POSITIONAL CLONING AND FUNCTIONAL ANALYSIS
OF THE SF3B1 GENE IN ZEBRAFISH

DISSERTATION

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By

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ABSTRACT

Zebrafish toast<sup>b460</sup> (tst) is a recessive embryonic lethal mutation, characterized by visible defects in both neural crest and blood development. tst is involved in the neural plate border development in zebrafish embryos, resulting in the complete lack neural crest derivatives and a decrease in the number of Rohon-Beard sensory neurons. In addition, lateral plate mesoderm development is also affected in tst mutants, as development of most of its derivatives are disrupted during hematopoiesis, vasculogenesis, and cardiogenesis. Other ectodermal and mesodermal derivatives develop normally in tst mutants.

The tst locus has been positionally cloned and encodes splicing factor 3b subunit 1 (sf3b1). Sequence analysis between wild-type and homozygous mutant genomic DNA identified the nucleotide mutation at 5′ splicing site in the 4th intron of sf3b1 genomic DNA where T is changed to G in the tst mutant gene. The nucleotide change causes abnormal splicing in tst homozygotes with variant transcripts. Normal sf3b1 transcripts are detected in tst mutants, whereas the other abnormal transcripts result in truncated proteins due to introduction of pre-mature stop codons. The truncations are predicted to occur in the extreme N-terminal region of the protein, thus eliminating essential functional regions and are therefore predicted to be non-functional. The presence of normal transcripts and the more severe phenotype of sf3b1 morphants compared to tst mutants indicate that the tst mutation is hypomorphic.
Zebrafish genome analysis shows that sf3b1 is a single-copy gene. The amino acid sequence of the zebrafish homolog of Sf3b1 protein is highly conserved among different species. It is expressed ubiquitously during early embryonic development in zebrafish.

Studies of interactions between sf3b1 and key transcriptional regulators of neural crest development and hematopoiesis reveal that deficiency of sf3b1 function not only causes disruption in the expression levels of genes required for sublineage fate specification, but also results in abnormal pre-mRNA splicing of some of these genes. As a result, the survival, migration, and differentiation of cells derived from both neural plate border and lateral plate mesoderm are severely disrupted. These results demonstrate that the ubiquitous and essential pre-mRNA processing gene sf3b1 is required to different degrees by specific embryonic cell populations during development. Thus, while some genes are required by all cell types, the timing and degree of the requirement for such "housekeeping" genes differs for specific embryonic cell population during embryogenesis.
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CHAPTER 1

INTRODUCTION

The neural crest is a distinct embryonic cell population that arises at the neural plate border between the neural and non-neural ectoderm (Fig. 1.1A). It is a cell population uniquely found in all vertebrate embryos. During neurulation, the cells in the neural plate border bend to form neural folds and are eventually located within the dorsal portion of the neural tube. Subsequently, neural crest cells undergo an epithelial to mesenchyme transition (EMT), delaminating from the neuroepithelium and migrating far and wide throughout the embryo along different pathways. These cells then differentiate into a broad array of cell types, including neurons and glia of the peripheral nervous system (PNS), pigment cells, craniofacial cartilage and skeleton, chromaffin cells of the adrenal gland, smooth muscle of the heart, and other cell types. Because of its diversity of derivatives and the numerous processes involved in the regulation of its development, including embryonic induction, cell fate specification, migration, proliferation, differentiation and cell survival, the neural crest is a informative system for studying molecular mechanisms of development. Recent advances have been made by scientists on the role of different signals during neural crest development although the regulation of neural crest development remains incompletely understood.
Similar to the neural crest, lateral plate mesoderm also involves multiple development mechanisms of cell diversification and thus provides another excellent system for studying embryonic development. toast$^{b460}(tst)$ plays key roles not only in neural crest development, but also in the hematopoiesis, vasculogenesis and cardiogenesis. Therefore, through identification and functional analysis of the gene that is disrupted in $tst$ mutant embryos, we may elucidate important molecular mechanisms regulating neural crest and lateral plate mesoderm development and thus potentially provide genetic insights into birth defects related to these tissues.

1.1 Neural plate border induction and neural crest formation and development

Neural crest induction is a multi-step process which starts at early gastrula stages. Embryological and molecular evidence from experiments carried out in *Xenopus*, chick, and mouse shows that neural crest progenitors are induced at the neural plate border. During gastrulation, the epidermal ectoderm secretes bone morphogenetic proteins (BMPs), which promote epidermal fate and suppress neural fate. Concurrently, the dorsal mesoderm secretes BMP antagonists, such as Noggin and Chordin, which directly bind to BMPs and prevent signaling from their receptors. Therefore, the neural ectoderm is induced as a default ectodermal fate as a result of blocking BMP signals [126, 129, 140]. The ectoderm is patterned by a gradient of BMP activity, in which high BMP activity specifies epidermal fate, low activity specifies neural fate, and intermediate activity specifies the neural plate border from which neural crest cells arise (Fig. 1.1B, see [129]). Zebrafish mutational analysis suggests an important role for the different components of BMP pathway in neural crest induction,
such as \textit{bmp2b} (\textit{swirl}, see [140]), \textit{bmp7} (\textit{snailhouse}; see [139]), and \textit{smad5} (\textit{somitbun}, see [178]).

Figure 1.1: A: Diagram of neural crest formation (adapted from Nat Rev Genet. 2002 Jun; 3(6):453-61). B: Patterning of ectoderm by BMP morphogen gradient.

Cells in the neural plate border are a heterogeneous population. The derivatives of a single labeled cell in the neural plate border can develop into different cell types, including neural crest cells, central nervous system (CNS) neurons or epidermal cells [34, 183]. Further, experiments carried out in \textit{Xenopus} show that intermediate levels
of BMP signaling alone can not induce neural crest in *Xenopus* ectodermal explants. This suggests that although an intermediate level of BMP signaling is an essential step for neural crest induction, additional signals are also required for neural crest induction. These additional signals required for neural crest induction have been identified as FGFs, Wnts, and retinoic acid (RA) from the epidermis and/or paraxial mesoderm [98, 208]. Based on experiments carried out in *Xenopus*, these signals work as posteriorizing signals that pattern the anterior-posterior axis of the neural fold. Thus, LaBonne and Bronner-Fraser (1998) propose a two-signal model of neural crest induction [98]. Initial neural crest induction is dependent on a BMP activity gradient which is established in the ectoderm. The neural plate border that is induced by the BMP activity gradient has anterior character. Then, posteriorizing signals transform the induced anterior neural plate border into neural crest progenitors [98]. Blocking FGF function by injection of dominant negative FGF receptor did prevent the expression of neural crest specific genes [125]. *Xenopus* animal cap assays suggest that FGF-8 is sufficient to induce the expression of neural crest specific marker genes. Overexpression of either *Wnt-1* or *Wnt-3a* expand the neural crest domain of the ectoderm and increase the number of neural crest cells [173]. Other experiments in chick and *Xenopus* also suggest that Wnt signaling is required for neural crest formation. Therefore, the requirements for Wnt, FGF, and retinoic acid during neural crest induction indicate that posteriorizing signals are involved in neural crest induction.

As a consequence of extracellular signaling on the ectoderm, a set of genes, *Msx* (muscle segment homeobox genes), *Dlx* (Distal-less), and *Zic* (odd-paired homolog, *Drosophila*) family genes are activated in cells of the neural plate border (Fig. 1.2)
Msx and Dlx genes are initially expressed in the non-neural ectoderm, and later they are restricted to the neural plate border in chick, Xenopus, and zebrafish. Studies in Xenopus suggested that the expression of Msx1 is regulated by intermediate levels of a BMP gradient and Msx1 can enhance the expression of some neural crest regulatory genes, such as snail, slug and Foxd3 [202]. If Msx1 expression is blocked, the expression of these genes is reduced consequently. In zebrafish, msxBCE morphants display down regulated expression of some neural crest regulatory genes, such as snai1b and sox10, whereas, expression of foxd3 is normal. Subsequently, deficiencies in neural crest cell migration and defects in the differentiation of neural crest derivatives including melanocytes and cranial facial cartilages occur [152]. Although the effects of blocking Msx1 and msxBCE in Xenopus and zebrafish respectively differ in the transcription factors affected, disruption in the development of neural crest derivatives such as melanocytes in both species supports a conserved role for Msx family genes in neural crest development. Dlx genes have been shown to play important roles in patterning the ectoderm, including neural crest and placodal cells. Studies in zebrafish suggest a mutual antagonism between MsxB and Dlx proteins, indicating that these two genes play key roles in positioning the neural plate border [152]. Zic is expressed in a more restricted region of the ectoderm than Msx and Dlx genes, in the neural plate and neural plate border [64, 199]. Zic family genes encode transcription factors that contain zinc finger domains. Experiments carried out in Xenopus embryos demonstrated that Zic family genes, except Zic4, can mediate neural crest development by inducing the expression of critical transcription factors in neural crest, such as slug [136, 137, 135].
As neural crest cells are induced at the neural plate border, expression of key transcriptional regulators of further neural crest development are activated (Fig. 1.2). Through gain- and loss-of-function experiments in chick, *Xenopus*, mouse, and zebrafish, researchers have found that different classes of transcription factors, including *Fox*, Activator Protein 2 (*AP2*), *Snail*, and *Sox* families, play important roles during neural crest development [100, 145, 174, 155, 192, 132, 96, 95, 11, 144, 112, 141, 195, 26, 30, 47, 29, 223]. However, the precise function and hierarchical relationships between these regulators remain unclear.

*foxd3* (forkhead box transcription factor D3) is a forkhead domain transcriptional repressor [174]. It is characterized by an evolutionarily conserved winged helix DNA-binding domain and was originally referred to a *Genesis* and *Hfh2* in mouse [100], *CWH3* in chick [97], *XFD6* in *Xenopus* [174, 155], and *fkd6* in zebrafish. The expression of its homologs is conserved across different species including *Xenopus*, zebrafish and mice [174, 145, 100]. During late gastrulation and early segmentation, *foxd3* is expressed in the presumptive neural crest domain of the ectoderm and in premigratory neural crest cells. Later, *foxd3* expression in post migratory neural crest cells is restricted to presumptive satellite glial cells associated with cranial ganglion neurons and dorsal root ganglion (DRG) neurons [155, 89]. In chick embryos, *foxd3* has been implicated in establishing the neural crest melanophore sublineage [97]. This result is also supported by the loss- and gain-of-function experiments carried out in *Xenopus* [174]. Sasai et al. (2001) also propose that *slug* induction by Zic factors requires *Foxd3*-related signaling, but no evidence suggests that *slug* is needed for *Foxd3* induction. However, a different group has shown that overexpression of *Foxd3* inhibits neural crest formation and migration [155]. The difference in the results of these
studies may be due to differences in the timing and concentrations of Foxd3 mRNA injection: early with high dose injection expanded the neural plate at the expense of neural crest [155], but later with low dose injection induced the expression of specific genes of both populations [174]. In zebrafish, expression of foxd3 in premigratory neural crest cells overlaps with another neural crest marker, snai1b [195], formerly snail-2, and crestin [119], and it is down-regulated before migration. Mutational analysis and knock down analysis of foxd3 in zebrafish embryos indicate that it may not be required for neural crest induction, but it is required for subsequent regulation of neural crest cell fate specification and survival. Disruption of foxd3 function causes defects in most neural crest sublineages, such as cranial cartilages, DRG neurons, glia, and enteric neurons whereas chromatophore development is largely unaffected [192, 113].

The AP2 (tfap2, activator protein 2) family of genes encode transcription factors that contain a highly conserved DNA-binding domain [73], Several members of the AP2 family of genes, Tcfap2a, b, c and g have been found in mouse and human. The homologs of these genes are TFAP2a in Xenopus, and tfap2a, 2b in zebrafish. The transcription factor AP2 is implicated in the regulation of the genes involved in a variety of important developing tissues, including keratinocyte specific genes, tyrosine kinase receptor gene C-KIT [79], transforming growth factor-a [211], and c-myc [57]. The expression of AP2a is highly conserved among different vertebrates. It is initially expressed in the non-neural ectoderm, then becomes gradually restricted to the neural plate border, and later in migrating neural crest cells in Xenopus, chick and zebrafish [121, 144]. Mutant analysis in mice has shown that the elimination of Tcfap2a function causes the defects in development of the cranial neural tube,
anterior limb, body wall and craniofacial skeleton and cranial ganglia, the latter being in part derived from the neural crest. Loss-of-function analysis by morpholino knockdown of AP2α activity in Xenopus suggests that AP2α plays a key role during early neural crest cell fate specification [120]. Studies in zebrafish tfap2a mutants (lockjaw and mont blanc) and tfap2a morphants indicate that the deficiency of tfap2a causes defects in early neural crest cell fate specification and survival as well as in the later differentiation of neural crest derivatives, such as craniofacial cartilages, melanophores, and sympathetic neurons [96, 95, 11]. Thus, AP2α is also a key neural crest regulator that plays critical roles during early and late neural crest development.

The function of Snail family genes has been most thoroughly analyzed in Xenopus and chick embryos [112, 9]. Slug and Snail are members of the Snail family of zinc-finger transcriptional repressors related to Drosophila snail [51, 126]. As neural crest markers, these genes are expressed specifically in the premigratory and migrating neural crest in Xenopus [112], chick [141], zebrafish [195] and mouse embryos [26]. In chick and Xenopus, loss- and gain-of-function experiments have shown that snail and slug play very important roles during neural crest development, including cell fate specification and migration [99, 42, 9, 29]. In Xenopus, snail is the earliest neural crest marker that is expressed in the neural plate border, before other neural crest specific genes are detected, such as slug and Foxd3 which are expressed in the prospective neural crest domain in Xenopus [112, 9, 174, 155], and functions upstream of these transcription factors in the hierarchy of neural crest specification. However, there is no evidence of a similar hierarchical relationship in other vertebrates, including zebrafish.
Sox proteins are transcriptional activators and are involved in several processes during embryogenesis [207, 220, 91, 40, 47, 29, 223]. The members of the Sox family have been classified into A to H groups [18]. Among these genes, some Sox genes are expressed in neural crest progenitors at some time point following neural crest induction, such as Sox8, Sox9, and Sox10 which comprise the SoxE group [213, 106, 30, 47, 187, 28, 5, 146]. However, the temporal and spatial expression pattern of these genes in different species, such as mouse, chick, zebrafish and Xenopus are slightly different (Fig 1.2.) [106, 30, 47, 187, 28, 5, 146]. Sox9 and Sox10 are the Sox family genes that have been studied the most in neural crest development. In human, there are two congenital diseases, campomelic dysplasia and Waardenburg-Shah syndrome that are associated with Sox9 and Sox10, respectively [154, 148, 160]. Patients with Waardenburg-Shah syndrome have hypopigmentation of skin, aganglionic megacolon, deafness, and severe demyelination. Mutant analysis in mice suggests that elimination of Sox9 activity causes dramatic cell death in the trunk neural crest region before and after delamination [29] and loss of Sox10 function results in dominant defects similar to the Waardenburg-Shah syndrome, primarily in neural crest derived peripheral neurons, glia and melanocytes [70]. Zebrafish sox10 homozygous mutants, colourless (cls), show a similar phenotype to mouse Sox10 null mutants [47]. Single-cell analysis of neural crest cells in cls-/- mutants indicates that sox10 plays a critical role in neural crest cell fate specification, including subsets of all peripheral neurons, glia and pigment cells. In zebrafish, there are two orthologs of sox9, sox9a and sox9b probably due to genome duplication [30]. sox9b is expressed in the presumptive neural crest domain of the ectoderm as early as other early neural crest specific genes, such as foxd3. Zebrafish sox9b^{J971} terminal deletion mutants showed
a similar phenotype to human campomelic dysplasia [209], a curly-down body axis and cranial pharyngeal cartilage defects. However, sox9a<sup>hi1134</sup> mutants have an even more severe phenotype in cranial pharyngeal cartilages suggesting that sox9a may play a more important role in cranial pharyngeal cartilage development than sox9b [223]. In addition, in double mutants of sox9b-/- and sox9a-/- all the cranial cartilages are absent. Mutant analysis and misexpression showed potential roles of sox9 co-orthologos in the regulation of other early neural crest regulatory genes, including foxd3, snai1b, sox10, and crestin [223].

Zebrafish crestin is expressed in all or most premigratory and migrating neural crest cells and its expression overlaps with other neural crest markers, such as foxd3 and tfap2a in zebrafish embryos [171, 119, 90]. It is a specific pan-neural crest marker gene from specification of the neural crest domain of the ectoderm to the overt differentiation of neural crest derivatives. The expression of crestin is dramatically reduced in several zebrafish mutants that affect neural crest development [11, 96, 95, 192, 132]. Zebrafish crestin is a retroelement and thus is likely non-functional. However, it is used extensively as a neural crest marker.

In summary, neural crest formation, cell fate specification and differentiation are complex processes that are controlled by a large number of key regulatory genes, including Dlx, Msx, Zic, Fox, AP-2, Sox, and Snail family genes. Although the study of these genes and their function during neural crest induction, specification, differentiation has begun to emerge, hierarchical relationships between these known genes remain unclear. Many gaps due to uncharacterized novel genes in these genetic circuits that coordinate such complex developmental processes need to be filled.
Figure 1.2: Several families of transcription factors are expressed in overlapping domains in the ectoderm. Shortly after neural crest induction, these factors are either limited to the neural crest tissue (red), or their domains of expression also extend into the non-neural ectoderm (orange), or into the neural plate (purple), or spans all three regions of the ectoderm (green). When available, the neural crest expression of these genes in mouse (M), Chick (C), Xenopus (X), or zebrafish (Z) embryos is indicated. From Saint-Jeannet and Huang, Dev. Biol. 274(2004) 1-11

### 1.2 Lateral Plate Mesoderm Development

Lateral plate mesoderm gives rise to the circulatory system including blood cells, blood vessels, and the heart which provides nourishment necessary for the developing vertebrate embryo (Fig. 1.3 and Fig. 1.4). Thus, the production and maintenance of this tissue is essential for vertebrate health and survival. Defects during
hematopoiesis, vasculogenesis and cardiogenesis cause many congenital diseases. An understanding of developmental mechanisms involved in lateral plate mesoderm development may lead to an understanding of the pathology of many genetic diseases.

During gastrulation, members of the TGF-β-related BMP family play critical roles in mesodermal dorsal-ventral axis patterning and are well conserved in vertebrates (reviewed in [62, 80]). The lateral plate mesoderm is segregated from other mesodermal tissues by this dorsal-ventral patterning and gives rise to different cell types during embryonic development (Fig. 1.4). Studies in a variety of model systems have shown that injection of BMP antagonists from the organizer that block BMP function causes a reduction in intermediate and lateral plate mesodermal cell fate and an expansion of dorsal mesodermal cell fate [68, 138, 94]. In contrast, the inhibition of BMP antagonists causes an expansion of ventral mesoderm (lateral plate mesoderm) including blood cells [67].

Figure 1.3: Diagram of mesoderm (A, From Gilber S.F: 1996, Developmental biology 6th) and fate map of mesoderm (B).
Zebrafish hematopoiesis has been shown to be very similar to that of mammals and other vertebrates, with the formation of analogous blood cell types, including erythroid, myeloid, and lymphoid lineages. Primitive hematopoiesis in zebrafish occurs in different regions compared with mammals (extraembryonic yolk sac blood island): the intraembryonic locations in the trunk, called the intermediate cell mass (ICM), and in the anterior lateral plate mesoderm of embryos [43]. The ICM contains primitive hematopoietic precursor cells and is divided into two regions, the anterior trunk domain and the posterior blood island. Cells from the anterior ICM migrate anteriorly and populate the dorsal mesentery (DM) before entering the circulation [43]. Later, some cells exit from the DM to the yolk sac and differentiate into erythrocytes and establish the embryonic circulation around 24 hpf [43]. The anterior
lateral plate mesoderm is the site of origin for early development of myeloid precursor cells including macrophages, granulocytes and other blood cell types [71, 110]. Like other vertebrates, zebrafish hematopoiesis also occurs in two phases, primitive and definitive phases. However, the sites of hematopoiesis are not well conserved during vertebrate development (reviewed in [10]). Definitive hematopoiesis in zebrafish embryos occurs in pronephros in larvae and kidney in adult fish. In contrast, in mammalian definitive hematopoiesis occurs in the dorsal mesentery (aorta, gonad, mesonephros region, AMG) and liver in the fetus, and bone marrow in adults.

