CHARACTERIZATION OF A NOVEL PRONEURAL BASIC HELIX LOOP HELIX TARGET GENE IN THE MOUSE NERVOUS SYSTEM

by

Wei Chen

A dissertation submitted to the faculty of
The University of Utah
in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

Neuroscience Program

The University of Utah

May 2011

The University of Utah Graduate School

STATEMENT OF DISSERTATION APPROVAL

The dissertation of **Wei Chen**

has been approved by the following supervisory committee members:

Monica Vetter	, Chair	**March 8th, 2011** Date Approved
Richard Dorsky	, Member	**March 8th, 2011** Date Approved
Sabine Fuhrmann	, Member	**March 8th, 2011** Date Approved
Suzanne Mansour	, Member	**March 8th, 2011** Date Approved
Thomas Parks	, Member	**March 8th, 2011** Date Approved

and by **Mary T. Lucero**, Chair of the Department of **Neuroscience Program**

and by Charles A. Wight, Dean of The Graduate School.

ABSTRACT

The vertebrate nervous system contains a diversity of neuronal and glial cell types, which are generated in the embryonic neural tube at appropriate times and locations. Proneural basic helix-loop-helix (bHLH) transcription factors are essential regulators in initiating neurogenesis and specifying different cell types, which are dependent upon inducing downstream target genes to regulate core neuronal differentiation program. Shared bHLH target gene 1 (SBT1) is a shared downstream target of multiple bHLH factors and plays critical roles in neurogenesis in the *Xenopus* open neural plate and retina.

Mouse *Sbt1* (Mus musculus RIKEN cDNA 3110035E14 gene, NCBI reference sequence: NM_178399.4) encodes a hypothetical protein LOC76982 (NP_848486), with uncharacterized expression and function. To determine whether *Sbt1* is involved in mouse neurogenesis, I examined its temporal and spatial expression in the mouse nervous system. I found that *Sbt1* is expressed in the developing cortex, brainstem, spinal cord and retina, and its expression in the spinal cord and cortex is maintained through adulthood. Moreover, its expression in the early embryonic brainstem and retina is regulated by *Ngn2*, suggesting that it acts as a downstream target of proneural bHLH factors in mammals.

To further analyze *Sbt1* function in nervous system development, I generated an *Sbt1-eGFPCre* knockin mouse. I followed GFP expression in the *Sbt1-eGFPCre* cortex,

and found that it is mainly expressed in cortical neurons. I compared neuronal versus glial cell numbers in the *Sbt1* mutant cortex and spinal cord with control. I also examined major cell types differentiation in the *Sbt1* mutant retina. So far, no significant differences have been found. These results suggest that unlike its important function in *Xenopus* neurogenesis, *Sbt1* function may be dispensable for mouse nervous system development. However, based on its dynamic expression in cortical neurons, it may be involved in neuronal maturation, which can be addressed in the future.

Collectively, this work provides significant insight into our understanding of SBT1, a novel bHLH target, expression and function in the mouse nervous system.

This thesis is dedicated to my parents, Zhaorong Lin and Zhengren Chen, my husband ChangJiang Zou, and my children Jeffrey and Chloe Zou.

TABLE OF CONTENTS

LIST OF FIGURES

ACKNOWLEDGMENTS

During the past years in graduate school, I got tremendous help and support from people around me. First, I would like to thank my mentor Dr. Monica Vetter, for her scientific guidance, generosity and optimism. She always encourages me to keep going on. Without her efforts, this dissertation would not have been possible.

I thank my committee members Drs. Richard Dorsky, Sabine Fuhrmann, Suzanne Mansour and Thomas Parks, for their advice and encouragement. They always helped me when I got troubles and used their broad knowledge to overcome difficulties.

I thank the people in Monica's lab, including the current members Kathy Moore, Ale Bosco, Mike Steele, Jianmin Zhang, Yangsook Green, Issam Al Diri, Kevin Breen, Derek Bunch, Joy Corley, Carly Mugleston and Cesar Romero, and the previous lab members Connie Dooly, Mary Logan, Minde Willardsen, Erin Callahan, Guoying wang and Jiying Song. They make the Vetter lab a wonderful place to work.

I extend special thanks to my parents Zhaorong Lin, Zhengren Chen, my husband ChangJiang Zou, my children Jeffrey and Chloe Zou, my friends Sen Wu, Yuanyuan Wu, and Li Jiang. Their love and support encourage me to pass the most difficult time, and gave me energy to finish my thesis.

CHAPTER 1

INTRODUCTION

The vertebrate central nervous system (CNS) contains a vast array of neuronal and glial cell types that are generated in the stereotyped times and positions. How they arise from a single layer of neuroepithelium has challenged scientists for years. Proneural basic helix-loop-helix (bHLH) transcription factors are critical regulators of neuronal differentiation and subtype specification. However, the molecular and cellular mechanisms of how proneural bHLH factors govern neurogenesis are still unclear. In this introduction, I will first discuss vertebrate neurogenesis in the retina and elucidate extrinsic and intrinsic signals contributing to neural differentiation. Then, I will focus on the expression and function of proneural bHLH factors in cell fate specification, and how bHLH factors regulate neurogenesis through inducing downstream target genes. Finally, I will focus on a novel proneural bHLH downstream target, SBT1, in *Xenopus laevis* neurogenesis.

Vertebrate neurogenesis

The vertebrate central nervous system (CNS) comprises of an extraordinary diversity of neural cell types, which are generated in the neural tube at specific times and locations. During early development, the neural tube acquires positional identity under the influence of patterning proteins, and divides into distinct domains. Within these domains, neurogenesis begins with progenitors exiting the cell cycle and differentiating into postmitotic neurons and glia (Guillemot, 2007). Later, the newly formed neurons and glia migrate to specific locations and further differentiate into mature cells, possessing differing functions (Huang and Scheiffele, 2008; Merot et al., 2009). It has been shown that cell extrinsic signals, including the Notch/Delta signaling pathway, intrinsic signals, including homeodomain transcription factors, bHLH transcription factors and cell cycle

regulators, and neural-specific genes, play essential roles in this process (Bally-Cuif and Hammerschmidt, 2003; Guillemot, 2007; Louvi and Artavanis-Tsakonas, 2006; Ono et al., 2010). The final step in neural development is the formation of functional synapses and neural circuitry, which are dependent upon environmental signals and neuronal activity (Berlucchi and Buchtel, 2009; Buchtel et al., 2009; Huang and Scheiffele, 2008; Navarro, 2009; Surmeier et al., 2009).

Several regions of the CNS have been extensively studied for neurogenesis. For example, the vertebrate retina is a favorite model for the study of cell fate determination. It originates from evagination of the early diencephalon, and ultimately contains six types of neurons, including retinal ganglion cells (RGCs), amacrine cells, horizontal cells, bipolar cells, rod and cone photoreceptors, and one type of glia, the Müller glia. Retinal neurons and glia are located in three neuronal layers including the ganglion cell layer (GCL), inner nuclear layer (INL) and outer nuclear layer (ONL). During retinogenesis, RGCs are generated first, at around E11.5 in mouse (Hufnagel et al., 2010), other neuronal cell types are produced in a conserved overlapping sequence, and Müller glia are born last, from E18 to postnatal stages. (Hatakeyama and Kageyama, 2004; Levine and Green, 2004; Livesey and Cepko, 2001; Young, 1985) (Figure 1.1).

An important question for understanding neurogenesis is determining how the neurons and glia are generated from the neuroepithelium with accurate numbers and cell types. For example, in the retina, how are the six types of neurons and Müller glia generated in a conserved order? Previous evidence has shown that intrinsic transcriptional programs play essential roles in this process. They promote progenitors to

leave the cell cycle and acquire specific cell fates with the influence of extrinsic signals (Guillemot, 2007).

Extrinsic and intrinsic signals in vertebrate cell fate specification

Vertebrate neurogenesis is well controlled by cooperation of extrinsic and intrinsic signals, but the relative contribution of each set of factors varies with cell type and developmental time. For example, Fibroblast growth factor (FGF) family members are required for neural induction and patterning at early stages, and they also contribute to regulate bHLH factor expression at late stages (Gonzalez-Quevedo et al., 2010; Martinez-Morales et al., 2005; Masai et al., 2000; Patel and McFarlane, 2000).

Extrinsic signals

Extrinsic signals are soluble factors that are present in the cell local environment and act through a nonautonomous manner to regulate cell fate (Edlund and Jessell, 1999; Livesey and Cepko, 2001; Yang, 2004). The best understood extrinsic signal in the numerous system is the Notch/Delta signaling pathway, which is critical for many processes, with a particular role in generating distinct cell fates (Kageyama et al., 2009; Liu et al., 2010). Delta is a single-pass transmembrane ligand targeting the Notch receptor, another single-pass transmembrane protein. Delta has two activities: it transactivates Notch in neighboring cells and inhibits Notch expression in its own cell (Dorsky et al., 1997; Sprinzak et al., 2010). The cells expressing a high level of Delta and a low level of Notch activate the expression of proneural genes and adopt a neuronal fate. On the neighboring cells, Delta binds to the Notch receptor, and Notch is activated and the intracellular domain of Notch (NICD) is cleaved and translocated to the nucleus. In

the nucleus, NICD induces Notch target genes and prevents the transcription of proneural bHLH factors, so that the cell is inhibited from acquiring a neuronal cell fate. This is the model of lateral inhibition (Artavanis-Tsakonas et al., 1999; Brennan and Moses, 2000; Chitnis and Kintner, 1996; Kunisch et al., 1994; Liu et al., 2010; Ma et al., 1998; Ma et al., 1996; Schneider et al., 2001).

During early developmental stages, Notch/Delta signaling regulates the transition from proliferation to neural differentiation in progenitors. Inhibition of Notch signaling leads to precocious neurogenesis (Dorsky et al., 1997). As development proceeds, Notch signaling is also involved in specific cell fate determination. Conditional deletion of *Notch1* in the mouse retina expands cone photoreceptor genesis, indicating it is required for inhibition of photoreceptor cell fate (Jadhav et al., 2006; Yaron et al., 2006). During late stages of development, introduction of Notch signaling into the vertebrate retina favors the development of Müller glial cell fate at the expense of neurons (Furukawa et al., 2000; Hojo et al., 2000).

In addition to Notch signaling pathway, other extrinsic factors also play important roles in vertebrate retina development. For example, in the *zebrafish* retina, the secreted factor Sonic hedgehog (SHH) is necessary for the propagation of the neurogenic RGC wave (Neumann and Nuesslein-Volhard, 2000) and Wnt signaling pathway controls differentiation of the retinal pigment epithelium by regulating *Mitf* and *Otx2* expression (Westenskow et al., 2009).

These data suggest that extrinsic signaling plays multiple roles in retinal cell fate determination.

Intrinsic signals

Although extrinsic signals are critical for neuronal cell fate determination, they interact and rely on intrinsic programs to perform their function. Intrinsic signals include a number of transcription factor families, such as the homeodomain factors (Guillemot, 2007; Hatakeyama and Kageyama, 2004; Mathers and Jamrich, 2000), basic helix-loop-helix (bHLH) factors (Hatakeyama and Kageyama, 2004; Ross et al., 2003; Vetter and Brown, 2001), early B-cell factor (*ebf*)/olfactory neuronal transcription factors (*olf*) (Garcia-Dominguez et al., 2003; Pozzoli et al., 2001; Wang et al., 1997), and Gli/Zic zinc-finger families (Aruga, 2004; Lamar et al., 2001; Zhang et al., 2002).

Some of the best-understood intrinsic signals are the bHLH transcription factors. They are essential mediators of neuronal differentiation and subtype specification. Proneural bHLH factors refer to proteins that are necessary and sufficient to initiate the development of neuronal lineages and to promote the generation of progenitors that are committed to differentiation (Bertrand et al., 2002). The vertebrate proneural bHLH factors are the homologs of *Drosophila achaete-scute* and *atonal* families (Bertrand et al., 2002). They act through antagonizing Notch signaling, promoting cell cycle withdrawal, or controlling neural-specific gene expression to initiate neurogenesis and regulate specific neuronal subtypes (Guillemot, 2007).

Proneural bHLH factors are expressed in the vertebrate nervous system and govern cell fate specification

Proneural bHLH factors are expressed in progenitors and their derivatives in the vertebrate nervous system, including the cortex, spinal cord and retina, with spatial and temporal patterns that correspond with the generation of different neuronal subtypes.

For example, in the developing cortex, *Ngn1/2* and *Ash1* are expressed in progenitors and increase their expression during neurogenesis (Nieto et al., 2001). *Ash1* is required for ventral telencephalon GABAergic interneuron specification, and *Ngns* mediate the differentiation of glutamatergic neurons (Jo et al., 2007; Wilson and Rubenstein, 2000). *Ngn1/2* and *Ash1* are also expressed in the mouse ventricular zone in the ventral midbrain, where midbrain dopaminergic (DA) neurons are born (Kele et al., 2006).

In addition, proneural bHLH factors are expressed in the spinal cord progenitor domains that give rise to different types of neurons, covering the entire dorsal-ventral axis (Gomez-Skarmeta et al., 2003). These bHLH factors interact with other molecules to regulate specific neuronal subtypes, such as *Ash1* and *Ngn1/2*, which regulate motor neuron and interneuron genesis, and *Olig2* and *Ngn3*, which are involved in oligodendrocyte development (Helms et al., 2005; Mizuguchi et al., 2006; Sugimori et al., 2007; Wu et al., 2006; Zhou et al., 2000). Interestingly, a bHLH transcription factor, stem cell leukaemia (SCL) is also required for astrocyte development in the ventral spinal cord (Muroyama et al., 2005).

In the developing retina, proneural bHLH factors are dynamically expressed in progenitors and postmitotic neurons, as summarized in Table 1.1. Gene mutational analyses have shown that these bHLH factors coordinately regulate specific retinal cell fates. For example, *Math3* is expressed in bipolar cells, but deletion of *Math3* does not affect bipolar cell development. However, in *Mash1/Math3* double mutants, virtually all bipolar cells are abolished, suggesting that *Mash1* and *Math3* cooperatively regulate bipolar cell fate (Tomita et al., 2000). In addition, proneural bHLH factors coordinate

with homeodomain genes to influence cell fate acquisition. For example, misexpression of *Mash1* or *Math3* is not sufficient for bipolar cell specification. In contrast, misexpression of *Vsx2 (Chx10)* with *Mash1* or *Math3* significantly promotes the generation of bipolar cells (Hatakeyama et al., 2001). This result indicates that co-expression of bHLH factors and homeodomain genes are essential for neuronal subtype determination.

Proneural bHLH factors mediate neurogenesis through three mechanisms

A large body of evidence has shown that bHLH factors are essential regulators of neurogenesis. The next question is determining through which mechanism they exert their functions. One important role is that bHLH factors overcome Notch mediated lateral inhibition. For example, Delta is a downstream target for proneural bHLH factors. Overexpression of proneural bHLH factors can induce ectopic Delta expression, which has the potential effect to autonomously repress Notch signaling (Chitnis and Kintner, 1996; Heitzler et al., 1996; Kunisch et al., 1994; Ma et al., 1998; Ma et al., 1996; Schneider et al., 2001). Also, in the *Xenopus* open neural plate, *Ngnr-1* can regulate the expression of *X-MyT1*, a C2HC-type zinc finger protein. *X-MyT1* is able to promote ectopic neuronal differentiation and to confer insensitivity to lateral inhibition, in cooperation with bHLH transcription factors, such as *Xenopus Ath5* (Bellefroid et al., 1996; Moore et al., 2002; Schneider et al., 2001).

