heat-inducible constructs were produced by microinjection of plasmid DNA into the mitotic germline of hermaphrodites according to the method of Mello et al. (1991). For all experiments, 40-50 ng/µl experimental clone was co-injected with 15 ng/µl pDP#MM016 (unc-119(+)) plasmid; Maduro and Pilgrim, 1995) into unc-119(e2498) animals. The resultant extrachromosomal transgenes were integrated into the chromosome by treating transgene-bearing animals with 3.0-3.5 kRad gamma irradiation, and selecting for stable transgene transmission in subsequent generations as described by Inoue et al. (2002).
Integrated transgenes were back-crossed several times, and tested for protein expression level following heat induction of the transgene using western blotting (Figure 2.1). Protein expression was visualized by subjecting mixed-stage animals to a 35°C heat-shock for 30 minutes. After 30 minutes recovery at room temperature, worms were washed twice in M9 buffer, and frozen in liquid nitrogen. Thawed pellets were sonicated in SDS-PAGE buffer, boiled for 5 minutes, and separated by SDS-PAGE. Proteins were transferred to membrane, blocked overnight with 5%(W/V) non-fat dried milk in PBS plus 0.1% tween, then washed 1x with PBS tween. Proteins were detected with anti-FLAG and anti-γ-tubulin antibodies (Sigma) diluted 1:100.

2.2.3 Functional assays of ectopically expressed EGL-38 proteins

The activity of ectopically expressed EGL-38 proteins was assessed by characterizing its impact on lin-48 transactivation and on embryonic viability. For lin-48 transactivation, 50 ng/µl of the lin-48::gfp clone pTJ1157 (Johnson et al., 2001) was co-injected with 180 ng/µl pRF4 (rol-6(d); Mello et al., 1991) into animals bearing the integrated hsp::egl-38 transgenes. Expression of lin-48::gfp in the expected pattern was confirmed in the resulting transgenic lines. Transgene-bearing hermaphrodites were allowed to lay eggs for five to twelve hours, and then removed from the plate. The eggs were subjected to a 35°C heat shock for 30 minutes, followed by incubation at 15°C for 10-11 hours, and analysis for expression of GFP. The data in Table 2.1 are all from embryos prior to elongation but with clear differentiation and alignment of intestinal cells.
For viability, transgene-bearing hermaphrodites were allowed to lay eggs for five hours, removed from the plate, and the eggs were counted. The eggs were then subjected to a 35°C heat-shock for 30 minutes. On the second day, the eggs and resulting larvae were subjected to a second heat-shock treatment. On the fourth day, the number of larvae at different developmental stages was counted, and the number of dead eggs was inferred by comparing resulting larva to number of eggs counted on the first day. The data in Figure 2.2 result from three independent trials. Each trial included 50 or more eggs for a given genotype. We have found that all embryos are sensitive to heat shock. However, heat treatment for 30 minutes at 35°C induces transgene expression yet permits significantly greater embryonic survival than other heat treatment protocols such as those used for larvae (32-33°C for 1-2 hours). Nevertheless, about half of control embryos do not survive heat-shock treatment.

2.3. RESULTS

2.3.1. Mutant alleles of egl-38 differentially affect its activity in vivo

Although each mutant allele disrupts certain functions of egl-38, they cannot be ordered into an allelic series, going from weakest to strongest allele (Zhang et al., 2005). Instead, the alleles exhibit cell or tissue preferential defects compared to each other. For example, in egl-38(gu22) and egl-38(sy287) mutants, tail functions including male tail development and lin-48 expression are disrupted, whereas a high level of function in the egg-laying system is retained (Zhang et al., 2005). In contrast, egl-
38(n578) mutants are defective in the egg-laying system, but retain a high level of activity in the tail. One possible interpretation of the loss-of-function genetics is that there are differences in tissue-specific stability, or subtle differences in protein activity for the different protein variants. To further investigate the differences among the mutants, we assessed the function of EGL-38 proteins when they ectopically expressed in animals under control of a heat-inducible promoter. We find that induction of wild-type EGL-38 results in ectopic expression of the target gene lin-48 and causes a stringent lethality effect in embryos (Figures 2.1, & 2.2). We tested the mutant proteins in these ectopic expression assays, and found that the different mutations alter EGL-38 activity to different extents (Table 2.1, Figures 2.1, & 2.2). For example, the protein corresponding to the egl-38(n578) allele (G69E; amino acid position indicates position within the DBD) retains a high level of activity in the lin-48::gfp transactivation assay, but is defective in the viability assay. In contrast, protein corresponding to the egl-38(sy294) allele (G33V) retains more activity in the viability assay than it does in the lin-48::gfp transactivation assay. We speculate that the lethality results from inappropriate expression of genes downstream of EGL-38. Although we do not know what gene or genes may be mediating the effect, the functional differences between the different mutant proteins in the two ectopic expression assays suggest it is not mediated through lin-48. Taken together, the loss- and gain-of-function genetic assays indicate that different mutations affecting the EGL-38 DNA binding domain can preferentially affect distinct functions of the protein.
2.4. DISCUSSION

2.4.1. Alterations to a Pax protein DNA binding domain can affect its function in a tissue-preferential manner

DNA binding affinity is a key component of transcription factor function. Results with EGL-38 indicate that mutations in the DNA binding domain of Pax proteins can alter their DNA binding and their in vivo activity (Zhang et al., 2005). Many mutations that affect the DNA binding domain of Pax proteins have been characterized previously (Tang et al., 1997; DeStefano et al., 1998; Vilain et al., 2001; Jumlongras et al., 2004). However, since we have characterized several alleles and functions of egl-38, we have been able to demonstrate that the alleles that affect the DBD of EGL-38 can preferentially affect its activity in certain functions, but not others. Our results with EGL-38 suggest one of two possible models to explain the tissue-preferential effects. One possibility is that the different mutant alleles affect the ability of EGL-38 to bind to certain sequences, but not others. This model predicts that either there is one or a few key targets in each cell type, or that there are specific response element sequences that are associated with all the targets for EGL-38 in a particular cell type. An alternative model is that, although the mutations affect DNA binding, their tissue preferential effects result from their impact on EGL-38 function in combination with other proteins that influence target sequence recognition in vivo. This model predicts that there would be cell-specific co-factors that function with EGL-38 and participate in identifying which sequences correspond to an appropriate DNA binding site or that enhance EGL-38 transactivation activity. It also suggests that the interaction
between the co-factor and EGL-38 would be mediated, or otherwise affected, by the EGL-38 DBD. Although we favor the second model, clearly distinguishing between the two will require identification of proteins that act with EGL-38 and a large collection of cell-specific EGL-38 target genes.
Figure 2.1: Ectopic expression of EGL-38 induces ectopic lin-48::gfp expression. A. Heat-treated embryos bearing a lin-48::gfp transgene exhibit a low level of GFP in the intestine. This low-level intestine expression is observed in all lin-48::gfp-bearing animals under normal culture conditions. B. Heat-treated embryos bearing both a hsp::egl-38(+) and a lin-48::gfp transgene show a high level of GFP, corresponding to ectopic expression of the lin-48::gfp transgene. The ectopic expression is commonly restricted to the posterior ectoderm (as in this embryo). This restricted ectopic expression may correspond to cells that contain the appropriate additional factors to promote lin-48 expression. C. Western blot of hsp::egl-38 transgene-bearing strains to demonstrate expression of proteins tagged with a FLAG epitope. Although the hsp::egl-38(sy387) strain exhibits lower protein levels than the others, this strain exhibits a high level of egl-38 activity in functional assays (Fig. 2.2 and Table 2.1).
Figure 2.2: Ectopic expression of EGL-38 affects embryonic viability. The graph shows the percentage of eggs that hatch following heat-shock treatment. The bars correspond to the average percentage of survivors from three independent trials, with brackets indicating the standard error of the mean. Induced ectopic expression of wild-type egl-38 results in embryonic lethality (hsp::egl-38(+)). This activity is disrupted to different extents by mutations that correspond to the different genetic alleles. Wild-type embryos (no transgene) exhibit some lethality which results from the heat-shock treatment.
Table 2.1: Ectopic expression of EGL-38 induces ectopic expression of \textit{lin-48}.

<table>
<thead>
<tr>
<th>Transgene</th>
<th>Ectopic lin-48::gfp (%)</th>
<th>( N )</th>
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</thead>
<tbody>
<tr>
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<td>16</td>
</tr>
<tr>
<td>\textit{hsp::egl-38(+)}</td>
<td>73</td>
<td>26</td>
</tr>
<tr>
<td>\textit{hsp::egl-38(sy287)}</td>
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<td>25</td>
</tr>
<tr>
<td>\textit{hsp::egl-38(sy294)}</td>
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<td>14</td>
</tr>
<tr>
<td>\textit{hsp::egl-38(n578)}</td>
<td>60</td>
<td>20</td>
</tr>
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CHAPTER 3

A SCREEN FOR FACTORS THAT REGULATE LIN-48::GFP EXPRESSION IN THE HINDGUT IDENTIFIES SPTF-3 AND OTHER POSSIBLE MODULATORS

3.1. INTRODUCTION

lin-48 encodes a C2H2 zinc finger protein similar to the Drosophila ovo gene (Johnson et al., 2001). A transcriptional reporter, lin-48::gfp, is expressed in multiple tissues in the animal, such as the head neurons, excretory duct cell, hindgut, and the phasmid (Johnson et al., 2001). lin-48 is expressed and is important for the cell fate specification of the same subset of hindgut cells as one of the C. elegans Pax2/5/8 genes, egl-38 (Johnson et al., 2001). Specifically, lin-48 is a direct target of EGL-38 in the hindgut, but not in other tissues, such as the egg-laying system (Johnson et al., 2001). Previously, two redundant cis regulatory elements important for lin-48 hindgut expression (Ire1 and Ire2) were shown to be sensitive to EGL-38 activity in vivo (Johnson et al., 2001). However, EGL-38 only binds to Ire2 in vitro (Johnson et al., 2001). Because of the in vivo sensitivity of both elements to egl-38 and the inability of
EGL-38 to bind to lre1 *in vivo*, we hypothesized that EGL-38 can form a complex with other unidentified factors to mediate hindgut *lin-48::gfp* expression through the lre1 element. This is of particular interest because although the DNA binding sites for Pax factors have been identified, how these factors identify their appropriate binding sites and how they regulate different genes in different cellular contexts remains poorly understood.

In this study, I used *lin-48* as a model target gene to further our understanding of how Pax factors act in combination with other proteins to mediate tissue specific gene expression. For this purpose, I first further delineated the lre1 element. I determined the minimum amount of sequences that were necessary for hindgut *lin-48::gfp* expression. Next, in order to identify factors that mediate hindgut *lin-48* expression through the lre1 element, I conducted a genetic screen. The screen utilized animals carrying a *lin-48::gfp* transgene with a mutant lre2 sequence. These animals express GFP normally since lre1 is intact. I screened for mutants that lack hindgut expression to identify genes that act with EGL-38 through the lre1 element. This screen yielded two separate genes, *sptf-3*, a Sp1-related transcription factor, and *lex-3* that are important for hindgut *lin-48::gfp* expression.
3.2. MATERIALS AND METHODS

3.2.1. Strains

Nematode strains were cultured according to standard techniques (Brenner, 1974; Sulston and Hodgkin, 1988). The following mutations were used:

Linkage group (LG) I: \textit{dpy-5(e61)}, \textit{unc-29(e1072)}, \textit{unc-13(e1091)}, \textit{hT2}

LGII: \textit{tra-2(q276)}

LGIII: \textit{unc-119(e2498)}

LGV: \textit{dpy-11(e224)}, \textit{him-5(e1490)}

LGX: \textit{lon-2(e678)}, \textit{unc-18(e81)}, \textit{dpy-6(e14)}, \textit{lin-2(e1309)}, \textit{unc-9(e101)}, \textit{yDp5, yDp6, yDp9}

The following transgenes were used: \textit{salIs14} (\textit{lin-48::gfp}; Johnson, et al., 2001), \textit{guIs9} (\textit{lin-48::gfp}), \textit{guIs29} (\textit{lin-48::gfp}) and \textit{guIs1} (\textit{nac-2::gfp}; Sewell et al., 2001).

3.2.2. Mutagenesis analysis

To further delineate the sequences necessary for \textit{lre1} activity \textit{in vivo} and to test whether they are necessary for hindgut \textit{lin-48::gfp} expression, I mutagenized the sequences flanking the previously defined \textit{lre1} element. These sequences were labeled
as the lre1a, lre1b, lre1c and lre1d elements (Figure 3.1). Point mutations in these elements were generated using a two-step, PCR-based method (Ausubel et al., 2000). The cloned PCR fragments were sequenced to verify that the clone is correct.

Briefly, complementary forward and reverse primers that mutated the target sequences were designed. Next, each of these primers was used in a separate PCR reaction with an outside primer. In this case, the plasmid PTJ1157 (lin-48::gfp) was used as template DNA. The products from these PCR reactions were themselves used as templates for a new PCR reaction that utilized the outside primers. Finally, the mutated and amplified final PCR product was introduced back into the vector backbones. These vectors either were wild-type lre2 (PTJ1157) to create constructs that were single mutants in one of the newly defined lre1 areas (lre1a, b, c or d), or were mutant lre2 (9.1C PTJ1157) to create constructs that were double mutant in one of the newly defined lre1 areas and lre2. Fifty ng/µl of these plasmids were co-injected with 15 ng/ul of *unc-119* marker DNA into *unc-119(e2498), him-5(e1490)* animals.

### 3.2.3. Genetic Screen to isolate mutations in factors that are important for hindgut lin-48::gfp expression

Mutations in factors important for hindgut *lin-48::gfp* expression were isolated using EMS mutagenesis. We used the CM753 {*unc-119(e2498); guIs9*} strain. The *guIs9* transgene contains a mutant lre2 sequence and a wild type lre1 sequence. Hindgut *lin-48::gfp* expression was thus mediated entirely through the lre1 element. We predicted that by screening for animals with reduced or abolished hindgut *lin-48::gfp* expression.
expression, we would be selecting for mutations in factors that directly or indirectly function through the lre1 element. Fourth larval stage (L4) P0 hermaphrodites were mutagenized using 50mM EMS for four hours (Sulston and Hodgkin, 1988), individually placed on agar plates at 20°C and allowed to grow for two generations. The F2 progeny were screened under a dissecting microscope for defects in hindgut $\text{lin-48::gfp}$ expression. Animals that exhibited reduced or abolished hindgut expression were individually picked into fresh plates and allowed to breed. From a screen of about 12,000 mutagenized gametes, four mutations (gu84, gu85, gu86, gu90) that affected hindgut $\text{lin-48::gfp}$ expression were recovered. Only the gu84 and gu85 mutations specifically affected hindgut expression, whereas both the gu86 and gu90 mutations reduced $\text{lin-48::gfp}$ expression in all the tissues where it is expressed. Because we wanted to isolate factors important for $\text{lin-48}$ hindgut expression, we restricted our analysis to gu84 and gu85.