A number of transcription factors have been shown to have important roles during hematopoiesis by both expression studies and mutant studies. These genes include \textit{gata-1}, \textit{gata-2}, \textit{scl}, \textit{lmo2}, and \textit{flk-1} (reviewed in [41]). A study in mice homozygotes for a \textit{gata-2} null mutation has shown that \textit{gata-2} is required for the proliferation of hematopoietic progenitor cells and it is also expressed in endothelial cells [203]. Erythroid cells originally arise from posterior lateral plate mesoderm. \textit{gata-1} is expressed primarily in erythrocytes and megakaryocytes and is essential for erythrogenesis based on mouse knockout experiments [186]. In zebrafish, the homologs of these genes have been identified. Transcription factors \textit{scl}, \textit{lmo2}, and \textit{gata-2} that drive key aspects of embryonic hematopoiesis are expressed in the lateral plate mesoderm. Later in embryonic development, the cells in the posterior lateral plate mesoderm converge on the midline and form the ICM [43]. In addition, several lines of evidence from zebrafish and mice indicate that hematopoietic precursors and vascular endothelial precursors share common signal transduction molecules during development and are consistent with the notion of a common precursor for hematopoietic and endothelial cells, the hemangioblast [108, 31]. These hemangioblasts give rise to angioblasts, the
precursors of vascular endothelial cells, and to pluripotential hematopoietic precursor cells [108, 31]. Loss- and gain-of-function analysis in mouse and zebrafish indicate that *gata-2, scl, lmo2* are required for all hematopoietic sublineage development and endothelial development in vertebrates, and support the possibility that these genes mark the hemangioblasts [203, 212, 221, 45, 228].

Other molecules play important roles during other specific blood sublineage development. *PU.1* is a transcription factor that plays a critical role during formation of macrophages and granulocytes, known as myelopoiesis [127]. These cells comprise the early immune system which responds to bacterial infection and cellular debris in the body. *PU.1* is expressed in myeloid precursor cells at the anterior lateral plate mesoderm in zebrafish and it marks the rostral site of early myelopoiesis [110]. Rhodes et al. (2005) suggest that common myelo-erythroid progenitors present in zebrafish are functionally equivalent with mammalian common myeloid progenitors [166]. The autoregulation and reciprocally antagonistic gene regulation between *gata-1* and *PU.1* determine the myelo-erythroid progenitor cell fate [166]. For example, blocking translation of *PU.1* causes ectopic expression of *gata-1* in anterior lateral plate mesoderm and knockdown of *gata-1* results in the expansion of *PU.1* expression in the anterior lateral plate mesoderm and posterior lateral plate mesoderm. Experiments in mice and zebrafish suggest that *gata-1* reprograms myeloid cells to undergo erythroid and megakaryocyte differentiation [151, 84, 55]. Conversely, misexpression of *PU.1* does not lead to excessive myelopoiesis in the anterior lateral plate mesoderm and ectopic myelopoiesis in the posterior lateral plate mesoderm, or dramatically inhibit erythropoiesis in posterior lateral plate mesoderm [166].
All of these studies support the existence of common myelo-erythroid progenitors in zebrafish embryos. Several additional genes have been identified that are expressed in zebrafish myeloid lineage cells, including \textit{cfms} and \textit{l-plastin} expressed in macrophages [71, 147], and \textit{mpo} and \textit{c/ebp1} expressed in granulocytes [122, 109]. Lymphoid development is also conserved between zebrafish and other vertebrates. Zebrafish orthologs of several genes required for lymphoid development are expressed in the same spatio-temporal pattern as those found in higher vertebrates [200].

As noted above, vascular and hematopoietic cells are thought to share transient common precursors, hemangioblasts. During early somitogenesis, several transcription factors, such as \textit{scl}, \textit{lmo2} and \textit{flk-1} are expressed in presumptive hemangioblasts in the lateral plate mesoderm (reviewed in [41]). As development proceeds, specific genes start to be expressed in endothelial progenitors that distinguish themselves from hematopoietic progenitors. Vascular endothelial growth factor (VEGF) ligand and their receptors, the VEGFR tyrosine kinase (\textit{flt-1}, \textit{flk-1}), have been identified in zebrafish [53, 107]. In mammals, the VEGF signaling pathway plays an essential role during embryonic vasculogenesis and adult vascular morphogenesis [130, 19]. VEGF not only plays a central role in endothelial cell differentiation, but also in hematopoiesis based on experiments carried out in zebrafish [107]. These gene expression and mutant studies in different model systems suggest that molecular mechanisms of vasculogenesis are highly conserved across species.

The heart is the first organ to form and function during vertebrate embryogenesis. The embryonic heart consists of an outer muscular (myocardial) layer and an inner endothelial (endocardial) layer. Myocardial and endocardial cells both originate from bilateral populations of the anterior portion of the lateral plate mesoderm close to
the midbrain-hindbrain boundary (MHB; see [162]). During early somitogenesis, myocardial precursors, along with other cell types in the lateral plate mesoderm, converge on the midline [188, 189]. Based on zebrafish mutation studies, four genes, \textit{gata-5}, \textit{fgf8}, \textit{bmp2b}, and \textit{oea} have been shown to play critical roles in the initial induction of \textit{nkh2.5} expression, a transcription factor that is considered as a marker gene of precardiac mesoderm in many vertebrates [164, 165, 162]. In zebrafish, most, but not all of the \textit{nkh2.5} expressing-cells ultimately differentiate into myocardiocytes [225]. Only \textit{nkh2.5} expressing-cells that are anterior to the top of the notochord initiate the expression of the genes \textit{cmlc1} and \textit{cmlc2} that encode cardiac sarcomere proteins [164, 225]. During embryonic development, two heart primordia start to fuse and initiate heartbeats around 24 hpf in zebrafish embryos [225]. After the completion of somitogenesis, cardiac looping starts, then two heart chambers including ventricle and atrium form under the control of different genes, such as \textit{nkh2.5}, \textit{tbx5}, and \textit{hand2} [27, 104, 12, 226]. Compared with the extensive work on myocardial induction and differentiation discussed above, endocardial induction and differentiation are less well understood in zebrafish and other model systems.

Although advanced studies of hematopoiesis, vasculogenesis and cardiogenesis carried out in a variety of vertebrate model systems and human genetic diseases lead us to understand molecular and cellular pathways of regulating aspects of their development, many fundamental questions about lateral plate mesoderm development, such as mesoderm patterning, specification of different cell types in lateral plate mesoderm, regulation of their proliferation, survival and terminal differentiation, still remain unclear. To date, more and more zebrafish mutants with different blood, heart, or vascular phenotypes have been isolated based on large-scale mutation screening
Analysis of these mutants will identify a number of genes (novel or known genes with unknown function) involved in these developmental processes and should bring exciting new insights into vertebrate lateral plate mesoderm development.

1.3 Splicing Factor 3b Subunit 1 (sf3b1)

The removal of introns from pre-mRNA is an essential component of pre-mRNA processing. The precise excision of introns of pre-mRNA is catalyzed by a highly dynamic protein complex, the spliceosome [22]. It is assembled on pre-mRNA by the stepwise association with small nuclear ribonucleoproteins (SnRNPs; reviewed in [88]). The components of the major spliceosome (U2-dependent) are U1, U2, U5, and U4/U6 snRNP particles (Splicing associate protein, SAP; reviewed in [88]). Two forms of the U2 snRNP have been found, an inactive 12S form and an active form 17S U2 snRNP [13, 14]. The sequential interaction among the splicing factors SF3b and SF3a with 12S produces the 17S U2 snRNP [21, 20]. SAP155 (Sf3b1) is the largest protein in the SF3b protein complex [22]. In addition to being a component of the U2 snRNP, Sf3b1 is also found in the minor spliceosome (U12-dependent, see [217]). Its amino acid sequence is highly conserved among different species, including Schizosaccharomyces pombe (S. pombe), Caenorhabditis elegans (C. elegans), Xenopus, mouse and human [82]. Two kinds of repeating sequence, TP and RWD are found in the Sf3b1 amino-terminus [210, 182, 82]. These TP repeats closely resemble CDK2 phosphorylation sites. RWD repeats may serve as protein-protein interaction domains with specific proteins [61]. The human homolog of SAP155 is phosphorylated in the splicing pathway and phosphorylated SAP155 contacts the branch site during splicing catalysis [210, 17]. Moreover, phosphorylation of SAP155 is only in the functional
spliceosome [210, 17]. These findings indicate that SAP155 is critical for pre-mRNA splicing and phosphorylation of SAP155 may have an important role in splicing [210, 17]. Isono et al. (2001) isolated the mouse homolog of SAP155 (Sf3b1) and determined its chromosomal position [82], in the central part of chromosome 1 (chr.1). Through Northern blot and whole-mount in situ hybridization analysis, they found that Sf3b1 is expressed ubiquitously in adult mouse tissue and in the mouse embryo [82]. All these findings, high conservation and the ubiquitous expression pattern of Sf3b1, indicate that Sf3b1 plays essential roles in all eukaryotic cells. However, research carried out in mice showed that Sf3b1 is required for the polycomb-mediated repression of Hox gene expression and subsequent skeletogenesis, indicating a differential requirement for Sf3b1 in a specific component of development [83]. Sf3b1 physically interacts with polycomb group proteins (PcG), Zfn144 and Rnf2. Sf3b1 +/- heterozygous mice showed skeleton defects along the anterior-posterior axis. The expression of the Hox genes, Hoxb6 and Hoxb8, are affected in heterozygotes. In mice Sf3b1 -/- homozygous mutants die very early, around the 16- to 32- cell stage [83]. More, recently it also has been found that RNA splicing plays a critical role in adult neurogenesis, and specifically an essential role for RNA splicing factor Sf3b1 [111]. Thus, the degree to which Sf3b1 is required at specific times by specific tissues is not invariant, raising the possibility of Sf3b1 playing specific roles in development.
CHAPTER 2

ZEBRAFISH \textit{tst} IS REQUIRED FOR NEURAL PLATE BORDER AND LATERAL PLATE MESODERM DEVELOPMENT

2.1 Introduction

The neural crest is a distinct population of embryonic precursor cells that give rise to a variety of cell types including pigment cells, peripheral neurons, glia, smooth muscle and craniofacial cartilages. The neural crest is induced during gastrulation at the neural plate border by multiple signals including BMP and posteriorizing factors (Wnt, FGF, and Retinoic acid) from adjacent ectoderm and/or mesoderm. As a consequence, a set of gene families, \textit{Msx}, \textit{Dlx}, and \textit{Zic}, are activated in the neural plate border. Then, a growing set of neural crest transcriptional regulators mediate further neural crest development. These key regulators include the zinc-finger transcription repressor \textit{snail}, \textit{slug} [112, 141, 195, 26], forkhead transcription factor D3 (\textit{foxd3}, see [174, 145, 100, 192, 113]), Activator protein AP2 (\textit{tfap2a}, see [73, 120, 96, 95, 11]), and SRY-like HMG box SOX proteins [47, 30, 187, 28, 29, 223]. In zebrafish, \textit{foxd3}, \textit{tfap2a}, \textit{snai1b}, \textit{sox10} and \textit{sox9b} are expressed by most if not all neural crest progenitors and premigratory neural crest cells [195, 145, 47, 106, 96, 11]. Zebrafish mutant analysis of multiple alleles of \textit{foxd3} (\textit{sym1}, \textit{mos}; see [192, 113]), \textit{sox10} (\textit{cls}; see [89, 47]),
tfap2a (*low, mont blanc*; see [96, 11]), and sox9b (*b971, hi1134*; see [223]) shows that these genes are required for sublineage cell fate specification, migration, survival and differentiation of distinct and overlapping neural crest derivatives. In addition, the zebrafish retroelement *crestin* is also expressed in most premigratory and migrating neural crest cells [171, 119, 96] and its expression overlaps with the transcription factors cited above. Although *crestin* is expressed in most neural crest cells including premigratory and migrating cells, no evidence has been shown that *crestin* displays a role during neural crest development. However, it is a good marker gene for neural crest cells in zebrafish. In general, neural crest formation is a multi-step process including induction, cell fate specification, migration, survival, and differentiation that is controlled by a large number of genes including the genes mentioned above. Molecular studies of these genes and their functions during neural crest formation have begun to emerge, but hierarchical relationship between these known genes still remains unclear. Because of the diversity of neural crest derivatives and regulatory processes during neural crest development, neural crest is a good model system for studying development in general.

Like neural crest, hematopoiesis also requires multiple developmental processes (induction of ventrolateral mesoderm, hematopoietic sublineage cell fate specification, migration, survival, and differentiation), and thus provides another excellent model system for studying embryonic cell diversification. During gastrulation, members of the TGF-β-related BMP family play critical roles during mesodermal dorsal-ventral axis patterning and are well conserved in vertebrates (reviewed in [62, 80]). The lateral plate mesoderm is segregated from mesoderm by this dorsal-ventral patterning and not only gives rise to hematopoietic cell lineages, but also gives rise
to vascular endothelium and cardiac cells during embryonic development. Zebrafish hematopoiesis is well conserved among the vertebrates with the formation of analogous blood cell types, including erythroid, myeloid, and lymphoid lineages. Intensive study of hematopoiesis in vertebrates determined the functions of a large numbers of transcription factors in the regulation of hematopoietic development, such as gata-1, gata-2, scl, lmo2, flk-1, ikaros, PU.1, mpo, l-plastin, etc. However, many fundamental questions about lateral plate mesoderm development, such as mesoderm patterning, specification of different cell types in lateral plate mesoderm, and regulation of their proliferation, survival and terminal differentiation, still remain unresolved.

Taken together, both neural crest development and hematopoiesis are multiple step processes regulated by a large number of genes. Many congenital diseases and clinically relevant conditions are associated with abnormal neural crest development and hematopoiesis in humans. For example, disruption of neural crest development results in aberrant pigmentation, craniofacial defects and neuropathies; and disruption of hematopoiesis results in leukemia, anemia, and immunodeficiency. Now, more and more zebrafish mutants with different neural crest, blood, cardiac, or vascular phenotypes have been isolated based on large-scale and small-scale mutation screening. Analysis of these mutants will reveal and identify a number of genes (novel or known genes with unknown function) involved in these developmental processes and should bring exciting new insights into vertebrate neural crest development and lateral plate mesoderm development, and thus potentially provide genetic mechanisms of birth defects related to these processes. Studies carried out in mice mutants that have both hematopoietic and neural crest phenotypes suggest that there may be common pathways or molecular mechanisms involved in hematopoiesis and neural crest
development. For example, based on mouse mutant studies, the functions of the c-kit receptor tyrosine kinase (c-kit) and its ligand, steel factor (SLF), are required for hematopoiesis and neural crest-derived melanogenesis [58, 35, 52, 78, 219]. Whether additional genetic commonalities exist is not known. tst was isolated from an ENU mutation screen based on abnormal neural crest development [69]. tst mutants completely lack neural crest-derived melanophores. In addition, no red blood cells are found in the circulation of tst mutants and heart development is abnormal as evidenced by no looping and a slow heart rate. The phenotype of tst indicates that tst is selectively involved in both neural crest and lateral plate mesoderm development. Characterization of tst mutants will help us to elucidate tst function during neural crest and lateral plate mesoderm development.

2.2 Materials and Methods

2.2.1 Zebrafish strains and maintenance

Zebrafish embryos and adults were raised and maintained and staged in the Ohio State University zebrafish facility as described [216, 92]. The toast$^{b460}$ (tst) mutation was induced in the wild-type *AB genetic background by ENU (ethyl-N-nitrosourea) mutagenesis. tst WIK lines were obtained by outcrossing with wild-type WIK background fish for genetic mapping and genotyping. Heterozygous mating pairs (*AB background or WIK background) were used to produce embryos (wild-type and mutant) for all experiments.
2.2.2 Whole-mount in situ hybridization and immunohistochemistry

Whole-mount single and double in situ hybridization was performed as described [86, 85] with minor modification. Embryos were fixed overnight in 4% paraformaldehyde (PFA) in 1X PBS (phosphate-buffered saline solution) at 4°C. Embryos older than 24 hpf were treated with PTU (1-phenyl-2-thiourea) to prevent melanin synthesis. Prehybridization and hybridization steps were carried out at 65°C. The following digoxigenin (DIG) labeled antisense RNA probes were used in single in situ hybridization: crestin [119], foxd3 [145], snai1b (snail-2, [195], sox10 [47], tfap2a [96], sox9b [30], dlx2 [2], islet-1 [81], islet-2 [7], huC [149], dct and mitf [114], gata-1[43], gata-2 [43], flk-1 [197], scl [150], PU.1 [15], mpo [15], l-plastin [15], ikaros [218], nkd2.5 [104], myoD [214], ntl [180], and pax2.1 [185]. Whole-mount double in situ hybridization was performed by using DIG labeled myoD antisense RNA probe and fluorescein labeled crestin antisense RNA probe as described [85] with minor modification.

Immunohistochemistry was performed as described [69]. Embryos were fixed in 4% PFA for 2 h at room temperature or 24 h at 4°C. Antibodies were used at the following dilutions: zn-12 [201], 1:4000; acetylated tubulin (Sigma), 1:200; goat anti-mouse, 1:200; PAP, 1:200.

2.2.3 TUNEL assay

Whole-mount TUNEL staining was performed on zebrafish embryos fixed overnight with 4% PFA in 1X PBS at 4°C, permeabilized with methanol, and rehydrated. TdT/digoxigenin or fluorescein-dUTP reaction (Roche) was done for 1 h on ice, followed by 1 h at 37°C. After labeling with digoxigenin or fluorescein-dUTP, anti-digoxigenin or
anti-fluorescein-AP (Roche) was used to bind digoxigenin or fluorescein. NBT/BCIP was used for developing blue color. For whole-mount in situ hybridization and TUNEL double labeling, TUNEL assay was performed first by using fluorescein-dUTP and NBT/BCIP for developing color. After TUNEL staining, the embryos were incubated in 2mg/ml glycine (pH 2.9) for 10 min at room temperature to inactivate anti-fluorescein-AP, washed with 1X PBS to recover pH. Then, whole-mount in situ was performed as described. Fast red was used for detection.

### 2.2.4 Genetic mosaic assay

Genetic mosaic assays were performed by transplanting cells between wild-type and mutant embryos at the late blastula stage as described [74]. The embryos obtained from *tst* heterozygous carriers were divided into two groups, a donor group and a host group. Donor embryos were injected with rhodamine dextran (10³ MW; Molecular Probes) at the 1- to 4-cell stage. Rhodamine dextran-labeled cells from donor embryos were transplanted into different regions of host embryos at the same developmental stage (dome stage 3.7-4 hpf) according to the zebrafish embryo fate map [93]. The host and donor embryos were grown under the same conditions (28.5°C, warm plate). After 24 hpf, wild-type and *tst* mutants embryos were identified based on phenotype. Since *tst* mutants completely lack melanophores and red blood cells in the circulation, it allowed us to easily assess the terminal cell fate of transplanted donor cells. Transplanted cells were visualized using fluorescence microscopy on a Zeiss Axioplan/DIC microscope. Images were captured with a Photometrics SPOT camera.
2.3 Results

2.3.1 Live phenotype of the \( tst \) mutant

The zebrafish \( tst \) mutation was isolated in an ethyl-N-nitrosourea (ENU) mutagenesis screen for mutations that affect neural crest derivatives \cite{69}. \( tst \) is a recessive embryonic lethal mutation and mutant embryos die by 48 hpf. \( tst \) mutants completely lack neural crest-derived melanophores (Fig. 2.1). No mature erythrocytes are present in the circulation of \( tst \) mutant embryos (Fig. 2.1). In addition, heart development is also affected in \( tst \) mutants, showing no looping and a very slow heart rate. Subsequently, massive cell death is clearly present in CNS resulting in embryo death. Because of the complete absence of neural crest-derived melanophores and erythrocytes in \( tst \) mutant embryos, potential early embryonic patterning phenotypes and later differentiation phenotypes during the neural plate border development and lateral plate mesoderm development were studied to investigate the potential molecular mechanisms which result in these abnormalities.