The second role of bHLH proteins is to promote cell cycle exit. They influence the timing of the last cell division to determine cell fate. For example, in the *zebrafish* retina, *Ath5* is expressed in progenitors at the G2 phase, promoting a single division and

generating one RGC and one non-RGC daughter cell (Poggi et al., 2005). In mouse, loss of *Ath5* dramatically reduces *p27/Kip* (cyclin dependent kinase inhibitor protein 1) expression, which causes the failure of progenitors to exit the cell cycle and a significant increase of *Ath5* lineage cells remaining in the cell cycle (Feng et al., 2010; Le et al., 2006). Moreover, in the *zebrafish* retina, overexpression of *NeuroD* causes cells to withdraw from the cell cycle, upregulates the expression of the cell cycle inhibitors, and downregulates the cell cycle progression factors (Ochocinska and Hitchcock, 2009). However, in some contexts, forcing neural progenitors to leave the cell cycle is not sufficient to induce neuronal differentiation. Overexpression of *p27/Xic1*, in *Xenopus* retinal progenitors, does not force precocious or increased neuronal differentiation (Ohnuma et al., 2002). It is possible that some critical factors are absent which are essential to coordinate bHLH factors with cell cycle withdrawal.

The third role is that bHLH factors regulate neural-specific gene expression. There are numerous molecules that are expressed by neural progenitors downstream of bHLH factors to regulate neuronal terminal differentiation. For example, in the *Xenopus* and mouse retina, *Ath5* regulates the expression of the POU-homeodomain transcription factor *Brn3* in differentiating RGCs (Hutcheson and Vetter, 2001; Liu et al., 2001), which can activate genes encoding cytoskeletal and presynaptic molecules (Mu et al., 2004). Thus, *Brn3* is required for RGC terminal growth and survival but is not sufficient to promote RGC genesis (Erkman et al., 1996; Pan et al., 2005).

Collectively, proneural bHLH factors regulate neurogenesis by overcoming Notch mediated inhibition, promoting cell cycle exit and inducing neural-specific gene expression.

Proneural bHLH factors govern downstream targets to perform their function

Proneural bHLH factors share a common ability to promote neuronal differentiation. For example, misexpression of *Ngn* and *NeuroD* in the *Xenopus* open neural plate promotes dramatic ectopic neuron induction (Lee et al., 1995; Ma et al., 1996). Furthermore, in the *Mash1* knockout spinal cord, cells cannot become neurons and instead become immature glia, demonstrating that *Mash1* has the intrinsic capacity to specify neuronal differentiation (Battiste et al., 2007). However, how the proneural genes exert their "proneural" activity is not well understood. It has been proposed that proneural bHLH factors may regulate the production of diverse neuronal subclasses by initiating a core neuronal differentiation program, as well as activating certain genes that are required for the acquisition of particular cell fates (Brunet and Ghysen, 1999; Powell and Jarman, 2008). In this model, multiple bHLH factors target a set of lineage independent genes that are important for neuronal differentiation, while select bHLH factors are able to upregulate genes specific to particular lineages. It has been shown that bHLH factors select target gene sets by binding to different cofactors or selectively interacting with specific enhancers in the target gene regulatory sequences (Powell and Jarman, 2008). However, our understanding of how the proneural bHLH factors regulate core neuronal differentiation programs as well as specific targets is still limited.

To identify the downstream targets of proneural bHLH factors, several groups have determined gene expression profile in bHLH mutant animals by microarray analysis. For example, Matter and colleagues compared the gene expression profiles between wild type and *Ngn2* mutant cortical neurons (Mattar et al., 2004), and Mu and his group revealed that numerous genes are expressed downstream of *Math5* (Mu et al.,

2005). However, this technology cannot distinguish between direct and indirect targets. Previous studies in our laboratory demonstrated a set of genes that are directly regulated by *Ath5* and *NeuroD* in *Xenopus* animal cap explants, identifying genes involved in regulation of transcription, cell cycle modification, neuronal-specific exocytosis, and several novel targets (Figure 1.2) (Logan et al., 2005).

Expression of these target genes in the *Xenopus* retina falls into two basic patterns. Some genes are restricted to progenitors and early differentiating cells in the ciliary marginal zone (CMZ), highly overlapping with that of *Ath5* and other bHLH factors expression, while others are expressed in late-differentiating cells as they migrate to their final positions in the mature retinal layer, virtually identical to *Brn3d*, a previously described target of *Xenopus Ath5* and *NeuroD*. These distinct patterns suggest that some targets are involved in initiation of differentiation, while others function in neuronal terminal differentiation (Logan et al., 2005). Similar to our analysis, Seo and his group screened for direct targets of *Ngn* and *NeuroD* in both *Xenopus* and mouse (Seo et al., 2007). Their data suggest that *Ngn* and *NeuroD* directly coordinate target genes regulating neuronal cell fate, morphology and migration. Moreover, *Ngn* and *NeuroD* regulate similar targets in both *Xenopus* and mouse, suggesting that these genes are evolutionarily conserved core mediators of neurogenesis (Seo et al., 2007). In addition, several shared downstream targets of *Ngn* and *NeuroD* act as critical links connecting proneural bHLH factors with other signaling pathways, such as *Gadd45-gamma*, a cell cycle regulator, and *MyT1*, which is involved in inhibiting Notch signaling pathway. However, among the downstream targets of *NeuroD*, no genes are directly related to neuronal functions such as coding for neuropeptides, neurotransmitter receptors, or

channels, indicating proneural factors may regulate neuronal differentiation by inducing transcription factor targets, which then subsequently inducing genes controlling neuronal function. It is also possible that the cell context used for this screen has enriched for targets regulated by *NeuroD* in committed and differentiating primary progenitors, while unfavorable to detect the targets for late development process (Seo et al., 2007). Interestingly, there are several targets that are found in both analyses. For example, *Ebf2* and *Gadd45-gamma* are shared targets of *Ngn*, *Ath5* and *NeuroD*, suggesting that they act as common effectors to mediate proneural bHLH function.

Taken together, the downstream targets of proneural bHLH factors span multiple processes during development, and many of these targets are transcription factors involved in early differentiating effects. These data suggest that proneural bHLH factors act at the top of a regulatory cascade to initiate neurogenesis.

Interestingly, in each screen, there are a large number of novel targets with uncharacterized expression and function in the developing nervous system. Understanding the function and regulation of these novel targets will greatly benefit our understanding of neural development.

SBT1 is a novel bHLH target and acts as a critical regulator of *Xenopus* neurogenesis

Shared bHLH target gene 1 (SBT1) was identified as a shared transcriptional target of *atonal*-related bHLH factors *Ath5* and *NeuroD* in *Xenopus laevis* animal caps (Logan et al., 2005). It was also found as a direct target of *Ngn* and *NeuroD* (Seo et al., 2007). Blast analysis identified related protein sequences in several species, including *Xenopus tropicalis*, chick, mouse and human with no known functional domains or

motifs. However, the amino acid identity between *Xenopus laevis* and mouse is only 25% (Logan, 2006) (Figure 1.3).

In *Xenopus*, *Sbt1* is first detected in scattered cells of differentiating primary neurons at the open neural plate stage, which is consistent with the spatial expression of known proneural bHLH targets (Figure 1.4 A). *Sbt1* expression is found throughout the neural tube and is present in the optic vesicle shortly after the onset of retinal neurogenesis (Figure 1.4 B) (Logan, 2006). In the mature retina, *Sbt1* expression is restricted to the central ciliary marginal zone (CMZ), indicating it may be transiently expressed in differentiating neurons (Figure 1.4 C, D). This expression pattern is in contrast to other targets, such as *Brn3d* and *Ebf3*, which are expressed in later differentiating cells and mature retinal neurons, suggesting their functions in neuronal terminal differentiation. In addition, SBT1 protein localizes to the nucleus and cell membrane, suggesting it may shuttle between different cellular compartments. Interestingly, a candidate nuclear localization sequence (NLS) is conserved in the SBT1 sequence in several species, suggesting a nuclear function.

To understand the function of *Sbt1* in neurogenesis, we performed gain-of-function experiments by *Sbt1* mRNA injection and loss-of-function experiments by antisense morpholino oligo nucleotide injection (Figure 1.5). We found that *Sbt1* promotes neuronal differentiation in the open neural plate and retina in *Xenopus* embryos. Especially in the retina, overexpression of *Sbt1* promotes differentiation of early born cell types, including RGCs, horizontal cells and probably cone photoreceptors, at the expense of later born cell types, such as bipolar cells and Müller glia. Blocking *Sbt1* function by antisense morpholino injection strongly promotes Müller glial or neuroepithelial cell fates

at the expense of virtually all neuronal cell types in the *Xenopus* retina (Figure 1.5). These results reveal that *Sbt1* is an important contributor to neural differentiation in *Xenopus laevis* (Logan, 2006).

Importantly, misexpression of mouse *Sbt1* in cleavage stage *Xenopus* embryos has similar effects as *Xenopus Sbt1*. It localizes to both nucleus and membrane in the animal cap ectoderm, and promotes neural differentiation in the open neural plate. In the *Xenopus* retina, overexpression of mouse *Sbt1* promotes early born cell types at the expense of later cell types, strikingly similar to *Xenopus Sbt1* overexpression. These results suggest that *Sbt1* function is conserved between *Xenopus* and mouse.

However, *Sbt1* expression and function are completely uncharacterized in mouse, and the mouse presents experimental advantages for the in vivo analysis of gene function. For example, retinal neurogenesis in mouse takes place over a 2-week window, as opposed to 24 hours in *Xenopus*, so there is better spatial and temporal resolution to retinal histogenesis and the expression of genes regulating these events. In addition, *Sbt1* was identified as a shared target for multiple bHLH factors. However, we do not know which factors specifically regulate its expression, and this question is very difficult to address in *Xenopus*. In mouse, we can analyze *Sbt1* expression in various bHLH mutants to answer whether its expression depends upon specific proneural bHLH factors.

The goal of this thesis was to determine the expression and function of *Sbt1* gene in mouse neural development. In Chapter 2, I analyze the spatial and temporal expression of *Sbt1* in the mouse nervous system, and find it is expressed in the developing spinal cord, cortex, brainstem and retina, and its expression in the spinal cord and cortex persists to adulthood. Furthermore, *Sbt1* expression in the early embryonic brainstem and retina

are transiently regulated by *Ngn2*, confirming it is a downstream target of bHLH factors. To further understand *Sbt1* function, I generated an *Sbt1-eGFPCre* knockin mouse. In Chapter 3, I compare neuronal versus glial cell numbers in the *Sbt1* mutant cortex and spinal cord. I also examine major retinal cell fates in the *Sbt1* mutant retina. Surprisingly, our evidence suggests that *Sbt1* is not required for mouse neurogenesis, especially major cell fate specification, indicating that other gene(s) may have redundant functions with *Sbt1* in mutant animals. However, further analysis is needed to elucidate the function of *Sbt1* in generation of small subclasses of neurons in nervous system development, as well as effects on neuronal maturation.

References

Artavanis-Tsakonas, S., Rand, M. D., and Lake, R. J. (1999). Notch signaling: cell fate control and signal integration in development. Science *284*, 770-776.

Aruga, J. (2004). The role of Zic genes in neural development. Mol Cell Neurosci *26*, 205-221.

Bally-Cuif, L., and Hammerschmidt, M. (2003). Induction and patterning of neuronal development, and its connection to cell cycle control. Curr Opin Neurobiol *13*, 16-25.

Battiste, J., Helms, A. W., Kim, E. J., Savage, T. K., Lagace, D. C., Mandyam, C. D., Eisch, A. J., Miyoshi, G., and Johnson, J. E. (2007). Ascl1 defines sequentially generated lineage-restricted neuronal and oligodendrocyte precursor cells in the spinal cord. Development *134*, 285-293.

Bellefroid, E. J., Bourguignon, C., Hollemann, T., Ma, Q., Anderson, D. J., Kintner, C., and Pieler, T. (1996). X-MyT1, a Xenopus C2HC-type zinc finger protein with a regulatory function in neuronal differentiation. Cell *87*, 1191-1202.

Berlucchi, G., and Buchtel, H. A. (2009). Neuronal plasticity: historical roots and evolution of meaning. Exp Brain Res *192*, 307-319.

Bertrand, N., Castro, D. S., and Guillemot, F. (2002). Proneural genes and the specification of neural cell types. Nat Rev Neurosci *3*, 517-530.

Bhattacharya, S., Dooley, C., Soto, F., Madson, J., Das, A. V., and Ahmad, I. (2004). Involvement of Ath3 in CNTF-mediated differentiation of the late retinal progenitors. Mol Cell Neurosci *27*, 32-43.

Brennan, C. A., and Moses, K. (2000). Determination of Drosophila photoreceptors: timing is everything. Cell Mol Life Sci *57*, 195-214.

Brown, N. L., Kanekar, S., Vetter, M. L., Tucker, P. K., Gemza, D. L., and Glaser, T. (1998). Math5 encodes a murine basic helix-loop-helix transcription factor expressed during early stages of retinal neurogenesis. Development *125*, 4821-4833.

Brown, N. L., Patel, S., Brzezinski, J., and Glaser, T. (2001). Math5 is required for retinal ganglion cell and optic nerve formation. Development *128*, 2497-2508.

Brunet, J. F., and Ghysen, A. (1999). Deconstructing cell determination: proneural genes and neuronal identity. Bioessays *21*, 313-318.

Buchtel, H. A., Berlucchi, G., and Mascetti, G. G. (2009). Behavioural and electrophysiological analysis of strabismus in cats: modern context. Exp Brain Res *192*, 359-367.

Chitnis, A., and Kintner, C. (1996). Sensitivity of proneural genes to lateral inhibition affects the pattern of primary neurons in Xenopus embryos. Development *122*, 2295-2301.

Dorsky, R. I., Chang, W. S., Rapaport, D. H., and Harris, W. A. (1997). Regulation of neuronal diversity in the Xenopus retina by Delta signalling. Nature *385*, 67-70.

Edlund, T., and Jessell, T. M. (1999). Progression from extrinsic to intrinsic signaling in cell fate specification: a view from the nervous system. Cell *96*, 211-224.

Erkman, L., McEvilly, R. J., Luo, L., Ryan, A. K., Hooshmand, F., O'Connell, S. M., Keithley, E. M., Rapaport, D. H., Ryan, A. F., and Rosenfeld, M. G. (1996). Role of transcription factors Brn-3.1 and Brn-3.2 in auditory and visual system development. Nature *381*, 603-606.

Feng, L., Xie, Z. H., Ding, Q., Xie, X., Libby, R. T., and Gan, L. (2010). MATH5 controls the acquisition of multiple retinal cell fates. Mol Brain *3*, 36.

Furukawa, T., Mukherjee, S., Bao, Z. Z., Morrow, E. M., and Cepko, C. L. (2000). rax, Hes1, and notch1 promote the formation of Muller glia by postnatal retinal progenitor cells. Neuron *26*, 383-394.

Garcia-Dominguez, M., Poquet, C., Garel, S., and Charnay, P. (2003). Ebf gene function is required for coupling neuronal differentiation and cell cycle exit. Development *130*, 6013-6025.

Gomez-Skarmeta, J. L., Campuzano, S., and Modolell, J. (2003). Half a century of neural prepatterning: the story of a few bristles and many genes. Nat Rev Neurosci *4*, 587-598.

Gonzalez-Quevedo, R., Lee, Y., Poss, K. D., and Wilkinson, D. G. (2010). Neuronal regulation of the spatial patterning of neurogenesis. Dev Cell *18*, 136-147.

Guillemot, F. (2007). Spatial and temporal specification of neural fates by transcription factor codes. Development *134*, 3771-3780.

Guillemot, F., Lo, L. C., Johnson, J. E., Auerbach, A., Anderson, D. J., and Joyner, A. L. (1993). Mammalian achaete-scute homolog 1 is required for the early development of olfactory and autonomic neurons. Cell *75*, 463-476.