3.2.4. Polymorphism and genetic mapping to Linkage Groups (LGs) and complementation tests.

All mutations were backcrossed twice to wild-type stocks and frozen. To determine whether gu84 and gu85 were mutations in the same gene, I conducted a complementation test. For that purpose, the guIs1 transgene (nac-2::gfp), which expresses in the excretory cell, was used as a marker for cross. Males carrying that transgene were crossed into gu85; guIs9 mutants. In turn, guIs1 carrying, gu85 and guIs9 heterozygote males were crossed in to the guIs9, gu84 strain. Cross progeny were
identified by the presence of the nac-2::gfp, picked and assessed for hindgut expression. I expected to observe equal number of wild-type and mutant animals if gu84 and gu85 failed to complement, whereas I expected to observe only wild-type animals if gu84 and gu85 complemented each other. The complementation test revealed that gu84 and gu85 complement each other and thus are mutations in different genes.

I used Single Nucleotide Polymorphism (SNP) mapping to determine the chromosome to which the gu84 and gu85 mutations were linked (Wicks et al., 2001). snip-SNPs are SNPs that alter a restriction enzyme site and can be detected following PCR, restriction digest, and electrophoresis. For that purpose, the confirmed polymorphisms between two closely related C. elegans isolates, Bristol(N2) and Hawaiian (HI or CB4856), were used (Wicks et al., 2001). First, we crossed the guIs9 (lin-48::gfp) transgene into the wild-type HI strain ten times. This strain was used a control HI strain and was tested for all polymorphisms utilized. To create recombinants for linkage analysis, wild-type (N2) males were mated with the guIs9, gu84 or gu85, guIs9 hermaphrodites. Cross males were next mated with hermaphrodites from the guIs9 (HI) strain. Cross hermaphrodites were transferred to fresh agar plates, where they were allowed to self cross. Mutant progeny were picked and allowed to self cross. Animals from each plate were harvested and frozen at -80°C in worm lysis buffer (50mM KCl, 10mM Tris-HCl pH8.3, 2.5mM MgCl2, 0.45% NP-40, 0.45% Tween 20, 0.01% gelatine and freshly added 60µg/ml proteinase K). The tubes were thawed on ice and lysed by incubation for one hour at 60°C and fifteen minutes at 95°C. PCR reactions were set up using primers that amplify three different polymorphisms located at the right, center and left arms of all six linkage groups (Wicks et al., 2001). The PCR
products were digested with the enzymes that allow for observation of the SNP for two hours and the digest was electrophoresed on 2% (W/V) agarose gels to determine whether the DNA is of HI or N2 origin. This analysis showed that \textit{gu84} is linked to LGX and that \textit{gu85} is either linked to LGI or LGX.

To determine whether \textit{gu85} is X-linked, I mated wild-type males (N2) with the \textit{gu85; guIs9} strain. The cross males were mated with \textit{gu85, guIs9, dpy-11} hermaphrodites. If \textit{gu85} is X-linked, then I expected the progeny of this cross to all be \textit{gu85}, whereas if \textit{gu85} is autosomally-linked, then I expected an equal number of wild-type and \textit{gu85} cross progeny. This set of crosses showed that \textit{gu85} is not linked to LGX.

### 3.2.5. Fine mapping of \textit{gu84} on LGX

Three factor crosses, SNP mapping, and rescue by duplications were used to further map the \textit{gu84} mutation on LGX.

#### 3.2.5.1. Three factor mapping of \textit{gu84} on LGX

I performed several three factor crosses to further map \textit{gu84} on LGX. First \textit{gu84} was mapped to the right arm of LGX by using the EM122 strain \{\textit{stDp2(X;II)/+II; him-5(e1490)V; unc-18(e81), dpy-6(e14) X}\}. The genetic positions of \textit{unc-18} and \textit{dpy-6} on LGX are -1.39 and 0.00, respectively. Males from the EM122 strain were mated with \textit{guIs9, gu84} hermaphrodites. From the heterozygous F1 cross animals, Unc non-Dpy
and Dpy non-Unc recombinants were picked and scored for the \textit{gu84} mutant phenotype. All the Unc non-Dpy recombinants picked the \textit{gu84} phenotype, while none of the Dpy non-Unc recombinants did. This result indicated that \textit{gu84} lies is very close to, or to the right of the \textit{dpy-6} locus.

To further map \textit{gu84} on the right arm of LGX, I performed a second three factor cross that utilized the SP928 strain \{\textit{dpy-6(e14), unc-9(e101)}\} (Figure 3.2). The genetic position of \textit{unc-9} on LGX is 10.25. Since both males generated from \textit{guIs9; gu84 or dpy-6(e14), unc-9(e101)} do not mate, we used \textit{tra-2(q276)} males. In \textit{tra-2(q276)} mutants, XX females are transformed into fertile males. \textit{tra-2} males were mated with \textit{dpy-6(e14), unc-9(e101)} hermaphrodites. The cross hermaphrodites (Non Dpy, Non-Unc) were allowed to self-cross. \textit{tra-2} males which have two X chromosomes and which were heterozygote for \textit{dpy6(e14) and unc-9(e101)} were crossed into \textit{guIs9, gu84}. From the heterozygote F1 hermaphrodites, Dpy non-Unc and Unc non-Dpy recombinants were picked and scored for the \textit{gu84} mutant phenotype. Since both types of recombinants picked the \textit{gu84} mutation, I concluded that \textit{gu84} lies between the \textit{dpy-6} and \textit{unc-9} loci.

Finally, in an attempt to further map \textit{gu84} on LGX, I performed a third three factor cross. In this cross, I used the \textit{lin-2} locus (Figure 3.2), whose genetic position on LGX is 7.04. The \textit{lin-2(e1309)} hermaphrodites are vulvaless and exhibit an egg-laying defective (Egl) phenotype. \textit{lin-2(e1309)} males that are heterozygous for the \textit{guIs9} transgene were mated with \textit{guIs9; gu84, unc-9(e101)} hermaphrodites. From the
heterozygous F1 hermaphrodites, gu84 non-Unc and Unc non-gu84 recombinants were picked and scored for their egg-laying ability. The data from this cross indicated that the gu84 mutation is to the left of both lin-2 and unc-9 and that gu84 is closely linked to lin-2.

3.2.5.2. snip-SNP mapping of gu84 on LGX

To map gu84 on LGX, several snip-SNP were used as markers. These snip-SNP included pkP6129, uCE6-1068, snp_F41D9[1], pkP6158, pkP6114, pkP6130 and R03E1(Figure 3.2). To create recombinants for snip-SNP analysis, I mated HI males with guIS9(HI) hermaphrodites. Cross males were then mated with guIS9, dpy-6, gu84 hermaphrodites. Dpy non-gu84 and gu84 non-Dpy recombinants were picked from the progeny of the cross F1 hermaphrodites. The same crossing steps were used to obtain unc-18 non-gu84, gu84 non-unc-18, gu84 non-unc-9 and unc-9 non-gu84 recombinants. The recombinant chromosomes were homozygosed, and then the recombinant animals were harvested and analyzed for snip-SNPs as described in section 3.2.4. Data from this analysis revealed that gu84 is to right of pkP6114 and to the left of R03E1. The gu84 mutation could not be separated from the pkP6130 snip-SNP.
3.2.5.3. Duplication rescue as a means of mapping gu84 on LGX

To map gu84 on the right arm of LGX, rescue by three duplications (yDp5, yDp6 and yDp9) was tested (Figure 3.2). All three duplications rescued unc-9(e101) but not lon-2(e678). The following strains were used: TY1910 {yDp5(X;A); lon-2(e678), unc-9(e101)X}, TY1911 {yDp6(X;A); lon-2(e678), unc-9(e101)X} and TY1914 {yDp9(X;A); lon-2(e678), unc-9(e101)X}. To test for rescue, I designed a series of crosses. First, guIs9 heterozygous males were mated with hermaphrodites from the above mentioned strains. Cross males were identified as having guIs9 and being Lon. These males were mated with guIs9, gu84, unc-9(e101) hermaphrodites. From cross hermaphrodites (non-Unc non- gu84), non unc and non Lon and non Lon segregating progeny were picked. If the duplications rescue the gu84 mutant phenotype, then all of the non-Unc progeny should be wild-type and all of the Unc progeny are predicted to be mutant. If the duplications do not rescue, then both non-Unc and Unc are predicted to be mutant.
3.2.6. Fine mapping of gu85 on LGI

Three factor crosses, and snip-SNP mapping were used to further map the gu85 mutation on LGI.

3.2.6.1. Three factor mapping of gu85 on LGI

I performed a three factor cross to further map gu85 on LGI. guIs9 heterozygote males were crossed into dpy-5(e61), unc-29(e1072) hermaphrodites obtained from the TB1 strain {ceh-6(mg60)/dpy-5(e61), unc-29(e1072)} (Figure 3.3). Males from this cross (dpy-5(e61), unc-29(e1072)/+, +; guIs9/+) were mated into the gu85; guIs9 animals. The cross hermaphrodites were identified as non-gu85 (wild-type hindgut lin-48::gfp expression) and allowed to self-cross. Plates that segregated the dpy-5(e61), unc-29(e1072) genotype were selected and Dpy non-Unc and Unc non Dpy recombinants were picked and screened for the presence of gu85.

3.2.6.2. snip-SNP mapping of gu85 on LGI

To map gu85 on LGI, several snip-SNP were used as markers. These snip-SNP included K04F10, T23G11, K02B12, PKP1121, PKP1123, CE1-187, PKP1074, pkP1066, dbP1, pkP1073, and pkP1071 (Figure 3.3). To create recombinants for snip-SNP analysis, I mated HI males with guIs9(HI) hermaphrodites. Cross males were then mated with guIs9, dpy-5(e61), gu85 hermaphrodites. Dpy non-gu85 and gu85 non-Dpy
recombinants were picked from the progeny of the cross F1 hermaphrodites. The recombinant chromosomes were homozygosed, and then the recombinant animals were harvested and analyzed for snip-SNPs as described in section 3.2.4. Data from this analysis revealed that gu85 is to right of pkP1073 and to the left of pkP1071. Screening of the RNAi phenotypes in WormBase.org provided Y40B1A.4 or sptf-3 as a candidate gene (Chapter 4 of this document will provide evidence that indeed gu85 is a mutation in the above-mentioned gene).

3.2.7. The effect of the gu85 mutation on different lin-48::gfp transgenes’ hindgut expression

We introduced the other lin-48::gfp transgenes into the sptf-3(gu85) mutant background. These transgenes included salIs14, which has both wild-type lre1 and lre2 elements, and guIs29, which has the mutant lre1 and a wild type lre2. To create the lines salIs14; him-5 males were crossed into sptf-3(gu85) hermaphrodites. Heterozygote cross hermaphrodites were identified by the presence of lin-48::gfp expression. These animals were allowed to self cross and the F2 from the self cross were picked. gu85 animals were identified according to their growth patterns and phenotypes. The crosses were performed in a similar fashion for the guIs29 transgene.
3.2.8. Determine whether SPTF-3 interacts with EGL-38 in vivo and in vitro through \textit{lre1}

3.2.8.1. \textit{In vitro gel shift assays}

Production of recombinant EGL-38 DNA binding domain (EGL-38 DBD) or SPTF-3 full length protein in \textit{E. coli} was carried out as described previously (Johnson \textit{et al.}, 2001). Briefly, cDNAs coding for amino acids 29-156 of EGL-38 or the entire cDNA coding for SPTF-3 were amplified using RT-PCR and cloned into vector pET-32 (Novagen). Recombinant EGL-38 DBD and SPTF-3 were expressed in \textit{E. coli} strain BL21 (DE3) (Novagen). Cells were lysed with sonication in buffer Z (Fitzsimmons \textit{et al.}, 2001) in the case of EGL-38 DBD or Sp1 gel shift buffer [5 \(\mu\text{M ZnSO}_4\), 50 mM KCl, 2 mM DTT, 12 mM HEPES-KOH (pH 7.5), 6 mM MgCl\(_2\), 0.1% NP-40 and 50% (V/V) glycerol] in the case of SPTF-3. Recombinant protein expression was confirmed with SDS-PAGE, and expressed protein amount was estimated by comparing dilutions to known quantities of BSA. For the experiments, lysates were utilized that included similar levels of expressed recombinant protein. Cell lysates, rather than purified protein, were used for DNA binding assays since previous experiments have found that purification of Pax proteins significantly reduces their activity (Fitzsimmons \textit{et al.}, 2001). In all cases, lysates derived from cells expressing the pET-32 vector with no cDNA insert was used as a negative control. The oligomers used for this experiment were obtained by PCR amplification (PR 75 and 1392) of approximately 100 bp including the \textit{lre1} element followed by purification from a 12% polyacrylamide gel and
ethanol precipitation. The oligomers (200ng) were then end labeled with DIG-11-ddUTP (Roche). The EGL-38 DBD and the SPTF-3 proteins were separately and combinatorially incubated with the Ire1 area oligomers for thirty minutes. The reactions were run on a 15% polyacrylamide gel in a Tris-Glycine-EDTA (TGE) running buffer (10X buffer: 0.25M Tris-Base, 1.9M Glycine, 10mM EDTA, pH=8.3) for 6-7 hours at 100 volts. The gel was transferred in 1X TGE buffer overnight. The nucleic acids on the membrane were fixed by cross-linking with UV, then washed with washing buffer (0.1M Maleic Acid, 0.15M NaCl; pH 7.5; 0.3% v/v Tween 20) for 2 minutes, blocked for 30 minutes with 1X blocking solution (Roche), incubated for 30 minutes with the 1:10000 Anti-Digoxigenin AP antibody, washed twice for 15 minutes, and finally detected in CSPD (Roche).