2.3.2 Abnormal trunk neural crest cell development at early stages in \( tst \) mutants

Since \( tst \) mutants completely lack neural crest-derived melanophores, the expression of pan-neural crest marker genes, such as \textit{crestin}, transcription factors \textit{foxd3}, \textit{tfap2a}, \textit{sox10}, \textit{sox9b}, and \textit{snai1b} were examined in \( tst \) mutant embryos. \textit{foxd3} is a forkhead domain transcriptional repressor that is expressed in the presumptive neural crest domain of the ectoderm (neural plate border, NPB) and premigratory neural crest cells during early somitogenesis \cite{174}. At 11 hpf, \textit{foxd3} expression is almost absent in the trunk region of the NPB in \( tst \) mutants compared to wild-type siblings (Fig. 26).
2.2A-B). At 12.5 hpf, a similar pattern of the expression of crestin is also found in tst mutants in the trunk region (Fig. 2.2C-D). Zebrafish crestin is normally expressed in most premigratory and migrating neural crest cells [171, 119]. The expression of sox9b is also dramatically reduced at this stage in tst mutants (Fig. 2.2E-F). A reduction is found in the expression of snai1b and sox10 in tst mutants in the trunk region at 15 hpf and 16 hpf (Fig. 2.2 G-J). However, the expression of tfap2a was not distinguishable at 13 hpf and 14 hpf between tst mutants and wild-type siblings. Subsequently, a mild reduction in the expression of tfap2a is observed in tst mutants at 18 hpf (Fig. 2.2K-L) and the expression of foxd3, sox10 and snai1b remains reduced at this stage. These transcription factors, foxd3, tfap2a, snai1b, sox10 and sox9b, are key regulators of neural crest cell fate specification, survival, migration, and differentiation. In tst mutants, the expression of these key regulators is down regulated.
at slightly different time points and to different extents. This suggests that the *tst* gene is required for the establishment and/or maintenance of the normal expression patterns of these key regulators. Interestingly, the expression of *foxd3*, *sox10*, *snai1b* and *tfap2a* in the cranial region appears normal in *tst* mutants. The difference in the expression of these genes between cranial and trunk neural crest in *tst* mutant suggests that some molecular mechanisms underlying cranial neural crest formation and trunk neural crest formation may be different. In addition, analysis of cell death using TUNEL and acrodine orange revealed dying cells in the neural plate border and subsequently dying premigratory neural crest cells (data not shown). This suggests that *tst* is also required either directly or indirectly for the survival of at least some neural crest progenitors.

2.3.3 Regulatory neural crest cells emerge, but fail to migrate and differentiate, and undergo programmed cell death during development in *tst* mutants

*crestin* is expressed not only by premigratory neural crest cells, but also by migrating neural crest cells [119]. Based on *crestin* expression, I found that neural crest cells emerge during late development in the trunk region where the neural crest cells are almost absent during earlier stages in *tst* mutants (Fig. 2.3A-B). These neural crest cells are defined as regulatory neural crest cells [175, 158, 206]. At 20 hpf and 22 hpf, the expression of *crestin* in wild-type embryos clearly shows the migration of neural crest cells along the trunk region (Fig. 2.3A). As compared with wild-type, these regulatory neural crest cells in *tst* mutants fail to migrate (Fig. 2.3A-B). Similar results are also observed based on the expression of *sox10* at 20 hpf and 22 hpf (data not shown). Since these regulatory neural crest cells fail to migrate, the later
Figure 2.2: Significant abnormalities in trunk neural crest expression of critical transcription factors at early stages in \textit{tst} mutants, but grossly normal expression in the cranial neural crest. Whole-mount in situ hybridization with \textit{foxd3} (A-B), \textit{crestin} (C-D), \textit{sox9b} (E-F), \textit{snai1b} (G-H), \textit{sox10} (I-J), and \textit{tfap2a} (K-L) antisense RNA probes. All embryos are dorsal view, anterior to top with the exception of K-L which is anterior to left.

differentiation of these regulatory neural crest cells was tested. By using the early melanophore precursor marker gene \textit{mitfa} and late melanophore precursor marker gene \textit{dct} antisense RNA probes, one of the neural crest derivatives, melanophores were examined. The expression of these two genes in \textit{tst} mutant embryos reveals the
absence of melanophore precursor cells (Fig. 2.3C-F). These results suggest that although these regulatory neural crest cells emerge in tst mutant embryos, they fail both to migrate and initiate differentiation. These late-developing regulatory neural crest cells are unlikely to result from a general developmental delay because cranial neural crest cells develop normally at early stages in tst mutant embryos as in wild-type siblings (Fig. 2.3A-B). Rather, this phenomenon may be the result of a regulatory interaction within the neural ectoderm. Indeed, embryological experiments in chick [175] and zebrafish [158, 206] have demonstrated that the cells in the ventral neural tube are able to generate such regulatory neural crest cells. However, while the regulatory neural crest cells produced in these experiments are able to differentiate, this is not the case in tst mutants. Double-labeling with TUNEL for apoptotic cells and crestin antisense RNA probe for neural crest cells demonstrated that these regulatory cells undergo programmed cell death in tst mutants (Fig. 2.4 A-C) which is not found in wild-type embryos.

2.3.4 The number of precursor cells of craniofacial cartilages is dramatically decreased in tst mutants

Although cranial neural crest appears normal at early somitogenesis stages, crestin expression in the cranial region is almost absent in tst mutant embryos at 22 hpf as compared to wild-type embryos (Fig. 2.3A-B). dlx2 is expressed by neural crest cells in the pharyngeal arches at early stages, and hand2 is also expressed by the neural crest in the arches at later stages in zebrafish embryos. The expression of dlx2 and hand2 revealed a marked deficit in the number of cranial neural crest cells in tst mutant embryos and the absence of all posterior arches and only limited development
Figure 2.3: The regulatory neural crest cells in *tst* mutants fail to migrate and differentiate into melanophores. *crestin* expressing cells are present in the *tst* mutant embryo (B). However, these cells fail to migrate in the *tst* mutant (B) as compared with wild-type embryos (A). There is no expression of *mitfa* (C-D) and *dct* (E-F) in *tst* mutants. All whole-mount in situ embryos are lateral view and anterior to left.

Figure 2.4: The regulatory neural crest cells that emerge in *tst* mutants belatedly undergo programmed cell death (arrows). Double labeling with TUNEL (NBT/BCIP) and *crestin* whole-mount in situ hybridization (fast red). Transverse section is from the trunk region, dorsal is up. A: DIC image; B: fluorescence image of the same section; C: A and B merged.
of arches 1 and 2 (Fig. 2.5). This result is consistent with crestin expression in tst mutant embryos (Fig. 2.3A-B).

Figure 2.5: Pharyngeal arches are dramatically reduced in tst mutant embryos. A-D: lateral view of whole-mount in situ embryos with dlx2 antisense RNA probe. E-F: dorsal view of flat-mounted in situ embryos with hand2 antisense RNA probe.

2.3.5 The cranial ganglia are also affected in tst mutants

The cranial sensory organs derive from two embryonic structures, the ectodermal placodes and the neural crest. Both placodes and neural crest cells arise from the
The cranial ganglia are also affected in *tst* mutants. A-B: whole-mount antibody staining with zn-12. Arrows show the trigeminal ganglia. C-H: whole-mount antibody staining with anti-acetylated tubulin antibody. Arrows in C-D show the decreased and disorganized trigeminal ganglia in *tst* mutants compared to wild-type siblings. Arrows in E-H show the decreased posterior lateral line ganglion in *tst* mutants compared to wild-type siblings. Arrowheads show that the projections of neurons from the posterior lateral line ganglion are stunted in *tst* mutants compared to wild-type siblings.

At the most rostral extreme of the neural axis ectodermal placodes are directly adjacent to the neural plate [177], whereas neural crest cells are absent from this region [103]. In *tst* mutants, the number of neurons in the trigeminal ganglia is decreased (Fig. 2.6A-B) and later the trigeminal ganglia are decreased in size and disorganized (Fig. 2.6C-D). In addition, the posterior lateral line ganglion is also reduced in size (Fig. 2.6E-H) and the projections of neurons in the posterior
lateral line ganglion are stunted in \textit{tst} mutants (Fig. 2.6E-F). These data suggest that the \textit{tst} mutation also disrupts the sublineages in the most anterior neural border.

### 2.3.6 Rohon-beard sensory neuron development is disrupted in \textit{tst} mutant embryos

In addition to neural crest cells, Rohon-Beard sensory neurons, another cell type that arises from the neural plate border, are also affected in \textit{tst} mutant embryos. During early neurulation, \textit{huc} expression demarcates three longitudinal stripes of primary neurons including: primary motor neurons (medial line), interneurons (mediolateral line), and Rohon-Beard sensory neurons (lateral line) within the neural plate. \textit{islet-1} is expressed by Rohon-Beard sensory neurons and primary motor neurons in the neural plate and neural plate border during early development. Based on whole-mount in situ hybridization with \textit{huc} and \textit{islet-1} antisense RNA probes, the deficiency of Rohon-Beard sensory neuron precursors was found in \textit{tst} mutant embryos at 11.5 hpf (Fig. 2.7A-B) while the ventral cell types, primary motor neurons and interneurons are unlikely affected compared to wild-type siblings (Fig. 2.7A-B). This finding is consistent with evidence that neural crest progenitors and Rohon-Beard sensory neuron precursors form an equivalence group in the neural plate border during neurulation [8, 36]. Antibody labeling with \textit{zn-12} also provides evidence of a deficiency in the numbers of Rohon-Beard sensory neurons in \textit{tst} mutant embryos during development (Fig. 2.7C-D). In addition, antibody labeling with acetylated tubulin shows that Rohon-Beard sensory neurons develop abnormally with fewer and disorganized cell bodies and branched or stunted axons at later stages (Fig. 2.7E-F).
2.3.7 The induction of the neural plate border is normal in \textit{tst} mutants

As a result of extracellular signals during ectoderm patterning, \textit{Msx} and \textit{Dlx} genes are expressed in the neural plate border. In \textit{Xenopus}, \textit{Msx1} is regulated by an intermediate level of BMP activity and enhances the expression of neural crest transcriptional regulators, including \textit{snail-2}, \textit{slug}, and \textit{Foxd3} [202]. Morpholino knockdown
msxBCE in zebrafish causes a deficiency of migrating neural crest cells and defects in melanophores and craniofacial cartilages [152]. Dlx genes play an important role in positioning the neural plate border together with msxB during zebrafish embryonic development [152]. During early gastrulation, gata-2 is expressed in the non-neural ectoderm of zebrafish embryos. There is no difference in the expression of gata-2 and bmp-2b (data not shown) between tst mutants and wild-type embryos at 8 hpf which suggests that the epidermal ectoderm (non-neural ectoderm) is not affected in tst mutants (Fig. 2.9). Because of the significant disruption of gene expression in the trunk neural crest domain of the ectoderm during early somitogenesis in tst mutants, the expression of msxB and dlx3 at the neural plate border was tested between tst and wild-type embryos. The expression of msxB and dlx3 at the neural plate border was indistinguishable between tst mutants and wild-type siblings (Fig. 2.8). These results provide strong evidence that although the tst gene is required for the normal expression patterns of neural crest regulatory genes in the neural plate border, it is not likely to play a role in the induction of the neural plate border and ectoderm patterning.

2.3.8 Disrupted tst function causes specific defects during lateral plate mesoderm development

Red blood cells are absent in the circulation of live tst mutant embryos (Fig. 2.1). There are at least three possibilities underlying the absence of red blood cells in tst mutant embryos: defects during hematopoiesis, vasculogenesis and/or cardiogenesis. As we know, lateral plate mesoderm gives rise to the circulatory system including blood cells, vessels, and the cardiac tissue which provides the nourishment necessary
Figure 2.8: Induction of the neural plate border occurs normally in \textit{tst} mutants. Whole-mount in situ hybridization with \textit{msxB} (A-B) and \textit{dlx3} (C-D).

Figure 2.9: Non-neural ectoderm appears normal in \textit{tst} mutant embryos. Whole-mount in situ hybridization with antisense RNA probe \textit{gata-2} at 8 hpf.

for developing vertebrate embryos. Because of similarities in gene expression by blood and vasculature initially, a common precursor of blood and vascular endothelium is
Figure 2.10: Whole mount in situ hybridization shows that most derivatives of lateral plate mesoderm are affected in \textit{tst} mutant embryos. A-H, K-L, and Y-Z are dorsal view, anterior to top. I-J are dorsal view of flat-mounted embryos, anterior to left. M-N and Q-X are lateral view, anterior to left. O-P are dorsal view, anterior to left. A-H: The deficiency of hemangioblasts in \textit{tst} mutants (A-D: \textit{gata-2}, E-F: \textit{flk-1}, and G-H: \textit{scl}). I-J and M-P: The number of erythroid progenitors is decreased in \textit{tst} mutants and cells in ICM fail to migrate into dorsal mesenteric region (DM) based on \textit{gata-1} expression. K-L: the number of myeloid precursor cells is decreased in \textit{tst} mutants (\textit{PU.1}). Q-T: Remaining myeloid precursor cells fail to differentiate into granulocytes (Q-R) and macrophages (S-T) in \textit{tst} mutant embryos. U-V: The expression of \textit{ikaros} is reduced in \textit{tst} mutant embryos. W-X: Expression of \textit{flk-1} is decreased in \textit{tst} mutants, especially in the caudal vein. Y-Z: The number of cells in the heart anlage is decreased in \textit{tst} mutants. \textit{nkk2.5} marks the paired cardiac primordia.

proposed as the hemangioblast [31, 80]. A number of transcription factors, including \textit{scl}, \textit{flk-1} and \textit{gata-2}, have been shown to play important roles during hematopoiesis
and vasculogenesis, and are expressed in both blood progenitor cells and vascular endothelial cells. At 11.5 hpf and 13 hpf, the expression of flk-1, scl and gata-2 in posterior lateral plate mesoderm is reduced in tst mutant embryos as compared with wild type siblings (Fig. 2.10A-H). These results suggest that a deficiency in the number of common precursor cells of blood and vascular endothelium, the hemangioblast, in tst mutant embryos. gata-1 is expressed primarily in erythrocytes and megakaryocytes and it is essential for erythrogenesis based on mouse and zebrafish experiments. In tst mutants, gata-1 expression has revealed that the number of erythroid precursor cells is greatly reduced (Fig. 2.10 I-J, M-P) and the remaining gata-1 expressing cells do not migrate to the dorsal mesenteric region (DM) through which blood cells enter the circulation [43]. TUNEL analysis reveals that cells in the ICM undergo programmed cell death in tst mutants (Fig. 2.11). Analysis of the myeloid
lineage with *PU.1* whole-mount in situ hybridization revealed a reduced number of myeloid progenitors (Fig. 2.10K-L). In addition, the remaining *PU.1* expressing cells subsequently fail to differentiate into macrophages (*l-plastin\(^+\)*, Fig. 2.10 Q-S) and granulocytes (*mpo\(^+\)*, Fig. 2.10 R-T), components of the immune system. TUNEL analysis for apoptotic cells in *tst* mutants revealed programmed cell death in anterior lateral plate mesoderm (data not shown) and indicates that cell death may be a causative factor in the failure of differentiation of the remaining myeloid precursor cells. Another blood lineage, lymphoid precursors, are also reduced in number in *tst* mutants based on *ikaros* expression (Fig. 2.10U-V). However, *tst* mutants die too early to examine whether remaining lymphoid precursor cells differentiate into T-cell or B-cell. Overall, in *tst* mutants, the deficiency in hemangioblasts causes the decrease in the number of progenitors of different blood lineages, and further causes the defects in all derivatives of blood lineages. Therefore, the decrease in the number of hemangioblasts may be the primary defect in *tst* mutants.

The development of other cell types derived from lateral plate mesoderm, vascular endothelium and myocardial cells are also disrupted as shown by reduced numbers of precursors of these cells in *tst* mutant embryos (Fig. 2.10W-Z). The expression of *flk-1*, a gene that is expressed in endothelial cells later and is essential for vasculogenesis, is dramatically reduced in *tst* mutants (Fig. 2.10W-X). These data are consistent with a deficiency of hemangioblasts in *tst* mutant embryos and support the notion of transient common precursors of blood cells and vascular endothelial cells [31]. Analysis of *nkx2.5* expression indicates that the number of cells in myocardial mesoderm (heart anlage) is reduced and suggests abnormal cardiac development in *tst* mutants (Fig.
2.10Y-Z). In live *tst* mutant embryos, it is readily apparent that the heart is reduced in size, fails to loop, and fails to form chambers.

### 2.3.9 The early development of other mesodermal cell populations occurs normally in *tst* mutants

In addition to lateral plate mesoderm, cell types derived from other mesodermal cell populations were examined by testing the expression of different genes which are involved in the development of chorda mesoderm (notochord), paraxial mesoderm (muscle), and intermediate mesoderm (pronephros). The expression of *ntl*, a notochord marker gene, *myoD*, a gene involved in somitogenesis, and *pax2.1*, a gene expressed in the pronephros, appears normal in *tst* mutant embryos (Fig. 2.12). The normal expression of these genes revealed that the cell types from other mesodermal populations develop normally in *tst* mutants. Thus, these results suggest that the *tst* gene selectively functions during lateral plate mesoderm development.

### 2.3.10 The *tst* mutation functions cell-autonomously during neural crest development and non-cell autonomously during hematopoiesis

Disruption of *tst* gene function results in defects during the neural plate border development and lateral plate mesoderm development. The question is how the *tst* gene functions during embryonic development. Is it an intrinsic cue or an extracellular signal? To answer this question, genetic mosaic analysis was performed by transplanting cells between wild-type and *tst* mutant embryos at the same developmental stage. Since *tst* mutants completely lack melanophores and red blood cells, and melanophores and red blood cells are easily observed in live embryos after 24
Figure 2.12: The derivatives from paraxial mesoderm, chordamesoderm, and intermediate mesoderm respectively, are not affected in \textit{tst} mutants. A-B: dorsal view of double in situ with \textit{crestin} (red) and \textit{myoD} (blue), anterior to top. C-F: lateral view of whole-mount in situ with \textit{ntl}, anterior to left. G-H: lateral view of whole-mount in situ with \textit{pax2.1}, anterior to left.
Figure 2.13: Genetic mosaic assay between *tst* mutants and wild-type embryos. A: melanophores derived from WT donor cells on the yolk of a *tst* mutant host (DIC image). B: fluorescence image of the same cells. C: A and B merged. D: a WT donor. E: a *tst* mutant host.

hpf, these cells allow us to assess whether the transplanted cells differentiated into melanophores or red blood cells. In mutant hosts, transplanted cells from wild-type embryos did differentiate into melanophores which are normally absent in *tst* mutants (Fig. 2.13). This demonstrates that *tst* function cell-autonomously during neural crest development. However, the red blood cells were not found in *tst* mutant hosts. Interestingly, red blood cells differentiated from transplanted *tst* mutant donor cells were observed in wild-type hosts (data not shown). Therefore, these data reveal that *tst* functions non-cell autonomously during hematopoiesis and cell-autonomously during neural crest development.
2.4 Discussion

2.4.1 Within ectoderm-derived tissues, the *tst* gene is specifically required for the neural plate border development

Since *tst* mutants completely lack neural crest derivatives and neural crest cells arise from the neural plate border, the function of the *tst* gene during the neural plate border development was examined. During embryonic development, secreted signaling molecules (BMP, Wnt, FGF, Notch, and RA) pattern the ectoderm and have been demonstrated to regulate neural crest induction (reviewed in [98]). The expression of transcription factors *msxB* and *dlx3* is activated in the neural plate border during late gastrulation and early somitogenesis. Once neural crest is induced in the neural plate border, key transcriptional regulators of neural crest development including *foxd3*, *tfap2a*, *sox10*, *snai1b* and *sox9b* are expressed in the prospective neural crest domain of the ectoderm (reviewed in [191]). Analysis of the expression of these transcription factors in *tst* mutants during early somitogenesis stages reveals the requirement for *tst* gene function in early neural crest development in the trunk region. The *tst* mutation disrupts the expression of these genes at early stages in the trunk region.

In contrast to the trunk region, the expression of *foxd3*, *sox10*, *sox9b*, *snai1b*, and *tfap2a* in the cranial region appears grossly normal in *tst* mutants in contrast to *foxd3*/*sym1* and *tfap2a*/low [192, 96] mutant embryos. In *tfap2a*/low mutants, *foxd3* expression is reduced in hindbrain neural crest, but normal in the more anterior cranial crest and in the trunk neural crest region. In *foxd3*/*sym1* mutants [192], the expression of *sox10* and *snai1b* is significantly reduced at the neural plate border at 11 hpf and is pronounced in cranial and trunk regions at 14 hpf. Among these mutants,
the expression of crestin is almost absent or is dramatically reduced. Taken together, these data may suggest that initial cranial and trunk neural crest development may be regulated by different molecular mechanisms. Studies in mice and zebrafish have shown that there are differences in the genetic regulation of cranial and trunk neural crest development [227, 193, 36, 96, 95]. In dlA mutants, the number of trunk neural crest cells and their derivatives is reduced. In contrast, cranial neural crest and their derivatives are present in dlA mutants. Like dlA mutants, we found tst mutants show a significant disruption of trunk neural crest cell development whereas cranial neural crest development is comparatively normal at early stages. However, during subsequent development, cranial neural crest cells are almost absent or dramatically reduced based on crestin and sox10 expression. Further, the expression of hand2 and dlx2 reveals the deficiency of cranial chondrogenic crest cells in tst mutants and the absence of all posterior pharyngeal arches. These data indicate differences in the genetic regulation of cranial and trunk neural crest development and a differential terminal requirement for tst function by different neural crest cell populations.