Hatakeyama, J., and Kageyama, R. (2004). Retinal cell fate determination and bHLH factors. Semin Cell Dev Biol *15*, 83-89.

Hatakeyama, J., Tomita, K., Inoue, T., and Kageyama, R. (2001). Roles of homeobox and bHLH genes in specification of a retinal cell type. Development *128*, 1313-1322.

Heitzler, P., Bourouis, M., Ruel, L., Carteret, C., and Simpson, P. (1996). Genes of the Enhancer of split and achaete-scute complexes are required for a regulatory loop between Notch and Delta during lateral signalling in Drosophila. Development *122*, 161-171.

Helms, A. W., Battiste, J., Henke, R. M., Nakada, Y., Simplicio, N., Guillemot, F., and Johnson, J. E. (2005). Sequential roles for Mash1 and Ngn2 in the generation of dorsal spinal cord interneurons. Development *132*, 2709-2719.

Hojo, M., Ohtsuka, T., Hashimoto, N., Gradwohl, G., Guillemot, F., and Kageyama, R. (2000). Glial cell fate specification modulated by the bHLH gene Hes5 in mouse retina. Development *127*, 2515-2522.

Huang, Z. J., and Scheiffele, P. (2008). GABA and neuroligin signaling: linking synaptic activity and adhesion in inhibitory synapse development. Curr Opin Neurobiol *18*, 77-83.

Hufnagel, R. B., Le, T. T., Riesenberg, A. L., and Brown, N. L. (2010). Neurog2 controls the leading edge of neurogenesis in the mammalian retina. Dev Biol *340*, 490-503.

Hutcheson, D. A., and Vetter, M. L. (2001). The bHLH factors Xath5 and XNeuroD can upregulate the expression of XBrn3d, a POU-homeodomain transcription factor. Dev Biol *232*, 327-338.

Jadhav, A. P., Mason, H. A., and Cepko, C. L. (2006). Notch 1 inhibits photoreceptor production in the developing mammalian retina. Development *133*, 913-923.

Jasoni, C. L., and Reh, T. A. (1996). Temporal and spatial pattern of MASH-1 expression in the developing rat retina demonstrates progenitor cell heterogeneity. J Comp Neurol *369*, 319-327.

Jo, A. Y., Park, C. H., Aizawa, S., and Lee, S. H. (2007). Contrasting and brain region-specific roles of neurogenin2 and mash1 in GABAergic neuron differentiation in vitro. Exp Cell Res *313*, 4066-4081.

Kageyama, R., Ohtsuka, T., Shimojo, H., and Imayoshi, I. (2009). Dynamic regulation of Notch signaling in neural progenitor cells. Curr Opin Cell Biol *21*, 733-740.

Kele, J., Simplicio, N., Ferri, A. L., Mira, H., Guillemot, F., Arenas, E., and Ang, S. L. (2006). Neurogenin 2 is required for the development of ventral midbrain dopaminergic neurons. Development *133*, 495-505.

Kunisch, M., Haenlin, M., and Campos-Ortega, J. A. (1994). Lateral inhibition mediated by the Drosophila neurogenic gene delta is enhanced by proneural proteins. Proc Natl Acad Sci U S A *91*, 10139-10143.

Lamar, E., Kintner, C., and Goulding, M. (2001). Identification of NKL, a novel Gli-Kruppel zinc-finger protein that promotes neuronal differentiation. Development *128*, 1335-1346.

Le, T. T., Wroblewski, E., Patel, S., Riesenberg, A. N., and Brown, N. L. (2006). Math5 is required for both early retinal neuron differentiation and cell cycle progression. Dev Biol *295*, 764-778.

Lee, H. Y., Wroblewski, E., Philips, G. T., Stair, C. N., Conley, K., Reedy, M., Mastick, G. S., and Brown, N. L. (2005). Multiple requirements for Hes 1 during early eye formation. Dev Biol *284*, 464-478.

Lee, J. E., Hollenberg, S. M., Snider, L., Turner, D. L., Lipnick, N., and Weintraub, H. (1995). Conversion of Xenopus ectoderm into neurons by NeuroD, a basic helix-loop-helix protein. Science *268*, 836-844.

Levine, E. M., and Green, E. S. (2004). Cell-intrinsic regulators of proliferation in vertebrate retinal progenitors. Semin Cell Dev Biol *15*, 63-74.

Liu, J., Sato, C., Cerletti, M., and Wagers, A. (2010). Notch signaling in the regulation of stem cell self-renewal and differentiation. Curr Top Dev Biol *92*, 367-409.

Liu, W., Mo, Z., and Xiang, M. (2001). The Ath5 proneural genes function upstream of Brn3 POU domain transcription factor genes to promote retinal ganglion cell development. Proc Natl Acad Sci U S A *98*, 1649-1654.

Livesey, F. J., and Cepko, C. L. (2001). Vertebrate neural cell-fate determination: lessons from the retina. Nat Rev Neurosci *2*, 109-118.

Logan, M. A. (2006) Mechanisms of neuronal differentiation in Xenopus Laevis, Ph.D, The University of Utah.

Logan, M. A., Steele, M. R., Van Raay, T. J., and Vetter, M. L. (2005). Identification of shared transcriptional targets for the proneural bHLH factors Xath5 and XNeuroD. Dev Biol *285*, 570-583.

Louvi, A., and Artavanis-Tsakonas, S. (2006). Notch signalling in vertebrate neural development. Nat Rev Neurosci *7*, 93-102.

Ma, Q., Chen, Z., del Barco Barrantes, I., de la Pompa, J. L., and Anderson, D. J. (1998). neurogenin1 is essential for the determination of neuronal precursors for proximal cranial sensory ganglia. Neuron *20*, 469-482.

Ma, Q., Kintner, C., and Anderson, D. J. (1996). Identification of neurogenin, a vertebrate neuronal determination gene. Cell *87*, 43-52.

Ma, W., and Wang, S. Z. (2006). The final fates of neurogenin2-expressing cells include all major neuron types in the mouse retina. Mol Cell Neurosci *31*, 463-469.

Martinez-Morales, J. R., Del Bene, F., Nica, G., Hammerschmidt, M., Bovolenta, P., and Wittbrodt, J. (2005). Differentiation of the vertebrate retina is coordinated by an FGF signaling center. Dev Cell *8*, 565-574.

Masai, I., Stemple, D. L., Okamoto, H., and Wilson, S. W. (2000). Midline signals regulate retinal neurogenesis in zebrafish. Neuron *27*, 251-263.

Mathers, P. H., and Jamrich, M. (2000). Regulation of eye formation by the Rx and pax6 homeobox genes. Cell Mol Life Sci *57*, 186-194.

Mattar, P., Britz, O., Johannes, C., Nieto, M., Ma, L., Rebeyka, A., Klenin, N., Polleux, F., Guillemot, F., and Schuurmans, C. (2004). A screen for downstream effectors of Neurogenin2 in the embryonic neocortex. Dev Biol *273*, 373-389.

Merot, Y., Retaux, S., and Heng, J. I. (2009). Molecular mechanisms of projection neuron production and maturation in the developing cerebral cortex. Semin Cell Dev Biol *20*, 726-734.

Mizuguchi, R., Kriks, S., Cordes, R., Gossler, A., Ma, Q., and Goulding, M. (2006). Ascl1 and Gsh1/2 control inhibitory and excitatory cell fate in spinal sensory interneurons. Nat Neurosci *9*, 770-778.

Moore, K. B., Schneider, M. L., and Vetter, M. L. (2002). Posttranslational mechanisms control the timing of bHLH function and regulate retinal cell fate. Neuron *34*, 183-195.

Morrow, E. M., Furukawa, T., Lee, J. E., and Cepko, C. L. (1999). NeuroD regulates multiple functions in the developing neural retina in rodent. Development *126*, 23-36.

Mu, X., Beremand, P. D., Zhao, S., Pershad, R., Sun, H., Scarpa, A., Liang, S., Thomas, T. L., and Klein, W. H. (2004). Discrete gene sets depend on POU domain transcription factor Brn3b/Brn-3.2/POU4f2 for their expression in the mouse embryonic retina. Development *131*, 1197-1210.

Mu, X., Fu, X., Sun, H., Beremand, P. D., Thomas, T. L., and Klein, W. H. (2005). A gene network downstream of transcription factor Math5 regulates retinal progenitor cell competence and ganglion cell fate. Dev Biol *280*, 467-481.

Muroyama, Y., Fujiwara, Y., Orkin, S. H., and Rowitch, D. H. (2005). Specification of astrocytes by bHLH protein SCL in a restricted region of the neural tube. Nature *438*, 360-363.

Navarro, X. (2009). Chapter 27: Neural plasticity after nerve injury and regeneration. Int Rev Neurobiol *87*, 483-505.

Neumann, C. J., and Nuesslein-Volhard, C. (2000). Patterning of the zebrafish retina by a wave of sonic hedgehog activity. Science *289*, 2137-2139.

Nieto, M., Schuurmans, C., Britz, O., and Guillemot, F. (2001). Neural bHLH genes control the neuronal versus glial fate decision in cortical progenitors. Neuron *29*, 401-413.

Ochocinska, M. J., and Hitchcock, P. F. (2009). NeuroD regulates proliferation of photoreceptor progenitors in the retina of the zebrafish. Mech Dev *126*, 128-141.

Ohnuma, S., Hopper, S., Wang, K. C., Philpott, A., and Harris, W. A. (2002). Co-ordinating retinal histogenesis: early cell cycle exit enhances early cell fate determination in the Xenopus retina. Development *129*, 2435-2446.

Ono, Y., Nakatani, T., Minaki, Y., and Kumai, M. (2010). The basic helix-loop-helix transcription factor Nato3 controls neurogenic activity in mesencephalic floor plate cells. Development *137*, 1897-1906.

Pan, L., Yang, Z., Feng, L., and Gan, L. (2005). Functional equivalence of Brn3 POU-domain transcription factors in mouse retinal neurogenesis. Development *132*, 703-712.

Patel, A., and McFarlane, S. (2000). Overexpression of FGF-2 alters cell fate specification in the developing retina of Xenopus laevis. Dev Biol *222*, 170-180.

Poggi, L., Vitorino, M., Masai, I., and Harris, W. A. (2005). Influences on neural lineage and mode of division in the zebrafish retina in vivo. J Cell Biol *171*, 991-999.

Powell, L. M., and Jarman, A. P. (2008). Context dependence of proneural bHLH proteins. Curr Opin Genet Dev *18*, 411-417.

Pozzoli, O., Bosetti, A., Croci, L., Consalez, G. G., and Vetter, M. L. (2001). Xebf3 is a regulator of neuronal differentiation during primary neurogenesis in Xenopus. Dev Biol *233*, 495-512.

Ross, S. E., Greenberg, M. E., and Stiles, C. D. (2003). Basic helix-loop-helix factors in cortical development. Neuron *39*, 13-25.

Schneider, M. L., Turner, D. L., and Vetter, M. L. (2001). Notch signaling can inhibit Xath5 function in the neural plate and developing retina. Mol Cell Neurosci *18*, 458-472.

Seo, S., Lim, J. W., Yellajoshyula, D., Chang, L. W., and Kroll, K. L. (2007). Neurogenin and NeuroD direct transcriptional targets and their regulatory enhancers. Embo J *26*, 5093-5108.

Sprinzak, D., Lakhanpal, A., Lebon, L., Santat, L. A., Fontes, M. E., Anderson, G. A., Garcia-Ojalvo, J., and Elowitz, M. B. (2010). Cis-interactions between Notch and Delta generate mutually exclusive signalling states. Nature *465*, 86-90.

Sugimori, M., Nagao, M., Bertrand, N., Parras, C. M., Guillemot, F., and Nakafuku, M. (2007). Combinatorial actions of patterning and HLH transcription factors in the spatiotemporal control of neurogenesis and gliogenesis in the developing spinal cord. Development *134*, 1617-1629.

Surmeier, D. J., Plotkin, J., and Shen, W. (2009). Dopamine and synaptic plasticity in dorsal striatal circuits controlling action selection. Curr Opin Neurobiol *19*, 621-628.

Takebayashi, K., Takahashi, S., Yokota, C., Tsuda, H., Nakanishi, S., Asashima, M., and Kageyama, R. (1997). Conversion of ectoderm into a neural fate by ATH-3, a vertebrate basic helix-loop-helix gene homologous to Drosophila proneural gene atonal. Embo J *16*, 384-395.

Tomita, K., Moriyoshi, K., Nakanishi, S., Guillemot, F., and Kageyama, R. (2000). Mammalian achaete-scute and atonal homologs regulate neuronal versus glial fate determination in the central nervous system. Embo J *19*, 5460-5472.

Tomita, K., Nakanishi, S., Guillemot, F., and Kageyama, R. (1996). Mash1 promotes neuronal differentiation in the retina. Genes Cells *1*, 765-774.

Vetter, M. L., and Brown, N. L. (2001). The role of basic helix-loop-helix genes in vertebrate retinogenesis. Semin Cell Dev Biol *12*, 491-498.

Wang, S. S., Tsai, R. Y., and Reed, R. R. (1997). The characterization of the Olf-1/EBF-like HLH transcription factor family: implications in olfactory gene regulation and neuronal development. J Neurosci *17*, 4149-4158.

Wang, S. W., Kim, B. S., Ding, K., Wang, H., Sun, D., Johnson, R. L., Klein, W. H., and Gan, L. (2001). Requirement for math5 in the development of retinal ganglion cells. Genes Dev *15*, 24-29.

Westenskow, P., Piccolo, S., and Fuhrmann, S. (2009). Beta-catenin controls differentiation of the retinal pigment epithelium in the mouse optic cup by regulating Mitf and Otx2 expression. Development *136*, 2505-2510.

Wilson, S. W., and Rubenstein, J. L. (2000). Induction and dorsoventral patterning of the telencephalon. Neuron *28*, 641-651.

Wu, S., Wu, Y., and Capecchi, M. R. (2006). Motoneurons and oligodendrocytes are sequentially generated from neural stem cells but do not appear to share common lineage-restricted progenitors in vivo. Development *133*, 581-590.

Yang, X. J. (2004). Roles of cell-extrinsic growth factors in vertebrate eye pattern formation and retinogenesis. Semin Cell Dev Biol *15*, 91-103.

Yaron, O., Farhy, C., Marquardt, T., Applebury, M., and Ashery-Padan, R. (2006). Notch1 functions to suppress cone-photoreceptor fate specification in the developing mouse retina. Development *133*, 1367-1378.

Young, R. W. (1985). Cell proliferation during postnatal development of the retina in the mouse. Brain Res *353*, 229-239.

Zhang, F., Nakanishi, G., Kurebayashi, S., Yoshino, K., Perantoni, A., Kim, Y. S., and Jetten, A. M. (2002). Characterization of Glis2, a novel gene encoding a Gli-related, Kruppel-like transcription factor with transactivation and repressor functions. Roles in kidney development and neurogenesis. J Biol Chem *277*, 10139-10149.

Zhou, Q., Wang, S., and Anderson, D. J. (2000). Identification of a novel family of oligodendrocyte lineage-specific basic helix-loop-helix transcription factors. Neuron *25*, 331-343.

Table 1.1. Proneural gene expression and cell fate specification in the vertebrate retina.

Proneural bHLHs	Expression window	Expression cells	Regulate specific cell fate	References
Ath5	E11 to P0	Retinal progenitors	RGC	(Brown et al., 1998) (Brown et al., 2001) (Wang et al., 2001)
Ath3	E12.5 to adult retina	Retinal progenitors and HZ, AM and BP	HZ, AM, BP	(Bhattacharya et al., 2004; Hatakeyama et al., 2001; Takebayashi et al., 1997) (Hatakeyama and Kageyama, 2004)
Ngn2	E11 to P0	Retinal progenitors RGCs	?	(Brown et al., 1998) (Lee et al., 2005) (Ma and Wang, 2006)
NeuroD	E13.5 to adult retinas	Retinal progenitors, AM, photoreceptors	AM, cone and rod photoreceptors	(Brown et al., 1998) (Morrow et al., 1999)
Ash1	E14.5 to P9	Retinal progenitors	BP, cone and rod photoreceptors	(Guillemot et al., 1993) (Jasoni and Reh, 1996; Tomita et al., 2000) (Tomita et al., 1996)

RGC: retinal ganglion cell; HZ, horizontal cell; AM: amacrine cell; BP: bipolar cell.