3.2.8.2. EGL-38 and SPTF-3 coimmunoprecipitation experiments

cDNAs coding for full-length SPTF-3, EGL-38, and GFP were amplified from RNA isolated from wild-type worms and cloned downstream of the hsp16–41 promoter in the vector pPD49.83 (a gift from A. Fire). Sequences corresponding to the cMyc were introduced in-frame at the 3′ end of the sptf-3,  and gfp cDNAs to allow detection of the expressed proteins by the anti-Myc antibody. Sequences corresponding to the FLAG epitope were introduced in-frame at the 3′ end of the egl-38 cDNA (Zhang et al. 2005) to allow detection of the expressed proteins by the Anti-FLAG antibodies. Transgenes for the heat-inducible constructs were produced by co- injection of 50 ng/µl of the abovementioned plasmid DNAs with 15 ng/µl pDP#MM016 (unc-119(+)) plasmid; Maduro and Pilgrim, 1995) into unc-119(e2498) animals.
Protein expression and coimmunoprecipitations studies were performed as described by Wang et al., 2006. Protein expression was induced by subjecting mixed-stage animals to a 35°C heat-shock for 30 min. After 30-min recovery at room temperature, worms were harvested and washed with M9 twice and then equilibrated with 1 ml lysis buffer (50 mM HEPES–NaOH, pH 7.6; 1 mM EDTA; 140 mM NaCl; 0.5% NP-40; 10% glycerol; 5 mM DTT; protease inhibitor cocktail (Calbiochem). Worms were then centrifuged, and the supernatant was discarded. Following addition of another 500 µl lysis buffer with fresh protease inhibitor cocktail, lysates were transferred to −80°C freezer overnight. Worm pellets were thawed and sonicated eight times, 10 s each. Following sonication, worm lysates were centrifuged, and the supernatant was transferred to a fresh tube. 500 µl of supernatant was used for the immunoprecipitation, and 10 µl was reserved for the input control. Supernatant was pre-cleared by equilibrated anti-Flag M2 agarose affinity gel beads (Sigma), and then incubated with equilibrated anti-Flag beads on a rotator for 2 h at 4°C. The beads were washed twice with the same lysis buffer as above and then boiled with 2X SDS buffer for 5 min. 16 µl of each sample was separated by SDS-PAGE. Proteins were transferred to a nitrocellulose membrane (Bio-Rad), blocked overnight with 5% non-fat dried milk in PBS plus 0.1% Tween, then washed twice with PBS Tween. Proteins were detected with anti-FLAG and anti-cMyc antibodies (Sigma and Santa Cruz Biotechnology) diluted 1:100 and 1:700 respectively.
3.3. RESULTS

3.3.1. Mutagenesis analysis defines 33 bps as being necessary for lre1 activity in vivo

To define the sequences necessary for lre1 activity in vivo, I scored animals lines that carried lin-48::gfp transgenes, which are either only mutated in only one of the lre1 elements (a, b, c or d) or are mutated in both lre1 elements and lre2. I observed that a single mutation in any of these elements did not have any effect on hindgut lin-48::gfp expression (Figure 3.4). Similarly, mutations in either lre1a or lre1d in a lre2 mutant background showed wild-type hindgut expression. Conversely, mutations in lre1b, lre1 and lre1c in a lre2 mutant background abolished hindgut lin-48::gfp expression. This result indicated that a 33 bp sequence is necessary for lin-48::gfp hindgut expression and functions redundantly with the lre2 element in vivo (Figure 3.4). In addition, this result suggests that a protein complex that includes EGL-38 mediates hindgut lin-48::gfp expression since Pax factors are known to bind to 20 bp (Xu et al., 1995). An alternative interpretation is that EGL-38 does not act through the lre1 element, but that some other protein does.

3.3.2. Genetic screen identifies two different factors that affect lin-48::gfp hindgut expression

From a genetic screen, I isolated four mutations, gu84, gu85, gu86 and gu90 that affected guIs9 (lin-48::gfp) hindgut expression. The genetic screen was designed to
identify factors that directly or indirectly mediate hindgut \textit{lin-48::gfp} expression through the lre1 element as the \textit{guIs9} transgene had a wild type lre1 element and a mutant lre2 element. The lre1 and lre2 elements are necessary and redundant for hindgut \textit{lin-48::gfp} expression (Johnson \textit{et al}., 2001). Mutations affecting one of these elements do not affect hindgut expression, whereas mutations affecting both of them abolish hindgut expression. We reasoned that in a transgene that has a wild-type lre1 element and a mutant lre2 element (\textit{guIs9}), all hindgut expression is mediated through the lre1 element. Screening for reduced or abolished hindgut expression in this case will potentially identify factors that function either directly or indirectly through the lre1 element (Figure 3.5).

From the mutations isolated in the screen, only the \textit{gu84} and \textit{gu85} mutations specifically reduced hindgut expression, while maintaining a wild-type pattern and level of expression in the other \textit{lin-48::gfp} expressing tissues, such as the head cells, excretory duct cell, and the phasmid neuron (Figure 3.5). The \textit{gu86} and \textit{gu90} mutations reduced \textit{lin-48::gfp} expression in all expressing tissues (Data not shown). Because I was interested in identifying factors that specifically affect hindgut \textit{lin-48::gfp} expression, I restricted my analysis to the \textit{gu84} and \textit{gu85} mutations. We named the gene containing the \textit{gu84} mutation, \textit{lex-3}. \textit{gu85} turned out to be a mutation in the \textit{sptf-3} gene (refer to chapter 4).

Since both \textit{gu84} and \textit{gu85} shared the same phenotype, I first tested whether they are alleles of the same gene by a complementation test. By scoring hindgut \textit{lin-48::gfp} expression of the cross animals (refer to materials and methods section for the genotype of these animals), I expected to observe an equal number of wild-type and
mutant animals if gu84 and gu85 failed to complement each other. On the other hand, if gu84 and gu85 complemented each other, I expected to score only wild-type animals. I only observed wild-type animals in this case. This revealed that gu84 and gu85 complemented each other and thus are mutations in separate genes. snip-SNP analysis, using three separate polymorphisms located on the right, center and left arms of the six chromosomes, placed gu84 on chromosome X and gu85 on either chromosome I or X (Refer to section 3.2.4) .

To determine whether gu85 is autosomally or X-linked, I designed a series of crosses (material and method section). If gu85 is X-linked, then I expected the progeny of this cross to all be gu85, whereas if gu85 is autosomally-linked, then I expected an equal number of wild-type and gu85 cross progeny. I observed the latter scenario and thus confirmed that gu85 is not X-linked, but rather linked to chromosome I.

3.3.3. Mapping of the gu84 mutation

Three factor crosses enabled us to further map the lex-3(gu84) locus on Chromosome X. unc-28 and dpy-6 mapping placed the gu84 mutation either very close to or to the right of the dpy-6 locus (Table 3.1). dpy-6 and unc-9 mapping placed the gu84 mutation in between the two loci with a predicted genetic position of 3.4 (Table 3.1). Finally unc-9 and lin-2 mapping placed gu84 very close to and to the left of the lin-2 locus at a predicted genetic position of 6.6 (Table 3.1).
Duplication rescue experiments solved the discrepancy between the predicted genetic position obtained from the *dpy-6, unc-9* and the *lin-2, unc-9* mapping. The two yDp5 and yDp9 duplications whose left breakpoints are 5.6 and 2.3 respectively rescued the *lex-3*(*gu84*) phenotype whereas the yDp6 whose left breakpoint is 8.6 did not (Table 3.1 and Figure 3.2). The duplication data alone placed the *gu84* mutation to a genetic interval between 5.6 and 8.6. This suggested that the predicted position from the *lin-2, unc-9* mapping is probably more accurate than that of the *dpy-6, unc-9* mapping.

Finally snip-SNP mapping revealed that that *gu84* is to the right of pkP6114 at a predicted position of 5.36. In addition, in a total of fifty eight different recombinants, the *gu84* mutation could not be separated from the pkP6130 polymorphism located at position 7.01 (Table 3.2).

Taken together the above data place *gu84* in an area extending from genetic position 5.6 to 7.07. In addition, *gu84* is tightly linked to the pkP6130 polymorphism located at position 7.01.

### 3.3.4. Mapping of the *gu85* mutation

Three factor mapping using *dpy-5* and *unc-29* placed the *gu85* mutation to the right of the *dpy-5* locus (Table 3.3). Snip-SNP mapping was then used to further map *gu85* on the right arm of chromosome I. Multiple snip-SNPs were used and this analysis revealed that *gu85* is to the right of pkP1173 and to the left of pkP1071, placing *gu85* in the genetic interval extending from 17.1 to 23.4. By scanning the area for candidate genes, we observed that the RNAi phenotype of one particular gene *Y40B1A.4* or *sptf-3*
matches phenotypes observed in the gu85 mutants. For example, both sptf-3 RNAi and gu85 animals show vulval defects and lethality (embryonic and larval). Further analysis revealed that gu85 is indeed a mutation in the above-mentioned gene (refer to chapter 4).

3.3.5. SPTF-3 specifically functions through the lre1 element to mediate lin-48::gfp hindgut expression

The genetic screen in which we isolated the gu85 mutation was designed to obtain factors that acted through the lre1 element to mediate hindgut lin-48::gfp. To determine whether SPTF-3 specifically mediates hindgut lin-48::gfp expression through the lre1 element, we tested its effect on different lin-48::gfp transgenes, one with both wild-type lre1 and lre2 elements (saIs14) and one with a mutant lre1 sequence and wild-type lre2 sequence (guIs29). We predicted that if SPTF-3 acts only through the lre1 element to mediate hindgut expression, then having a transgene with a wild-type copy of the lre2 element will be sufficient for hindgut lin-48::gfp expression in a sptf-3(gu85) mutant background. Unlike the case of the guIs9 transgene, the gu85 mutation does not have the same effect on the hindgut expression of these two transgenes (Figure 3.6).

This argues that SPTF-3 mediates hindgut lin-48::gfp expression either directly or indirectly through the lre1 element. For example, SPTF-3 could directly bind to the lre1 element either alone or as part of a complex with EGL-38. Alternatively, SPTF-3 could indirectly function through the lre1 element by regulating the expression of a factor that can bind to this element alone or as part of a complex with EGL-38.
3.3.6. SPTF-3 does not form a complex with EGL-38 DBD on the lre1

To test the hypothesis that SPTF-3 can bind to the lre1 region directly either alone or as part of a complex with EGL-38, I performed a gel shift experiment (Figure 3.7). The results of this experiment revealed that the EGL-38 DBD can bind to the lre1 containing sequences. This was in contrast to previous reports that showed that the EGL-38 DBD binds to lre2, but not the lre1 sequences (Johnson et al., 2001). However, in this case the DNA oligo used contained additional sequences than were shown to be necessary for lre1 activity in vivo. SPTF-3, on the other hand, did not bind to lre1 sequences either alone or as part of a complex with the EGL-38 DBD (Figure 3.7). The interpretation of this result is complicated because we did not use the full length EGL-38 protein for this experiment. A possibility remains that the portions of the EGL-38 protein that are important for interaction with SPTF-3 are outside of the DBD. In addition, there could be other yet unidentified factors that are necessary for complex formation. An alternative explanation is that SPTF-3 mediates lin-48::gfp hindgut expression through the lre1 element in an indirect manner, for example by regulating the expression of factors that can bind to these sequences.

3.3.7. SPTF-3 and EGL-38 can not interact in vivo

To determine whether SPTF-3 and EGL-38 can interact in vivo, we ectopically expressed a myc-tagged SPTF-3 and flag-tagged EGL-38 under the control of the heat shock promoter and used anti-flag antibody to immunoprecipitate EGL-38 (Figure 3.8).
We detected a weak interaction between EGL-38 and SPTF-3. In this case as well, the lack of overexpression of other unidentified factors important and limiting for the interaction between these two proteins could complicate the interpretation of the results. Alternatively, the weak interaction detected could be an artifact from the immunoprecipitations and no interaction between these proteins occurs in vivo.

3.4. DISCUSSION

In this chapter, I have described my attempts to identify proteins that function with one of the C. elegans Pax 2/5/8 factors, EGL-38. My main interest was to try to understand how Pax factors can have different targets in different cell types and to understand how they mediate their tissue specific functions. I hypothesized that the tissue specific function and gene target regulation of EGL-38 is achieved through its cooperation with different transcription factors. To identify these factors, I conducted a genetic screen. This screen was designed to identify factors that would potentially interact with EGL-38 at a particular DNA element, lre1, in a hindgut specific target gene, lin-48. This screen identified two separate genes, lex-3 and sptf-3. The molecular identity of the lex-3 gene remains unknown. SPTF-3 is a C. elegans Sp1-related factor. It is proposed to function as a transcription factor. Contrary to our expectations, we could not detect an interaction between SPTF-3 and EGL-38 in vitro nor in vivo.
3.4.1. The lre1 element comprises of 33bp necessary for lin-48 hindgut expression

Mutagenesis analysis of the sequences flanking the previously defined lre1 element (Johnson et al., 2001) revealed that a 33 bp sequence is required for hindgut lin-48::gfp expression. Since it was previously shown that the lre1 element is sensitive to egl-38 activity in vivo (Johnson et al., 2001) and that Pax factors can only bind to 20 bp (Xu et al., 1995), we hypothesized that EGL-38 functions through the lre1 element in a protein complex with yet unidentified factors. This was also supported by the inability of the EGL-38 DBD to bind to the lre1 element in an EMSA experiment, whereas that same DBD was capable of robustly binding to the lre2 element (Johnson et al., 2001). To identify members of the proposed complex, we conducted a genetic screen.