Based on crestin and foxd3 expression data in tst mutants, trunk neural crest development is severely disrupted at early stages. During later development, we found crestin expressing cells emerging in the trunk region where crestin expression is almost absent at early stages. These newly emerged neural crest cells are defined as regulatory neural crest. The appearance of these cells does not indicate a general developmental delay because cranial neural crest cells (Fig. 2.2A-B) and the expression of tfap2a appear normal in tst mutant embryos at early somitogenesis stages. Regulatory neural crest has been shown in chick [175], amphibians [133], and zebrafish ([158, 206]; and reviewed in [205]). Experiments carried out in chick
demonstrated that the cells in the ventral neural tube give rise to neural crest cells after neural fold ablation in the cranial region and these regulatory neural crest cells migrate and differentiate into cranial mesenchyme as normal cranial neural crest cells [175]. Although the neural fold is removed, the regulatory neural crest cells are still in the wild-type environment and have normal intrinsic signals. Therefore, these cells are able to differentiate normally. In contrast, the regulatory cells that emerge later in \textit{tst} mutant embryos fail to migrate and differentiate into melanophores. Based on TUNEL analysis, we found these cells undergo programmed cell death (Fig. 2.4).

Since \textit{tst} mutation acts cell autonomously during neural crest development and results in abnormal expression of critical neural crest regulatory genes and neural crest cell death, we suspect the death of regulatory neural crest cells results from a similar defect in the establishment of appropriate gene expression by these cells.

In addition to neural crest cells, Rohon-Beard sensory neurons also arise from the neural plate border. Evidence has shown that Rohon-Beard sensory neurons and neural crest progenitor cells comprise an equivalence group in the neural plate border and that Rohon-Beard sensory neurons via Delta signaling are the primary fate in the equivalence group [8, 36]. In \textit{dlA} mutants, loss of Delta signaling results in supernumerary Rohon-Beard sensory neurons, decrease in the number of trunk neural crest cells and absence of trunk neural crest derivatives [36]. Neurogenic gene \textit{Delta/Notch} appears to regulate lateral inhibition by repressing proneural gene Neurogenin1 (\textit{ngn1}) function [6, 37]. \textit{ngn1} is required for the development of Rohon-Beard sensory neurons. In \textit{tst} mutants, not only the number of Rohon-Beard sensory neurons is dramatically reduced at early stages, but also the trunk neural crest development is also severely disrupted. Similarly, zebrafish narrowminded (\textit{nrd})
mutants also show the defects in both Rohon-Beard sensory neurons and neural crest cells with elimination of Rohon-Beard sensory neurons and reduction of neural crest cells during early stages [8]. *nrd* encodes *prdm1* gene which functions downstream of the BMP inducing signal [72]. The reduction of *dlx3* expression (absence of expression of *dlx3*) in *prdm1/nrd* mutants suggests that loss of *prdm1* function results in the defect in the formation of the neural plate border and *prdm1* functions upstream of *dlx3* gene [8, 72]. In *tst* mutants, although the development of both sublineages within the neural plate border, neural crest cells and Rohon-Beard sensory neurons, are affected in *tst* mutants, the induction of the neural plate border appears normal. The expression of *msxB* and *dlx3* which are expressed in the neural plate border did not show any difference between *tst* and wild-type embryos. This suggests that *tst* is not likely to play a role in the induction of the neural plate border. *tst* gene functions downstream of *msxB* and *dlx3* gene, which is different from *prdm1*. These data reveal that cell fate specification of neural crest and Rohon-Beard sensory neurons in the neural plate border may take place after the induction of the neural plate border. *tst* mutation causes the deficiency in Rohon-Beard sensory neuron precursors during early stages, as a consequence, Rohon-Beard sensory neurons form abnormally with disorganized cell bodies and branched or stunted axons. In addition, non-neural ectoderm appears normal in *tst* mutants and ventral cell types in neural plate, primary motor neurons and interneurons, are not likely affected in *tst* mutants. These data further demonstrate that *tst* specifically functions in the neural plate border of the ectoderm.
2.4.2 Within mesoderm-derived tissue, the \textit{tst} gene is specifically required for lateral plate mesoderm development

Analysis of \textit{tst} mutant embryos at early stages of development revealed decreased expression of \textit{scl}, \textit{gata-2}, and \textit{flk-1}, indicating that the \textit{tst} mutation results in a deficiency in common precursors of blood and endothelial cells, hemangioblasts. Further, we analyzed the different blood lineages in \textit{tst} mutant embryos later in development. Analysis of the erythroid-specific transcription factor \textit{gata-1} in posterior lateral plate mesoderm revealed a deficiency of erythroid precursor cells in \textit{tst} mutant embryos and the remaining \textit{gata-1} expressing cells fail to migrate to the dorsal mesenteric region. The cells in the ICM undergo programmed cell death in \textit{tst} mutants. Transcription factor \textit{PU.1} is essential for myelopoiesis and is expressed in the anterior lateral plate mesoderm where myelopoiesis takes place. Analysis of \textit{PU.1} expression in \textit{tst} mutants suggests a dramatic decrease of myeloid precursor cells. Further, the remaining \textit{PU.1} expressing cells fail to differentiate into macrophages and granulocytes based on the expression of the macrophage and granulocyte specific genes \textit{l-plastin} and \textit{mpo}. Programmed cell death was also observed in the anterior lateral plate mesoderm based on the TUNEL assay in \textit{tst} mutants. This result may suggest that the remaining \textit{PU.1} expressing cells undergo programmed cell death. [166] suggest that common myeloid-erythroid progenitors present in zebrafish are functionally equivalent to mammalian common myeloid progenitors. The autoregulation and reciprocally antagonistic gene regulation between \textit{gata-1} and \textit{PU.1} determine the myeloid-erythroid progenitor cell fate. For example, blocking translation of \textit{PU.1} causes ectopic expression of \textit{gata-1} in the anterior lateral plate mesoderm and knockdown of \textit{gata-1} results in the expansion of \textit{PU.1} expression in the anterior lateral plate mesoderm. Experiments in mice
and zebrafish suggest that *gata-1* reprograms myeloid cells to undergo erythroid and megakaryocyte differentiation [151, 84, 55]. All of the data above support the idea that common myeloerythroid progenitors are present in zebrafish embryos. Our data shows that not only *gata-1* expression is down regulated, but *PU.1* expression is also down regulated in *tst* mutants, indicating the decrease of common myeloerythroid progenitor cells. The loss of myeloid sublineage derived macrophages and granulocytes, and the failure of migration of the remaining *gata-1* expressing cells indicates that the *tst* gene is necessary for cell migration and myeloid differentiation. Rhodes et al. (2005) found that knockdown of *gata-1* or *PU.1* does not affect the expression of *gata-2* and *scl* [166]. In *tst* mutants, we found that *gata-2*, *scl*, and *flk-1* are down regulated. This suggests that *tst* is also responsible for the normal expression of key regulators that are required for sublineage specification during lateral plate mesoderm development. In addition to erythroid and myeloid hematopoietic lineages, lymphoid precursors are also affected in *tst* mutants based on *ikaros* expression, indicating that all blood lineages are affected due to the *tst* mutation. In addition to hematopoietic lineages, the *tst* mutation also results in defects during vascular endothelium development indicated by *flk-1* expression and cardiac development by *nkx2.5* expression. The expression patterns of different genes involved in chordamesoderm (notochord), paraxial mesoderm (muscle, somite), and intermediate mesoderm (pronephros) development are not affected in *tst* mutants (Fig. 2.12), suggesting that *tst* is specifically required for lateral plate mesoderm development. The *tst* mutation disrupts the expression of the key regulators that are required for sublineage specification of lateral plate mesoderm, that likely results in subsequent defects in the migration, survival and differentiation of lateral plate mesoderm-derived cells.
CHAPTER 3

MOLECULAR IDENTIFICATION OF THE \textit{tst} GENE AS \textit{sf3b1} AND FUNCTIONAL ANALYSIS DURING THE NEURAL PLATE BORDER AND LATERAL PLATE MESODERM DEVELOPMENT

3.1 Introduction

Forward genetics is a classical genetic approach generally comprised of three phases: isolation and analysis of mutations induced by a mutagen, identification of the genes affected in these mutants using positional cloning and/or a candidate gene approach, and elucidation of the functions of these genes to understand molecular mechanisms during embryonic development and biology. This approach has been used extensively to elucidate many fundamental developmental mechanisms in different species, from invertebrates, \textit{C. elegans} and \textit{Drosophila melanogaster}, to vertebrates, mouse and zebrafish. Zebrafish as a model system for the study of genetic mechanisms underlying vertebrate development has been widely used. Through large and small-scale mutation screening, thousands of mutants with specific phenotypes have been isolated [134, 46, 69]. Since 2001, the Sanger Institute has been sequencing the zebrafish genome and has generated whole genome assemblies. Although the zebrafish genomic sequencing project is still ongoing, it makes the time consuming positional cloning of zebrafish mutated genes easier less time consuming. Molecular
and functional identification of these mutated genes will provide the basis for elucidating novel genetic pathways and insights into vertebrate embryonic development, physiology, and behavior. The zebrafish \textit{tst} mutation was isolated in an ethyl-N-nitrosourea (ENU) mutagenesis screen for mutations affecting neural crest development \cite{69}. Characterization of \textit{tst} mutants described in the previous chapter reveals that disruption of the \textit{tst} gene results in selective defects during the neural plate border and lateral plate mesoderm development. Although much has been learned about the formation of the neural crest and the specification of different sublineages from zebrafish, \textit{Xenopus}, mice and chick, these processes still remain incompletely understood. Hematopoiesis in a variety of vertebrates and a number of human disease associated with blood development have been well studied at the cellular and molecular levels. A large numbers of transcription factors and growth factors have been identified that regulate specification, proliferation, and terminal differentiation during hematopoiesis. However, many important questions still need to be addressed, such as specification and regulation of hematopoietic stem cell development and identification of novel critical regulators of hematopoietic sublineage diversification. As a mutant with defects of both neural crest development and hematopoiesis, identification of the \textit{tst} gene by positional cloning and analysis of its function may provide new insights into the molecular mechanisms regulating the neural plate border and lateral plate mesoderm development.
3.2 Materials and Methods

3.2.1 Zebrafish strains and maintenance

As described in chapter 2, section 2.2.1. The hi3394a (sf3b1) mutant line was kindly provided by the Hopkins’s lab from MIT [4].

3.2.2 Genetic mapping and positional cloning

$tst^{b460}$ heterozygous carriers (*AB background) were crossed to wild-type strain WIK to produce a mapping line. Female heterozygous F1 generation fish were used for the in vitro production of parthenogenetic diploid F2 embryos. Homozygosity at most loci was achieved by suppressing the second meiotic division by application of hydrostatic pressure [216]. Based on live phenotype, F2 diploid embryos were divided into two groups, homozygous mutants and wild-type embryos including homozygous and heterozygous wild-type embryos. The $tst$ locus was mapped to its corresponding chromosome using linkage analysis with simple sequence length polymorphisms (SSLPs). Through recombination frequency analysis using haploid F2 mutants and WT embryos, several closely linked SSLPs and simple-strand conformation polymorphisms (SSCPs) were identified and a critical region defined using recombinant analysis (1cM=1 recombinant per 100 meioses, 1cM=600 kb). Bacterial artificial chromosome (BAC, commercially available from RZPD) and expressed sequence tags (ESTs, commercially available from RZPD) were searched for within this critical region through the zebrafish genome database at Welcome Trust Sanger Institute (http://www.sanger.ac.uk/cgi-bin/Projects/D_rerio/mapsearch). SSCP markers were used for fine genetic mapping:

c tg9339_566337,
5′-CTGGAAACCCCATGCTAA-3′ and
5′-AGGTGCAAGGCAGTTAGC-3′;
zk83M22SP6
5′- GGTTCATAGTAGCTGAAGACACCTG-3′ and
5′-GTCAGTACATTACAGCAGAACATTCC-3′;
zV4.5
5′-CCACGTGCATCTCCCGAGTA-3′ and
5′-GTGCCTCTTACGCTCCTACAT-3′.

Zebrafish Microsatellite markers used were z60982, z21824, z54324, z35323.

3.2.3 BAC microinjection and phenotype rescue analysis in zebrafish embryos

BAC DNA clones overlapping with the defined critical region used for injection were isolated using a Qiagen protocol for large plasmid isolation with Qiagen midi-prep kit. For phenotype rescue assays, the BAC DNA was microinjected into a single blastomere of 1- to 2-cell stage embryos obtained from heterozygote intercrosses. The BAC DNA was diluted to 100-120 ng/µl as a working stock in sterile 1% phenol red. Injected embryos were scored according to live phenotype. Since tst mutant embryos completely lack melanophores and do not have any red blood cells in the circulatory system and differentiated melanophores and red blood cells are easily observed in live embryos after 24 hpf, this allowed us to easily and accurately assess phenotypic rescue. Potential rescued mutants were genotyped with highly linked SSLP markers (z21824, z54324) to confirm mutant genetic status. The embryos for injection of BACs were obtained by intercrossing heterozygous parents that are generated by crossing tst heterozygotes on Oregon *AB background to WIK, a zebrafish strain from India with
a different genetic background. Therefore, \textit{tst} homozygous mutants can be identified by genotyping with polymorphic SSLP markers.

\textbf{3.2.4 cDNA sequencing analysis in \textit{tst} mutant and wild-type embryos}

Total RNA from 18-24 hpf AB wild-type embryos and \textit{tst} mutants was isolated using TRIzol reagent (Sigma). Qiagen one-step RT PCR kit was used for RT-PCR with AB wild-type and \textit{tst} mutant total RNA as templates. Primers used for \textit{sf3b1} cDNA fragments are:

- \textit{sf3b1} (1-2703 bp),
  5'-AAATGGGCAGATCGCCA AAA-3' and
  5'-CGGCACCAAGGTTACCCTATGATTT-3';
- \textit{sf3b1a} (1-1659 bp),
  5'-AAATGGGCAGATCGCCA AAA-3' and
  5'-ACCAAACTCCCTGCGC TTATCTGT-3';
- \textit{sf3b1b} (1486-3159 bp),
  5'-TGCTGGTGCAAGTTGATGAGTCCA-3' and
  5'-TGCAAGTCTCCTGCACTTTCATC-3';
- \textit{sf3b1c} (2934-4393 bp),
  5'-AAGCTTATGGGCCATTGTGGGTGTG-3' and
  5'-AAGAGCGAACTTGACAGACCAGGA-3';
- \textit{sf3b1aa} (46)F,
  5'-ATGACATTGGGCTCAGATC CTGG-3';
- \textit{sf3b1aa} (579)R,
  5'-TCACCGCTTTTCA GCTCCTCCAGC TT-3';
The RT-PCR programs used were described as in the Qiagen protocol (OneStep RT-PCR kit). The fresh RT-PCR products were cloned into PCRII vector by TA cloning (Invitrogen). The cDNAs obtained from RT-PCR were sequenced by Ohio State University (OSU) Plant-Microbe Genomics Facility. The sequencing analysis was performed using Clustal X 1.81 and sequence analysis program from http://www.ncbi.nlm.nih.gov.

3.2.5 cDNA misexpression analysis in zebrafish embryos

The wild-type full length cDNA of sf3b1 was assembled with wild-type sf3b1 cDNA fragment 1-2703 bp and EST clone fb99f09. The EST clone fb99f09 (RZPD) was sequenced by OSU Plant-Microbe Genomics Facility and it contains a 3′-sf3b1 cDNA fragment which overlaps with the sf3b1 cDNA fragment 1-2703 bp. The 2.5 kb fragment from sf3b1 cDNA fragment 1-2703 bp in PCRII vector (digested with XhoI/ClaI) and the 1.9 kb fragment from fb99f09 EST clone (digested with ClaI/XbaI) were cloned into the XhoI/XbaI site of pCS2+ vector as pCS2+sf3b1. The sf3b1 full length cDNA from construct pCS2+sf3b1 with XhoI (blunt)/XbaI site was cloned into ClaI (blunt)/XbaI site of the heatshock vector pCSHSP [66, 47] as hsp>sf3b1. DNA of hsp>sf3b1 was diluted to a final concentration of 25-30 ng/µl in 0.5% phenol red. tst mutant embryos and their wild-type siblings from intercrosses of the tst WIK line were injected with hsp>sf3b1 at the 1- to 2- cell stage and raised at 28.5°C. Embryos were heatshocked 2 or 3 times at 6-7 hpf, 11-12 hpf, and 23-24 hpf time points for 1 hour at 37°C.
hs>sox10 [47] was injected into a single blastomere of tst mutant embryos at 1- to 2- cell stages and their wild-type siblings from intercrosses of tst WIK line. The injected embryos were heatshocked as described [47].

The injected embryos were scored as in the BAC DNA injection experiments, and genotyped by the highly linked SSLP markers (z21824 and z54324).

3.2.6 Whole-mount in situ hybridization

Whole-mount in situ hybridization was performed as described [86] with minor modification. Embryos were fixed overnight in 4% PFA (paraformaldehyde) in 1X PBS (phosphate-buffered saline solution) at 4°C. Embryos older than 24 hpf were treated with PTU to prevent melanin synthesis. Prehybridization and hybridization steps were carried out at 65°C. sf3b1 antisense and sense RNA probes were synthesized corresponding to 3′-sf3b1 around 1.6 kb (2934-4393 bp) from PCRIIf3b1c construct linearized with NotI or BamHI separately and transcribed with SP6 or T7 separately. Other sf3b1 antisense and sense RNA probes were made corresponding to 5′-sf3b1 around 1.6 kb (1-1659 bp) from PCRIIf3b1a construct linearized with NotI and HindIII and transcribed with SP6 and T7 separately.

3.2.7 mRNA misexpression experiments

Synthetic sense-polyA-capped mRNAs were transcribed in vitro from linearized templates using the mMESSEGE mMACHINE kit (Ambion). After transcription, the mRNAs were recovered as described (mMESSEGE mMACHINE kit manual). pCS2+foxd3 [192], pCS2+tfap2a [96], and pCS2+bcl2 [101], were digested with NotI. The linearized DNA was used as template for in vitro transcription by SP6 polymerase. The RNA for injection was diluted to a final concentration range from 50-100
ng/µl and injected into a single blastomere of *tst* mutant and wild-type embryos from intercrosses of *tst* WIK line at the 1- to 4- cell stage. The injected embryos were scored as described above. The embryos injected with *bcl2* were fixed at 17 hpf and used for the TUNEL assay to detect programmed cell death as well as subsequently (see above) for phenotype rescue assessment.

### 3.2.8 TUNEL assay

Whole-mount TUNEL staining was performed on zebrafish embryos fixed overnight with 4% PFA in 1X PBS at 4°C, permeabilized with methanol, and rehydrated. TdT/Digoxigenin or fluorescein-dUTP reaction (Roche) was done for 1 h on ice, followed by 1 h at 37°C. After labeling with Digoxigenin or fluorescein-dUTP, anti-Digoxigenin or anti-fluorescein-AP (Roche) was used to bind Digoxigenin or fluorescein. NBT/BCIP was used for developing the blue color reaction product.

### 3.2.9 Morpholino phenocopy analysis

Two Morpholino antisense oligonucleotides (MO) were designed and acquired from Gene Tools: one is sf3b1MO3rd that targets pre-mRNA splicing at the 3rd exon (ATGAATCC-TCGTCATCATCCTAAAA), and one is sf3b1ATGmo that blocks translation of *sf3b1* (GGCGATCTGCGCCATTTCGTGCTG). Each morpholino was injected separately into one blastomere of wild-type embryos at the 1- to 4- cell stage to interfere with either pre-mRNA splicing of *sf3b1* or to block translation, respectively. Wild-type embryos for morpholino injections were divided into two groups: morphants (embryos injected with morpholinos), uninjected wild-type embryos. Efficiency of sf3b1MO3rd against the splice site was evaluated by RT-PCR with primers.
sf3b1aa (46)F and sf3b1aa (579)R. Western blot with mouse Sf3b1 monoclonal antibody [75] was used to determined the efficiency of sf3b1ATGmo.