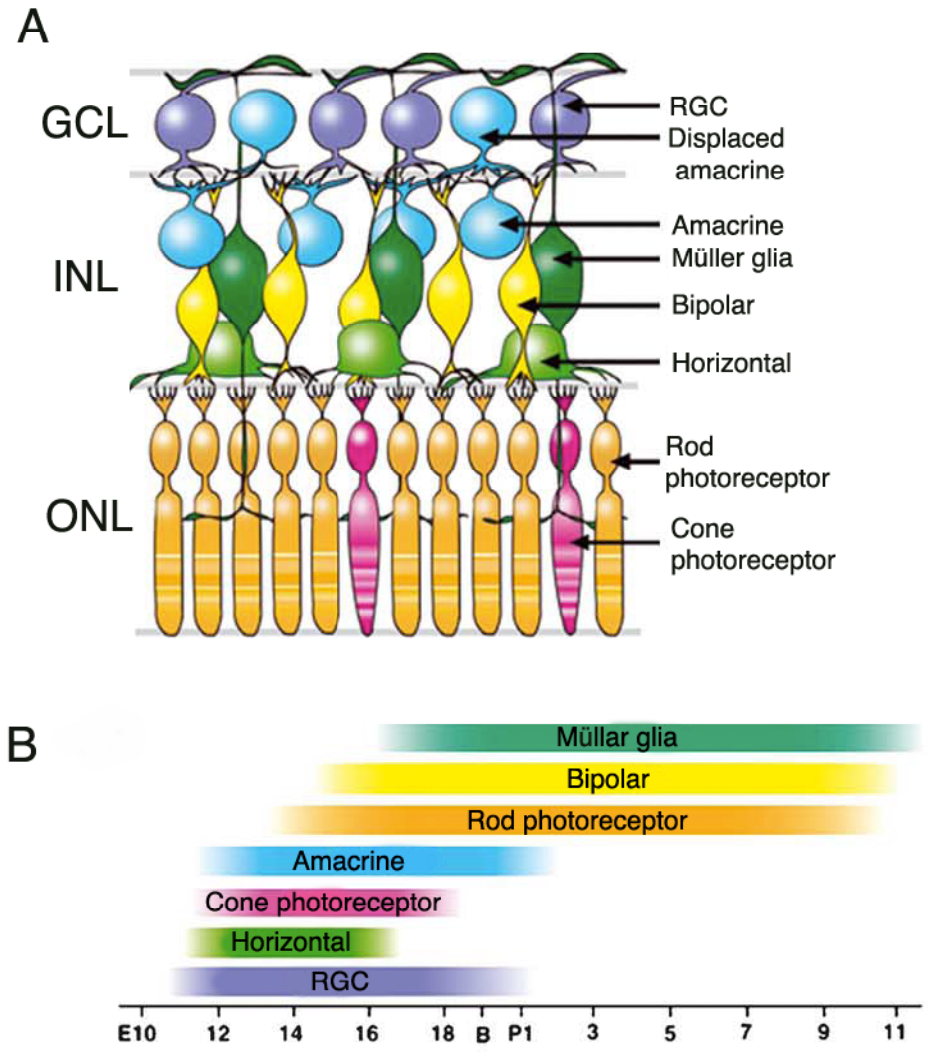


Figure 1.1 Schematic structure of the neural retina.
(A) The vertebrate retina consists of three layers and seven major cell types. (B) The retinal cell types are generated in a conserved order. GCL, Ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer; RGC, retinal ganglion cell (adapted and reprinted with permission from Hatakeyama and Kageyama 2004).

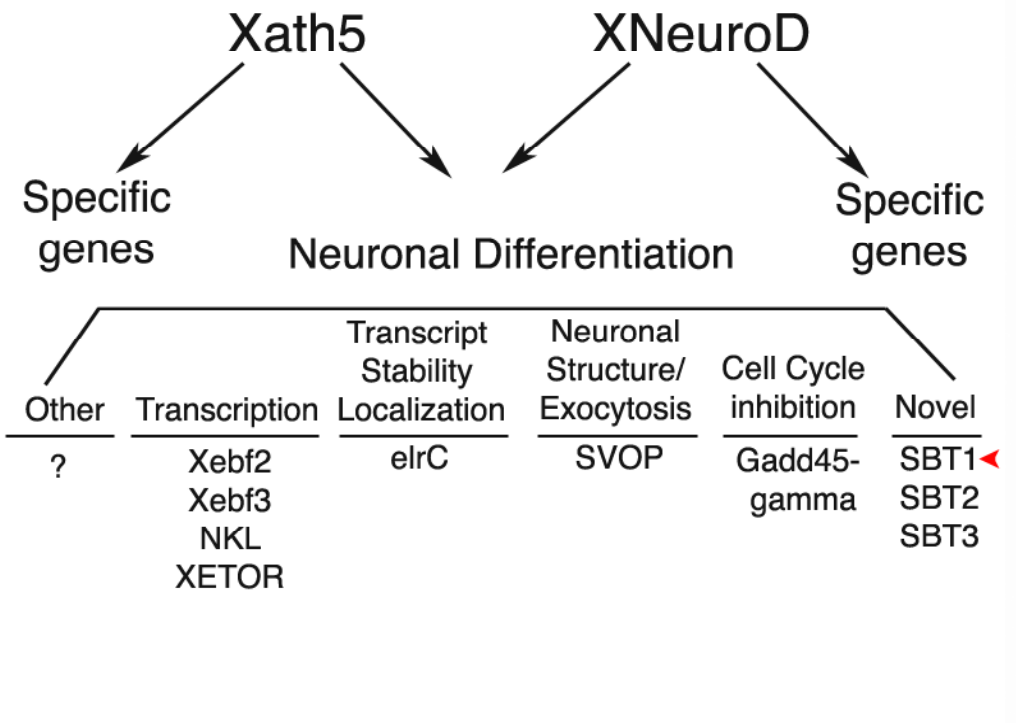
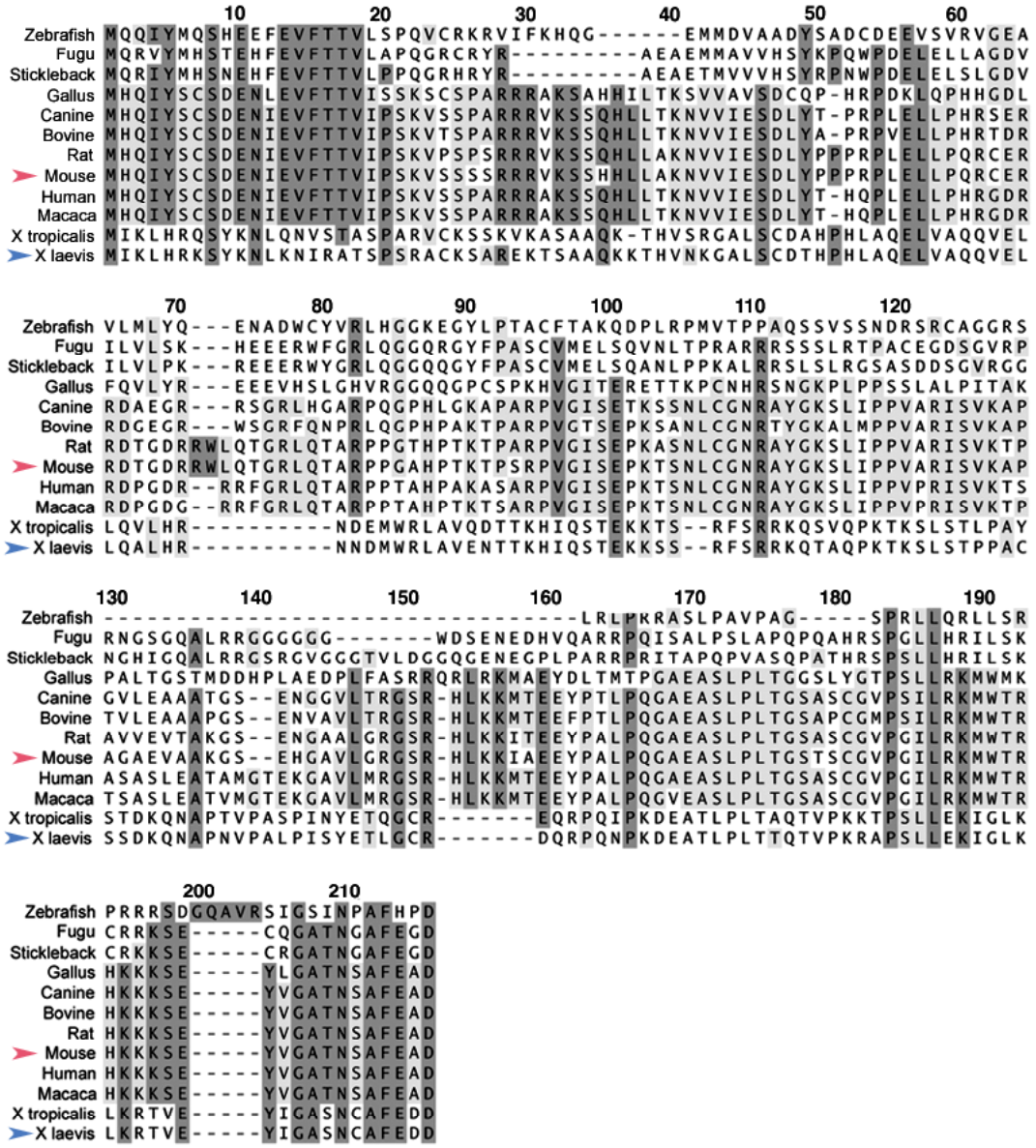


Figure 1.2 *Xenopus Ath5* and *NeuroD* activate a set of shared target genes involved in neuronal differentiation as well as specific targets (adapted and reprinted with permission from Logan 2006).

Figure 1.3 Alignment of SBT1 proteins. The predicted protein sequence of *Xenopus laevis* SBT1 (Translation of Accession Number BJ029724) was aligned to predicted SBT1 sequences from Danio rerio (Accession XP_699157), Takifugu rubripes, Gasterosteus aculeatus (Translation of Accession DW671154), Gallus gallus (Accession XM_001231371), Canis familiaris (XP_852534), Bos taurus (NP_001069943), Rattus norvegicus (Accession XP_575752), Mus musculus (Accession NP_848486), Human (Accession NP_689978), Macaca mulatta (Accession XM_001094918), and Xenopus tropicalis (Translation of Accession CX393883) using the ClustalW program. Residues conserved in at least 70% of the aligned sequences are indicated with dark gray shading while light gray shading indicates residues that are 50-69% conserved. Red arrowhead indicates *Xenopus laevis* sequence and blue arrowhead indicates mouse sequence (adapted with permission from Monica Vetter).


10 20 30 40 50 60
Zebrafish MQQIYMQSHEEFEVFTTVLSPQVCRKRVIFKHQG------EMMDVAADYSADCDEEVSVRVGEA
Fugu MQRVYMHSTEHFEVFTTVLAPQGRCRYR---------AEAEMMAVVHSYKPQWPDELELLAGDV
Stickleback MQRIYMHSNEHFEVFTTVLPPQGRHRYR---------AEAETMVVVHSYRPNWPDELELSLGDV
Gallus MHQIYSCSDENLEVFTTVISSKSCSPARRRAKSAHHILTKSVVAVSDCQP-HRPDKLQPHHGDL
Canine MHQIYSCSDENIEVFTTVIPSKVSSPARRRVKSSQHLLTKNVVIESDLYT-PRPLELLPHRSER
Bovine MHQIYSCSDENIEVFTTVIPSKVTSPARRRVKSSQHLLTKNVVIESDLYA-PRPVELLPHRTDR
Rat MHQIYSCSDENIEVFTTVIPSKVPSPSRRRVKSSQHLLAKNVVIESDLYPPPRPLELLPQRCER
Mouse MHQIYSCSDENIEVFTTVIPSKVSSSSRRRVKSSHHLLAKNVVIESDLYPPPRPLELLPQRCER
Human MHQIYSCSDENIEVFTTVIPSKVSSPARRRAKSSQHLLTKNVVIESDLYT-HQPLELLPHRGDR
Macaca MHQIYSCSDENIEVFTTVIPSKVSSPARRRAKSSQHLLTKNVVIESDLYT-HQPLELLPHRGDR
X tropicalis MIKLHRQSYKNLQNVSTASPARVCKSSKVKASAAQK-THVSRGALSCDAHPHLAQELVAQQVEL
X laevis MIKLHRKSYKNLKNIRATSPSRACKSAREKTSAAQKKTHVNKGALSCDTHPHLAQELVAQQVEL
70 80 90 100 110 120
Zebrafish VLMLYQ---ENADWCYVRLHGGKEGYLPTACFTAKQDPLRPMVTPPAQSSVSSNDRSRCAGGRS
Fugu ILVLSK---HEEERWFGRLQGGQRGYFPASCVMELSQVNLTPRARRRSSSLRTPACEGDSGVRP
Stickleback ILVLPK---REEERWYGRLQGGQQGYFPASCVMELSQANLPPKALRRSLSLRGSASDDSGVRGG
Gallus FQVLYR---EEEVHSLGHVRGGQQGPCSPKHVGITERETTKPCNHRSNGKPLPPSSLALPITAK
Canine RDAEGR---RSGRLHGARPQGPHLGKAPARPVGISETKSSNLCGNRAYGKSLIPPVARISVKAP
Bovine RDGEGR---WSGRFQNPRLQGPHPAKTPARPVGTSEPKSANLCGNRTYGKALMPPVARISVKAP
Rat RDTGDRRWLQTGRLQTARPPGTHPTKTPARPVGISEPKASNLCGNRAYGKSLIPPVARISVKTP
Mouse RDTGDRRWLQTGRLQTARPPGAHPTKTPSRPVGISEPKTSNLCGNRAYGKSLIPPVARISVKAP
Human RDPGDR--RRFGRLQTARPPTAHPAKASARPVGISEPKTSNLCGNRAYGKSLIPPVPRISVKTS
Macaca RDPGDG--RRFGRLQTARPPTAHPTKTSARPVGISEPKTSNLCGNRAYGKSLIPPVPRISVKTP
X tropicalis LQVLHR----------NDEMWRLAVQDTTKHIQSTEKKTS--RFSRRKQSVQPKTKSLSTLPAY
X laevis LQALHR----------NNDMWRLAVENTTKHIQSTEKKSS--RFSRRKQTAQPKTKSLSTPPAC
130 140 150 160 170 180 190
Zebrafish ----------LRLPKRASLPAVPAG-----SPRLLQRLLSR
Fugu RNGSGQALRRGGGGGG-------WDSENEDHVQARRPQISALPSLAPQPQAHRSPGLLHRILSK
Stickleback NGHIGQALRRGSRGVGGGTVLDGGQGENEGPLPARRPRITAPQPVASQPATHRSPSLLHRILSK
Gallus PALTGSTMDDHPLAEDPLFASRRQRLRKMAEYDLTMTPGAEASLPLTGGSLYGTPSLLRKMWMK
Canine GVLEAAATGS--ENGGVLTRGSR-HLKKMTEEYPTLPQGAEASLPLTGSASCGVPSILRKMWTR
Bovine TVLEAAAPGS--ENVAVLTRGSR-HLKKMTEEFPTLPQGAEASLPLTGSAPCGMPSILRKMWTR
Rat AVVEVTAKGS--ENGAALGRGSR-HLKKITEEYPALPQGAEASLPLTGSASCGVPGILRKMWTR
Mouse AGAEVAAKGS--EHGAVLGRGSR-HLKKIAEEYPALPQGAEASLPLTGSTSCGVPGILRKMWTR
Human ASASLEATAMGTEKGAVLMRGSR-HLKKMTEEYPALPQGAEASLPLTGSASCGVPGILRKMWTR
Macaca TSASLEATVMGTEKGAVLMRGSR-HLKKMTEEYPALPQGVEASLPLTGSASCGVPGILRKMWTR
X tropicalis STDKQNAPTVPASPINYETQGCR-------EQRPQIPKDEATLPLTAQTVPKKTPSLLEKIGLK
X laevis SSDKQNAPNVPALPISYETLGCR-------DQRPQNPKDEATLPLTTQTVPKRAPSLLEKIGLK
200 210
Zebrafish PRRRSDGQAVRSIGSINPAFHPD
Fugu CRRKSE-----CQGATNGAFEGD
Stickleback CRKKSE-----CRGATNGAFEGD
Gallus HKKKSE-----YLGATNSAFEAD
Canine HKKKSE-----YVGATNSAFEAD
Bovine HKKKSE-----YVGATNSAFEAD
Rat HKKKSE-----YVGATNSAFEAD
Mouse HKKKSE-----YVGATNSAFEAD
Human HKKKSE-----YVGATNSAFEAD
Macaca HKKKSE-----YVGATNSAFEAD
X tropicalis LKRTVE-----YIGASNCAFEDD
X laevis LKRTVE-----YIGASNCAFEDD