3.4.2. Genetic screen to identify binding partners for EGL-38 yields two genes, lex-3 and sptf-3

We designed a genetic screen that would allow for the identification of potential binding partners for EGL-38 at the lre1 element in the lin-48 gene. This screen utilized a specific lin-48::gfp transgene called guIs9. This transgene contains wild-type lre1 sequences and mutant lre2 sequences; as a result, the entire hindgut lin-48::gfp expression should be mediated through the lre1 element in animals carrying the guIs9 transgene. By identifying mutant animals that had reduced hindgut expression, we predicted that we would be identifying mutations in factors that could interact with
EGL-38 at the lre1 element. Other potential categories of genes that could result from such a screen include transcription factors that regulate the expression of the potential EGL-38 binding partners, as well as proteins that play a general role in transcription, such as chromatin modifying enzymes or general transcriptional machinery. Our screen yielded two separate genes, *lex-3* that maps to the chromosome X and whose molecular identity remains unknown and *sptf-3* that maps to chromosome I and that encodes a Sp1-related transcription factor. Since Sp1-related factors have been shown to interact with the general transcriptional machinery (Gill *et al.*, 1992; Chiang and Roeder, 1995) and to recruit chromatin modifying enzymes such as HDAC (Lee *et al.*, 2004; Subramanian *et al.*, 2004), I wanted to ensure that SPTF-3 is specifically regulating hindgut *lin-48::gfp* expression through the lre1 element and not by modulating transcription or modifying chromatin accessibility. For that purpose, I studied the expression of the different *lin-48::gfp* transgenes in the *sptf-3(gu85)* background. These studies revealed that hindgut expression is not affected in the same way when the lre2 element is intact. They also revealed that hindgut expression is significantly affected when the entire expression is mediated through the lre1 element. These results argue that SPTF-3 specifically functions through the lre1 element.

### 3.4.3. SPTF-3 can not interact with EGL-38 in vivo or in vivo

To test our hypothesis that SPTF-3 interacts with EGL-38 to mediate hindgut *lin-48::gfp* expression through the lre1 element, we tested whether these proteins can interact and form a complex *in vitro* and *in vivo*. EMSA studies using a probe that
included all the sequences necessary for lre1 activity \textit{in vivo} revealed that EGL-38 DBD can bind to these sequences; however, no complex with SPTF-3 could be detected. Similarly, overexpressing these two proteins in the animals and performing co-immunoprecipitations experiments revealed that there is a weak interaction between these two proteins. Taken together, these results could argue that these two proteins do not interact and that SPTF-3 functions through the lre1 element by modulating the expression of a yet unidentified factor that forms a complex with EGL-38. Even though such a prediction is simple, our data do not exclude the possibility that there are other members of the complex that are necessary for us to detect a strong and stable interaction between EGL-38 and SPTF-3 \textit{in vivo}. In addition, since our EMSA results used the DBD of EGL-38 instead of the full length protein, domains important for the interaction of these two proteins could have been missing in our experiment. Finally, Sp1-related factors have been shown to mediate transcription by looping of the DNA (Su \textit{et al.}, 1991; Mastrangelo \textit{et al.}, 1991; Sun and Hurley, 1994; Sakata-Sogawa \textit{et al.}, 1998; Kim \textit{et al.}, 1999; Vergeer \textit{et al.}, 2000). It remains a possibility that SPTF-3 binds elsewhere in the \textit{lin-48} regulatory sequences and interacts with proteins that bind to the lre1 element causing looping of the DNA.

In conclusion, we have identified two factors, \textit{lex-3} and \textit{sptf-3} that are important for tissue specific \textit{lin-48} expression. In the next chapter, I will further describe the roles of SPTF-3 in organ development and Wnt-related processes.
Figure 3.1: Sequence alignment between the lre1 element of *C. elegans* and its closely related species *C. briggsae*. The previously defined lre1 element is displayed in the orange box (Johnson *et al.*, 2001). The sequences that I mutated are labeled as the lre1a, lre1b, lre1c and lre1d elements.
**Figure 3.2:** Genetic map of chromosome X that includes the loci, the snip-SNPs, and the duplications used for *gu84* mapping.
Figure 3.3: Genetic map of chromosome I that includes the loci and the snip-SNPs used for gu85 mapping.
Figure 3.4: 

<table>
<thead>
<tr>
<th>lin-48 sequences in transgene</th>
<th>Expression in hindgut (percent of cells)</th>
<th>Head cell expression (average no.)</th>
<th>N</th>
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*Figure 3.4:*  lre1 mutational analysis. Single Mutations in lre1a, lre1, lre1b, lre1c, lre1d and lre2 do not have an effect on hindgut *lin-48::gfp* expression. Double mutants comprising either lre1a and lre2 or lre1d and lre2 do not affect hindgut expression either. Double mutants between lre1 or lre1b or lre1c and lre2 completely abolish hindgut *lin-48::gfp* expression. This indicates that the area extending from lre1 to lre1c is necessary for hindgut *lin-48::gfp* expression. White circles are used to mark the mutant elements. Hindgut expression is calculated as percentage of cells expressing. Head cell expression is used as an internal control for the presence of the transgene.
Figure 3.5: *lin-48::gfp* expression in animals. Wild-type *lin-48::gfp* expression in the hindgut and tail of the animals. *gu84* and *gu85* animals have reduced hindgut expression while maintaining wild-type phasmid expression.
<table>
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**Figure 3.6:** Comparison of the expression of different lin-48::gfp transgenes in wild-type and gu85 animals. Wild-type and gu85 animals carrying a transgene that is wild-type for both the lre1 and lre2 elements (sal14) have normal hindgut expression. Wild-type animals carrying a transgene mutant in the lre2 element (guls9) have normal hindgut expression whereas gu85 animals have reduced hindgut expression. Wild-type and gu85 animals carrying a transgene mutant in the lre1 element (guls29) have normal hindgut expression. These data indicate that the gu85 mutation is specific to the lre1 element.
Figure 3.7: EMSA gel showing that the EGL-38 DBD can bind to the lre1 area sequence. The SPTF-3 protein can not bind to the lre1 area either alone or in a complex with the EGL-38 DBD.
Figure 3.8: EGL-38 and SPTF-3 Co-immunoprecipitation experiments. EGL-38::FLAG was either ectopically expressed with SPTF-3::MYC or GFP::MYC under the control of the heat shock promoter. A. The EGL-38::FLAG was immunoprecipitated with Anti-Flag resins. B. Very little to no SPTF-3::MYC was detected in the immunoprecipitates. No GFP::MYC was detected in the immunoprecipitates. GFP::MYC served as a negative control for the specificity of the interaction.
<table>
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<th>Heterozygote</th>
<th>Recombinants</th>
<th>Results</th>
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Table 3.1
Table 3.2

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Table 3.3

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CHAPTER 4

THE C. ELEGANS SP-RELATED TRANSCRIPTION FACTOR, SPTF-3,
FUNCTIONS IN WNT-MEDIATED DEVELOPMENTAL DECISIONS

4.1. INTRODUCTION

Sp-related transcription factors play essential roles during animal development (Reviewed by Zhao and Meng, 2005). Moreover, they mediate the functions of signaling pathways to promote normal development and disease progression, including cancer (Reviewed by Safe and Abdelrahim, 2005). Even though transcriptional regulation by Sp-related transcription factors has been extensively studied, our understanding of the in vivo roles of these proteins remains limited. This is mainly due to the diverse and complicated mechanisms of action of these proteins as well as due to limited studies on how these factors interact with different signaling pathways in vivo to mediate animal development. To explore how Sp-related transcription factors interact with signaling pathways to promote cell fate specification and organ development, we used C. elegans to study the role of and the interaction between a Sp1-related transcription and the Wnt signaling pathway.
The mammalian Sp1 family of transcription factors is comprised of nine members (Sp1-Sp9) (reviewed by Suske, 1999; Bouwman et al., 2002; Kaczynski et al., 2003). These all have three highly conserved C2H2 zinc fingers that make up the DNA binding domain and that are located at the proteins’ C-terminus (reviewed by Suske, 1999; Bouwman et al., 2002; Kaczynski et al., 2003). These factors bind to GC or GT boxes in proximal promoters and in enhancers to regulate the transcription of a wide array of target genes (Kadonaga et al., 1986; Kadonaga et al., 1988; Bucher, 1990; Mastrangelo et al. 1991, Su et al., 1991). Sp-related transcription factors are divided into different subgroups based on similarities in their N-terminus. The N-terminus of these proteins is more variable and may include the activation domains in some cases (Sp1-Sp4) as well as an inhibitory domain (Sp3) (reviewed by Safe and Abdelrahim, 2005). Sp-related transcription factors function as activators or repressors depending on the promoter they bind to and on the cofactors they interact with (Hagen et al., 1994; Dennig et al., 1995; Kwon et al., 1999). For example, Sp1 activates transcription by binding to DNA and interacting with general transcriptional machinery proteins, such as the TATA binding protein (TBP) and TATA binding protein associated factors (TAFs) or with other transcription factors (Hoey et al., 1993; Seto et al., 1993; Gill et al., 1994; Emili et al., 1994; Chiang and Roeder, 1995; Datta et al., 1995; Lin et al., 1996; Billon et al., 1999; Doetzlhofer et al., 1999; Suzuki et al., 2000; Lee et al., 2002).

Repression by Sp-related transcription factors occurs either through the competition of the repressors for the GC boxes or through recruitment of chromatin modifying enzymes such as HDAC (Birnbaum et al. 1995; Hagen et al. 1995; Majello et al., 1997; Won et al. 2002; Yu et al. 2003; Moorefield et al. 2004; Phan et al. 2004).
In *C. elegans*, three genes have been annotated as Sp-related transcription factors, *sptf-1*, *sptf-2*, and *sptf-3*. The roles of these genes in animal development have not been described. We were interested in characterizing the role of *sptf-3* in *C. elegans* cell fate specification and organ development as well as the interaction of this gene with signaling pathways important for these processes.

Wnt signaling is critical for early embryonic patterning, generation of cell polarity, cell fate specification and organ development (Logan and Nusse, 2004). Hyper-activation of the Wnt pathway is associated with many diseases, such as human colon cancer and Wilms tumor (reviewed by Koesters *et al.*, 1999; Nusse, 2005 and Nusse, 2007). Moreover, Wnt signaling plays an important role in stem cell and progenitor cell maintenance and renewal (Reya and Clevers, 2005). Because the Wnt pathway plays critical roles in normal development and disease progression, it is important to understand how this pathway mediates its tissue-specific functions as well as how it is regulated. As a result, deciphering how the expression of Wnt pathway members is regulated as well as identifying tissue-specific targets of the this pathway, may result in therapeutic strategies.

One mechanism by which Wnt signaling influences development is through regulation of gene transcription. Wnt signaling can influence transcription through regulating the activity of the TCF/LEF transcription factor. Wnt ligands bind to receptors (Frizzled or Ryk) to initiate an intracellular signaling cascade (Bhanot *et al.*, 1996; Kroither *et al.*, 2001; Yoshikawa *et al.*, 2003; Lu *et al.*, 2004). This cascade leads to the stabilization of the β-catenin protein, its accumulation in the cytoplasm, followed by its nuclear translocation. In the nucleus, β-catenin interacts with TCF/LEF
transcription factor to regulate target gene expression (Molenaar et al., 1996). Even though, the Wnt pathway has been extensively studied, many of its tissue specific target genes remain unidentifed. In addition, very little is known about the transcriptional regulation of Wnt pathway members.

In this study, we report the identification of a mutant allele of the C. elegans Sp1-related gene, sptf-3. We show that this gene mediates multiple developmental decisions and is broadly expressed throughout the animals’ life cycle. Defects observed in sptf-3 mutants are similar to those observed in Wnt pathway mutants, including abnormal vulval lineage polarization, abnormal gonad arm migration, and defects in cell specification and asymmetric cell division. In addition, we observe a sensitivity of sptf-3(gu85) mutants to the dose of the lin-17/frizzled Wnt receptor, suggesting a potential role for SPTF-3 in regulating the function of LIN-17-dependent developmental decisions. These results provide insight on how Sp-related factors cooperate with signaling pathways to mediate developmental decisions.

4.2. MATERIALS AND METHODS

4.2.1. Genetic Strains

Nematode Strains were cultured according to standard techniques (Brenner, 1974; Sulston and Hodgkin, 1988). The following mutations were used:

Linkage group (LG) I: lin-17(n671), dpy-5(e61), unc-29(e1072), unc-13(e1091), hT2, sptf-3(gu85)
LGIII: *unc-8(e49), lin-3(e1417), lin-3(n378), dpy-20(e1362), unc-119(e2498)*

LGV: *him-5(e1490)*

LGX: *lin-18(e620)*

The following transgenes were used: *guls9 (lin-48::gfp); ayIs4 (egl-17::gfp; Burdine et al., 1998); syIs63 (cog-1::gfp; Inoue et al., 2004); syEx615 (egl-5::gfp (pLG7); Teng et al., 2004).*

**4.2.2. Strain Construction**

To transfer the *ayIs4, syIs63* or *syEx615* transgenes into the *gu85* mutant background, we mated males carrying the transgenes with L4 *gu85* hermaphrodites. F1 cross hermaphrodites {*gu85/++; ayIs4/+ or gu85/++; syIs63/+ or gu85/++; syEx615*} were individually picked and allowed to self fertilize. In the F2, animals homozygous for *gu85* were picked, and maintained to retain the transgene. We confirmed that the animals were indeed homozygous for the *gu85* mutation by observing their growth rate, and brood size.

To create the *sptf-3(gu85); lin-18(e620)* double mutants, we first mated *hT2(qIs48)* heterozygous males with *sptf-3(gu85); guIs9* hermaphrodites. F1 cross male progeny were mated with *lin-18(e620)* hermaphrodites. *lin-18(e620)* F2 cross males carrying the *sptf-3(gu85)* chromosome (identified by the absence of the *qIs48* transgene) {*sptf-3(gu85)/++; guIs9/+; lin-18*} were selected and mated with *lin-18(e620)* hermaphrodites. From the resultant F3 offspring, animals carrying the *guIs9* transgene were selected {*sptf-3(gu85) or +/+; guIs9/+; lin-18(e620)/lin-18(e62)}.

These animals
were self-crossed, and *gu85* homozygotes, identified as animals with reduced *guIs9* hindgut expression, were selected from among the F4 progeny to establish the *sptf-3(gu85); lin-18(e620)* strain.