### 3.2.10 Western blotting

Western blot analysis was performed as described [131] with some modification. Dechorinated zebrafish embryos (12 hpf -24 hpf) were dissolved in blending buffer (10% SDS, 50mM Tris HCl, pH 6.8, 10mM EDTA, µl/each embryo) and incubated in boiling water for 5-10 min. BAC protein assay kit (PIERCE) was used for measuring protein concentration. 80 µg of protein from different embryos (Wild-type embryos, tst homozygous mutants, hi3394a homozygous mutants, sf3b1ATGmo morphants, hsp>sf3b1 injected tst mutant embryos with phenotype rescue) mixed with an equal volume of sample buffer (62.5mM Tris HCl, pH 6.8, 10% glycerol, 0.1% Bromophenol blue, 10% β-mercaptoethanol) was loaded and electrophoresed on 8% polyacrylamide gel. Then, samples were transferred to nitrocellulose membrane (Whatman GmbH). TBS Blotto A (Santa cruz biotechnology, Inc.) was used as a blocking reagent. Primary antibodies for western blotting are mouse monoclonal antibodies, anti- Sf3b1 (kindly provided by Isono, see [75]) and anti-β-actin as protein loading amount control (Abcam Inc.). HRP-conjugated secondary antibody was used to bind primary antibody and detected by chemiluminescence system (ECL western blotting analysis system, Amersham Bioscience).

### 3.3 Results

#### 3.3.1 Genetic and physical mapping

By linkage analysis, the tst locus was mapped to chromosome 9 using SSLP marker z6430. Through recombination frequency analysis of a mapping panel consisting of
figure 3.1: critical region between closely linked sslp and sscp markers. 1cm=600 kb.

2018 meioses (1cm=1 recombinant per 100 meioses), several closely linked sslps (z21824, z54324, and z35323) and ssbps (zk83m22t7, ctg9339_566337, zk83m22sp6 and zv4.5) were identified and the final critical region was determined to be 0.164cm (fig. 3.1) defining a genetic distance around 100 kb (a genetic distance of 1cm represents an average 600 kb of dna; see [156]). several bacs, dkey-83m22, dkey-16o6, dkey-15p9, dkey-14p3, and ch211-218o17, were identified that encompass this critical region from http://www.sanger.ac.uk. rescue experiments by bac dna injection were performed. tst mutant embryos completely lack melanophores and do not have any red blood cells in the circulatory system. the melanophores and red blood cells are easily observed in live embryos. it allows me to easily and accurately assess phenotypic rescue. i found three bacs, dkey-83m22, dkey-14p3,
and CH211-218O17 that rescue melanophores in \textit{tst} mutants at a frequency ranging from 57-100% (Fig. 3.2). The melanophores which are normally absent in \textit{tst} mutants were present in the injected \textit{tst} mutants with DKEY-83M22 BAC DNA (Fig. 3.2C-F). The injected embryos were genotyped with highly linked SSLP markers to confirm mutant and wild-type genetic status. Among these BACs, DKEY-14P3 didn’t show rescue activity in \textit{tst} mutants (not shown). These BAC rescue results suggested that the three BACs with rescue activity contain the \textit{tst} gene. These 3 BACs are around 170 kb-290 kb (RZPD). With these results, physical distance of critical region of \textit{tst} mutation was reduced to around 170 kb-290 kb.

\subsection*{3.3.2 Candidate gene identification}

One of three BACs that displayed phenotype rescue activity, DKEY-83M22, had been completely sequenced by the Sanger Institute. Using the GENESCAN program, potential candidate genes were identified, including \textit{sf3b1}, \textit{pip5k3}, Serine/threonine protein kinase and an Ankryin repeat domain containing potential gene. Specific primers were designed for these predicted potential candidate genes. cDNAs of these candidate genes were obtained by RT-PCR using specific primers and total RNA from wild-type embryos and \textit{tst} homozygous mutants, the cDNA were sequenced and sequences were compared. Through cDNA sequence analysis, 4 variant transcripts of splicing factor 3b subunit 1 (\textit{sf3b1}) in \textit{tst} mutants were identified as compared to a single transcript from *AB wild-type embryos (Fig. 3.3A). The pair of primers used here are \textit{sf3b1aa}(46)F and \textit{sf3b1aa}(579)R. Based on sequence analysis, tran1 shows wild-type \textit{sf3b1} cDNA. tran2 shows the 4th exon missing. tran3 shows partial 2nd, and complete 3rd and 4th exons missing with partial 4th intron added between
the 2nd and 5th exons. tran4 shows complete loss of the 3rd and 4th exons (Fig. 3.3B). All three of these transcripts result in premature stop codons that likely cause truncated proteins with deletion of RWD or TP and PP2A-like repeats (Fig. 3.3B and Fig. 3.4) and are predicted to be non-functional. These results suggested that sf3b1 may be the gene that is disrupted in tst mutant embryos.

3.3.3 Cloning of sf3b1 and identification of the tst gene as sf3b1

The zebrafish EST database at WashU-Zebrafish Genome Resources Project and http://www.sanger.ac.uk/cgi-bin/Projects/D_rerio/mapsearch was analyzed. fb99f09 EST clone was found with partial sequence of sf3b1, about 2.9 kb insert containing poly (A) + sequence downstream of the base position 4385 bp, the AATAAAA sequence (4366) as the polyadenlyation signal. According to sequence analysis of DKEY-83M22 BAC DNA by the GENESCAN program and the DNA sequence of fb99f09 EST clone, I designed the primer sets, sf3b1(1-2703), sf3b1a(1-1659), sf3b1b(1486-3159), sf3b1c(2934-4311). cDNA fragments of sf3b1 were isolated by RT-PCR using these primer sets and total RNA from wild-type embryos as template and TA cloned into PCRII vector.

To determine whether sf3b1 is the gene disrupted in tst mutants, the full length cDNA was made from sf3b1 fragment (1-2703 bp) obtained from RT-PCR using sf3b1 (1-2703) primer sets and wild-type total RNA as a template, and fb99f09 insert, and cloned into the XhoI/XbaI site of pCS2+ vector. Then, the full length cDNA insert of sf3b1 in pCS2+ was cloned into the ClaI (blunt)/XbaI site of heatshock vector pCSHSP [47]. The full length cDNA of sf3b1 is 4393 bp. To further test whether the sf3b1 gene is responsible for the tst phenotype, phenotype rescue experiments
using misexpression of sf3b1 in tst mutant embryos were performed. Like BAC DNA injection, misexpression of wild-type sf3b1 by hsp>sf3b1 DNA injection results in the rescue of melanophores in the injected tst mutants at a frequency of 80% (the number of total injected mutants is 92, Fig. 3.5B). Melanophores normally are absent in tst mutants (Fig. 3.5A). Genotyping of injected embryos confirmed the injected embryos genetic status as wild-type or homozygous mutants. In addition, western blot analysis shows that the protein level of Sf3b1 in hsp>sf3b1 injected tst mutants embryos with melanophore phenotype rescue is much higher than in uninjected tst mutant embryos and is lower than the protein level in wild-type embryos (Fig. 3.6). These results further support the possibility that the tst locus encodes sf3b1. Although the protein level of Sf3b1 is elevated in tst mutant embryos by injection of hsp>sf3b1, no blood phenotype rescue was observed in hsp>sf3b1 injected tst mutant embryos. Western blot analysis shows the protein level in the hsp>sf3b1 injected tst mutant embryos is lower than in wild-type embryos because the injected construct is activated only under heatshock condition (Fig. 3.6). Genetic mosaic assay reveals that tst functions non-cell autonomously during lateral plate mesoderm. Therefore, there is possibly insufficient sf3b1 protein in the tst enviroment to rescue tst blood phenotype.

To further test whether the tst locus encodes sf3b1, knockdown experiments with morpholino antisense oligonucleotide against sf3b1 were performed. Both sf3b1MO3rd, that targets sf3b1 splicing, and sf3b1ATGmo, that blocks translation of sf3b1 mRNA, showed phenocopy of tst mutants (Fig. 3.7). 4-6.4 ng/embryo of sf3b1MO3rd was sufficient to phenocopy the tst mutant phenotype at an 80-99% frequency (Fig. 3.7B). RT-PCR was performed to test the efficiency of sf3b1MO3rd using sf3b1aa(46)F and sf3b1aa(579)R primers. Sequence analysis shows that two transcripts are present in
sf3b1MO3rd morphants (Fig. 3.8). One is a wild-type transcript. The other one shows that the 2nd intron fails to splice causing a premature stop codon in the 2nd intron (Fig. 3.8). The results of RT-PCR demonstrate that sf3b1MO3rd can efficiently and specifically block the sf3b1 pre-mRNA splicing at the 3′ splice site of the 2nd intron. The injection of sf3b1ATGmo causes a more severe phenotype compared to tst mutants (Fig. 3.7C) when the doses are at 1-8 ng/embryo. Western blot analysis with mouse Sf3b1 monoclonal antibody [75] shows the protein level of sf3b1 in zebrafish sf3b1ATGmo morphants is dramatically reduced as compared with uninjected wild-type embryos (Fig. 3.9). Phenocopy of tst mutants by knockdown sf3b1 gene using both morpholinos provides further evidence that sf3b1 is encoded by the tst locus.

Results of complementation testing between tst heterozygous carriers and hi3394a heterozygous carriers [4] provide evidence that sf3b1 is disrupted in tst mutants. hi3394a is an insertional mutation found in a large insertional mutagenesis screen that disrupts sf3b1 with the insertion of virus DNA [4]. Failure to complement between tstb460 heterozygous carriers and hi3394a heterozygous carriers is consistent with both tstb460 and hi3394a being mutations in the same locus. Total RNA from hi3394a homozygous mutant embryos at 20-24 hpf was isolated and RT-PCR was performed using sf3b1aa (46)F and sf3b1aa (579)R primers set. After TA cloning and sequence analysis, two variant transcripts were found in hi3394a homozygous mutant embryos including one normal transcript and the other abnormal transcript which has a viral insertion between the 1st and 2nd exons that causes a premature stop codon in the 2nd exon (Fig. 3.8B). Western blot analysis shows the dramatic decrease of the protein level of Sf3b1 in hi3394a mutant embryos (Fig. 3.6). Thus, like tst, the presence
of normal transcripts and some normal Sf3b1 proteins in hi3394a mutant embryos suggests that hi3394a mutation is a hypomorphic allele too.

The most direct demonstration that sf3b1 is the tst gene is to identify the nucleotide mutation in the sf3b1 gene of tst mutants. Since tst mutants contain a wild-type transcript, and other abnormal variant transcripts with premature stop codons all upstream of the 5th exon, the genomic DNA fragments of sf3b1 before the 6th exon were obtained by PCR using different specific primer sets and extracted genomic DNA from *AB wild-type and tst homozygous mutant with the same genetic background as templates. Sequence analysis of the resulting products revealed a point mutation at the 5′ splice site of the 4th intron (Fig. 3.10; Fig. 3.11) where thymidine (T) is changed to guanine (G), causing a disruption of pre-mRNA splicing. The U1 snRNP recognizes the 5′ splice site to form a complex that commits the pre-mRNA to spliceosome assembly (reviewed in [142]). The recognition of the 5′ splice site involves base-pairing between the conserved nucleotides at the 5′ end of U1 and the 5′ splice site. This specific nucleotide mutation at 5′ splice site of the 4th intron in tst mutants may cause failure of base-pairing between the consensus sequences of U1 and the 5′ splice site of the 4th intron, resulting in the abberant transcripts, tran2 with loss of the 4th exon, tran3 with loss of partial 2nd exon and complete 3rd and 4th exons and insertion of partial 4th intron, and tran4 with loss of the 3rd and 4th exons (Fig. 3.3).

Taken together, abberant sf3b1 transcripts in tst mutants, phenotype rescue by overexpression of sf3b1, phenocopy of tst mutants by knockdown sf3b1, complementation test between tst heterozygous carriers and hi3394a heterozygous carriers, and
identification of a nucleotide point mutation in \textit{tst} mutant genomic DNA demonstrate that \textit{tst} mutant locus encodes \textit{sf3b1}.

![Figure 3.2: Injection of BACs, DKEY-83M22 partially rescues the \textit{tst} mutant melanophore phenotype. (A,B) uninjected \textit{tst}^{b460} homozygous mutant embryos; (C-F) BAC, DKEY-83M22 injected \textit{tst}^{b460} homozygous mutant embryos, showing partial rescue of the melanophore phenotype](image)

3.3.4 Characterization of the \textit{sf3b1} gene

Genome analysis reveals that the zebrafish \textit{sf3b1} genomic DNA is around 17 kb and is a single-copy gene on chromosome 9. It has 25 exons and its complete cDNA sequence has 4393 bp. Zebrafish \textit{sf3b1} cDNA predicts the protein contains 1315 amino acids (Fig. 3.4). The amino acid sequence of the zebrafish Sf3b1 homolog is nearly the same as human, mouse and \textit{Xenopus} Sf3b1 homologs (92\% identity) and the carboxy-terminal three fourths of Sf3b1 shows even higher conservation (97.5\%, Fig. 65).
Figure 3.3: Variant transcripts of sf3b1 in tst mutants. A: RT-PCR products were amplified between exon 1 and exon 6 of sf3b1 with 20-24 hpf AB wild-type and tstb460 homozygous mutant total RNA as template using sf3b1aa (46)F and sf3b1aa (579)R primers. AB wild-type embryos showed RT-PCR product of size 534 bp (tran1). tstb460 homozygous mutants showed 4 different transcripts, tran1(534 bp), and variant transcripts, tran2(412 bp), tran3(390 bp), and tran4(312 bp). B: diagram of variant transcripts in tstb460 homozygous mutants.

3.12). Even in C. elegans and S. pombe, the amino acid sequence of Sf3b1 also shows high conservation compared to vertebrates [82]. This high conservation among different species suggests that sf3b1 is an essential gene for all eukaryotic cells. Pre-mRNA must be efficiently and accurately processed via splicing and 3′-end processing before becoming mature mRNA that are transported to the cytoplasm and direct the synthesis of proteins. Pre-mRNA splicing is catalyzed by spliceosome. Two unique spliceosomes, U2-dependent spliceosome and U12-dependent spliceosome, coexist in
most eukaryotic cells. Splicing factors SF3b and SF3a interact with an inactive form 12s U2 snRNP, producing an active form 17S U2 snRNP [21, 20]. Sf3b1 is the largest protein in the SF3b complex [22] and is also found in the minor U12-dependent spliceosome [217]. Sf3b1 is phosphorylated in the splicing pathway and the phosphorylated Sf3b1 with the spliceosome contacts the branch site [210]. Therefore, as a splicing factor, Sf3b1 is critical for pre-mRNA splicing, which is an essential process for all eukaryotic cells. According to the amino acid sequence of mouse and human Sf3b1 homologs [210, 83], the TP and RWD repeat sequences were identified in zebrafish sf3b1 amino-terminus (Fig. 3.4). TP repeats closely resemble CDK2 phosphorylation sites and RWD repeats may serve as protein-protein interaction domains with specific proteins [182, 61]. The tst mutation causes the abnormal sf3b1 pre-mRNA splicing, resulting in different abnormal variant transcripts with pre-mature stop codons. Sequence analysis reveals the truncated tst Sf3b1 proteins completely lack the TP and RWD repeats and are thus almost certainly non-functional.

3.3.5 Expression of zebrafish sf3b1

To determine the sf3b1 expression pattern in zebrafish embryos, a sf3b1 antisense RNA probe around 1.6 kb against 3′- sf3b1 (2934-4393 bp) was made and whole mount in situ hybridization was performed at different stages from 3-48 hpf. During early stages, sf3b1 is expressed ubiquitously in zebrafish embryos. Later in development, its expression may be slightly higher in the brain, dorsal neural tube and ventral trunk region (Fig. 3.13) although the different cellular composition of these areas compared to others may be responsible for the apparent difference. The sf3b1
expression pattern in zebrafish is consistent with its expression in mice except for expression in the ventral trunk region in zebrafish [83]. I also compared the expression of sf3b1 in wild-type embryos and tst mutant embryos and did not find differences between tst mutants and wild-type embryos. Since sf3b1 abnormal variant transcripts in tst mutants only affects the 2nd, 3rd, and 4th exons and spare of the rest sequence of these variants, the detection of sf3b1 expression using antisense RNA probe targeting 3'-sf3b1 (2934-4393 bp) shows no distinguishable difference between tst and wild-type embryos. In addition, the antisense probe was made to against 5'-sf3b1 (1-1659 bp) and whole mount in situ showed no difference between wild-type and tst mutants (data not shown). This suggests that tst mutation does not affect the overall RNA expression level or distribution of sf3b1 transcripts including abnormal variant transcripts. However, western blot analysis reveals that the protein level of Sf3b1 is dramatically reduced in tst mutants as compared with wild-type embryos (Fig. 3.6), further demonstrating tst mutation is a hypomorphic allele.

3.3.6 The functions of different genes whose expression are disrupted in tst mutants and are normally involved in neural crest development and cell survival in tst mutants

The tst mutation disrupts the expression of essential transcriptional regulatory genes involved in sublineage development in the neural plate border, such as sox10, tfap2a, and foxd3. The question addressed here is whether the tst phenotype is rescued if the expression of these genes is restored via misexpression. Melanophores that are normally absent in tst mutants were observed in tst mutants injected with hs>sox10 DNA (Fig. 3.14C-D). Similar phenotype rescue was found in tst mutants
injected with tfap2a mRNA (Fig. 3.14F). These data reveal that misexpression of sox10 and tfap2a result in phenotype rescue in injected tst mutant embryos (Fig. 3.14 C-F) and indicate sox10 and tfap2a are sufficient for neural crest melanophore sublineage specification and further development. However, the injection of foxd3 mRNA did not show melanophore phenotype rescue in the injected tst mutants (data not shown). Mutational analysis in zebrafish reveals that disruption of foxd3 function causes defects in DRG neurons, enteric neurons, glia, and cranial cartilages, whereas melanophores are largely normal [192, 113]. Thus, foxd3 may not be sufficient for melanophore development. Negative results for tst phenotype rescue by foxd3 mRNA injection indirectly supports this possibility. Through TUNEL analysis, we found

Figure 3.4: Amino acid sequence of zebrafish sf3b1 protein.
programmed cell death in *tst* mutant embryos. Although misexpression of zebrafish *bcl2* did block programmed cell death in *tst* mutants (Fig. 3.14I-J), the phenotype of injected *tst* mutants was not rescued (Fig. 3.14G-H). This suggests that only blocking cell death is not sufficient to rescue the *tst* phenotype, presumably because multiple regulatory genes involved in sublineage cell fate specification, migration, and differentiation are down regulated in *tst* mutants. Last, co-injection of *hs>sox10* and *bcl2* mRNA did not exhibit more rescue based on phenotype than *hs>sox10* injection alone.
Figure 3.6: Western blot analysis of Sf3b1 protein levels in wild-type, \textit{tst} mutant and \textit{hi3394a} mutant embryos at 24 hpf. Lane 1: wild-type embryos; Lane 2: \textit{tst} homozygous mutants; Lane 3: \textit{hi3394a} homozygous mutants; Lane 4: \textit{hsp}>sf3b1} injected \textit{tst} mutants with melanophore phenotype rescue.

3.3.7 The function of different genes whose expression is disrupted in \textit{tst} mutants that are normally required for development of lateral plate mesoderm

Almost all sublineages derived from lateral plate mesoderm are affected in \textit{tst} mutants as discussed in chapter 2. During hematopoiesis, the \textit{tst} mutation disrupts the expression of key regulatory genes that are required for sublineage cell fate specification and further development. As a consequence, the numbers of erythroid, myeloid and lymphoid precursor cells are dramatically reduced in \textit{tst} mutants (Fig. 2.10), the remaining \textit{gata-1} expressing erythroid cells fail to migrate to the mesenteric region, and remaining \textit{PU.1} expressing myeloid cells fail to differentiate into granulocytes and macrophages. To test whether sublineage development is restored by misexpression of these regulatory genes, including \textit{scl}, \textit{gata-2}, and \textit{PU.1}, the mRNA of these genes was synthesized and injected into wild-type and \textit{tst} mutant siblings. Misexpression of \textit{scl} restored the expression of \textit{gata-1}, which is normally required for erythroid development, in \textit{tst} mutants compared to uninjected \textit{tst} mutants and wild-type embryos (Fig.
Figure 3.7: Phenocopy of \textit{tst} mutant embryos using morpholino knockdown analysis. A: lateral view of a live uninjected wild-type embryo at 30 hpf. B: lateral view of a live sf3b1MO3rd morphant shows the absence of melanophores and massive CNS cell death at 30 hpf. C: lateral view of a live sf3b1ATGmo morphant showing phenocopy of the \textit{tst} mutant at 30 hpf. D: lateral view of a \textit{tst} homozygous mutant at 30 hpf.

3.15). \textit{tst}/\textit{sf3b1} is required for the normal expression of \textit{scl} during lateral plate mesoderm development, and this result indicates \textit{scl} regulates \textit{gata-1} expressing erythroid
Figure 3.8: Variant transcripts in sf3b1MO3rd morphants (A) and hi394 mutants (B).