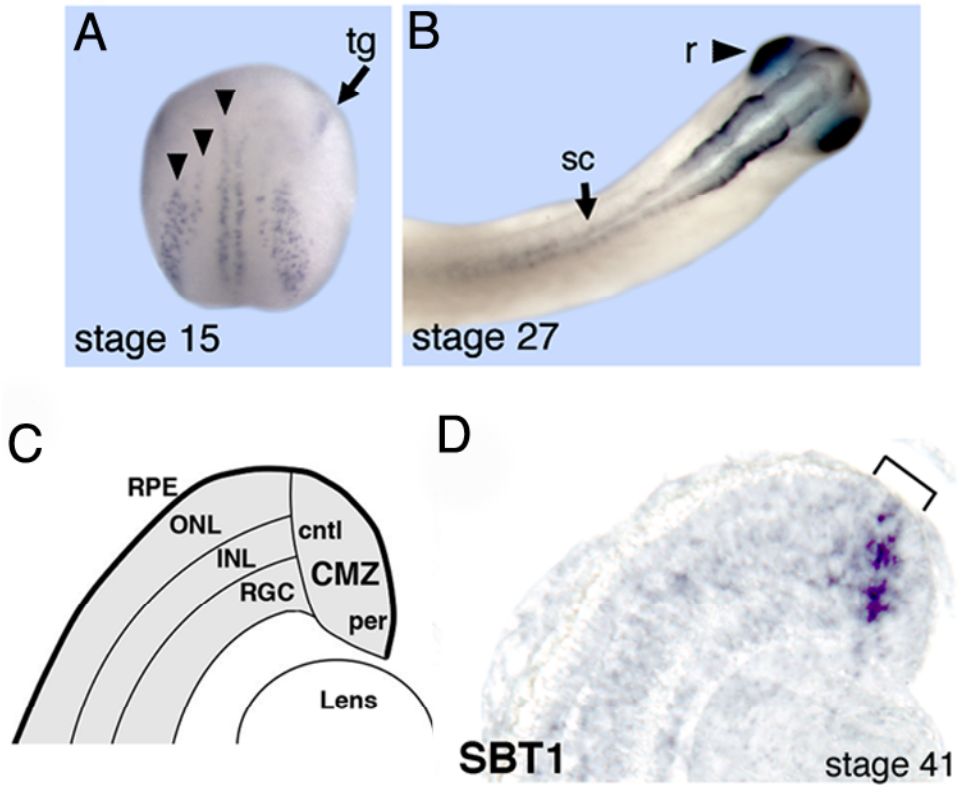
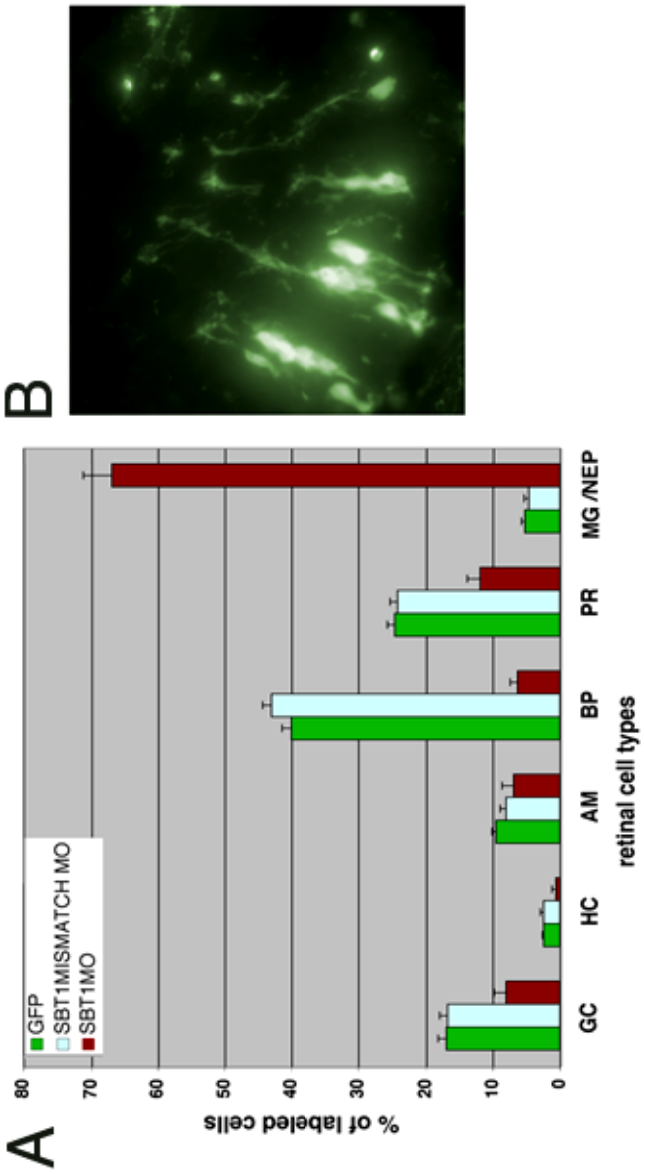


Figure 1.4 *Sbt1* is expressed in the *Xenopus* developing nervous system. (A) *Sbt1* is expressed in the primary neurons (arrowheads) and presumptive trigeminal ganglia (tg) at open neural plate stage. (B) *Sbt1* is expressed in the spinal cord (sc) and anterior neural structures including retina (r) at late stage. (C) Schematic of mature retinal sections. The central retina contains three layers of postmitotic retinal cells: retinal ganglion cell (RGC) layer, INL and ONL. The ciliary marginal zone (CMZ) contains retinal stem cells within the most peripheral region (per) and early differentiating progenitors within the central region (ctl). RPE, retinal pigment epithelium. (D) *Sbt1* expression are restricted to the central CMZ (bracket) and excluded from the most peripheral CMZ (adapted and reprinted with permission from Logan 2006).

Figure 1.5 SBT1 is required for retinal neurogenesis. (A) SBT1 MO injection suppressed differentiation of all retinal neuronal cell types and promoted non-neural Müller glia and neural epithelial cell type. A control 5bp mismatch MO (SBT1MIS MO) had no effect. (B) SBT1MO and GFP injection increased number of GFP-positive cells showing Müller glia and neural epithelial cell morphology. GC: ganglion cells; HC, horizontal cells; AM, amacrine cells; BP, bipolar cells, PR: photoreceptor cells; MG/NEP: Müller glia and neural epithelium cells (Kathy Moore unpublished data).


A
B
GFP
SBT1MISMATCH MO
SBT1MO
% of labeled cells
0
10
20
30
40
50
60
70
80
GC
HC
AM
BP
PR
MG /NEP
retinal cell types

CHAPTER 2

DYNAMIC EXPRESSION OF PRONEURAL TARGET GENE SBT1 IN THE MOUSE NERVOUS SYSTEM

Abstract

Proneural bHLH transcription factors are required for initiation of neurogenesis and cell fate specification in the vertebrate nervous system. SBT1 (shared bHLH target gene 1) was identified as a shared downstream target of proneural bHLH factors *Ath5* and *NeuroD* in *Xenopus laevis*, with amino acid sequence conserved among vertebrate species. However, its expression and function are completely unknown in mammals. In this study, we report that *Sbt1* (Mus musculus RIKEN cDNA 3110035E14 gene, NCBI reference sequence: NM_178399.4) is expressed in the developing spinal cord, cortex, brainstem, and retina, and its expression is maintained in the adult cortex and spinal cord in mouse. It is not only expressed in early differentiating cells, but also expressed in postnatal cortical neurons. In addition, *Sbt1* expression is regulated by *Ngn2* in the embryonic retina and brainstem, suggesting it acts as a downstream target of proneural bHLH factors in mouse.

Introduction

Proneural basic helix-loop-helix *(*bHLH*)* proteins are transcription factors, involved in cell cycle exit, cell migration, neuronal differentiation and subtype specification (Bertrand et al., 2002; Gomez-Skarmeta et al., 2003; Ross et al., 2003; Vetter and Brown, 2001). In *Drosophila*, proneural bHLH genes belong to the *achaete-scute* (*asc*) and *atonal* (*ato*) families. The vertebrate homologs of the *achaete-scute* family includes *Ash1* (in mouse, chick, zebrafish and *Xenopus*) Mouse *ash2, Xenopus ash3* and Chicken *ash4*. The *atonal*-related genes include *Ath1, Ath5*, *Ngns*, *NeuroDs* and

Olig genes. Most of these factors are expressed in the developing nervous system and act as transcriptional activators; only *Olig2* acts as a repressor (Bertrand et al., 2002).

Previous studies have shown that proneural bHLH factors are essential regulators of vertebrate neurogenesis. For example, in the developing spinal cord, *Ath1, Ash1, Ngns* and *Oligs* are expressed in non-overlapping populations of progenitors that will give rise to different types of neurons. Among them, *Ngn1/2* are expressed in both dorsal and ventral spinal cord, and impart identity to dorsal and ventral interneurons and motor neurons (Helms et al., 2005; Parras et al., 2002). In the retina, *Ath5* is required for retinal ganglion cell and optic nerve formation (Brown et al., 1998; Brown et al., 2001; Wang et al., 2001).

Although specific proneural bHLH factors are required for different subsets of neurons, they share a common ability to promote neuronal differentiation, which suggests that proneural bHLH factors may regulate both shared target genes for neurogenesis and unique targets for neuronal subtype characteristics. Defining the gene cascades that are common to proneural bHLH factors will be of great value to understand how transcriptional programs mediate neurogenesis.

Previously we showed that in *Xenopus*, the proneural bHLH factors, *Ath5* and *NeuroD*, share a group of downstream targets, including genes involved in transcription, posttranscriptional regulation, cell cycle modification and neuronal specific exocytosis, as well as several novel genes with unknown functions. *Sbt1* is one of these novel genes that is directly regulated by *Ath5* and *NeuroD* (Logan et al., 2005). It is conserved among vertebrate species including mouse and human, but there are no orthologs in invertebrate species. In *Xenopus*, it is expressed in the developing neural tube and anterior neural

structures, including the developing retina. Overexpression of either *Ath5* or *NeuroD* mRNA in the *Xenopus* neural plate induces ectopic expression of *Sbt1*, while blocking proneural function reduces endogenous *Sbt1* expression, revealing that its expression is regulated by proneural bHLH factors (Logan et al., 2005). However, there have been no studies of *Sbt1* expression and function in mammals. It is unclear whether its expression or regulation is conserved. Combining in situ hybridization and immunostaining, we found that *Sbt1* expression is restricted to the mouse nervous system, including spinal cord and cortex, from embryonic stages to adult, brainstem from embryonic day 11.5 (E11.5) to E13, and transiently in retina from E11.5 to postnatal day 14 (P14), and that this expression is correlated with neurogenesis as well as regions of proneural gene expression (Brown et al., 1998; Helms et al., 2005; Wilson and Rubenstein, 2000). Since *Sbt1* expression is remarkably similar to *Ngn2* in the midbrain, spinal cord and retina, we asked whether *Ngn2* regulates *Sbt1* expression in mouse. We examined *Sbt1* expression in *Ngn2* mutants and demonstrate that *Sbt1* expression is regulated by *Ngn2* specifically in the retina and ventral midbrain at E11.5, but not in the spinal cord and cortex, indicating that *Sbt1* is a downstream target of bHLH factors in mouse, and its regulation depends on timing and cellular context.

Materials and methods

Animals

Timed pregnant CD-1 mice were obtained commercially from Charles River. For postnatal studies, we used C57BL/6J mice from Jackson Laboratory. *Ngn2* mutant mice were maintained in a CD-1 background. PCR genotyping was performed as previously

described (Hufnagel et al., 2010). Embryos were considered as E0.5 at noon on the day at which a vaginal plug was observed.

In situ hybridization and antibody staining

Embryos or isolated retinas were fixed in 4% paraformaldyhyde (PFA) in phosphate buffered saline (PBS) for 2 hours at room temperature or overnight at 4^{o}C. Whole-mount in situ hybridization was performed as previously described (Henrique et al., 1995). Digoxigenin-labeled RNA probe was prepared from image clone (ID # 336055) containing the 3' UTR of mouse *Sbt1* sequence, using a DIG RNA labeling kit (Roche 1277073). Hybridization was performed at 65 ^{o}C. After in situ hybridization, embryos or tissues were postfixed in 4% PFA overnight and mounted in 15% sucrose/PBS, then 30% sucrose/PBS. Vibratome sections at 50 μm were prepared after whole mount in situ hybridization of E11.5 embryos. For other experiments, embryos were embedded in O.C.T (Tissue Tek 4583) before or after in situ hybrizidation, and were cryosectioned at 20 μm. To detect mitotic cells, a rabbit anti-pHH3 antibody (Upstate Biotechnology) was used at 1:500 dilution following HRP conjugated secondary antibody.

Results and discussion

Dynamic expression of *Sbt1* in the ventral spinal cord

During spinal cord development, newly formed neurons migrate out of the ventricular zone to their final positions, and incorporate into the neural circuitry. There are multiple bHLH factors and homeodomain genes governing the expression of particular target in this process (Helms et al., 2005; Parras et al., 2002; Scardigli et al.,

2001). To determine where *Sbt1* is expressed in the nervous system, We performed in situ hybridization (ISH) on mouse embryos. We found that *Sbt1* began to be expressed in the midbrain (MB), hindbrain (HB) and rostral spinal cord (SC) at E9.0 (19 somites) (Figure 2.1 A), and its expression spread caudally at E9.5 (25 somites) (Figure 2.1 B), which is consistent with spinal cord neurogenesis, which spreads from rostral to caudal at these stages. *Sbt1* was expressed in the ventral lateral spinal cord at E9.0 (Figure 2.1 C), and later on its expression was restricted to two limited domains (Figure 2.1 D), similar to the locations of the progenitors for v2 and v3 ventral interneurons (Muroyama et al., 2005). Through combining in situ hybridization for *Sbt1* with immunostaining for phosphohistone H3 (pHH3), a mitotic cell marker, we found that *Sbt1* expression was excluded from pHH3-positive cells in the ventricular zone, indicating that *Sbt1* is only expressed in postmitotic cells (Figure 2.1 E). At E13, *Sbt1* expression spread to the whole ventral spinal cord (Figure 2.1 F), and later on to the entire spinal cord (Figure 2.1 G). This expression pattern persisted to adulthood, where *Sbt1* was expressed in the grey matter with scattered expression in the white matter (Figure 2.1 H).