To create the *lin-17(n671), sptf-3(gu85)/hT2(qIs48), sptf-3(gu85)* strain, we first crossed *him-5(e1490)* males with *unc-13(e1091) sptf-3(gu85)/hT2(qIs48)* *sptf-3(gu85); guIs9/guIs9* hermaphrodites. F1 Cross males \{hT2(qIs48) sptf-3(gu85)/+ +; guIs9/+; him-5(e1490)/+\} were next mated with *lin-17(n671); him-5(e1490)* hermaphrodites. F2 cross hermaphrodites \{hT2(qIs48) sptf-3(gu85)/ lin-17(n671) +; guIs9/+; him-5/him-5 or +\} were identified by the presence of both the *qIs48* and *guIs9* transgenes. These animals were allowed to self fertilize and *lin-17(n671) sptf-3(gu85)/ hT2(qIs48) sptf-3(gu85); guIs9/guIs9; him-5/him-5 or +* recombinants were identified among the F3 offspring based on the reduced *guIs9* hindgut expression characteristic of *sptf-3(gu85)* mutants.

To create the *sptf-3(gu85); lin-3(e1417)* double mutants, we mated wild-type males with *sptf-3(gu85)/hT2(qIs48); unc-8(e49) dpy-20(e1362)/ unc-8(e49) dpy-20(e1362)* hermaphrodites. F1 males from this cross \{sptf-3(gu85)/+; unc-8(e49) dpy-20(e1362)/+ +\} were mated with *hT2(qIs48)/+; lin-3(e1417)/lin-3(e1417)* hermaphrodites. Cross F2 hermaphrodites of *sptf-3(gu85)/ hT2(qIs48);+ lin-3(e1417)+/unc-8(e49) + dpy-20(e1362)* genotype were identified by following their segregation. F3 *sptf-3(gu85); lin-3(e1417)* homozygotes were recovered by selecting against the GFP associated with hT2, and against segregation of Unc-8 Dpy-20 animals.
4.2.3. Phenotypic analysis

We used Nomarski DIC microscopy to perform phenotypic analysis studies (Sulston and Horvitz, 1977). Animals were mounted on agar pads containing sodium azide (10-25mM). A Zeiss Axioscope (Carl Zeiss, Inc.) equipped with a Spot-RT CCD camera and software (Diagnostic Instruments, Inc.) was used to identify, observe and photograph the different cells and organs described below.

To quantify vulval morphology, mid-L4 animals were examined. Wild-type L4 animals display a single mirror symmetrical vulval lumen that is ventrally and centrally located (Figure 4.5). Animals displaying an ectopic vulval lumen posterior to the main vulva were scored as having a Bivulva (Biv) phenotype (Ferguson et al., 1987; Inoue et al., 2004; Deshpande et al, 2005)

To quantify vulval induction, live L3 animals were examined with Nomarski optics (Han et al., 1990). In wild-type animals, six Pn.p cells (P3.p –P8.p) have the potential to produce vulval tissue and are known as the vulval precursor cells. Only three of these cells (P5.p, P6.p and P7.p) however are induced to produce vulval tissue. The uninduced cells divide only once before fusing with the hypodermis. The induced cells divide more than once to produce cells of the vulva. Induction can be scored by determining the number of time these cells divide compared to the uninduced Pn.p cells. All the Pn.p cells were accounted for and the numbers of cells that have divided more than once were counted. Finally, the animals were recovered and grown at 20°C until they reached the L4 stage when they were scored a second time to determine whether they exhibit the Biv phenotype.
To quantify gonad migration, mid-L4 animals were examined with Nomarski optics. In wild-type hermaphrodites, two distal tip cells lead two gonad arms in their anterior and posterior extension. After migrating away from the mid-body, the gonad arms reflex or turn. This is followed by growth back towards the center of the animal (Kimble and Hirsh, 1979). We scored animals for defects in gonad arm migration and turning (Kimble and Hirsh, 1979; Hedgecock et al., 1990; Nishiwaki et al., 1999). We categorized animals with abnormal turning of at least one gonad arm as defective in gonad migration. Specifically, animals in which one gonad arm failed to reflex, but instead continues to migrate away from the center of the animal (forming an S-shaped gonad arm) were scored as defective.

To quantify P11/P12 cell fates, L3 or early L4 animals were examined with Nomarski optics. The cell fate was determined by examining the nuclear size and position of the P11.p and P12.pa cells (Jiang and Sternberg, 1998). In wild-type animals, the P12.pa cell has a small nucleus size and is located near the hindgut lumen. In contrast, the P11.p cell has a larger nucleus size and is located anterior to the hindgut lumen. We used these criteria to determine the cell fate of these cells in our animals.

To quantify B cell asymmetric division, L1 or L2 males were examined. Relative nuclear size of the two B cell daughters was used a marker for asymmetric/symmetric cell division (Herman and Horvitz, 1994; Herman et al., 1995). In wild-type animals, the B cell divides to produce a large anterior daughter cell (B.a) and a small posterior daughter cell (B.p) (Sulston et al., 1980).
4.2.4. Isolation of sptf-3(gu85)

gu85 was isolated in the background of the CM753 (unc-119(e2498); guIs9) strain. The intact lin-48::gfp reporter transgene includes two redundant elements (lre1 and lre2) important for hindgut expression (Johnson et al., 2001). The guIs9 transgene contains a mutant lre2 sequence and a wild-type lre1 sequence. Hindgut lin-48::gfp expression is thus mediated entirely through the lre1 element in this transgene. We predicted that by screening in the background of the CM753 strain for animals with reduced or abolished hindgut lin-48::gfp expression, we would select for mutations in factors that directly or indirectly function through the lre1 element. gu85 was isolated using EMS. Fourth larval stage (L4) P0 hermaphrodites were mutagenized using 50 mM EMS for four hours (Sulston and Hodgkin, 1988), individually placed on agar plates at 20°C and allowed to grow for two generations. The F2 progeny were screened for defects in hindgut lin-48::gfp expression, using a dissecting microscope with epifluorescence. Animals that exhibited reduced or abolished hindgut expression were individually picked into fresh plates and allowed to self-cross.

4.2.5. Genetic mapping and gene identification for gu85

Single Nucleotide Polymorphism (snip-SNP) mapping was used to determine that gu85 is linked to LGI (Wicks et al., 2001; Chapter 3). Three factor crosses, and snip-SNP mapping were used to determine that gu85 maps to the right of pkP1073 (LGI: 17.1) and the left of pkP1071 (LGI: 23.4) (Table 3.3), with a map position of
approximately 18.5. To find candidate genes in this region, we screened the data in WormBase.org for the RNAi phenotypes of genes located in the area extending from positions 17.5 to 20 on LGI. The area we focused on is predicted to contain 23 genes (WormBase.org). This candidate approach targeted genes in the area with an RNAi phenotype similar to the phenotypes we observe in gu85 animals. In particular, we focused our search for genes reported to show vulval defects, such as the protruding vulva (Pvl) phenotype and exploding through the vulva (Rup) phenotype. RNAi of two of these 23 genes results in vulval defects characteristic of the gu85 mutants: snr-2 and sptf-3 (Fraser et al., 2000; Simmer et al., 2003). snr-2 codes for a small nuclear ribonucleoprotein, located at position 18.1, and sptf-3 codes for a Sp-related transcription factor, located at position 19.2 (WormBase.org). Since our genetic screen was focused on the transcription of a transgene, we focused on the sptf-3 locus. Transgenic rescue, RNAi phenocopy, and DNA sequencing (below) allowed us to confirm that gu85 is an allele of sptf-3.

**4.2.6. Plasmid and transgene generation**

We used a heat-inducible promoter to express the wild-type sptf-3 cDNA in gu85 mutant animals. To create these constructs, the cDNA coding for full-length, wild-type SPTF-3 protein was amplified using RT-PCR, and was cloned downstream of the hsp16-41 promoter in the pPD49.83 vector (a gift from A. Fire). The pPD49.83 vector was modified so that it includes sequences corresponding to the FLAG epitope at the 3’ end of the cDNA insertion site, allowing antibody detection of the expressed
protein. The clone was confirmed by sequencing to ensure that no mutations were introduced by PCR. Transgenes for the heat-inducible construct were produced by microinjection of plasmid DNA into the mitotic germline of hermaphrodites according to the method of Mello et al. (1991). For all experiments, 50 ng/µl experimental clone was co-injected with 15 ng/µl pDP#MM016 (unc-119(+)) plasmid; Maduro and Pilgrim, 1995) and 15 ng/µl of the cytoplasmically localized transformation marker (myo-2::gfp) (Okkema et al., 1993) into unc-119(e2498) animals. The resulting transgenic animals were crossed with gu85 mutant animals. For that purpose, wild-type males were crossed into these transgenic animals. F1 cross males were next mated with sptf-3(gu85); unc-119(e2498); guIs9 animals. F2 cross hermaphrodites were identified by the presence of the transgene and were allowed to self-fertilize. F3 sptf-3(gu85) animals carrying the transgene were maintained for further analysis.

To create the sptf-3::gfp construct, we amplified a 6.8 Kb fragment of the sptf-3 upstream sequences from wild-type C.elegans genomic DNA and introduced this fragment in-frame upstream to the gfp coding sequences in the pPD95.73 vector (a gift from A. Fire). To create transgenes for this construct, 125 ng/µl of the experimental clone was co-injected with 15ng/µl of pDP#MM016 (unc-119(+)) plasmid; Maduro and Pilgrim, 1995) ) into unc-119(e2498) animals.
4.2.7. Rescue of the gu85 phenotype by induced expression of SPTF-3

Heat-induced SPTF-3(WT) protein expression produced was visualized by Western blot. Mixed-stage animals were treated with a 35°C heat-shock for 30 minutes. After 30 minutes recovery at room temperature, worms were washed 2x in M9 buffer, and frozen in liquid nitrogen. Thawed pellets were sonicated in SDS-PAGE buffer, boiled for 5 minutes, and separated by SDS-PAGE. Proteins were transferred to membrane, blocked overnight with 5% non-fat dried milk in PBS plus 0.1% tween, then washed 1x with PBS tween. Proteins were detected with anti-FLAG antibody (Sigma) diluted 1:100. The activity of SPTF-3 proteins expressed in response to heat treatment was assessed by testing for rescue of the sptf-3(gu85) vulval and gonadal defects. Transgene-bearing hermaphrodites were allowed to lay eggs for five hours, removed from the plate, and the eggs were counted. The eggs were then subjected to a 35°C heat-shock for 30 minutes. On the second day, the eggs and resulting larvae were subjected to a second heat-shock treatment. Once the larvae reached the L4 stage, the animals were scored for the presence or absence of the transgene and for vulval (presence of an ectopic posterior lumen) and gonadal phenotypes (abnormal migration). The data in Figure 4.1A result from 3 independent trials. Each trial included 20 or more eggs for a given genotype.
4.2.8. RNAi experiments

cDNA coding for SPTF-3 was amplified using RT-PCR, and cloned into the RNAi feeding vector (pPD129.36, a gift from A. Fire). The resultant plasmids were transformed into the HT115 RNase-deficient strain of E. coli. (Kamath et al., 2001). The bacteria were grown in culture without induction at 37°C overnight. Next, the bacteria were seeded onto plates containing 25 µg/ml carbenicillin, 10 µg/ml tetracyclin and 100µM IPTG, and then incubated overnight at room temperature (Kamath et al., 2001). Four hermaphrodites were selected to these plates, allowed to lay eggs overnight, and then removed. The offspring were analyzed for vulval defects (presence of an ectopic posterior lumen) and gonadal phenotypes (abnormal migration). Plates seeded with bacteria carrying the empty vector (pPD129.36) were used a negative controls. We used 100µM IPTG instead of 1mM IPTG since induction with the latter was highly lethal and affected our ability to recover larvae for our post-embryonic assays (Kamath et al., 2001). This use of a diluted induction concentration may account for some of the variability observed in the RNAi experiments, as well as the weak RNAi effect.

4.2.9. DNA sequencing and cDNA analysis

To identify the mutation associated with the gu85 allele, we used PCR to amplify the genomic sequence of the sptf-3 (Y40B1A.4) gene from sptf-3(gu85) mutant animals. The PCR products were sequenced by the Plant-Microbe Genomics Facility at
The Ohio State University using an Applied Biosystems 3730 DNA Analyzer and BigDye cycle sequencing terminator chemistry. PCR products covering all predicted exons and introns were analyzed. In addition, RT-PCR, using RNA derived from both wild-type and \textit{sptf-3(gu85)} mutant animals, was utilized to amplify the cDNAs coding for the full length SPTF-3 protein. The PCR products were sequenced and a comparison of the sequencing results obtained from the cDNA amplified from wild-type RNA and \textit{gu85} RNA was done by nucleotide sequence alignment using the ClustalW program (Thompson \textit{et al.}, 1994)

\textbf{4.2.10. In vitro gel shift assays}

Production of recombinant SPTF-3(WT) or SPTF-3(F135I) full length proteins in \textit{E. coli} was carried out as described previously (Johnson \textit{et al.}, 2001). Briefly, the entire cDNA coding for SPTF-3(WT) or SPTF-3(F135I) was amplified using RT-PCR and cloned into vector pET-32 (Novagen). Recombinant SPTF-3 was expressed in \textit{E. coli} strain BL21 (DE3) (Novagen). Cells were lysed with sonication in Sp1 gel shift buffer [5µM ZnSO\textsubscript{4}, 50mM KCl, 2mM DTT, 12mM HEPES-KOH (pH 7.5), 6mM MgCl\textsubscript{2}, 0.1% NP-40 and 50% glycerol]. Recombinant protein expression was confirmed with SDS-PAGE, and expressed protein amount was estimated by comparing dilutions to known quantities of BSA. For the experiments, lysates were utilized that included similar levels of expressed recombinant protein. In all cases, lysates derived from cells expressing the pET-32 vector with no cDNA insert were used as a negative control. The probe used for this experiment is a mammalian consensus Sp1 binding site (Promega).
The probe (200ng) was end labeled with DIG-11-ddUTP (Roche). The SPTF-3 proteins were incubated with the probe for thirty minutes. The reactions were electrophoresed on a 15% polyacrylamide gel in a Tris-Glycine-EDTA (TGE) running buffer (10X buffer: 0.25M Tris-Base, 1.9M Glycine, 10mM EDTA, pH=8.3) for 6-7 hours at 100 volts. The gel was transferred in 1X TGE buffer overnight. The nucleic acids on the membrane were fixed by cross-linking with UV, then washed with washing buffer (0.1M Maleic Acid, 0.15M NaCl; pH 7.5; 0.3% v/v Tween 20) for 2 minutes, blocked for 30 minutes with 1X blocking solution (Roche), incubated for 30 minutes with the 1:10000 Anti-Digoxigenin AP antibody, washed twice for 15 minutes, and finally detected in CSPD (Roche).