Figure 3.9: Western blot analysis of Sf3b1 protein levels in uninjected wild-type embryos and sf3b1ATGmo morphants at 12 hpf. Lane 1: uninjected wild-type embryos; Lane 2: sf3b1ATGmo morphants (1ng).

progenitors. In addition, ectopic expression of gata-1 was observed in injected wild-type and tst mutant embryos compared to uninjected embryos, further demonstrating that scl is sufficient for hematopoietic sublineage development (Fig. 3.15).
The nucleotide mutation in the 5′ splice site of the 4th intron of *sf3b1* genomic DNA is identified in *tst* mutants. In *tst*, T is changed to G.

### 3.3.8 The *tst* mutation disrupts pre-mRNA splicing of some transcription factors required for the neural plate border and lateral plate mesoderm development

*Sf3b1* (SAP155) is an essential component of spliceosome activity site by cross-linking to pre-mRNA on both sides of branch sites [210]. Phosphorylation of Sf3b1 is coupled with splicing catalysis [210]. It is absolutely required for pre-mRNA processing. The zebrafish *tst* mutation of the *sf3b1* gene is at the 5′ splice site of the 4th intron. The mutation results in mis-splicing of *sf3b1*. Aberrant forms are predicted to be non-functional. However, a normal species is formed. Together, this suggests that the *tst* mutation results in reduced functional Sf3b1 protein levels. The question we then addressed is whether key regulatory gene pre-mRNAs that are required for the neural plate border and lateral plate mesoderm development in *tst* mutants, such as *tfap2a*, *snai1b*, *sox9b*, *gata-2*, *gata-1*, *scl*, and *PU.1*, are processed correctly in *tst* mutants. RT-PCR with gene specific primers using total RNA isolated from wild-type
Sequence analysis reveals that the transcripts of genes including *tfap2a* (BC060900), *scl* (NM_213237), *gata-1* (NM_131234), and *PU.1* (NM_198062) are spliced normally in *tst* mutants. No abnormal variant transcripts are found in *tst* mutants. There are two different kinds of transcripts of *snai1b* in *tst* mutants: one is the normal copy, the other one shows a deletion from 154 to 665 bp (NM_130989). *snai1b* has five zinc fingers [195]. The first three zinc fingers are deleted in this *snai1b* abnormal variant transcript in *tst* mutants. The normal transcript of *sox9b* was absent in *tst* mutants. Abnormal pre-mRNA splicing of *sox9b* in *tst* mutants causes the deletion from 110-1398 bp in the *sox9b* mRNA (NM_131644 and AF-277097). As a consequence, the
Figure 3.12: Comparison of amino acid sequence of *sf3b1* from different vertebrates, mouse (Mus), human (Homo), *Xenopus*, and zebrafish (Danio).

...whole coding region is deleted in this *sox9b* variant transcript. *gata-2* has two different transcripts in *tst* mutants. One is normal. The other one shows the 1st intron inserted between the 1st exon and the 2nd exon in *tst* mutants. The 1st intron insertion is in
Figure 3.13: Whole-mount in situ hybridization with sf3b1 antisense probe in wild-type embryos. The expression of sf3b1 appears elevated in the brain region, neural tube and ventral trunk region of at 24 hpf (lateral view, anterior to left).

Figure 3.15: Misexpression of scl in tst mutants. *scl* is normally required for hematopoietic sublineage development of lateral plate mesoderm. Whole-mount in situ hybridization with antisense RNA probe *gata-1*. A-B: uninjected wild-type and *tst* mutant siblings. C-D: wild-type and *tst* mutant siblings injected with *scl* mRNA. Ectopic expression is indicated by arrowheads.

the 5′-UTR of *gata-2* (NM_131233), so the start codon is not affected and transcripts contain the whole coding region. It is not clear whether *gata-2* protein levels are abnormal in *tst* mutant. However, the *gata-2* expression in *tst* mutants is normal in epidermal ectoderm during gastrulation (Fig. 2.9), but is dramatically reduced in the lateral plate mesoderm during early somitogenesis (Fig. 2.10A-D). Thus, the level of expression of *gata-2* transcripts is differently affected in *tst* mutants. Since the *tst* mutation is a hypomorphic mutation and *tst* mutants still have some functional sf3b1 proteins, some genes may only require low functional sf3b1 protein levels for their
normal RNA splicing. Therefore, pre-mRNA splicing of these genes is not affected in \textit{tst} mutants. These data reveal that \textit{sf3b1} is required for normal pre-mRNA splicing of some genes that are involved in the neural plate border development and lateral plate mesoderm development, and for the maintenance of normal levels of expression of multiple regulatory genes in a tissue specific manner.

3.4 Discussion
3.4.1 The \textit{tst} locus encodes \textit{sf3b1}

Through positional cloning, the \textit{tst} gene was identified as \textit{sf3b1}, a homolog of human Sf3b1 (SAP155) and mouse Sf3b1. Direct evidence for identification of the gene disrupted in a mutant includes identification of the nucleotide mutation. The lesion in \textit{tst} mutants is a point mutation at the 5' splice site of the 4th intron in \textit{sf3b1} genomic DNA where thymidine (T) is changed to guanine (G) (Fig. 3.10; Fig. 3.11), resulting in abnormal pre-mRNA splicing that causes variant transcripts of \textit{sf3b1} in \textit{tst} mutants (Fig. 3.3). Normal transcripts were detected in \textit{tst} mutants and other transcripts have premature stop codons near the N-terminus because of deletion of exons and/or the insertion of introns (Fig. 3.3B), causing truncated proteins with deletion of RWD, and TP. TP sequence has been shown to be a CDK2 phosphorylation site [182] and RDW repeats may serve as protein-protein interaction domains with specific proteins [61]. This suggests that truncated sf3b1 proteins with deletion of TP repeats and RWD motifs in zebrafish \textit{tst} mutants are likely to be non-functional. Further, injection of two different morpholinos that target a splice site of the \textit{sf3b1} 3rd exon and the AUG start site to block translation separately phenocopy \textit{tst} mutants (Fig. 3.7B-C). The injection of sf3b1ATGmo that blocks translation results in a
more severe phenotype compared to \textit{tst} mutants when doses are at 1-8ng/embryo (Fig. 3.7C-D), indicating together with the presence of normal \textit{sf3b1} transcripts in \textit{tst} mutants that the \textit{tst} mutation is hypomorphic. In mice, \textit{Sf3b1-/-} homozygous null mutants die very early, around the 16- to 32- cell stage [83]. In contrast, \textit{tst} homozygous mutants die in 48hpf, showing a less severe phenotype compared to mouse \textit{Sf3b1-/-} homozygous mutants. In addition, phenotype rescue of \textit{tst} mutants by misexpression of \textit{sf3b1} reveals that \textit{sf3b1} is responsible for the \textit{tst} phenotype (Fig. 3.5).

The other zebrafish allele of \textit{sf3b1} [4], \textit{hi3394a} results in a less severe phenotype compared to \textit{tst} mutants. \textit{hi3394a} homozygotes die by 4 dpf [4]. In contrast, \textit{tst} mutants die by 2 dpf. The \textit{hi3394a} allele is a viral insertional mutation in the 1st intron of the \textit{sf3b1} gene [4]. Through sequence analysis of \textit{sf3b1} cDNA obtained from wild-type and \textit{hi3394a} homozygous mutants total RNA by RT-PCR, two kind of variant transcripts in the mutants were identified (Fig. 3.8B), normal transcripts and abnormal transcripts with the insertion of the viral DNA sequence between the 1st exon and the 2nd exon. This result is consistent with the original identification of the insertional mutation \textit{hi3394a} [4]. As compared with \textit{hi3394a} allele, \textit{tst} is a point mutation at a splice site causing the disruption of pre-mRNA splicing. Pre-mRNA splicing in vertebrates is a very complicated process and it involves over 50 proteins and 5 small nuclear RNAs (snRNAs; see [22]). So, further study of this splicing mutation may help us to understand more about the mechanisms of pre-mRNA splicing in vertebrates.
3.4.2 *sf3b1* is highly conserved among different species and is ubiquitously expressed in zebrafish

U2snRNP proteins are components of two multiprotein complexes, Sf3a and Sf3b (splicing associated proteins, SAP). The Sf3b complex contains SAP114, SAP145 and SAP 155 (*sf3b1*, reviewed in [161]). It has been shown that the human homolog of Sf3b1 is phosphorylated concomitant with splicing catalysis and phosphorylated Sf3b1 contacts the branch sites during catalysis [210]. These results indicate that Sf3b1 is necessary for branch site recognition and is a critical component of the spliceosome active site. Zebrafish genome analysis shows that *sf3b1* is a single-copy gene on chromosome 9 (Fig. 3.1). The amino acid sequence of the zebrafish *sf3b1* homolog (Fig. 3.4) is highly homologous to human, mouse and *Xenopus* Sf3b1 homologs (92% identity). Further, Isono and co-workers [82] isolated the mouse Sf3b1 homolog and also found high similarity between mouse, and *C. elegans* and *S. pombe* species. These results suggest that Sf3b1 plays essential roles in all eukaryotic cells. Since pre-mRNA splicing is an essential cell function, *sf3b1* is considered to be a housekeeping gene.

Northern blot analysis and whole-mount in situ hybridization carried out in mouse [82] show that Sf3b1 is expressed ubiquitously during early embryonic development and is expressed at comparatively higher levels in the dorsal region of midbrain, hindbrain, and neural tube later. In zebrafish embryos, *sf3b1* is also expressed ubiquitously during early embryonic development. Later, expression of *sf3b1* appears to be expressed at slightly higher levels in the brain region, dorsal neural tube and the trunk region ventral to the notochord. This result is similar to the expression pattern of the mouse Sf3b1 homolog with the exception of the ventral trunk region (Fig. 3.13). Although the *tst* mutation results in aberrant pre-mRNA splicing of *sf3b1*, the total
expression level of sf3b1 mRNA is not significantly different between wild-type and tst mutants as assessed by whole-mount in situ with sf3b1 antisense probes against 5'-sf3b1 (1-1659 bp) and 3'-sf3b1 (2934-4393 bp).

3.4.3 sf3b1 is involved in the neural plate border development and lateral plate mesoderm development in zebrafish embryos

Although Sf3b1 can be considered to be a housekeeping gene, research carried out in mice shows that Sf3b1 is required for polycomb-mediated repression of Hox genes during skeletogenesis, indicating that Sf3b1 is differentially required for the development of distinct tissues [83]. Specifically, Sf3b1+/- heterozygous mice showed abnormal skeleton development along the anterior-posterior axis. The expression of the Hox genes, Hoxb6 and Hoxb8 are affected in the Sf3b1+/-heterozygous mice [83]. We found that in tst heterozygous fish the skeleton develops normally (data not shown). This apparent discrepancy is likely due to different gene dosage between heterozygous mice and tst mutants since tst is a hypomorphic mutation. Further evidence for a differential level of requirement for sf3b1 by different tissues also includes the the observation that RNA splicing has been reported as a prominent biological process regulating adult neurogenesis in the mammalian brain subventricular Zone (SVZ) by neurogenic stem cells [111, 54, 56]. mRNA splicing factors enriched in this region include Sf3b1. These results suggest that Sf3b1 not only plays ubiquitously essential roles as a splicing factor, but is also involved in specific developmental process including skeletogenesis and adult neurogenesis. However, its potential differential functions during embryogenesis have not previously been reported.
As described in chapter 2, characterization of zebrafish *tst* mutants reveals that the *tst* mutation severely and selectively disrupts the expression of key regulatory genes that are required for sublineage development in the neural plate border and lateral plate mesoderm, subsequently resulting in the complete lack of neural crest derivatives and a severe decrease in the number of Rohon-Beard sensory neurons, and pronounced defects in all derivatives of the lateral plate mesoderm. Recent studies of zebrafish mutants revealed that disruption of *foxd3*, *tfap2a*, and *sox10* results in distinct and overlapping neural crest phenotypes [192, 96, 95, 11, 89, 47]. *sox10* is required only in non-ectomesenchymal sublineages of neural crest including melanophores, peripheral neurons, and glia [89, 47]. Loss of *tfap2a* function in zebrafish results in varying degressed defects in most neural crest derivatives which is preceded by abnormal expression of *foxd3*, *sox9b*, and *snai1b*. These results suggest distinct transcription factor requirements for neural crest sublineage fate specification that are dependent on *tfap2a* [96, 95, 11]. Misexpression of *sox10* and *tfap2a* in *tst* mutants, which display reduced expression levels of each results in partial phenotype rescue of melanophores. These results, together with loss of function analysis, indicate that *sox10* and *tfap2a* promote melanophore development. In contrast, misexpression of *foxd3* did not result in phenotype (melanophore) rescue in *tst*. *foxd3* mutants (*sym1* and *mos*) and *foxd3* morphants display severe defects in most neural crest sublineages, including peripheral neurons (DRG neurons, sympathetic neurons, and enteric neurons) and craniofacial cartilages. However, melanophores appear normal in *foxd3* mutants [192, 132]. The negative result of *foxd3* misexpression in *tst* mutants is consistent with the possibility that *foxd3* is not sufficient for melanophore development. Because *tst* mutants die in 48 hpf, it is too early to test other neural crest derivatives, such peripheral neurons
and craniofacial cartilages in *tst* mutants injected with *foxd3* mRNA. Taken together, the defects of neural crest in *tst* mutants are due to the disruption of normal expression of some critical regulatory genes including *sox10* and *tfap2a*.

*scl* is one of the genes that are critical for hemangioblast development in lateral plate mesoderm [59, 45]. The expression of *scl* is dramatically reduced in *tst* mutants. Misexpression of *scl* to restore scl protein levels in *tst* mutants shows phenotype rescue of erythroid precursors. Specifically, *gata-1* expression is restored in the mRNA injected *tst* mutants compared to uninjected *tst* mutants. These results suggest that hematopoietic defects in *tst* mutants result at least in part from disregulation of *scl* expression in *tst* mutants.

### 3.4.4 The requirements for *sf3b1* for normal nuclear pre-mRNA splicing of different regulatory genes critical for sublineage development in the neural plate border and lateral plate mesoderm

Sequence analysis reveals that pre-mRNA splicing of some genes that are important for sublineage development in the neural plate border and lateral plate mesoderm are affected in *tst* mutants. Two kinds of different transcripts of *snai1b* were detected in *tst* mutants, normal transcripts and transcripts with a deletion of the first three zinc fingers. Transcription factor Snai1b has five zinc fingers [195]. The expression of *snai1b* is reduced in trunk neural crest of *tst* mutants whereas the expression in the cranial region appears normal. The size of *snai1b* antisense RNA probe for whole-mount in situ hybridization is around 400 bp against the region from the 5′-UTR to the zinc finger [195]. The abnormal *snai1b* transcripts in *tst* mutants deletes around 500 bp from 154-665 bp. Thus, the *snai1b* antisense RNA probe is likely to hybridize
with *snai1b* abnormal transcripts in *tst* homozygotes. If the hybridization with abnormal transcripts of *tst* occurs, the reduced level of *snai1b* expression in *tst* mutants observed includes both normal transcripts and abnormal transcripts, and the actual functional protein present is reduced even more in *tst* mutants than mRNA levels suggest. If the probe does not hybridize with the abnormal transcripts, the level of normal Snai1b proteins may be accurately reflected by the level of *snai1b* mRNA expression in *tst* mutants.

Normal transcripts of *sox9b* were not detected in *tst* mutants. Because of abnormal pre-mRNA splicing in *tst* mutants, the whole coding region of *sox9b* is deleted in the abnormal transcripts. The *sox9b* antisense RNA probe used is 945 bp, containing 404 bp of coding region and 541 bp of the 3′-UTR. The transcript detected in *tst* containing a deletion of whole coding region (111-1397 bp) overlaps with the *sox9b* antisense probe sequence at least 500 bp in the 3′-UTR. The reduced expression level of *sox9b* in *tst* mutants reveals that the *sox9b* antisense probe hybridizes with *tst* *sox9b* abnormal transcripts. However, it is likely that the efficiency of hybridization to the *tst* transcript is reduced and is reflected in reduced in situ detection. Finally, the possibility of the presence *sox9b* normal transcripts in *tst* mutants can not be ruled out because *sox9b* normal transcripts in *tst* could be present below the level of detection.

The hematopoietic transcription factor *gata-2* also shows two different kinds of transcripts in *tst* mutants, normal transcripts and transcripts with an insertion of the 1st intron between the 1st exon and the 2nd exon, which does not affect the coding region of *gata-2* (NM_131233). The functional effect of an insertion of 1st intron in the 5′-UTR of *gata-2* is not certain. As described previously, the expression of *gata-2*
in the epidermal ectoderm appears normal during gastrulation in \textit{tst} mutants whereas it is dramatically reduced in the lateral plate mesoderm during early somitogenesis.

Although the expression of other regulatory genes, such as, \textit{tfap2a}, \textit{scl}, \textit{gata-1}, and \textit{PU.1} is reduced to different degrees and at different stages in \textit{tst} mutants, the pre-mRNA splicing of these genes appears normal since no abnormal transcripts were detected in \textit{tst} mutants.

Because the \textit{tst} mutation is hypomorphic, some functional \textit{sf3b1} proteins are present in \textit{tst} mutants and may be sufficient for normal pre-mRNA splicing of certain genes, such as \textit{tfap2a}, \textit{gata-1}, \textit{scl}, and \textit{PU.1}. Taken together, our results indicate that \textit{sf3b1} is normally required to different degrees for normal pre-mRNA splicing of regulatory genes involved in sublineage development in the neural plate border and lateral plate mesoderm.

Although pre-mRNA splicing of \textit{tfap2a} and \textit{scl} genes is normal in \textit{tst} mutants, normal expression of these genes is disrupted in \textit{tst} mutants. Therefore, as key transcription factors during neural crest and lateral plate mesoderm development, mis-expression of these genes could rescue partial neural crest phenotype and restore the expression of downstream genes, respectively. \textit{sf3b1} gene not only functions as an essential gene, but also is specifically involved in different embryonic cell population development. The deficiency of \textit{sf3b1} function not only causes abnormal pre-mRNA splicing of some key regulatory genes at different degrees, but also result in down regulation of these genes. As a consequence, the following developmental processes during neural crest and lateral plate mesoderm development, including migration, survival and terminal differentiation, are severely disrupted in \textit{tst} mutant embryos.
CHAPTER 4

DISCUSSION AND CONCLUSIONS

Neural crest cells originate in the neural plate border and subsequently migrate throughout the embryo and form a wide array of different cell types including peripheral neurons, glia, pigment cells, craniofacial cartilages, chromaffin cells of the adrenal gland, and other cell types. Due to the vast array of different and functionally important cell types derived from the neural crest, many common congenital disorders in humans result from abnormal development of neural crest, such as *Waardenburg-Shah* syndrome and *Hirschsprung’s* disease. Neural crest development involves multiple processes such as tissue induction, cell fate specification, migration, survival and differentiation. Thus, neural crest is a good system for the study of the cellular and molecular mechanisms of development. Similarly, lateral plate mesoderm development involves the sequential diversification of different cell lineages that terminally differentiate into erythroid, myeloid, and lymphoid blood lineages, blood vessels and heart. Miscues during lateral plate mesoderm also result in a large number of clinically relevant conditions in humans, such as anemia, leukemia, immunodeficiency, and congenital heart disease.

Zebrafish is a powerful model system for the study of the molecular and cellular mechanisms of development, being amenable to forward and reverse genetic strategies.
The zebrafish *tst* mutant, isolated from ENU-mutagenesis screen based on abnormal neural crest development [69], has visible neural crest, blood and heart phenotypes during embryonic development. This dissertation work uses genetic strategies to elucidate the identity and function of the zebrafish *tst* locus to understand its selective requirement for neural plate border and lateral plate mesoderm development.