There are several bHLH factors expressed similarly to *Sbt1* in the spinal cord, including *Ngn2*, *Ngn3*, *Ash1*, and *Olig1/2* (Helms et al., 2005; Wu et al., 2006; Zhou et al., 2000). Among them, *Ngn2* and *Ash1* are expressed in both dorsal and ventral spinal cord, and are required for motor neuron and interneuron development, while *Ngn3* and *Olig1/2* are expressed ventrally from E9.5 to E12.5, and play roles in spinal cord neurogenesis and gliogenesis (Helms et al., 2005; Lee et al., 2003; Parras et al., 2002; Wu et al., 2006). *Sbt1* is expressed in the mouse ventral spinal cord, overlapping with domains required for interneuron and oligodendrocyte formation, revealing that *Sbt1*

might act as a mediator for proneural bHLH factors to balance neuronal versus glial genesis in the spinal cord.

Sbt1 is expressed in the mouse cortex during cortical neurogenesis and neuronal maturation

Mouse cortical neurogenesis starts from E10.5 and peaks at E13, with pyramidal neurons generated locally in the dorsal ventricular zone in an inside-out manner, to establish the six laminar layers, and GABAergic interneurons arising from the germinal zone of ventral telencephalon and migrating to their final destinations (Ross et al., 2003). In order to understand the potential role of *Sbt1* in cortical development, we performed in situ hybridization on mouse embryos at multiple stages. *Sbt1* was expressed in the mouse cortex beginning at E13 (Figure 2.2 A, B), which is the most active time for cortical neurogenesis (Mattar et al., 2004). Unlike proneural bHLH factors, which are expressed in the ventricular zone progenitors, *Sbt1* expression was found at the preplate (PP) where the postmitotic neurons are located, mirroring the expression of *Ngn2* downstream targets, *Bhlhb5*, *Mef2c*, *Dcc*, *EphA5*, and *Sema3C* (Figure 2.2 B) (Mattar et al., 2004). At postnatal stages, *Sbt1* expression was detected in deep layer neurons at P16, which then expanded to upper layer neurons (Figure 2.2 C, D), with this expression pattern being maintained in adult animals (Figure 2.2 E). The postnatal expression of *Sbt1* is consistent with a role in cortical neuronal maturation events, including axon guidance and synapse formation. Also in the adult, *Sbt1* was expressed in the hippocampal formation and dentate gyrus (data not shown). This result is consistent with the in situ hybridization analysis performed by Allen Brain Institute (http://www.alleninstitute.org).

Sbt1 is expressed in the developing brainstem

Within the vertebrate brainstem, dopaminergic neurons are generated, and these neurons are essential for multiple brain functions, including movement control and emotion. Dopaminergic neurons are born from E10.5 to E13, and *Ngn2*, *Lmx1a*, *Lmx1b* and *Ash1* act as early fate determinants (Kele et al., 2006). Early B-cell factor 1 (*Ebf1*), a downstream target of *Ngn2* is also expressed and required for terminal migration of these neurons to form the substantia nigra pars compacta (Yin et al., 2009). However, understanding of the role of proneural bHLH factors in brainstem development is still very limited. At E9, *Sbt1* was expressed in the midbrain and hindbrain (Figure 2.1 A, B). At E11.5, it was highly expressed in the ventral midbrain, the presumptive substantia nigra, where dopaminergic neurons are generated (Figure 2.3 A). In the ventral midbrain, *Ngn2* is the only known bHLH factor that is required for differentiation of ventricular zone progenitors into *Nurr1* positive dopaminergic neuron precursors (Kele et al., 2006), and *Sbt1* is expressed in an overlapping domain of high *Ngn2* expression (Figure 2.3 A). *Sbt1* expression was also detected at E11.5 in the rostral pons (Figure 2.3 B, C), which will give rise to the rostral raphe nuclei, where the serotonergic (5-HT) neurons are located. Serotonergic neurons are born between E10.5 and E12, and *Ash1* is the only known proneural protein that is expressed in this region (Pattyn et al., 2004). The high level of *Sbt1* expression at the peak time of serotonergic neurogenesis suggests that *Sbt1* could be involved in serotonergic neuron formation. *Sbt1* was also weakly expressed in medulla oblongata at E11.5 and this expression became stronger at E13 and expanded caudally (Figure 2.3 C, D, E, F). At both stages, *Sbt1* was expressed in the ventricular zone (Fig 2.3 A, D, E), consistent with a role in cell fate determination.

Sbt1 is transiently expressed in the developing retina

Sbt1 expression began in the dorsal retina at E11.5 (Figure 2.4 A), around the time when the first postmitotic neurons are generated, and by E13, *Sbt1* expression expanded to scattered cells throughout the retina (Figure 2.4 B). The first two proneural factors that are expressed in the mouse retina are *Ngn2* and *Ath5*. They begin to be expressed in the dorsal-central retina at E11.0 (43 somites), and are required for neurogenesis propagation and retinal ganglion cell (RGC) formation (Hufnagel et al., 2010). *Sbt1* expression followed the endogenous pattern of *Ngn2* and *Ath5*, but lagged behind by approximately a half day, consistent with its role as a proneural target gene (Hufnagel et al., 2010). By E15, *Sbt1* was expressed in cells in the outer neuroblast layer (NBL) and in differentiating RGCs in the inner retina (Figure 2.4 C), suggesting it may be expressed by newly formed neurons just leaving the cell cycle and migrating to the retinal ganglion cell layer (GCL). By P0, *Sbt1* was expressed within a subset of cells in the NBL and in the newly formed RGCs (Figure 2.4 D). From P3 to P7, *Sbt1* expression was maintained in the inner nuclear layer (INL) but no longer detected in the GCL (Figure 2.4 E, F), consistent with a role in RGC specification or differentiation but not in their maintenance. By P12, *Sbt1* was only weakly expressed in the peripheral retina, but was not detected in the central retina (Figure 2.4 G). *Sbt1* expression was absent from the retina after P14 (Figure 2.4 H).

In summary, *Sbt1* is expressed during retinal neurogenesis from E11.5 to P14 in early differentiating cells. This expression pattern suggests that it may be playing more than one role during retinogenesis, including cell fate specification, laminar formation, cell migration or others.

Sbt1 expression in the ventral midbrain and retina is regulated by *Ngn2*

Sbt1 expression appears to correlate both temporally and spatially with a number of proneural factors, including *Ath5, NeuroD, Ngn2* and *Ash1*. For example, in the developing spinal cord and brainstem, *Sbt1* is expressed in overlapping domains with both *Ngn2* and *Ash1* (Figure 2.1 and 2.3). In the retina, *Sbt1* expression follows that of *Ngn2* and *Ath5* at early stages. Later on, its expression in the NBL and RGCs are similar to *NeuroD* and *Ash1* expression at the same time. In the cortex, *Sbt1* is not expressed in the same ventricular zone progenitors as proneural bHLH factors, instead its expression pattern mimics that of known *Ngn2* downstream targets (Mattar et al., 2004). Notably, *Sbt1* expression is highly similar to the expression of *Ngn2* or its target genes in all nervous system tissues that we analyzed. Because of this consistency and because *Sbt1* expression is regulated by *atonal*-related bHLH factors in *Xenopus*, we asked whether *Sbt1* expression is regulated by *Ngn2* in mouse (Logan et al., 2005). To address this question, we assayed *Sbt1* expression in *Ngn2* mutants at multiple time points and tissues by in situ hybridization. However, we did not detect any differences in the *Ngn2* mutant spinal cord or cortex at either E9.5 or E11.5. This was not a complete surprise since *Sbt1* was identified as a target of multiple bHLH factors in *Xenopus*. Moreover, previous studies found that removal of one proneural factor may result in upregulation of another bHLH factor (Akagi et al., 2004; Le et al., 2006). It is possible that deletion *Ngn2* alone is not sufficient to alter *Sbt1* expression, since *Ash1* may partially rescue its effects in the spinal cord, and *Ngn1* may compensate for loss of *Ngn2* in the cortex. In addition, *NeuroD2*, which is expressed in the developing and postnatal cortex, may also contribute to regulate *Sbt1* expression (Ince-Dunn et al., 2006).

Interestingly, we found that *Sbt1* expression was significantly altered in the *Ngn2* mutant midbrain. By in situ hybridization on mouse embryos at E11.5, we found that *Sbt1* was expressed adjacent to the midline in the ventral midbrain in control embryos (Figure 2.5 A, B), but its expression in this domain was selectively lost in *Ngn2* mutants (Figure 2.5 C, bracket). The proliferating dopaminergic neuron progenitors are located in this region (Kele et al., 2006). Thus, the midbrain provides a good model to study *Sbt1* regulation by proneural bHLH factors, since it expresses high levels of *Ngn2* and low levels of *Ash1,* avoiding the high redundancy with other proneural genes that occurs in most neural tissues.

In addition, the *Sbt1* expression domain in the retina was dramatically reduced in *Ngn2* mutants as compared with control (Figure 2.5 D, E, F). In the E11.5 retina, *Sbt1* was expressed in a broader domain dorsally, corresponding to the location where neurogenesis begins in the retina (Figure 2.5 D, E). In contrast, the *Sbt1* expression domain was significantly reduced in *Ngn2* mutants, although it was still expressed dorsally with similar intensity (Figure 2.5 F). Recent evidence has shown that *Ngn2* has transient effects on peripheral expansion of retinal neurogenesis from E11.5 to E13.5, concomitant with reduced RGC specification and cell cycle exit in the *Ngn2* mutant retina (Hufnagel et al., 2010). Our results are consistent with the observed reduced propagation of neurogenesis in *Ngn2* mutants. Moreover, *Sbt1* expression was not completely absent in the *Ngn2* mutant retina, suggesting that genes other than *Ngn2* regulate *Sbt1* expression. Importantly, the *Ath5* expression domain in the *Ngn2* mutant is also restricted (Hufnagel et al., 2010), so that it is unclear whether *Ngn2* directly regulates *Sbt1* expression or whether it acts through regulating *Ath5* expression.

However, it will be complicated to analyze *Sbt1* regulation by using *Ath5* mutants, since *Ngn2* expression is upregulated in the *Ath5* mutant retina (Le et al., 2006). This increased level of *Ngn2* may compensate for the lack of *Ath5*. Thus, it will be useful to assess *Sbt1* expression in *Ath5* and *Ngn2* double mutants, then compare with *Ngn2* single mutants, to determine whether *Ath5* regulates *Sbt1* expression or not.

It is becoming clear now that proneural bHLH factors regulate both shared targets to promote neuronal differentiation and unique targets for specific neuronal subtypes. Our results are consistent with *Sbt1* acting as a downstream target of proneural bHLH factors, and we have shown that *Ngn2* regulates *Sbt1* expression depending on the timing and cellular context. However, in the mouse nervous system, proneural factors are redundantly expressed, and cross regulate each other. It is difficult to find an environment where is only one bHLH factor expressed, or any mutant tissue that can remove all proneural bHLH factors. For this reason, we cannot rule out the possibility that *Sbt1* is also regulated by other bHLH factors.

Conclusion

Mouse *Sbt1* is dynamically expressed in the nervous system, including the spinal cord, cortex, brainstem and retina. *Sbt1* expression is not restricted to early differentiating cells at embryonic stages, but it is also expressed in the postnatal spinal cord and cortical neurons, suggesting it has more than one function during development. *Sbt1* expression is regulated by proneural bHLH factor *Ngn2* in the ventral midbrain and retina at E11.5, consistent with its role as a proneural target gene.

Acknowledgments

We thank Tian Le, Robert Hufnagel and Dr. Nadean Brwon, for providing the *Ngn2* mutant embryos and for valuable discussions; Ranajeet Saund and Dr. Yukio Saijoh for technical advice on in situ hybridization; Drs. ChangJiang Zou and Kathryn Moore for comments on the manuscript. This work was supported by NIH grants EY012274 to M.L.V. and EY13612 to N.L.B.

References

Akagi, T., Inoue, T., Miyoshi, G., Bessho, Y., Takahashi, M., Lee, J. E., Guillemot, F., and Kageyama, R. (2004). Requirement of multiple basic helix-loop-helix genes for retinal neuronal subtype specification. J Biol Chem *279*, 28492-28498.

Bertrand, N., Castro, D. S., and Guillemot, F. (2002). Proneural genes and the specification of neural cell types. Nat Rev Neurosci *3*, 517-530.

Brown, N. L., Kanekar, S., Vetter, M. L., Tucker, P. K., Gemza, D. L., and Glaser, T. (1998). Math5 encodes a murine basic helix-loop-helix transcription factor expressed during early stages of retinal neurogenesis. Development *125*, 4821-4833.

Brown, N. L., Patel, S., Brzezinski, J., and Glaser, T. (2001). Math5 is required for retinal ganglion cell and optic nerve formation. Development *128*, 2497-2508.

Gomez-Skarmeta, J. L., Campuzano, S., and Modolell, J. (2003). Half a century of neural prepatterning: the story of a few bristles and many genes. Nat Rev Neurosci *4*, 587-598.

Helms, A. W., Battiste, J., Henke, R. M., Nakada, Y., Simplicio, N., Guillemot, F., and Johnson, J. E. (2005). Sequential roles for Mash1 and Ngn2 in the generation of dorsal spinal cord interneurons. Development *132*, 2709-2719.

Henrique, D., Adam, J., Myat, A., Chitnis, A., Lewis, J., and Ish-Horowicz, D. (1995). Expression of a Delta homologue in prospective neurons in the chick. Nature *375*, 787-790.

Hufnagel, R. B., Le, T. T., Riesenberg, A. L., and Brown, N. L. (2010). Neurog2 controls the leading edge of neurogenesis in the mammalian retina. Dev Biol *340*, 490-503.

Ince-Dunn, G., Hall, B. J., Hu, S. C., Ripley, B., Huganir, R. L., Olson, J. M., Tapscott, S. J., and Ghosh, A. (2006). Regulation of thalamocortical patterning and synaptic maturation by NeuroD2. Neuron *49*, 683-695.

Kele, J., Simplicio, N., Ferri, A. L., Mira, H., Guillemot, F., Arenas, E., and Ang, S. L. (2006). Neurogenin 2 is required for the development of ventral midbrain dopaminergic neurons. Development *133*, 495-505.

Le, T. T., Wroblewski, E., Patel, S., Riesenberg, A. N., and Brown, N. L. (2006). Math5 is required for both early retinal neuron differentiation and cell cycle progression. Dev Biol *295*, 764-778.

Lee, J., Wu, Y., Qi, Y., Xue, H., Liu, Y., Scheel, D., German, M., Qiu, M., Guillemot, F., Rao, M., and Gradwohl, G. (2003). Neurogenin3 participates in gliogenesis in the developing vertebrate spinal cord. Dev Biol *253*, 84-98.

Logan, M. A., Steele, M. R., Van Raay, T. J., and Vetter, M. L. (2005). Identification of shared transcriptional targets for the proneural bHLH factors Xath5 and XNeuroD. Dev Biol *285*, 570-583.

Mattar, P., Britz, O., Johannes, C., Nieto, M., Ma, L., Rebeyka, A., Klenin, N., Polleux, F., Guillemot, F., and Schuurmans, C. (2004). A screen for downstream effectors of Neurogenin2 in the embryonic neocortex. Dev Biol *273*, 373-389.

Muroyama, Y., Fujiwara, Y., Orkin, S. H., and Rowitch, D. H. (2005). Specification of astrocytes by bHLH protein SCL in a restricted region of the neural tube. Nature *438*, 360-363.