4.3 RESULTS

4.3.1. gu85 is an allele of the C. elegans Sp1-related gene sptf-3

In a genetic screen, we identified a mutation with a range of phenotypes. These phenotypes include slow growth, larval lethality, embryonic lethality, hindgut development defects, gonad migration defects and vulval defects (see materials and methods, and below). Through three-factor and Snip-SNP mapping, we placed gu85 to the right arm of linkage group I (TABLE 3.3). We screened the target area for candidate genes by comparing the published RNAi phenotypes from WormBase.org to phenotypes observed in gu85 animals. This analysis focused our initial efforts on the Sp-related transcription factor, sptf-3 (see materials and methods).
To test whether gu85 is an allele of sptf-3, we tested for rescue and RNAi phenocopy of gu85 by the sptf-3 gene. For the rescue assay, we created a transgene that expresses wild-type sptf-3 cDNA under control of the heat inducible promoter, hsp14-61. Heat treatment of animals bearing this transgene rescued the vulval and gonad migration defects observed in gu85 mutant animals (Figure 4.1; see below for further description of phenotypes associated with gu85). Introduction of sptf-3 double-stranded RNA by bacterial feeding showed similar phenotypes to those observed for gu85 mutant animals (Table 4.2 and Figure 4.5). For example, some sptf-3 RNAi animals have an ectopic vulval lumen posterior to the main vulva, a phenotype observed in gu85 mutant animals. In addition, sptf-3 RNAi animals show a decrease in hindgut guIs9 expression, the mutant phenotype for which gu85 was originally selected (Data not shown).

To determine the molecular lesion associated with gu85, we sequenced the exons from genomic sptf-3 DNA and cDNA obtained by RT-PCR from gu85 mutant animals. This sequence was compared to the sequence obtained from wild-type animals. The sequence analysis identified a missense mutation in exon 4, which codes for part of the DNA binding domain (Figure 4.2A). In the predicted protein product, this mutation converts Phe 135 to an Ile. Phe 135 is conserved across species in SPTF-3 related proteins (Figure 4.2B). To assess the significance of this mutation on SPTF-3 function, we tested whether it alters the DNA binding ability of SPTF-3 using EMSA. We find that wild-type SPTF-3 binds to a defined consensus for Sp1 with high affinity. In contrast, the mutant protein fails to bind this sequence. (Figure 4.3A & B). Taken together, these data show that gu85 is a mutation in the sptf-3 gene and that this mutation affects the ability of SPTF-3 protein to bind to target DNA sequence.
4.3.2. *sptf-3* is broadly expressed throughout development

To understand which cells express *sptf-3*, and the developmental timing of its expression, we created an *sptf-3::gfp* reporter transgene that includes 6.8 Kb of the *sptf-3* upstream sequences. We observed *sptf-3::gfp* expression in wild-type animals and found that the *sptf-3* gene is broadly expressed in embryos. Moreover, we observe that *sptf-3* is expressed in several tissues and cells in postembryonic development. Expression in these tissues persists through larval development into adulthood (Table 4.1). We observe expression in the intestine, hypodermis, vulva, rectum and the tail region as well cells in the head (Figure 4.4 and Table 4.1).

4.3.3. *sptf-3* plays a role in lineage polarization during vulval development

*C. elegans* hermaphrodites lay eggs through an opening on their ventral side called the vulva. The vulva is a mirror-symmetrical organ that is made up of the progeny from three cells and two cell types: P6.p (primary fate), and P5.p and P7.p (secondary fates) (Sternberg and Horvitz, 1989). Vulval development is mediated by three signaling pathways: the EGF pathway (Ferguson and Horvitz, 1985; Hill and Sternberg, 1992; Katz *et al.*, 1995), the Delta/Notch pathway (Greenwald *et al.*, 1983; Yochem *et al.*, 1988) and the Wnt pathway (Ferguson *et al.*, 1987; Sawa *et al.*, 1996; Inoue *et al.*, 2004; Desphande *et al.*, 2005). During the fourth larval stage (L4), the prospective vulval cells have completed all cell divisions, and they undergo
morphogenesis. In mid-L4, the cells invaginate, forming a lumen ventral to the gonad. In \textit{sptf-3(gu85)} animals, we sometimes observe the presence of a second lumen (19\%, n=129) posterior to the main vulval lumen (Table 4.2 and Figure 4.5). This phenotype is also observed in \textit{sptf-3} RNAi animals (13\%, n=31). We never observe this posterior ectopic lumen in wild type (0\%, n=120) or control RNAi animals (0\%, n=31). This anatomical defect can result from two possible developmental defects: defects in the vulval induction process or reversals in the P7.p lineage. We decided to determine which of the above defects occurs during the vulval development in \textit{sptf-3(gu85)} animals.

The vulva forms from ventral epidermal cells that develop in response to a gradient of the EGF signal, LIN-3, produced by the anchor cell (AC) in the gonad (Hill and Sternberg, 1992). The ventral epidermal cell closest to the AC (P6.p) is induced to form vulval tissue and adopts the primary cell fate. The two adjacent ventral epidermal cells (P5.p and P7.p) are also induced, but adopt the secondary cell fate (Sulston and Horvitz, 1977; Sulston and White, 1980). Delta/Notch signaling through LIN-12 mediates a lateral inhibition from the P6.p cell to promote secondary fates (Greenwald \textit{et al.}, 1983; Sternberg, 1988; Sternberg and Horvitz, 1989). Hyperactive EGF or Delta/Notch signaling can lead to additional cells being induced to form vulval tissue; as a result, animals exhibit a “Multivulva” phenotype (Ferguson and Horvitz, 1985 and Ferguson and Horvitz 1989). This phenotype can manifest itself in the appearance of additional vulval lumens in the L4. To determine whether the ectopic vulval lumens observed in the \textit{sptf-3(gu85)} and \textit{sptf-3} RNAi animals reflect a Multivulva defect, we assayed the total number of cells induced to form vulval tissue. We find that, as in wild-
type animals, \textit{sptf-3(gu85)} animals displayed a normal number of induced cells (3.0, n=20) (Table 4.3). This argues that the ectopic posterior lumen we observe in the mutant is not a Multivulva phenotype and is not due to a defect in vulval induction.

Since the ectopic vulval lumen observed in \textit{sptf-3(gu85)} animals is not due to defects in induction, we next investigated whether this phenotype results from a reversal in the P7.p lineage, a phenotype that is characteristic of mutants of the Wnt pathway receptors \textit{lin-17} and \textit{lin-18} (Ferguson et al., 1987; Sawa et al., 1996; Inoue et al., 2004). Normally, the P5.p and P7.p descendents have identical cell lineages, but are organized in opposite and mirror symmetrical orientations along the anterior-posterior axis. To achieve the mirror symmetry in the vulva, the Wnt signaling pathway reorients the cell division pattern of the P7.p lineage and the fate of its progeny. Mutations in Wnt receptor genes (\textit{lin-17} and \textit{lin-18}) cause a reversal in the P7.p lineage, leading the descendents of the P7.p cell to adopt the P5.p orientation (Ferguson et al., 1987; Deshpande et al., 2005). This reversal in the P7.p lineage is morphologically observed as an ectopic lumen posterior to the main vulval lumen, a phenotype referred to as Bivulva (Ferguson et al., 1987; Sawa et al., 1996; Inoue et al., 2004; Deshpande et al., 2005). Since the ectopic vulval lumen observed in \textit{sptf-3(gu85)} and \textit{sptf-3} RNAi animals always occurs posterior to the primary lumen, we investigated whether this phenotype results from a reversal in the P7.p lineage. For that purpose, we used the \textit{egl-17::gfp} and \textit{cog-1::gfp} reporters (Inoue et al., 2004). These reporters are normally expressed only in the anterior daughters of the P7.p lineage (Inoue et al., 2004). In Wnt receptor Bivulva animals showing complete reversal in the P7.p fate, the expression of these markers is observed in the posterior daughters and not the anterior daughters of
P7.p (Inoue et al., 2004). We find that, like Wnt pathway mutants, sptf-3(gu85) animals exhibit altered patterns of egl-17::gfp and cog-1::gfp expression consistent with reversals in the P7.p lineage (Table 4.5). We conclude that the secondary lumen in sptf-3(gu85) mutants is the Bivulva phenotype characteristic of Wnt pathway mutants.

Altogether, our results indicate that the vulval defects observed in sptf-3(gu85) mutants are not due to defects in vulval induction. This result argues against defective EGF signaling in these animals. Moreover, no defects in the P5.p lineage were observed in the egl-17::gfp and cog-1::gfp marker expression analysis (Data not shown). This result argues against defective Delta/Notch pathway signaling, since defects in this signaling pathway should affect both P5.p- and P7.p-derived secondary fates. Because our results indicate that the vulval defects observed in sptf-3(gu85) animals are similar to those observed in Wnt pathway mutants, we decided to explore whether there is a genetic interaction between sptf-3 and the Wnt pathway receptors and whether sptf-3 is involved in other Wnt dependent biological decisions.

4.3.4. Reducing the dose of lin-17 enhances the bivulva phenotype observed in sptf-3(gu85) animals

Wnt controls the orientation of the P7.p lineage by functioning through two receptors, LIN-17/FZD and LIN-18/Ryk (Ferguson et al., 1987; Sawa et al., 1996; Inoue et al., 2004; Deshpande et al., 2005), which function parallel to each other (Inoue et al., 2004). To explore how SPTF-3 acts with respect to the Wnt pathway, and specifically with respect to each of the receptors, we constructed double mutants
between *sptf-3*(gu85) and *lin-18*(e620) or *lin-17*(n671). We find that in double mutants, *sptf-3*(gu85) enhances the Biv phenotype of null *lin-18* mutants (Table 4.4). This result argues that regulating *lin-18* expression cannot be the only role for SPTF-3, since if that were the case the double mutants should resemble *lin-18* single mutants. In contrast, we found that *lin-17*(n671) *sptf-3*(gu85) double mutants are not viable. Because the early lethality observed in the double mutants did not allow us to assay P7.p development in these animals, we instead asked whether *sptf-3*(gu85) mutants are sensitive to *lin-17* genotype in animals heterozygous for *lin-17*(n671) and homozygous for *sptf-3*(gu85). We find that reducing the dose of *lin-17* enhanced the Biv phenotype observed in *sptf-3*(gu85) animals (Table 4.4). In other words, in contrast to wild-type animals, *sptf-3*(gu85) animals are sensitive to the dose of *lin-17*.

4.3.5. *sptf-3*(gu85) animals exhibit loss of the asymmetric B cell division

To test whether *sptf-3* functions broadly in Wnt-mediated developmental decisions in *C. elegans*, we asked whether other phenotypes associated with *sptf-3*(gu85) are similar to those observed in Wnt mutants. The Wnt pathway is important for the polarity and asymmetry of division of the male B cell (Sternberg and Horvitz, 1988; Herman and Horvitz, 1994). In late L1 males, the B cell divides asymmetrically to yield a larger anterior daughter cell (B.a) and smaller posterior cell (B.p). In *lin-17* mutants, the B cell polarity is lost (Sternberg and Horvitz, 1988). The cell divides symmetrically, and the daughters adopt similar B.a-like fates. We observed the morphology of B cell daughters in *sptf-3*(gu85) mutant males, and found that indeed the
asymmetric cell division is affected in these animals. Specifically, we observe equal size of B.a and B.p in 19% of sptf-3(gu85) mutants (n=41; Table 4.6, Figure 4.6 G, H). These data show that another Wnt pathway dependent developmental process, namely cell polarity, is sensitive to sptf-3 function.

4.3.6. sptf-3(gu85) animals exhibit a defect in P12 development

The P11 and P12 cells are the most posterior ventral epithelial cells. P11 and P12 are initially equivalent, but lin-3/EGF and lin-44/Wnt promote P12 fate (Jiang and Sternberg, 1998). These signals also promote the expression of the HOM-C gene, egl-5 in the more posterior cell, which confers P12 fate on that cell (Jiang and Sternberg, 1998). To determine whether sptf-3 played a role in P11/P12 development, we scored these cell fates in sptf-3(gu85) mutants. We observed that sptf-3(gu85) mutants show defects in P12 fate specification (Table 4.7, Figure 4.6 A and B) similar to those observed in Wnt pathway mutants (Jiang and Sternberg, 1998). To determine whether SPTF-3 functions in P12 fate specification by affecting the expression of egl-5, we studied egl-5::gfp reporter expression. We observed that indeed egl-5::gfp expression is reduced in the P12.pa cell of sptf-3(gu85) animals as compared to wild-type animals (Table 4.7, Figure 4.6 C, D, E and F). These data show that SPTF-3 affects P12 development by affecting the Wnt target egl-5 expression. In addition to reduction of egl-5::gfp expression in the P12.pa cell, we observe an overall reduction in the number of hindgut cells expressing egl-5::gfp as well as a reduction in the intensity of expression levels (Figure 4.6 E and F). This suggests that sptf-3 could affect P12 fate
by influencing Wnt signaling, or that the P12 defects reflect this broader effect on egl-5 expression level. It is unlikely that the observed reduction in egl-5::gfp expression is due to a general role for SPTF-3 in transgene expression. This is because expression of many extrachromosomal and integrated transgenes remains intact in the sptf-3(gu85) background (Data not shown and Figure 3.6).

4.4. DISCUSSION

4.4.1. gu85 is a mutation in the C. elegans sptf-3 gene

In a genetic screen, we identified the gu85 mutation and showed that it is a lesion that disrupts the C. elegans Sp-related gene sptf-3. We predict that this mutation is a non-null, but reduction of function mutation. Genetic tests indicate that the null phenotype of sptf-3 is lethal. Microinjection RNAi of the sptf-3 gene is completely lethal indicating that sptf-3 plays essential roles in animal development (Data not shown). In addition, a deletion allele of sptf-3 is homozygous lethal (Sun et al., 2007). The survival of sptf-3(gu85) animals indicates that this mutation does not completely abolish sptf-3 function. However, we show that this mutation significantly affects the protein’s ability to bind to DNA corresponding to a mammalian Sp1 consensus site. This suggests that the defects observed in sptf-3(gu85) animals result from the compromised ability of the mutant protein to bind to its responsive element in target gene regulatory sequences. Even though SPTF-3(F135I) doesn’t bind DNA in vitro, we maintain the argument that the gu85 allele is not null. One reason could be that in vivo,
other interacting proteins recruit the protein to DNA even though it can not bind sequences. Reduction of SPTF-3 binding to its targets in multiple tissues could explain the many pleiotropic and weakly penetrant phenotypes observed in \textit{sptf-3(gu85)} animals. In addition, compensatory mechanisms by the other SPTF-3 homologs, namely SPTF-1 or SPTF-2 can also account for the weakly penetrant phenotypes observed in the \textit{sptf-3(gu85)} animals.