Zebrafish *tst* is a recessive embryonic lethal mutation that dies by 48 hpf. *tst* mutants completely lack neural crest derived melanophores. Gene expression analysis reveals that trunk neural crest development is disrupted in *tst* mutants, however, cranial neural crest appears normal during early somitogenesis. In contrast, *foxd3/sym1* and *tfap2a/low* mutants show early defects in cranial as well as trunk neural crest during early somitogenesis [192, 96]. The expression of key neural crest regulatory genes, such as *foxd3, tfap2a, snai1b, sox10* and *sox9b*, as well as the neural crest marker gene *crestin*, are down regulated at slightly different time points and to different degrees in *tst*, suggesting different levels of requirement for the *tst* gene for the normal expression of these regulatory genes. Later in development, regulatory neural crest cells emerge in *tst* mutants. Experiments carried out in chick and zebrafish demonstrated that regulatory neural crest cells derived from ventral neural tube after neural fold ablation migrate and differentiate normally [175, 158, 206]. However, regulatory neural crest cells that emerge in *tst* mutants fail to migrate and differentiate. TUNEL assays reveal that these cells undergo programmed cell death. The failure of migration and differentiation and undergoing programmed cell death of regulatory neural crest cells are probably due to lack of appropriate gene expression in *tst* mutants. Although cranial neural crest appears normal during early stages, the number of precursor cells of craniofacial cartilages is dramatically reduced in *tst*. The
differences in phenotypes between trunk neural crest and cranial neural crest in \textit{tst} mutants early in development suggests distinct molecular regulatory mechanisms for trunk and cranial neural crest, which are consistent with previous studies in mouse, chick and zebrafish [227, 193, 36]. In addition to neural crest defects in \textit{tst} mutants, the number of Rohon-Beard sensory neurons, that also arise from the neural plate border, is reduced and the neurons that do form and have disorganized cell bodies and branched or stunted axons, giving further support for the common origin of neural crest and Rohon-Beard sensory neurons in the neural plate border [8, 36]. \textit{prdm1} functions downstream of BMP inducing signal and upstream of \textit{dlx3} gene. Loss of \textit{prdm1} function results in the defect in induction of neural plate border, therefore, causing the elimination of Rohon-Beard sensory neurons and reduction of neural crest cells [8, 72]. In contrast, although both cell types derived from the neural plate border are affected in \textit{tst} mutants, the \textit{tst} mutation does not disrupt the induction of the neural plate border and ectoderm patterning, suggesting that \textit{tst} functions downstream of \textit{msxB} and \textit{dlx3} genes and it is not likely to be involved in the neural plate border induction or patterning of the ectoderm generally. These data suggest that cell fate specification of neural crest and Rohon-Beard sensory neuron precursors may take place after the induction of the neural plate border.

Down regulated expression of key regulatory genes (\textit{scl}, \textit{gata-2}, \textit{flk-1}, \textit{PU.1}, \textit{ikaros}, and \textit{nkh2.5}) involved in sublineage development in lateral plate mesoderm reveals that \textit{tst} results in the deficiency of hemangioblasts, myeloid precursors and myocardiac precursors, and subsequently disrupts most derivatives of lateral plate mesoderm. The normal early development of other mesodermal tissues indicates the differential requirement for \textit{tst} gene function during lateral plate mesoderm development.
Taken together, *tst* mutation selectively disrupts the expression of the key regulatory genes required for sublineage development in the neural plate border and lateral plate mesoderm. As a result, the subsequent sublineage development in both neural plate border and lateral plate mesoderm are severely disrupted.

In this work, the *tst* locus was positionally cloned and identified as the zebrafish homolog of *sf3b1* (SAP155). Sequence analysis reveals the *tst* nucleotide mutation in the *sf3b1* gene is a point mutation at the 5′ splice site in the 4th intron where nucleotide T is changed to G. This nucleotide change causes abnormal pre-mRNA splicing probably due to failure of base-pairing between the consensus sequences of U1 and the 5′ splice site of the 4th intron, resulting in variant *sf3b1* transcripts in *tst* mutants. The presence of normal transcripts and proteins in *tst* mutants and the more severe phenotype of *sf3b1*ATGmo morphants compared to *tst* mutants demonstrate that *tst* mutation is hypomorphic although the mutation results in severe disruption in the development of the neural plate border and lateral plate mesoderm. In mice, Sf3b1-/- homozygous null mutants die very early, around the 16- to 32- cell stage. Compared to mouse null mutants, zebrafish *tst* mutants show a less severe phenotype and provide the opportunity to assess *sf3b1* function during embryonic development. Genetic mosaic analysis suggests that *tst* functions cell-autonomously during neural crest development and non-cell autonomously during hematopoiesis. Because *sf3b1* is a splicing factor in the nucleus, it is easy to understand that it functions cell-autonomously during neural crest development. However, since the *tst* mutation results in severe defects during embryonic development, wild-type transplanted cells can not give rise to blood cells due to severe defects in embryonic environment, showing the non-cell autonomous character during hematopoiesis. The other zebrafish
allele of sf3b1, hi3394a is a viral insertional mutation [4]. Because of complexity of pre-mRNA splicing process in vertebrates, many functional questions about mechanisms of pre-mRNA splicing are still unresolved. Further study of the tst mutation provides the opportunity for further understanding mechanisms of RNA splicing.

Amino acid sequence analysis shows that the zebrafish homolog of Sf3b1 is highly conserved among different vertebrates (92% identity). In zebrafish, sf3b1 is expressed ubiquitously early in development and later is slightly enriched in the brain region, dorsal neural tube and the trunk region ventral to notochord. This expression pattern in zebrafish is very similar to the expression pattern of Sf3b1 in mouse [82].

Although sf3b1 is an essential gene for pre-mRNA splicing, it has been shown to be involved in specific developmental process including skeletogenesis and adult neurogenesis [83, 111]. Functions of some other essential genes during embryonic development have been reported. For an example, knockdown of some ribosomal proteins results in specific abnormality in the brain, eyes, and ears [204]. Histone deacetylase 1 regulates different specific embryonic development including neurogenesis, oligodendrocyte specification, craniofacial, and pectoral fins [38, 153, 222, 39]. In addition, another splicing factor, sfpq (splicing factor, proline/glutamine rich), is required for normal brain development and loss of function results in the defects in neural crest, heart and muscle development [118]. However, the potential differential functions of sf3b1 during early development have not previously been reported. Through characterization of the tst phenotype and studies of interactions between sf3b1 and different key regulatory genes involved in sublineage development in the neural plate border and lateral plate mesoderm, we found that sf3b1 is specifically
required for normal expression of these genes and subsequent sublineage development. In addition, as a splicing factor, the \textit{tst} mutation not only results in abnormal pre-mRNA splicing of \textit{sf3b1}, but also causes abnormal pre-mRNA splicing of some regulatory genes of neural crest and lateral plate mesoderm development. Among these genes, the pre-mRNA splicing of \textit{snai1b}, \textit{sox9b} and \textit{gata-2} genes, that are key regulatory genes during neural crest and lateral plate mesoderm development appears abnormal in \textit{tst} mutants.

Two different transcripts of \textit{snai1b}, normal transcripts and aberrant transcripts with elimination of the first three zinc fingers are found in \textit{tst} mutant embryos. In addition, the expression of \textit{snai1b} is down regulated in the trunk neural crest in \textit{tst} mutants. Snail family member genes in different model systems, including \textit{Xenopus}, chick, zebrafish and mouse, encode transcriptional repressor related to Drosophila Snail, and are expressed in the premigratory and migrating neural crest cells [51, 141, 126, 195, 112, 26, 116]. Studies carried out in chick and \textit{Xenopus} have shown that \textit{snail} and \textit{slug} play very important roles during neural crest cell fate specification and migration. Analysis of \textit{snail} knockout mice [26] have shown that \textit{snail} plays critical roles during the epithelial-mesenchymal transition process which occurs at different stages during mouse development, including gastrulation and delamination of neural crest cells from the dorsal neural tube. In \textit{Xenopus}, \textit{snail} is the earliest neural crest marker that is expressed in the neural plate border before other neural crest specific genes are detected, such as \textit{slug} and \textit{Foxd3}, and functions upstream of these transcription factors [112, 9, 174, 155].

Abnormal pre-mRNA splicing of \textit{sox9b} was found and no normal transcripts of \textit{sox9b} are present in \textit{tst} mutants. The whole coding region is deleted in the aberrant
transcripts of sox9b. The expression of sox9b is also down regulated in tst mutants. Taken together, these data suggest that tst mutation may result in complete loss of sox9b activity. In zebrafish, double mutants of sox9b/- and sox9a/- completely lack cranial cartilages [223]. Mutant analysis and misexpression experiments reveal that zebrafish sox9 co-orthologs may be required for regulation of other neural crest genes, including foxd3, snail1b, sox10, and crestin, but not tfap2a [223]. In Xenopus, knockdown of sox9 function results in severe decrease or elimination in the expression of snail, slug, Foxd3 and AP2 [187, 105]. Analysis of mouse sox9 null mutants suggests that loss of sox9 activity causes dramatic cell death in the trunk neural crest region [29]. In tst mutants, disruption of sf3b1 function results in abnormal pre-mRNA splicing of snai1b and sox9b and causes deficiency or loss of function of Snai1b and Sox9b.

In contrast, the pre-mRNA splicing of tfap2a is not affected in tst mutants. However, the expression of tfap2a, as well as other specific neural crest transcription factors (foxd3 and sox10), is severely disrupted in tst mutants. Partial phenotype rescue by misexpression of some of these genes (sox10 and tfap2a) in tst mutants, further suggests that these genes function downstream of sf3b1. Studies reveal that sox10 directly regulates the expression of mitfa [48] and tfap2a regulates the expression of c-kit [79, 144]. Because the foxd3 gene is encoded by a single exon, disruption of sf3b1 in tst mutants does not affect pre-mRNA splicing of foxd3. However, expression of foxd3 is almost absent in trunk neural crest in tst mutants, suggesting foxd3 functions downstream of sf3b1. Misexpression of foxd3 in tst mutants did not rescue the tst melanophore phenotype. In foxd3/sym1 mutants, peripheral neurons, including DRG neurons, sympathetic neurons, and enteric neurons, glia, and cranial cartilages are
affected, whereas melanophores are largely normal [192]. This negative result in foxd3 mRNA injected tst mutants supports the possibility that foxd3 may not be sufficient for melanophore development. Based on our data, we propose that sf3b1 directly functions upstream of snai1b and sox9b by regulation of pre-mRNA splicing of these genes and indirectly functions upstream of sox10, foxd3, and tfap2a through snai1b and sox9b. In zebrafish, mutant analysis or knockdown analysis of snai1b has not been reported. Therefore, further knockdown analysis of snai1b function will reveal the interaction between snai1b and other specific regulators and its function in neural crest programmed cell death, providing evidence for this hypothesis. To further test this hypothesis we propose, we can knockdown both snai1b and sox9b and analyze the expression of foxd3, sox10, and tfap2a in double morphants. In addition, it is not known for now whether the pre-mRNA splicing of sox10 is or not affected in tst mutants because of the failure of RT-PCR with different sox10 specific primer sets. I will try to figure out the RT-PCR conditions and examine pre-mRNA splicing of sox10 in tst mutants.

During gastrulation and early somitogenesis, a set of transcription factors Msx, Dlx, and Zic family genes are activated in the neural plate border as a consequence of the convergence of extracellular signals on ectoderm, including BMPs, Wnts, FGF, RA and Notch. Intact expression of some of these genes, such as msxB and dlx3 in tst mutants suggests that sf3b1 functions downstream of these inductive signals for the neural plate border induction.

Taken together, we propose that sf3b1 functions downstream of the extracellular inductive signals and upstream of key regulatory genes involved in neural plate
border development by directly regulating snai1b and sox9b pre-mRNA splicing and indirectly regulating sox10, tfap2a, and foxd3 expression through snai1b and sox9b.

For mesoderm, members of the TGF-β related BMP family play critical roles in mesodermal dorsal-ventral axis patterning during gastrulation (reviewed in [62, 80]). The deficiency of sf3b1 in tst mutants results in the down regulation of key regulatory genes that specify sublineage cell fate in the lateral plate mesoderm, including scl, gata-2, flk-1, gata-1, nkx2.5, and PU.1. However, pre-mRNA splicing of scl that is expressed in hemangioblasts, regulates proliferation of hematopoietic cells and acts as a positive regulator of erythroid differentiation [45], gata-1 that is required for erythroid development [186], and PU.1 that is required for myeloid development [127], is not affected in tst. Misexpression of scl rescues the expression level of gata-1 in injected tst mutants, suggesting that scl functions downstream of sf3b1. Pre-mRNA splicing of the gata-2 gene, which is required for the proliferation of hematopoietic progenitor cells and is also expressed in endothelial cells ([203], and reviewed in [41]) is abnormal in tst mutants. Two different transcripts, normal transcripts and abnormal transcripts with an insertion of the 1st intron of gata-2 between the 1st exon and the 2nd exon are found in tst mutants. The 1st intron sometimes fails to be excised from pre-mRNA of gata-2 during the splicing process in tst. This insertion in the gata-2 5′-UTR does not affect the coding region, but the functional consequence of this insertion is not known. Since the expression of all these genes are reduced in tst mutants, these genes function downstream of sf3b1. Genetic mosaic assays analyzing hematopoiesis suggest that the deficiency of sf3b1 in tst mutants may result in the disruption of extracellular signals involved in lateral plate mesoderm patterning, causing deficiencies in the expression of key regulators that are critical for further
cell fate specification. As a consequence, the further development of all sublineages is severely disrupted. Thus, *sf3b1* may function upstream of extracellular signals required for lateral plate mesoderm patterning. For future study, members of BMP family as candidates involved in lateral plate mesoderm patterning will be examined for pre-mRNA splicing and expression pattern in *tst* mutants.

The disruption of *sf3b1* function in *tst* mutants results in programmed cell death in neural crest and ICM during somitogenesis. Later, massive programmed cell death appears especially in the brain and dorsal trunk region in *tst* mutants and causes *tst* mutant embryo death. Cell culture studies reveal that Sf3b1 protein regulates the alternative 5′ splice site selection of *Bcl-x* pre-mRNA by binding to ceramide-responsive RNA cis-element 1 [124], demonstrating that *Sf3b1* is directly involved in the regulation of programmed cell death. Through alternative splicing, two variant transcripts, proapoptotic *Bcl-x*(s) and antiapoptotic *Bcl-x*(L), are produced from the BCL-x gene. Down regulation of *Sf3b1* function increases the *Bcl-x*(s)/*Bcl-x*(L) ratio, therefore, sensitizing cells to undergo cell death [124]. Misexpression of zebrafish *bcl2* [101] and knockdown of *P53* function by injection of antisense P53 morpholinos [115] did block programmed cell death in *tst* mutants, but did not rescue the *tst* phenotype. Since the *tst* mutation results in defects in the expression and pre-mRNA splicing of multiple key regulatory genes involved in sublineage cell fate specification, migration, differentiation, and survival during neural plate border and lateral plate mesoderm development, blocking cell death only is not sufficient to rescue the *tst* phenotype. Thus, misexpression of *bcl2* or knockdown of *P53* function did not rescue *tst* phenotype.
In summary, using a forward genetic strategy *tst* was positionally cloned and the *tst* nucleotide mutation was identified in the *sf3b1* gene at a 5′ splicing site. Characterization and functional analysis of *sf3b1* reveals the ubiquitous and essential pre-mRNA splicing gene, *sf3b1* is required by different specific embryonic cell population to different degrees during development and provides a new paradigm for functions of ubiquitous and essential genes during specific embryonic development. Sf3b1 functions downstream of inductive signals in different cell populations and upstream of key transcriptional regulatory genes involved in sublineage cell fate specification in neural plate border and lateral plate mesoderm. As a consequence, disruption of *sf3b1* gene function results in severe defects in further migration, survival and terminal differentiation in neural plate border and lateral plate mesoderm.
CHAPTER 5

DIFFERENTIATION AND MATURATION OF ZEBRAFISH DORSAL ROOT AND SYMPATHETIC GANGLION NEURONS†

Abstract

The trunk neural crest of vertebrate embryos gives rise to dorsal root ganglion (DRG) sensory neurons and autonomic sympathetic neurons, among other derivatives. We have examined the development of DRG and sympathetic neurons during development in the zebrafish. We found that sensory neurons differentiate rapidly and that their overt neuronal differentiation significantly precedes that of sympathetic neurons in the trunk. Sympathetic neurons in different regions differentiate at different times. The most rostral population, which we call the cervical ganglion, differentiates several days before trunk sympathetic neurons. After undergoing overt neuronal differentiation, sympathetic neurons subsequently express the adrenergic differentiation markers dopamine β-hydroxylase and tyrosine hydroxylase. A second

population of adrenergic nonneuronal cells initially localized with cervical sympathetic neurons appears to represent adrenal chromaffin cells. In more mature fish, these cells were present in clusters within the kidneys. Individual DRG and sympathetic ganglia initially contain few neurons. However, the number of neurons in DRG and sympathetic ganglia increases continuously at least up to 4 weeks of age. Analysis of phosphohistone H3 expression and bromodeoxyuridine incorporation studies suggests that the increases in DRG and sympathetic ganglion neuronal cell number are due wholly or in part to the division of neuronal cells within the ganglia.

5.1 Introduction

The neural crest and neural crest-derived neurons have been studied extensively to learn about mechanisms that regulate cell fate specification, survival, proliferation, and differentiation. To date, most studies have utilized avian and rodent embryos and cultures. Although a great deal has been learned using these systems, zebrafish (Danio rerio) embryos provide many additional experimental opportunities. For example, many aspects of zebrafish development can be analyzed at the single cell level in both wild-type and mutant embryos. Because very little is currently known about peripheral nervous system development in zebrafish, we have examined the development of zebrafish trunk neural crest-derived sensory and sympathetic neurons.

Trunk neural crest cells in vertebrate embryos give rise to three major classes of derivatives: peripheral neurons, glial cells, and pigment cells [103]. Trunk neural crest-derived peripheral neurons include both sensory neurons of the dorsal root
ganglia (DRG) and autonomic sympathetic neurons [103]. In all species of vertebrates that have been studied, including zebrafish, both DRG and sympathetic neurons are derived from neural crest cells that migrate ventrally and not from the subpopulation of neural crest cells that migrate laterally between the somite and overlying ectoderm [117, 184, 159, 157]. In birds and rodents, the overt neuronal differentiation of DRG sensory neurons is virtually concomitant with terminal mitosis [25, 181, 168, 169, 50, 123]. In contrast, sympathoblasts that express some markers of overt neuronal differentiation can undergo cell division both in vitro and in vivo [33, 170, 169, 44, 16]. After undergoing their final cell divisions, both populations of neurons are reduced in number during a period of programmed cell death [25]. Thus, the final populations of both DRG and sympathetic neurons appear to be established during embryonic or early postnatal (hatching) development.

Soon after sympathetic neurons in birds and rodents undergo overt neuronal differentiation, they express genes required for adrenergic function. Two of these genes are tyrosine hydroxylase (TH), the rate-limiting enzyme in catecholamine biosynthesis and a useful marker for adrenergic differentiation, and dopamine B-hydroxylase (DβH). In the chick, sympathetic neurons first express TH when they colonize the region adjacent to the dorsal aorta [49, 65]. Although numerous studies have indicated the requirement of signals from midline structures, including ventral neural tube/floor plate, notochord, and dorsal aorta as well as somitic mesoderm, for adrenergic differentiation by sympathetic neurons [32, 143, 194, 76, 77, 190, 65, 163], the most compelling evidence to date indicates that dorsal aorta-derived bone morphogenetic proteins are required for sympathoadrenergic differentiation [179].
In zebrafish, we have determined the timing of overt neuronal differentiation of DRG sensory neurons and autonomic sympathetic neurons and have described the initial expression of $TH$ and $D\beta H$ by sympathetic neurons. We have also found that the number of DRG and sympathetic neurons increases well into early adulthood. Furthermore, we found that these increases in neuron number appear to be due, at least in part, to cell division by neuronal cells in DRG and sympathetic ganglia. We compare and contrast these results with the present understanding of the development of trunk neural crest-derived peripheral neurons in other vertebrates.

5.2 Materials and Methods

5.2.1 Animals

All fish and embryos were maintained at 28.5°C. Embryos were obtained from natural crosses and staged according to hours postfertilization or days postfertilization (hpf; dpf, respectively; see citeKimmel1995). All fish used in this study were Oregon *AB and were reared at the Ohio State University Zebrafish Facility.

5.2.2 Antibodies

To identify differentiated neurons, we used monoclonal antibody (Mab) 16A11 [123, 69]. 16A11 is a panneuronal marker that recognizes members of the Hu family of RNA binding proteins, which share a relatively high degree of homology to Drosophila ELAV [23, 24, 167, 224]. 16A11 is currently the earliest known panneuronal marker for differentiated trunk neural crest-derived neurons in zebrafish. To detect $TH$ protein, we used a rabbit polyclonal antiserum to $TH$ (Pel-Freeze, Rogers, AZ). $D\beta H$ was detected using a polyclonal antiserum (Chemicon, Temecula, CA). The
antibromodeoxyuridine (BrdU) antibody was obtained from Boehringer-Mannheim (Indianapolis, IN), and the antiphospho-histone H3 antiserum was obtained from Upstate Biotechnology (Lake Placid, NY). A variety of commercially available species- and isotype-specific secondary antisera conjugated to a fluorophore or horseradish peroxidase was used.