Parras, C. M., Schuurmans, C., Scardigli, R., Kim, J., Anderson, D. J., and Guillemot, F. (2002). Divergent functions of the proneural genes Mash1 and Ngn2 in the specification of neuronal subtype identity. Genes Dev *16*, 324-338.

Pattyn, A., Simplicio, N., van Doorninck, J. H., Goridis, C., Guillemot, F., and Brunet, J. F. (2004). Ascl1/Mash1 is required for the development of central serotonergic neurons. Nat Neurosci *7*, 589-595.

Ross, S. E., Greenberg, M. E., and Stiles, C. D. (2003). Basic helix-loop-helix factors in cortical development. Neuron *39*, 13-25.

Scardigli, R., Schuurmans, C., Gradwohl, G., and Guillemot, F. (2001). Crossregulation between Neurogenin2 and pathways specifying neuronal identity in the spinal cord. Neuron *31*, 203-217.

Vetter, M. L., and Brown, N. L. (2001). The role of basic helix-loop-helix genes in vertebrate retinogenesis. Semin Cell Dev Biol *12*, 491-498.

Wang, S. W., Kim, B. S., Ding, K., Wang, H., Sun, D., Johnson, R. L., Klein, W. H., and Gan, L. (2001). Requirement for math5 in the development of retinal ganglion cells. Genes Dev *15*, 24-29.

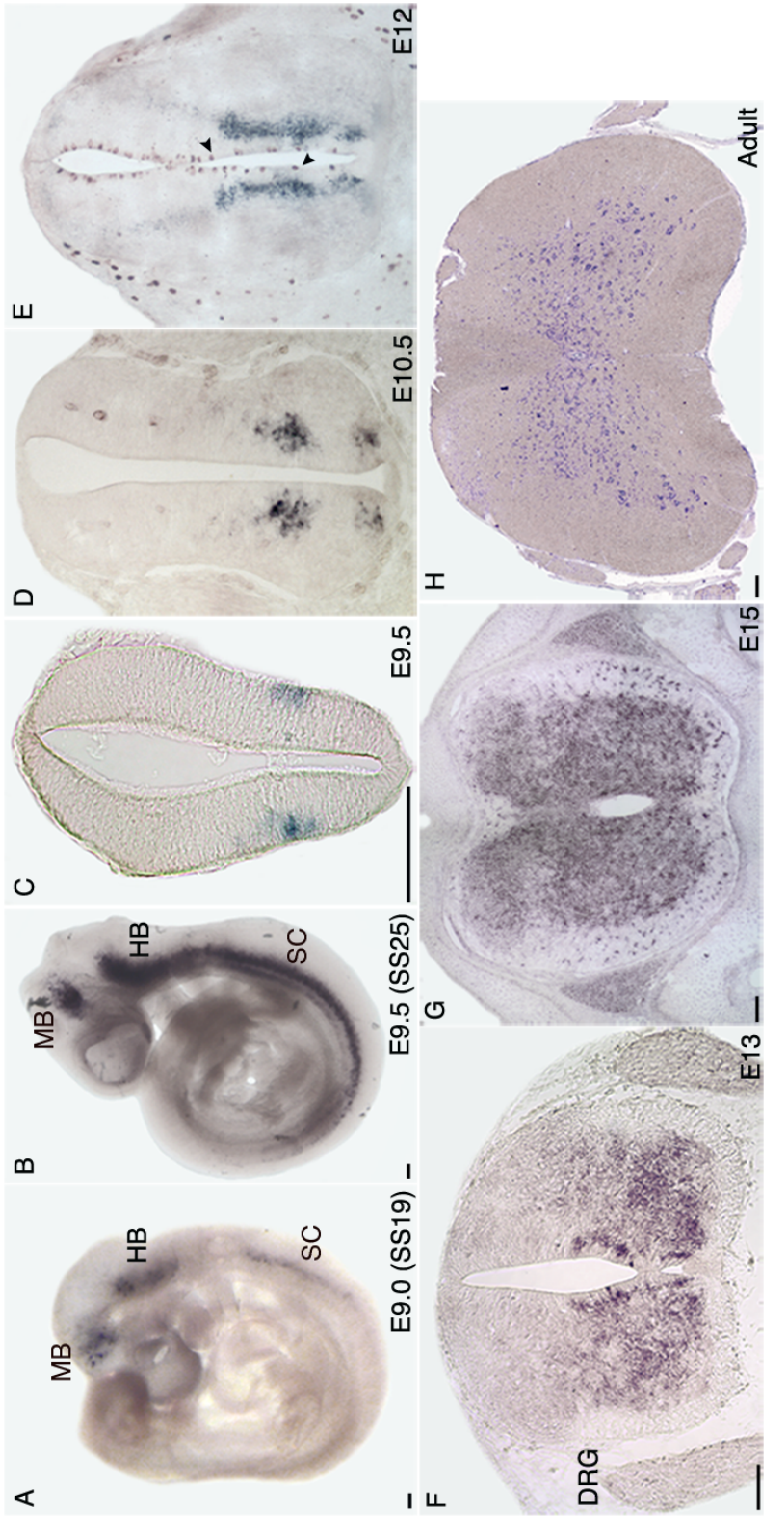
Wilson, S. W., and Rubenstein, J. L. (2000). Induction and dorsoventral patterning of the telencephalon. Neuron *28*, 641-651.

Wu, S., Wu, Y., and Capecchi, M. R. (2006). Motoneurons and oligodendrocytes are sequentially generated from neural stem cells but do not appear to share common lineage-restricted progenitors in vivo. Development *133*, 581-590.

Yin, M., Liu, S., Yin, Y., Li, S., Li, Z., Wu, X., Zhang, B., Ang, S. L., Ding, Y., and Zhou, J. (2009). Ventral mesencephalon-enriched genes that regulate the development of dopaminergic neurons in vivo. J Neurosci *29*, 5170-5182.

Zhou, Q., Wang, S., and Anderson, D. J. (2000). Identification of a novel family of oligodendrocyte lineage-specific basic helix-loop-helix transcription factors. Neuron *25*, 331-343.

Figure 2.l *Sbt1* is dynamically expressed in the mouse spinal cord.
(A, B) *Sbt1* is expressed in the presumptive MB, HB and spinal cord at E9.0 (A) and E9.5 (B). (C, D, E and F) Transverse sections through the mouse spinal cord show that *Sbt1* is restricted to the ventral spinal cord from E9.5 to E13, but is not expressed in the ventricular zone pHH3-positive cells (arrowhead in E). (G) *Sbt1* is expressed throughout the spinal cord at E15. (H) *Sbt1* expression is maintained in the adult spinal cord. SS, somite stage; MB, midbrain; HB, hindbrain; DRG, Dorsal root ganglion. Scale bar 100μm.


A
MB
HB
SC
E9.0 (SS19)
B
MB
HB
SC
E9.5 (SS25)
C
E9.5
D
E10.5
E
E12
F
DRG
E13
G
E15
H
Adult

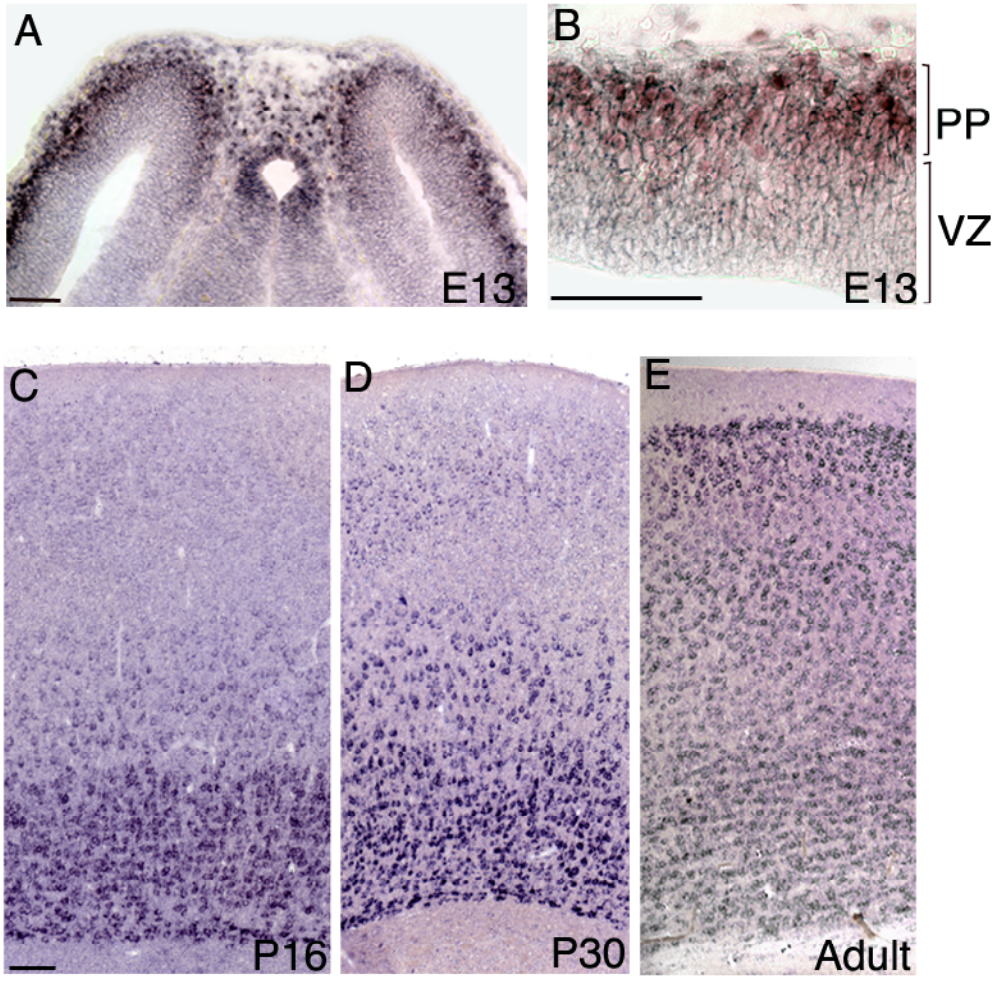


Figure 2.2 *Sbt1* is expressed in the mouse cortex during cortical neurogenesis and neuronal maturation.
(A) *Sbt1* is expressed in the E13 mouse cortex. (B) Higher magnification shows that *Sbt1* is not expressed in the ventricular zone (VZ) progenitors, but in the newly formed neurons in the preplate (PP). (C, D and E) *Sbt1* is expressed in deep layer neurons at P16 (C), then expands to upper layer neurons in late postnatal stages (D), and is maintained in the adult cortex (E). Scale bar 100μm.

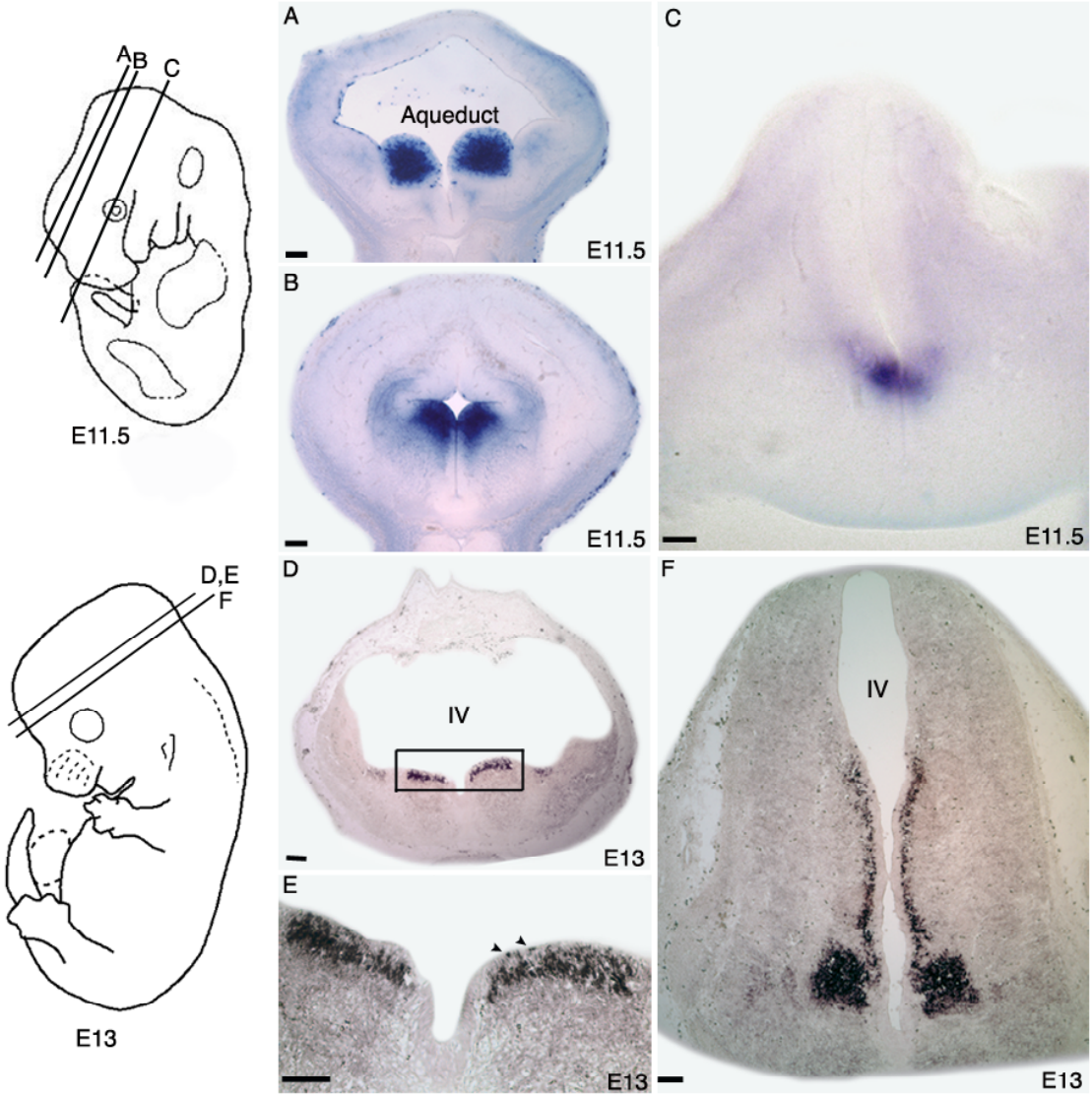
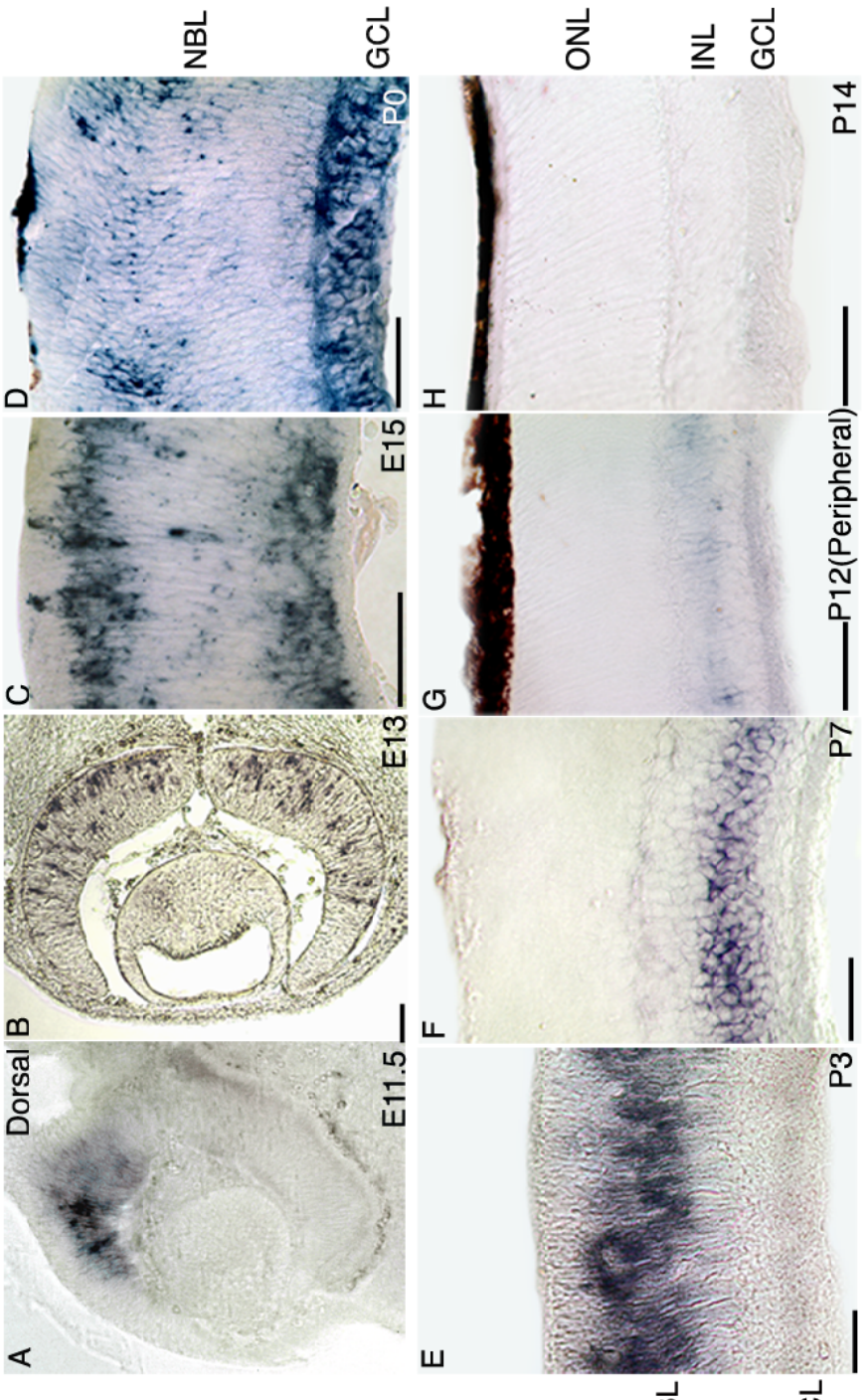