\textbf{4.4.2. sptf-3 expression is observed throughout animal development}

We created a transcriptional GFP reporter for the \textit{C. elegans sptf-3} gene. Expression of this reporter in the Pn.p cells, vulva of the hermaphrodite and hindgut cells is consistent with the observed SPTF-3 requirement in these tissues for their proper development. We observe a broad domain of \textit{sptf-3} expression; however, expression from this reporter is not ubiquitous in the animals. Even though some Sp-related transcription factors, such as human Sp1, Sp3 are ubiquitously expressed, others show tissue specific expression pattern (reviewed by Zhao and Meng, 2005). It is important to note however that since we do not have \textit{C. elegans} specific SPTF-3 antibodies, expression from our reporter may not reflect the complete endogenous expression of the \textit{sptf-3} gene. In addition, the continuous expression of \textit{sptf-3} in cells from early embryonic development throughout adulthood could implicate SPTF-3 in housekeeping functions as well as in maintenance of cell fate. Interestingly, such a role has been described for murine Sp1 (Marin et al., 1997).
4.4.3. SPTF-3 influences Wnt-mediated development in C. elegans

Our results indicate that SPTF-3 promotes several Wnt-mediated decisions during *C. elegans* development. These decisions include control of P7.p lineage orientation, P12.pa cell fate specification, and B cell asymmetric cell division. The control of P7.p orientation is mediated by the Wnt activation of signaling events in the P7.p cell and its descendents through two of its receptors, LIN-17/Frizzled and LIN-18/Ryk (Ferguson *et al.*, 1987; Sawa *et al.*, 1996; Inoue *et al.*, 2004). It has been proposed that a parallel pathway involved in this process is unlikely because the *lin-17; lin-18* double mutants show a completely penetrant bivulva phenotype (Inoue *et al.*, 2004; Desphande *et al.*, 2005). We used genetic analysis to determine whether *sptf-3* functions in one or both Wnt receptor pathways, or whether it independently functions to control P7.p orientation. For example, if the *sptf-3(gu85)* mutation does not enhance the Biv phenotype observed in the *lin-18* null mutants, then SPTF-3 can be placed in the *lin-18* receptor pathway. SPTF-3 can either function to regulate the expression of Wnt pathway members, including the *lin-18* gene itself, or it can be a downstream target of the Wnt pathway. Conversely, if the *sptf-3(gu85)* mutation enhances the Biv phenotype observed in the *lin-18* null mutants, then at least one conclusion that can be drawn from this result is that regulating the expression of the *lin-18* gene or any gene upstream to the *lin-18* and specific to this receptor pathway are not the only roles that allow SPTF-3 to affect P7.p orientation. Even though enhancement of the *lin-18(e620)* phenotype is observed in the *sptf-3(gu85); lin-18(e620)* double mutants and sensitivity to the dose of *lin-17* is observed in the *sptf-3(gu85)* animals, it remains possible that SPTF-3 is a
downstream target of the Wnt pathway. This is because the LIN-17 and LIN-18 receptor pathways converge at the level of POP-1/TCF-1 to control P7.p orientation; as a result, when one receptor signaling branch is affected, the other continues to contribute to P7.p orientation. However, if *sptf-3* is a downstream target of POP-1, then we expect that both branches of the pathway are affected in the double mutants or the sensitized background. Another possibility that can also explain the obtained results is that SPTF-3 regulates the expression of genes important for P7.p orientation. In this case, SPTF-3 specifically affects the expression of Wnt pathway members functioning in the LIN-17 branch of the pathway (Figure 4.7).

**4.4.4. SPTF-3 influences the development of hindgut cells**

The asymmetric division of the B blast cell in the male tail is controlled by a novel non-canonical Wnt pathway (Wu and Herman, 2006) that involves the asymmetric localization of LIN-17 and MIG-5/Dsh. *lin-17* mutants show a loss of symmetry in the B cell division, whereas *lin-44/Wnt* mutants show a reversal in symmetry (Herman and Horvitz, 1004). The defect observed in *sptf-3(gu85)* mutants is a loss of asymmetry similar to *lin-17* mutants. We predict that if SPTF-3 promotes asymmetric cell division by affecting the Wnt signaling, it will function to affect the expression of *lin-17* or Wnt pathway members downstream to it, rather than affect the expression of Wnt ligands. This is because the predominant phenotype observed in the *sptf-3(gu85)* mutants is similar to that of *lin-17* mutants and not *lin-44* mutants. It has been proposed that the rate at which the two different B daughter cell sizes are
established suggest that no novel transcription occurs (Wu and Herman, 2006). This argues against a role for SPTF-3 as a target for Wnt signaling in the dividing cell. Alternatively, SPTF-3 could promote asymmetric B cell division independently of the Wnt pathway. This can possibly be due to the reduction of egl-5 expression observed in the sptf-3(gu85) mutants. EGL-5 is important for proper B cell division and animals that carry a mutation in this gene show a loss of asymmetry in this division (RWJ and HMC submitted to MOD) (Figure 4.7).

The P12 fate is promoted by two signaling pathways, LIN-44/WNT and LIN-3/EGF that converge on egl-5/HOM-c expression (Jiang and Sternberg, 1998). We show that SPTF-3 also promotes the P12 fate, and alters egl-5 expression. We speculate that this regulation is indirect as we did not find any SPTF-3 binding sites in the sequences conserved in related Caenorhabditis species and included in the egl-5::gfp reporter used. The reduction of egl-5 expression observed in sptf-3(gu85) mutants is sufficient to explain the defects observed in P12 fate specification. This reduction can be achieved either through Wnt dependent or Wnt independent mechanisms. For example, SPTF-3 might promote egl-5 expression by affecting Wnt pathway activity. Specifically, SPTF-3 can regulate the expression of Wnt pathway members or the sptf-3 gene itself can be a target of the Wnt pathway, which in turn regulates egl-5 expression. Alternatively, SPTF-3 regulation of egl-5 expression could occur independently of the Wnt pathway as part of an undefined parallel pathway (Figure 4.7).
4.4.5. Sp-related transcription factors interact with the Wnt signaling pathway in vertebrate development

We have shown that proper SPTF-3 function is important for Wnt-dependent processes. There is evidence in vertebrates for an interaction between Sp-related transcription factors and the Wnt pathway. This interaction is critical for both normal animal development as well as disease progression. For example, Zebrafish Sp-5I and Sp5 were shown to be downstream of Wnt8 and important for mesodermal and neuroectodermal patterning (Weidinger et al., 2005). In addition, Sp5 was shown to be elevated in response to overexpression of beta-catenin in the mouse CNS and to repress Sp1 targets in response to Wnt.(Fujimura et al., 2007). Human Sp5 was shown to be a direct target of Wnt signaling. Sp5 is overexpressed in colorectal cancer and has been shown to deregulate negative inhibitors of the Wnt signaling pathway, such as p21\textsuperscript{WAF1} in these cells (Takahashi et al., 2005). Moreover, comparative studies show that mammalian FZD7 and FZD5 both have highly conserved Sp1 binding sites in their upstream regulatory sequences (Katoh and Katoh, 2007a and Katoh and Katoh, 2007b). These studies hint at a potential role for Sp-related transcription factors in regulation of Wnt receptor Frizzled expression. These results from vertebrates show that the Wnt pathway mediates a wide variety of its functions in development and disease progression through transcriptional regulation of the Sp-related transcription factors. This allows us to speculate that sptf-3 specifically interacts with the Wnt pathway in C. elegans rather than functions as a genetic enhancer or an enhancer of compromised signaling pathways. Uncovering the different ways that SPTF-3 affects Wnt pathway
function is of critical importance to better understand normal organ development and to find therapeutic targets for multiple diseases including cancer. In this study, we have shown that SPTF-3 plays important roles in organ development, cell fate specification and asymmetric cell division. Our studies suggest that many Wnt-dependent developmental decisions require SPTF-3 function.
Figure 4.1: Induced expression of SPTF-3::Flag rescues defects observed in *gu85* animals. (A) The vulval defect scored is the presence/absence of an ectopic posterior lumen and the gonadal defect scored is the presence/absence of S-shape gonadal migration. T-test analysis shows that rescue of both defects was statistically significant. p<0.05 for vulval rescue and p<0.01 for the gonad defect rescue. (B) Western blot using Anti-Flag antibody showing that SPTF-3::Flag expression is induced upon heat-shock.
**Figure 4.2:** sptf-3 allele isolated in a genetic screen. (A) Gene organization of sptf-3. Each box represents an exon and the regions coding for the DNA binding domain are shaded. The location of the gu85 allele is indicated with an asterisk (*). (B) Amino acid sequence alignment of the DNA binding domain of the *C. elegans* Sp1-related transcription factor, sptf-3 and other Sp1-related transcription factors. The amino acid substitution associated with the gu85 allele is marked with an arrow. The specific alteration associated with the gu85 allele is a Ttt (Phe) to Att (Ile) transition affecting codon 135.
Figure 4.3: Wild type SPTF-3 binds to the mammalian Sp1 consensus site, whereas the mutant protein does not. (A) Lysates including either SPTF-3(WT) or SPTF-3(F135I) were incubated with a mammalian Sp1 probe (5'ATT CGA TCG GGG CGG GGC GAG C 3'). When the SPTF-3(WT) protein is included in the reaction, a robust complex is formed; on the other hand, when SPTF-3(F135I) protein is included, no such complex is formed. Control lanes 1 and 2. 1 = probe alone. 2 = lysates of cells carrying an empty pET-32(b) vector. Different concentrations of the SPTF-3 proteins were tested (lanes 3-10). 3, 4, 5, and 6 = full concentration, 1:10, 1:20 and 1:50 dilutions of the lysate of cells expressing SPTF-3(WT) protein. 7, 8, 9, and 10 = full concentration, 1:10, 1:20 and 1:50 dilutions of the lysate of cells expressing SPTF-3(F135I) protein. (B) Coomassie staining showing that the soluble fraction of lysates expresses both WT and GU85 proteins at equivalent levels. The arrowhead indicates the position of the SPTF-3 proteins, whereas the asterisk indicates the position of the empty pET-32(b) vector.
Figure 4.4: *sptf-3::gfp* expression in wild-type animals. (A,C, E, G) Nomarski micrographs of larvae. (B,D, F, H) Epi-fluorescent micrographs of the same animals respectively. (A,B) *sptf-3::gfp* is expressed in the dividing Pn.p cells / vulval precursor cells (marked by arrowheads). It is also expressed in the somatic gonad, including in the anchor cell (marked by an asterisk). (C,D) *sptf-3::gfp* is expressed in the migrating distal tip cell (DTC) of the gonad (G,H) *sptf-3::gfp* is widely expressed in the hindgut and tail region throughout development (image is of a L4 animal). The expressing cells include P12.pa and the B cell.
Figure 4.5: *sptf-3*(gu85) and *sptf-3* RNAi animals exhibit an ectopic lumen posterior to the main vulval lumen or the bivulva phenotype. (A, B and C) Nomarski micrographs of the vulva of mid L4 animals. The animal in A is a wild-type control. The animal in B is a *sptf-3*(gu85) animal and the one in C is a *sptf-3* RNAi animal. The presence of the ectopic posterior lumen is indicated by the arrowhead.
Figure 4.6: Both P12.pa cell fate specification and B cell asymmetric division are affected in sptf-3(gu85) animals. (A,B,C,E,G,H) Nomarski micrographs of larvae. (D, F) Epi-fluorescent micrographs of the same animals as C and E respectively. (A,) wild-type animal showing normal P11.p and P12.pa cell fate specification as determined by nucleus size and location (cells marked by arrowheads). (B) sptf-3(gu85) animal that has two P11.p cells (marked by arrowhead). (C,D) In wild type animals, egl-5::gfp is expressed in several hindgut cells, including P12.pa. (E, F) In sptf-3(gu85) animals, hindgut (including P12.pa cell) egl-5::gfp expression is reduced (Exposure time in both D and F is 100ms). (G) In wild-type animals, the B cell divides asymmetrically. (H) In some sptf-3(gu85) animals, the B cell divides symmetrically.
Models for the mechanism by which SPTF-3 functions to affect Wnt dependent processes

**Figure 4.7:** In the first model, SPTF-3 specifically interacts with the Wnt pathway. It can either be a direct target of POP-1 or can itself regulate the expression of Wnt pathway members, such as *lin-17*. In the second model, SPTF-3 functions as a broad transcriptional activator, which controls the expression of multiple target genes, including that of the Wnt pathway targets. A lack of redundancy between the Wnt targets can account for the Wnt pathway phenotypes observed in *sptf-3(gu85)* animals.
Table 4.1: Expression pattern of sptf-3::gfp in animals. *sptf-3* expression is observed throughout larval development and adulthood. Specifically, consistent expression is observed in hindgut cells (F, U, B, K, K’ and P12,pa), Pn.p cells, vulva, intestine, head and tail cells. In addition, sporadic expression is observed in the DTC and the somatic gonad. All results in this table are shown as percentage of animals expressing *sptf-3*.