### 5.2.3 BrdU incorporation

Incorporation of BrdU during S phase of the cell cycle was used as one indicator of mitotic activity. Fish of various ages were placed in a petridish or 15 ml test tube filled with embryo medium containing 10 mM BrdU and 10% dimethyl sulfoxide (DMSO) for 15-30 minutes. Fish were then fixed with 4% paraformaldehyde overnight at 4°C and then sectioned with a cryostat and processed for immunocytochemistry to detect simultaneously cells that had incorporated BrdU (according to the manufacturers protocol; Boehringer Mannheim) and differentiated neurons (see below). All sections were also stained with Hoechst nuclear dye (Calbiochem, La Jolla, CA).

### 5.2.4 Immunocytochemistry

Whole mounts. Embryos were fixed in 4% paraformaldehyde (pH 7.4), washed extensively in phosphatebuffered saline containing 0.5% Triton X-100 (PBS-Tr; pH 7.0), and incubated in primary antibody diluted in PBS containing 1% bovine serum albumin (BSA), 1% DMSO, 0.5% Triton X-100, and 5% normal goat serum [dilution buffer (DB), pH 7.0] for a minimum of 4 hours at 4°C. Embryos were then washed extensively in PBS-Tr and incubated in secondary antibody diluted with DB. After they were washed in PBS-Tr, embryos incubated with a fluorescent secondary antibody were viewed, whereas those processed with a peroxidase-conjugated secondary
antibody were rinsed in PBS-Tr and incubated with a peroxidase-antiperoxidase complex, rinsed in 0.1 M phosphate buffer (pH 7.0), and incubated in a solution containing 0.05 M phosphate buffer, 0.05% diaminobenzidine, and 1% DMSO (pH 7.0). Embryos were then reacted by the addition of 0.001% hydrogen peroxide. After the desired degree of staining was achieved, the reactions were stopped by washing in PBS. Embryos were then equilibrated in a solution of PBS and glycerol (1:1) and viewed. Neuronal cell counts of DRG were performed using whole-mount preparations labeled with 16A11. At all stages, at least 10 ganglia from five different fish were counted. However, it is important to note that the neuronal numbers provided for each stage of development are approximations, because there is variability between individuals at any given axial level, particularly during larval stages, and because there is a great deal of variability in neuronal number between rostral and caudal ganglia.

Frozen sections. Frozen sections of embryos were collected on gelatin-coated slides and rinsed in PBS. Sections were then incubated for 1-12 hours at 4°C in primary antibody diluted in a buffer containing 0.5M NaCl, 0.01M phosphate buffer (pH 7.0), 2% BSA, 5% Triton X-100, and 5% normal goat serum. After extensive washing in PBS, the sections were incubated for 1-2 hours at room temperature with an appropriate secondary antibody diluted in the same buffer as the primary antibody. Sections were then washed in PBS, coverslipped with PBS/glycerol (1:1), and viewed.

5.2.5 RNA in situ hybridization

Analysis of TH mRNA expression was performed in whole-mount preparations according to Thesis et al. (196), with minor modifications. A detailed protocol will be provided upon request.
5.2.6 Figures

Photographs were made using a Zeiss MC 80 camera and 400 speed 35 mm film mounted on a Zeiss Axioplan II microscope equipped with epifluorescence. Processed slides were digitally scanned, and the figures were constructed using Adobe Photoshop 5.0.

5.3 Results

5.3.1 Timing of overt differentiation of sensory and sympathetic neurons

To determine when trunk neural crest-derived peripheral neurons initially undergo overt neuronal differentiation, embryos and frozen sections of embryos were processed for immunocytochemistry with Mab 16A11. 16A11 recognizes a subset of vertebrate ELAV-related RNA binding proteins that are specifically expressed by neurons [123] and are considered markers of overt neuronal differentiation [123]. DRG sensory neurons initially express 16A11 immunoreactivity (IR) between 36 and 40 hpf in a rostral-caudal sequence (Fig. 5.1b,c). However, in some cases, examination of serial transverse sections revealed that DRG neurons can differentiate in a more caudal segment prior to overt differentiation in a previous segment (not shown). By 45 hpf, 16A11-IR DRG neurons are present bilaterally in every trunk segment (Fig. 5.1a). Initially, one to three differentiated (16A11-IR) neurons are present in each bilateral “ganglion”. Although in most segments these neurons are in very close proximity, occasionally one or two neurons are located several cell body diameters away from their counterparts (not shown). This situation is most commonly observed in, but is not limited to, the most rostral six or seven ganglia and is not usually observed
in both ganglia of a bilateral pair. By 3 dpf, 16A11-IR DRG neurons also express other neuron-specific antigens, including neurofilament and neuron-specific enolase (not shown). Between 2 and 5 dpf, the average number of neurons in each DRG increases to approximately five to seven neurons, although the posteriormost ganglia located caudally to the anus usually contain only one or two additional neurons by 5 dpf. By 5 dpf, most ganglia are present adjacent to the ventrolateral portion of the neural tube, and most neurons are closely associated, although some individual neurons are often observed either more ventrolaterally along the ventral root or ventromedially adjacent to the notochord (Fig. 5.1 d). By 14 dpf, these “ectopic” neurons are rarely observed. Also by 14 dpf, the number of neurons in each DRG had increased to approximately 10-15 (Fig. 5.2a), with rostral ganglia containing more neurons than more caudal ganglia, and usually all the neurons in each ganglion were closely associated. The number of neurons per ganglion subsequently increases with development such that individual DRG in 28 dpf fish can contain over 100 neurons (Fig. 5.2b,c).

In contrast to the case with DRG sensory neurons, we found that autonomic sympathetic neurons initially differentiate much later in development, between 2 and 6 dpf. We define two populations of sympathetic neurons: cervical, which are the most rostral, and trunk, which are present immediately caudal to cervical sympathetic neurons and in segments as far posterior in the trunk as the anus. Presumptive cervical sympathetic neurons express 16A11-IR by 2 dpf (Fig. 5.3 a,b; see [159]), whereas trunk sympathetic neurons were found to express 16A11-IR initially between 4 and 8 dpf (Fig. 5.4a). Melanocytes were typically associated with both cervical and trunk sympathetic neurons. The initial expression of 16A11-IR in trunk sympathetic
neurons varied both from individual to individual and in rostral-caudal position. However, in general, more rostral neurons differentiated before more caudal neurons. Although presumptive cervical sympathetic neurons appeared to be organized in a single ganglion or in an hourglass-shaped complex of perhaps two ganglia; Fig. 5.3 c,d), trunk sympathetic neurons were initially present as two irregular rows of single neurons along the rostral-caudal axis located adjacent to the dorsal aorta ventral to

Figure 5.1: Initial differentiation of dorsal root sensory neurons. a: Whole-mount preparation of a 45 hpf embryo labeled with 16A11. The small brown dots along the ventral spinal cord indicated by arrows in some segments, most noticeable in the anterior trunk where spinal cord staining has yet to develop, are DRG. b-d: Transverse sections through the anterior gut tube region of the trunk of 38 hpf (b) and 45 hpf (c) zebrafish embryos stained with the panneuronal marker 16A11. Arrows indicate differentiated DRG neurons. d: Transverse section of the midtrunk of a 5 dpf embryo labeled with 16A11. The DRG indicated with the arrow represents the normal position of DRG at the midtrunk level, whereas the DRG indicated by the arrowhead is ectopically located adjacent to the notochord. Scale bars = 200 µm in a; 70 µm in c (applies to b-d).
Figure 5.2: The number of DRG neurons per ganglion increases during development. 

a,b: Transverse trunk sections of 14 dpf (a) and 28 dpf (b) fish labeled with 16A11. Arrows indicate differentiated DRG neurons. c: Higher magnification of DRG shown in b. By 14 dpf most DRG contain 10-15 neurons (a), with more rostral ganglia containing more neurons than more posterior ganglia. By 28 hpf, DRG can contain over 100 neurons (b,c). Star in b denotes a sympathetic ganglion. Scale bars = 90 µm in a, 150 µm in b, 125 µm in c.

the notochord (Figs. 5.4c,d, 5a). Subsequently, small groups of neurons were present in a more regular array corresponding to single segments, suggesting coalescence of existing and/or newly differentiated cells into primitive ganglia (Fig. 5.5b,c). As
was the case with DRG, the number of neurons in sympathetic ganglia increased significantly with development (Fig. 5.5d,e).

Figure 5.3: Early development of cervical sympathetic neurons. a,b: Side (a) and dorsal (b) views of a whole-mount in situ hybridization preparation to detect $TH$ mRNA expression in a 2 dpf embryo. Arrowheads indicate the cervical sympathetic ganglion complex. c,d: Expression of TH-IR in cervical sympathetic ganglia. Parasagittal section of a 3 dpf embryo labeled with anti-TH (c) and a higher magnification view (d) of the neurons indicated by arrows in c. Scale bars = 200 $\mu m$ in a (applies to a,b), 170 $\mu m$ in c, 75 $m$ in d.

5.3.2 Timing and regulation of adrenergic differentiation of sympathetic neurons

To determine whether zebrafish sympathetic neurons express adrenergic traits and, if so, when they undergo adrenergic differentiation, we examined the expression of $TH$ during development. TH is the rate-limiting enzyme in catecholamine biosynthesis and is generally, but not always, a marker for adrenergic function. TH-IR was
observed in presumptive cervical sympathetic neurons beginning at 2 dpf (Fig. 5.3 c,d). Likewise, \( TH \) mRNA was also readily detectable in cervical sympathetic neurons by 2 dpf (Fig. 5.3a,b). Most sympathetic neurons in the trunk, however, did not usually express detectable levels of TH-IR until after 7 dpf (Fig. 5.4a,b), although occasional rostrally located TH-IR sympathetic neurons were observed starting at 5 dpf. Thus, in general, TH protein expression is delayed by 1 day or more relative to the initial expression of 16A11-IR. In more caudal neurons, this delay was typically 3 or more days. In addition, not all neurons within an individual sympathetic ganglion initially expressed TH-IR simultaneously. However, by 10 dpf, the majority of sympathetic neurons expressed TH-IR.

Another enzyme involved in catecholamine biosynthesis is \( D\beta H \). Analysis of the expression of \( D\beta H \) during sympathetic neuron development revealed that \( D\beta H \) is initially expressed at detectable levels 1 day or more after TH-IR is detectable. In addition, at all times, \( D\beta H \)-IR was found to be much less intense than TH-IR in equivalent cell populations (Fig. 5.4c,d).

5.3.3 Development of chromaffin cells

When we examined the development of the cervical sympathetic ganglion, we found numerous adrenergic (TH\(^+\) and \( D\beta H^+ \)) nonneuronal (16A11) cells in the same region (Fig. 5.4e,f). These cells were typically found in clusters and were usually just ventral to sympathetic neurons. In addition, these cells expressed much more intense TH-IR and \( D\beta H \)-IR than did sympathetic neurons. The morphology of individual cells varied but was usually somewhat stellate, with more than one short, thin process, in contrast to the characteristic cell body morphology of sympathetic neurons (Fig.
Figure 5.4: Early development of trunk sympathetic neurons and identification of presumptive chromaffin cells. a,b: Transverse trunk section of a 7 dpf embryo labeled with 16A11 (a) and anti-TH (b) showing a trunk sympathetic neuron (arrowheads). Asterisks indicate position of the dorsal aorta. c,d: Transverse trunk section of a 10 dpf fish doubly labeled with 16A11 (c) and anti-DβH (d) showing trunk sympathetic neurons (arrows). e,f: Transverse section of a 10 dpf fish doubly labeled with 16A11 (e) and anti-TH (f) showing cervical sympathetic neurons (arrows) and presumptive chromaffin cells (TH⁺, 16A11⁻; asterisks). Scale bars = 90 µm in b (applies to a-d) 70 µm in e (applies to e,f).

5.4c). These cells were present only adjacent to the cervical sympathetic ganglion throughout embryonic development.
We believe, based on their adrenergic properties, their association with sympathetic neurons, and their location in the vicinity of the anterior pronephros (head kidney) associated with the caudal vein, and from comparison with other fish species [60, 176, 63], that these nonneuronal adrenergic cells are the zebrafish homologs of neural crest-derived chromaffin cells. In 28 dpf fish, a population of adrenergic non-neuronal cells was present in the kidneys (Fig. 5.4d). Because in some other species of fish chromaffin cells are known to move from the anterior pronephros to more posterior kidney [63], we suggest that these cells present in older fish represent mature chromaffin cells derived from the embryonic population in the head kidney.

5.3.4 Neuronal cell division in DRG and sympathetic ganglia

The number of neurons in both DRG and sympathetic ganglia increases with development. To determine whether cell division by existing neuronal (16A11-IR) cells contributes to the increase in neurons in DRG and sympathetic ganglia with age, we examined BrdU incorporation by 16A11-IR cells in 3-28 dpf fish. Briefly, fish were incubated in a solution containing BrdU for 15-30 minutes, quickly rinsed and then fixed, and sectioned and labeled with anti-BrdU and 16A11. At all ages examined, 16A11-IR cells that had incorporated BrdU were commonly observed in both DRG and sympathetic ganglia (Fig. 5.6), although a minority of ganglia in any individual fish contained double-labeled cells. Nonneuronal (16A11−) cells associated with both DRG and sympathetic ganglia that had incorporated BrdU were also observed and were more numerous than 16A11+/BrdU neuronal cells in both ganglia.
Figure 5.5: Late development of trunk sympathetic neurons. a: A parasagittal section in a region of the trunk labeled with anti-TH at 11 dpf illustrating the initial coalescence of trunk sympathetic neurons into segmental ganglia. These neurons are present just ventrally to the dorsal aorta and notochord. b: Parasagittal trunk section of a 17 dpf fish labeled with anti-TH shows the progressive condensation of neurons into segmental ganglia with development. c: Higher magnification of the region indicated by arrowheads in b showing the typical morphology of mature sympathetic neuron cell bodies. d,e: The number of sympathetic neurons per trunk ganglion increases dramatically with development. d: Transverse trunk section of a 28 dpf fish labeled with anti-TH (same section labeled with 16A11 in Fig. 5.2b) and a higher magnification view (e) of the trunk sympathetic ganglion in d indicated by the arrow. Trunk sympathetic ganglia at 28 dpf can contain 50 or more neurons. Top asterisk in d indicates the spinal cord, and the asterisk just below indicates the notochord. The asterisks below the sympathetic ganglion indicate groups of presumptive chromaffin cells. The surrounding tissue is believed to be primarily kidney. Scale bars =30 μm in a, 30 μm in b, 20 μm in c, 100 μm in d, 40 μm in e.

Although the period of time for which experimental fish were exposed to BrdU was short, in most cases 15 minutes, the possibility existed that DRG and sympathetic ganglion nonneuronal cells could have incorporated BrdU and then expressed...
16A11 during the pulse period and appeared as neuronal cells in S phase of the cell cycle. Therefore, we examined the expression of the M-phase mitosis marker phospho-histone H3 [1] in differentiated DRG and sympathetic neurons. Fish from 3 dpf to 28 dpf were fixed, sectioned, and doubly labeled with 16A11 and antiphospho-histone H3. At all developmental stages, double-labeled DRG (Fig. 5.7) and sympathetic neuronal cells (not shown) were observed. These results directly demonstrate the existence of dividing DRG and sympathetic neuronal cells and support the interpretation that BrdU-labeled neuronal cells also represent mitotically active differentiated neurons.
Figure 5.7: Neuronal cell division of DRG neurons. Two different (a,b and c,d) transverse trunk sections of 10 dpf fish labeled with 16A11 (a,c) and antiphospho-histone H3 (b,d). Arrows indicate cells (a,c) and nuclei (b,d) of DRG neuronal cells in M phase of the cell cycle (antiphospho-histone H3\(^+\)). M-phase sympathetic neurons were also observed (not shown). Scale bar = 45 \(\mu\)m in a.

5.4 Discussion

5.4.1 Delayed appearance of differentiated trunk sympathetic neurons relative to differentiated DRG neurons

We found that DRG sensory neurons differentiate beginning at approximately 36 hpf in rostral segments, which corresponds to about 1820 hours after the initiation of migration of neurogenic trunk neural crest cells [159, 157]. The rapid, overt neuronal differentiation by a subpopulation of neural crest-derived cells in zebrafish DRG is reminiscent of development in other teleosts [172, 102] and in the chick, in
which the neural crest subpopulation that contains neurogenic cells begins to migrate during embryonic day 2 (E2) [198, 117, 184], and the first cells in nascent DRG to express 16A11-IR are observed during E3 [123]. Likewise, the initial expression of 16A11-IR [123] and the neuronal marker SCG-10 [65] in chick sympathetic neurons also occurs during E3. The expression of TH by avian sympathetic neurons occurs after a brief delay relative to overt neuronal differentiation [33, 170, 169, 49, 65]. In contrast, differentiated zebrafish trunk sympathetic neurons were not detected until at least 2 days after the differentiation of DRG sensory neurons, and the expression of TH-IR was further delayed, usually by an additional 2 days or more. It is unclear why the differentiation of trunk sympathetic neurons is delayed relative to both DRG and cervical sympathetic neurons and in comparison with the chick [123, 49, 65].

Presumptive sympathetic precursors have been shown to colonize and subsequently differentiate in the cervical region [159], and neural crest-derived cells migrate to the region adjacent to the dorsal aorta long before we detected neuronal differentiation [172, 159, 157]. Although neuronal markers were not used in these studies to confirm the phenotype of presumptive trunk sympathetic precursors, it seems likely that many if not all sympathetic neurons arise from this precursor population. Alternatively, it is possible that some sympathetic neurons arise via a secondary migration of differentiated (16A11-IR) neuronal nonadrenergic (TH−; not shown) cells from the DRG, because 16A11-IR cells are commonly observed adjacent to the notochord, between nascent DRG and the future site of sympathetic ganglia, between 3 and 6 dpf (Fig. 5.1d). Presumably, in this case, these neuronal cells would subsequently acquire adrenergic traits in their new positions adjacent to the dorsal aorta. However, DRG neurons are also observed along the proximal ventral root at these times as well, and
a prototypical DRG is not necessarily observed until approximately 2 weeks of development. Thus, given that cervical sympathetic neurons colonize and differentiate in their final locations rapidly [159], it may be the case that local cues necessary for the overt neuronal differentiation of trunk sympathetic neurons are not present until 4-6 dpf in the trunk. Both of these possibilities, the secondary migration of neuronal cells from DRG to nascent sympathetic ganglia and the delayed differentiation of localized precursors, can be explored using long-term lineage analysis in combination with markers of neuronal differentiation.

5.4.2 Long-term proliferative activity of neuronal cells in DRG and sympathetic ganglia

The number of DRG and sympathetic neurons present in ganglia increases continuously during development. To examine how this might occur, BrdU incorporation was used as a marker for cell division by cells in the DRG and sympathetic ganglia. We observed neuronal (16A11-IR) cells in both DRG and sympathetic ganglia that had incorporated BrdU at all ages examined. Even as late as 28 dpf, the latest age examined, 16A11-IR cells that had incorporated BrdU were commonly observed. The incorporation of BrdU during S phase of the cell cycle by cells in peripheral ganglia that were subsequently identified by 16A11-IR suggests that dividing neuronal cells are present in DRG and sympathetic ganglia for at least 4 weeks and possibly throughout the life of zebrafish [215]. Consistent with this interpretation, we also observed numerous 16A11-IR cells in DRG and sympathetic ganglia labeled with the M-phase marker antiphospho-histone H3 at all stages of development. Thus, preexisting neuronal cells in peripheral ganglia can undergo cell division.
Dividing neuronal and nonneuronal cells were observed in both DRG and sympathetic ganglia. The identity of the nonneuronal cells is unclear, because we currently do not have robust markers for peripheral glia in zebrafish. Although we suspect that most or all of these cells are in fact glia, it is also possible, by analogy to other species, that some of these cells are undifferentiated neuroblasts (see, e.g., [169]). In addition, the presence of cycling neuronal cells in embryonic DRG and sympathetic ganglia has been described in chick and mouse [33, 170, 181, 168, 169, 50, 44, 16, 123]. Nevertheless, the presence of proliferating neurons in DRG and sympathetic ganglia throughout development and into adulthood, and possibly throughout life [215], is unlike the situation in birds and rodents, in which peripheral neurons are believed largely to cease cell division during embryonic (DRG; see [123]) or early postnatal (sympathetic ganglia) development. It remains unclear whether the cycling neuronal cells present in zebrafish peripheral ganglia represent a persistent pool of immature neuronal cells or whether mature peripheral neurons retain mitotic ability. Prolonged observation of individual dye-filled peripheral neurons may help to resolve this issue.

Acknowledgments

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