<table>
<thead>
<tr>
<th>Stage</th>
<th>Hindgut</th>
<th>Pn.p</th>
<th>Vulva</th>
<th>DTC</th>
<th>Somatic Gonad</th>
<th>Intestine</th>
<th>Head</th>
<th>Tail</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>L1-L2</td>
<td>100%</td>
<td>100%</td>
<td>N/A</td>
<td>0%</td>
<td>0%</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
<td>18</td>
</tr>
<tr>
<td>L3</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
<td>0%</td>
<td>23%</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
<td>17</td>
</tr>
<tr>
<td>L4</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
<td>10%</td>
<td>35%</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
<td>20</td>
</tr>
<tr>
<td>Adult</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
<td>0%</td>
<td>14%</td>
<td>100%</td>
<td>100%</td>
<td>95%</td>
<td>21</td>
</tr>
</tbody>
</table>
Table 4.2: *sptf-3*(*gu85*) and *sptf-3* RNAi animals exhibit an ectopic lumen posterior to the main vulva. (*) The full genotype used is *unc-119(e2498); guIs9*. (**) The full genotype used is *sptf-3*(gu85); *unc-119(e2498); guIs9*. Animals subjected to RNAi had the genotype: *unc-119(e2498); guIs9*.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Feed</th>
<th>% L4 animals with ectopic posterior lumen</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>+ *</td>
<td>-</td>
<td>0%</td>
<td>120</td>
</tr>
<tr>
<td><em>sptf-3</em>(gu85)**</td>
<td>-</td>
<td>19%</td>
<td>129</td>
</tr>
<tr>
<td>+ control</td>
<td></td>
<td>0%</td>
<td>31</td>
</tr>
<tr>
<td>+ sptf-3</td>
<td></td>
<td>13%</td>
<td>31</td>
</tr>
</tbody>
</table>

Table 4.2: *sptf-3*(*gu85*) and *sptf-3* RNAi animals exhibit an ectopic lumen posterior to the main vulva. (*) The full genotype used is *unc-119(e2498); guIs9*. (**) The full genotype used is *sptf-3*(gu85); *unc-119(e2498); guIs9*. Animals subjected to RNAi had the genotype: *unc-119(e2498); guIs9*. 
Table 4.3: *sptf-3*(gu85) animals that exhibit an ectopic posterior lumen have normal vulval induction. (*) The full genotype used is *unc-119*(e2498); *guIs9*. (**) The full genotype used is *sptf-3*(gu85); *unc-119*(e2498); *guIs9*.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Number of cells induced</th>
<th>Number of animals with an ectopic posterior lumen</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>+*</td>
<td>3.0</td>
<td>N/D</td>
<td>23</td>
</tr>
<tr>
<td><em>sptf-3</em>(gu85)**</td>
<td>3.0</td>
<td>4</td>
<td>20</td>
</tr>
</tbody>
</table>

Table 4.3: *sptf-3*(gu85) animals that exhibit an ectopic posterior lumen have normal vulval induction. (*) The full genotype used is *unc-119*(e2498); *guIs9*. (**) The full genotype used is *sptf-3*(gu85); *unc-119*(e2498); *guIs9*. 
<table>
<thead>
<tr>
<th>Genotype</th>
<th>WT</th>
<th>BIV</th>
<th>Abnormal</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>+</td>
<td>100%</td>
<td>0%</td>
<td>0%</td>
<td>120</td>
</tr>
<tr>
<td><em>lin-17(n671)/hT2</em></td>
<td>94%</td>
<td>0%</td>
<td>6%</td>
<td>31</td>
</tr>
<tr>
<td><em>lin-17(n671)/lin-17(n671)</em></td>
<td>46%</td>
<td>52%</td>
<td>2%</td>
<td>50</td>
</tr>
<tr>
<td><em>lin-18(e620)/lin-18(e620)</em></td>
<td>60%</td>
<td>40%</td>
<td>0%</td>
<td>30</td>
</tr>
<tr>
<td><em>sptf-3(gu85)/sptf-3(gu85)</em></td>
<td>79%</td>
<td>19%</td>
<td>2%</td>
<td>129</td>
</tr>
<tr>
<td><em>lin-17(n671), sptf-3(gu85)/hT2, sptf-3(gu85)</em></td>
<td>58%</td>
<td>42%</td>
<td>0%</td>
<td>23</td>
</tr>
<tr>
<td><em>sptf-3(gu85), lin-18(e620)</em></td>
<td>31%</td>
<td>68%</td>
<td>1%</td>
<td>71</td>
</tr>
</tbody>
</table>

*Table 4.4:* Reducing the dose of *lin-17* by half enhances the *sptf-3(gu85)* bivulva phenotype.
Table 4.5: *sptf-3*(*gu85*) mutants exhibit reversals in the P7.p lineage. This table was formatted according to Innoue et al., 2004. *sptf-3*(*gu85*) animals were scored as either normal or bivulva. Within the bivulva category, marker expression was recorded. P7.pa(+) indicates that at least one P7.pa daughter cell is expressing the markers. P7.pa(-) indicates that none of the P7.pa daughter cells are expressing the markers. P7.pp(-) indicates that none of the P7.pp daughter cells are expressing the markers. P7.pp(+) indicates that at least one P7.pp daughter cell is expressing the marker. The last column represents the rarest and most extreme case of P7.p reversals.

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Marker</th>
<th>Normal</th>
<th>Biv</th>
<th>Biv</th>
<th>Biv</th>
<th>Biv</th>
</tr>
</thead>
<tbody>
<tr>
<td>+</td>
<td>egl-17::gfp</td>
<td>23</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>sptf-3(<em>gu85</em>)</td>
<td>egl-17::gfp</td>
<td>60</td>
<td>14</td>
<td>2</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>+</td>
<td>cog-1::gfp</td>
<td>30</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>sptf-3(<em>gu85</em>)</td>
<td>cog-1::gfp</td>
<td>88</td>
<td>5</td>
<td>11</td>
<td>0</td>
<td>4</td>
</tr>
</tbody>
</table>
Table 4.6: *sptf-3(gu85)* animals exhibit defects in asymmetric B cell division. Images of animals showing the loss of asymmetry are shown in Figure 7. (*) Full genotype is *unc-119(e2498); guIs9; him-5(e1490)*. (**) Full genotype is *sptf-3(gu85); unc-119(e2498); guIs9; him-5(e1490)*.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>% WT Ba&gt;Bp</th>
<th>% Loss of asymmetry Ba=Bp</th>
<th>% Reversal of asymmetry Ba&lt;Bp</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>+*</td>
<td>100%</td>
<td>0%</td>
<td>0%</td>
<td>23</td>
</tr>
<tr>
<td><em>sptf-3(gu85)</em>*</td>
<td>76%</td>
<td>19%</td>
<td>5%</td>
<td>41</td>
</tr>
</tbody>
</table>

*Table 4.6: sptf-3(gu85) animals exhibit defects in asymmetric B cell division. Images of animals showing the loss of asymmetry are shown in Figure 7. (*) Full genotype is unc-119(e2498); guIs9; him-5(e1490). (**) Full genotype is sptf-3(gu85); unc-119(e2498); guIs9; him-5(e1490).*
Table 4.7: *sptf-3*(gu85) animals exhibit defects in P12.pa development and decreased expression of *egl-5::gfp* in that cell. N1 is the number of animals scored for P12.pa development and N2 is the number of animals scored for *egl-5::gfp* expression in the P12.pa cell.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>% WT animals</th>
<th>% animals with 2 P11.p</th>
<th>N1</th>
<th>%P12.pa cells expressing <em>egl-5::gfp</em></th>
<th>N2</th>
</tr>
</thead>
<tbody>
<tr>
<td>+</td>
<td>100%</td>
<td>0%</td>
<td>44</td>
<td>94%</td>
<td>34</td>
</tr>
<tr>
<td><em>sptf-3</em>(gu85)</td>
<td>92%</td>
<td>8%</td>
<td>87</td>
<td>56%</td>
<td>92</td>
</tr>
</tbody>
</table>
5.1. INTRODUCTION

The studies conducted in the preceding chapters as my part of my dissertation extend and elaborate on how a Pax factor can influence developmental decisions by regulating different target genes in different organs. They also provide the first report on how a Sp1-related transcription factor functions in C. elegans development. Finally, they provide evidence for the interaction of Sp1-related transcription factors with the Wnt pathway. In this chapter, I will discuss the implications of these studies as well as the conclusion that can be drawn for the functions of these transcription factors in organogenesis.

5.1. Alterations to a Pax protein DNA binding domain can affect its function in a tissue-preferential manner

In chapter 2 of this dissertation, I show that induced expression of the different
egl-38 DNA binding domain mutant alleles has different effects on embryo survival. For example, ectopic expression of wild-type egl-38 or the egl-38 (sy287) allele causes embryonic lethality, whereas ectopic expression of egl-38(n578) has no effect. These data, along with the loss of function characterization of the several alleles and functions of egl-38, demonstrate that the alleles that affect the DBD of EGL-38 can preferentially affect its activity in certain functions, but not others (Zhang et al., 2005). I also show that this ectopic expression of wild-type egl-38 can induce ectopic expression of its target gene, lin-48. This ectopic expression was restricted to the posterior ectoderm of the embryos. One explanation for this restricted ectopic expression of lin-48 is the presence of the EGL-38 cofactors necessary for lin-48 expression in the posterior ectoderm and their absence from the rest of the embryo.

DNA binding affinity is a key component of transcription factor function. Results with EGL-38 indicate that mutations in the DNA binding domain of Pax proteins can alter their DNA binding and their in vivo activity (Zhang et al., 2005). The results with EGL-38 suggest one of two possible models to explain the tissue-preferential effects. One possibility is that the different mutant alleles affect the ability of EGL-38 to bind to certain sequences, but not others. This model predicts that either there is one or a few key targets in each cell type, or that there are specific response element sequences that are associated with all the targets for EGL-38 in a particular cell type. An alternative model is that, although the mutations affect DNA binding, their tissue preferential effects result from their impact on EGL-38 function in combination with other proteins that influence target sequence recognition in vivo. This model predicts that there would be cell-specific co-factors that function with EGL-38 and
participate in identifying which sequences correspond to an appropriate DNA binding site or that enhance EGL-38 transactivation activity. It also suggests that the interaction between the co-factor and EGL-38 would be mediated, or otherwise affected, by the EGL-38 DBD. Although we favor the second model, clearly distinguishing between the two will require identification of proteins that act with EGL-38 and a large collection of cell-specific EGL-38 target genes.

5.1.3. Genetic Screen to identify cofactors for EGL-38

In an attempt to identify the cell specific EGL-38 cofactors, I designed a genetic screen. This genetic screen utilized the hindgut specific EGL-38 target, lin-48. The lin-48 regulatory sequences contain two elements (lre1 and lre2) necessary for its hindgut expression (Johnson et al., 2001). These two elements are sensitive to egl-38 function in vivo. In our screen, we utilized animals that carry lin-48::gfp transgenes that have a mutant lre2 sequence and intact lre1 sequence. As a result, the entire hindgut lin-48 expression is mediated through the lre1 element. By screening for mutant animals that did not have hindgut GFP expression, I hoped to identify the hindgut-specific EGL-38 cofactors, as well as cofactors that are specific to lre1. This screen identified two separate genes, lex-3 and sptf-3. The molecular identity of the lex-3 gene remains unknown. SPTF-3 is a C. elegans Sp1-related factor.

SPTF-3 is proposed to function as a transcription factor. Contrary to our expectations, we could not detect an interaction between SPTF-3 and EGL-38 in vitro nor in vivo. This result does not necessarily argue against the hypothesis that there cell-
specific co-factors that function with EGL-38 and participate in identifying which sequences correspond to an appropriate DNA binding site or that enhance EGL-38 transactivation activity. One reason we did not identify such factors is because we did not saturate our screen. In addition, even though we attempted to identify lre1 specific EGL-38 cofactors, our screen could potentially target genes that regulate egl-38 expression or the expression of other transcription factors that themselves regulate EGL-38 cofactor gene expression. Moreover, our screen could potentially target genes specifically involved in regulation of transgene expression as well as chromatin remodeling factors.

### 5.1.4. sptf-3 functions in Wnt-mediated processes

The above described screen identified SPTF-3 as important for expression of lin-48 in the hindgut of *C. elegans*. Since no reports described the role of *sptf-3* or any of its family members in *C. elegans* development, we were interested in characterizing its functions. We found that it promotes the development of several tissues. For example, it is involved in the polarization of the P7.p vulval lineage, B cell asymmetric cell division and P11/P12 cell fate specification. All these processes are also dependant on proper Wnt signaling. In addition, we showed a genetic interaction between *sptf-3* and *lin-17/frizzled* in vulval development. An interaction between the Wnt pathway and Sp1-related transcription factors has been reported in vertebrates. There is strong evidence that these factors are targets for TCF, the main effector of Wnt signaling, and that they mediate Wnt function during development and disease progression (Treichel *et*
al., 2003; Weidinger et al., 2005; Takahashi et al., 2005; Thorpe et al., 2005; Fujimura et al., 2007). In addition, there is some correlative evidence that these factors may regulate the expression of different frizzled receptors in mammals (Katoh and Katoh, 2007a; Katoh and Katoh, 2007b). Our results demonstrate a genetic sensitivity of *sptf-3* mutants to the dose of *lin-17*. We can speculate that SPTF-3 can function in the Wnt pathway through regulation of the expression of this gene, especially since all the Wnt dependent processes that SPTF-3 mediates require LIN-17 function. Another alternative that remains to be explored is the potential function of *sptf-3* as a broad genetic enhancer or an enhancer of compromised signaling pathways.

**5.2 CONCLUSIONS**

As part of my dissertation, I have examined how a Pax factor mediates tissue-specific functions by modulating the expression of different target genes in different cells. I have also studied the role of *sptf-3* in organ development and its interaction with the Wnt pathway.

With regards to the identification of EGL-38 cofactors, it remains important to clone the gene affected by the *gu84* mutation. This might lead to the identification of an EGL-38 cofactor that interacts with it to regulate tissue specific expression of *lin-48*. Moreover, additional biochemical experiments, such as EGL-38 immunoprecipitation followed by protein sequencing, may identify these cofactors. This approach is complicated by the lack of an EGL-38 antibody and the unavailability of target gene disruption methods that would allow for epitope tagging of the endogenous *egl-38*
With regards to the role of sptf-3 in animal development, it remains important to determine whether SPTF-3 is broad genetic enhancer or whether it specifically affects Wnt function. Even though our data suggest that SPTF-3 promotes Wnt dependent decisions, it remains unclear how SPTF-3 affects the Wnt pathway. It could either interact specifically with the Wnt pathway by regulating Wnt pathway member gene expression or by itself being a target for regulation by POP-1. On the other hand, it could function as a genetic enhancer that interacts with many signaling pathways. The sensitivity of the Wnt pathway to SPTF-3 function in this case could be explained by a possible lack of redundancy in Wnt target gene expression as compared to that of other pathways. Deciphering the difference between these possibilities will help us better understand how sptf-3 functions in animal development.
LIST OF REFERENCES


122