Figure 2.3 *Sbt1* is expressed in the mouse developing brainstem.
Left panels show schematic illustrations of section levels of E11.5 and E13 mouse embryos. (A, B) *Sbt1* is expressed in the ventral midbrain, at the level of aqueduct (A), and in the ventral rostral pons (B). (C) *Sbt1* is expressed in restricted domains in the ventral medulla oblongata. (D) *Sbt1* expression is maintained in the ventral midbrain at 4th ventricle level at E13. (E) Higher magnification of boxed area in D shows that *Sbt1* is expressed in cells in the VZ (arrowhead). (F) *Sbt1* is strongly expressed in the ventral nuclei in the medulla oblongata at E13. VZ: ventricular zone. Scale bar 50μm.

Figure 2.4 *Sbt1* is transiently expressed in early differentiating cells in the retina.
(A) *Sbt1* is expressed in the dorsal retina at E11.5. (B) *Sbt1* expression expands throughout the entire retina at E13. (C, D) *Sbt1* is expressed in the GCL and NBL at E15 (C) and E18 (D). (E) *Sbt1* expression is maintained in the NBL at P3, but not in the GCL. (F) *Sbt1* is only expressed in the INL at P7. (G) *Sbt1* expression is reduced in the peripheral retina, and absent from the central retina at P12. (H) *Sbt1* expression in the retina is not detected after P14. GCL: retinal ganglion cell layer; NBL: neuroblast cell layer; INL: inner nuclear layer. Scale bar 50μm.


A
Dorsal
B
E11.5
E13
C
D
NBL
GCL
E15
P0
E
F
G
H
ONL
INL
GCL
P3
P7
P12(Peripheral)
P14
NBL
GCL

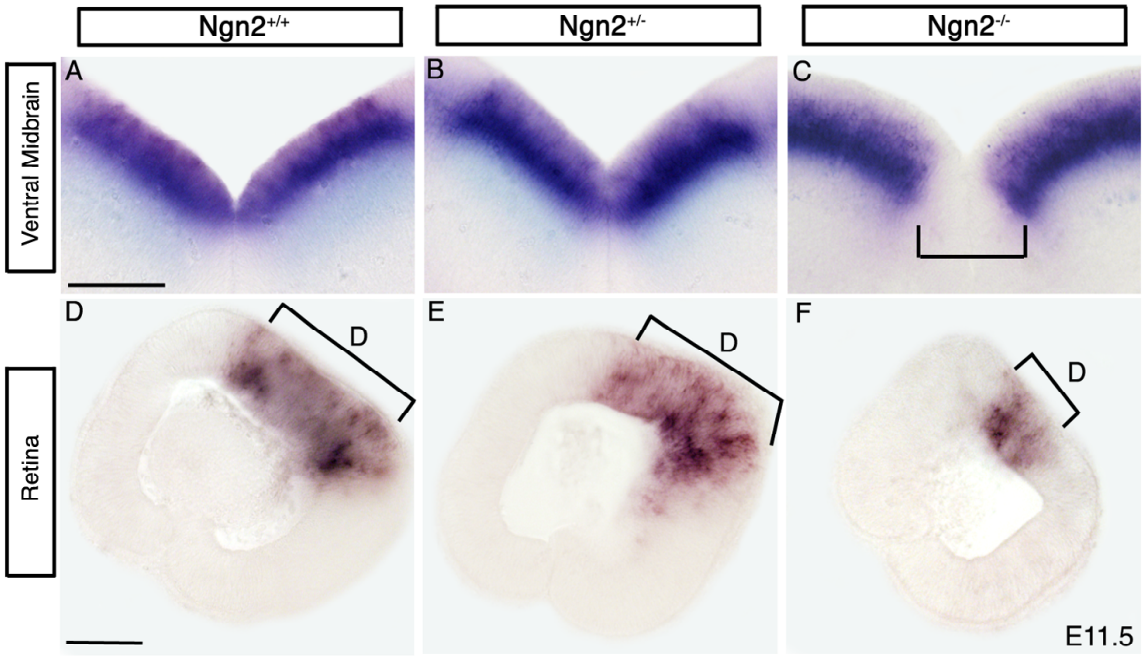


Figure 2.5 *Sbt1* expression in the retina and ventral midbrain is regulated by *Ngn2* at E11.5.
(A, B) Transverse sections show that *Sbt1* is expressed in the ventral midbrain, closely adjacent to the midline in the control animals. (C) *Sbt1* expression is selectively reduced in *Ngn2* mutants (bracket). (D, E) Sagittal sections show that *Sbt1* is expressed in a broad domain in the wild type (D) and heterozygous (E) dorsal retinas (bracket). (F) *Sbt1* expression domain is significantly reduced in the *Ngn2* mutant retina. n≥3 D, dorsal. Scale bar 100μm

CHAPTER 3

SBT1 FUNCTION IS DISPENSABLE IN MOUSE NEUROGENESIS

Abstract

SBT1 is a novel proneural bHLH target, which is identified in a screen to find the direct downstream target of *Ath5* and *NeuroD* in *Xenopus*. To assess *Sbt1* function in vertebrate neurogenesis, an *Sbt1-eGFPCre* knockin mouse was generated by replacing most of *Sbt1* coding sequence by an *eGFPCre* cassette. In $Sbt1^{eGFPCre/+}$ mice, GFP is mainly expressed in cortical neurons, corresponding to endogenous *Sbt1* expression in the cortex, and provides a useful tool to study *Sbt1*-expressing cells during cortical development. *Sbt1* null mice are viable, fertile, and generally indistinguishable from wild type (WT) littermates in appearance, body weight, overt behavior, and gross anatomy. Neuronal versus glial genesis was examined in the *Sbt1* mutant spinal cord and cortex, and specific neuronal subtype differentiation was examined in the *Sbt1* mutant retina. No dramatic differences were found in *Sbt1* mutant mice as compared with control. These results suggest that deletion of *Sbt1* alone in the mouse nervous system does not change major cell type genesis.

Introduction

The vertebrate central nervous system (CNS) is composed of three major classes of cells, including neurons, astrocytes and oligodendrocytes (Miller, 2002; Perters A., 1990; Rowitch, 2004; Rowitch and Kriegstein, 2010; Wang and Bordey, 2008). All these cell types are derived from the neuroepithelial cells located in the neural tube (Miller, 2002; Rowitch and Kriegstein, 2010). Among them, neurons are the most characteristic cells, and are primarily responsible for information transfer. Glia, including astrocytes and oligodendrocytes make up more than 90% of the cells in the human brain, and are

crucial for the complexity of neurological functions (Miller, 2002; Rowitch, 2004; Rowitch and Kriegstein, 2010). Classic studies suggest that in any particular region, neurons are the first-born cell type, followed by gliogenesis, including astrocytes, and oligodendrocytes (Altman and Bayer 1984). The only exception is that the neural retina of most mammalian organisms does not contain oligodendrocytes (Guldenagel et al., 2000), instead it has a specialized glial population, Müller glia. Also, retinal astrocytes do not generate in the retina, but instead, migrate in from the optic nerve.

One important question for vertebrate neurogenesis is how these different cell types derive from a monolayer of neuroepithelium. This question can be further separated into two levels, 1) how do progenitors decide to adopt a neuronal versus glial cell fate; and 2) how are subclasses of neuronal or glial cell types generated. Evidence shows that both extrinsic and intrinsic signals are involved in this process. Extrinsic signals including fibroblast growth factor (FGF), bone morpogenetic proteins (BMPs), and cytokines have the potential ability to influence the decision of progenitors to acquire a neuronal or a glial cell fate (Ghosh and Greenberg, 1995; Gross et al., 1996). Intrinsic factors, especially bHLH factors, are involved in regulating cell competence to respond to soluble factors and select a specific cell fate. It has been well documented that proneural bHLH factors regulate generation of different neuronal subtypes in the nervous system. In contrast, how proneural bHLH factors exert their “proneural” function to promote neuronal differentiation and inhibit gliogenesis is not completely understood. One hypothesis is that proneural bHLH proteins activate both shared targets to govern the core events for neurogenesis, and specific targets to control differentiation of specific lineages (Brunet and Ghysen, 1999; Logan et al., 2005). This hypothesis has been confirmed,

since it has been shown that *Ngn2* and *NeuroD* regulate shared target genes through the same enhancers, and these enhancers are sufficient for *Ngn2* and *NeuroD* mediated gene expression in neural tissues (Seo et al., 2007). Moreover, screening for downstream targets of proneural bHLH factors *Ath5* and *NeuroD* found that these two factors induce a complex gene cascade, governing neuronal cell fate, morphology, migration, neuronal function and signal transduction (Logan et al., 2005). These findings suggest that each proneural target may mediate a subset of proneural factor functions, which is dependent upon timing and cellular context.

Sbt1 was identified as a shared downstream target of proneural bHLH factors *Ngn2, Ath5* and *NeuroD* in *Xenopus laevis* (Logan et al., 2005; Seo et al., 2007). It is exclusively expressed in the developing nervous system in mouse (Chapter 2) and *Xenopus* (Logan, 2006), and localizes to both the nucleus and cell membrane in *Xenopus* animal cap ectoderm (Logan, 2006). Interestingly, in the *Xenopus* retina, it is expressed in early differentiating cells, overlapping with *Ath5* and other bHLH factors in the CMZ. There are two distinct expression patterns for proneural bHLH targets in the *Xenopus* retina. One group of targets, including *Sbt1*, *Gadd45-gamma* (cell cycle inhibitor), and *Xetor* (transcriptional repressor), are restricted to progenitors and early differentiating cells of the central CMZ, indicating they are involved in early differentiating programs. The other group of targets, including *Ebf3* (transcription factor), *ElrC* (RNA binding protein) and *Brn3d* (transcription factor, involved in RGC differentiation) are expressed in late differentiating cells and mature neurons in the retina, indicating they mediate terminal differentiation events (Logan et al., 2005). The function of *Sbt1* in neurogenesis was further analyzed by gain-of-function and loss-of-function experiments in the

Xenopus neural plate and retina. Overexpression of *Sbt1* by mRNA injection promotes neuronal differentiation in the neural plate and RGC generation in the retina, while blocking *Sbt1* expression by antisense morpholino injection inhibits the differentiation of early born neurons, and promotes Müller glial or neuroepithelial cell fate (Logan, 2006). These data suggest that *Sbt1* is an important mediator of proneural gene activity. However, there has been no analysis of *Sbt1* function in mouse development. Whether its effect is conserved in mouse neurogenesis or whether there are any species differences is uncharacterized.

In this study, I generated an *Sbt1-eGFPCre* knockin mouse to answer two basic questions, 1) whether *Sbt1* is required for balancing of neuronal versus glial cell numbers in the spinal cord and cortex; 2) whether *Sbt1* is involved in specific neuronal or glial cell fate genesis in the retina. So far, no differences in neuronal and glial cell types and numbers between *Sbt1* mutant mice and control have been seen. Also, there are no gross defects in cell fate determination in the *Sbt1* mutant retina. These findings suggest that deletion of *Sbt1* alone is not sufficient to alter mouse neurogenesis, especially major cell fates.

Materials and methods

Generation of *Sbt1* mutant mice by replacing exon 3,4 and 5 with an *eGFPCre* cassette

There are 6 exons in the mouse *Sbt1* gene, which is located on chromosome 1. The start codon is in exon1, while most of the coding sequence is located in exons 3, 4 and 5 (Figure 3.1A). To generate *Sbt1* mutant mice, a BAC (bacterial artificial chromosome) clone (RP23-433H6) containing *Sbt1* sequence of exons3, 4, 5 and 6 was

selected from the BACPAC Resources Center. A protocol of highly efficient recombination-based cloning method in bacteria (Wu et al., 2008) was adopted to generate the targeting vector. In this vector, most of *Sbt1* exon3, all of exons 4 and 5 were deleted, exon6 was frame shifted, and an *eGFPCre* cassette was inserted into the locus in frame with the start codon (Figure 3.1 A). The resulting *eGFPCre* protein is fused to 21 amino acid of SBT1 from exon1, 19 amino acids from exon2, and the first 5 amino acids from exon3. This insertion placed *eGFPCre* under the control of endogenous *Sbt1* regulatory sequence. The 5' and 3' homology arms were approximately 2kb and 8kb, respectively. The targeting sequence was shuttled into a TK (thymidine kinase) vector for selection, and the *Sbt1-eGFPCre* construct was electroporated into G4 mouse embryonic stem (ES) cells in collaboration with Dr. Capecchi's laboratory at the University of Utah, followed by positive-negative drug selection (Wu et al., 2008). After electroporation, more than 130 ES cell clones were screened by Southern blotting analysis. Following EcoRI digestion and Southern blot hybridization using the 3' probe (Figure 3.1 A), the targeted ES cell clone generated wild type and targeted bands at 7.4kb and 5.1kb, respectively (Figure 3.1B). This clone was further confirmed by PCR to amplify the GFP sequence (data not shown). This targeted ES cell clone was injected into C57BL/6J blastocysts to generate mouse chimeras. Then, the chimeric mice were bred with C57BL/6J to generate offspring. PCR was performed to genotype mice using genomic DNA from heterozygous mating (Figure 3.1C). PCR primers for genetyping are shown in Table 3.1.

Genotyping of *Rosa26* reporter strains (*R26R^{YFP}*, *R26R^{LacZ}*) and *Z/EG* mice

In this study, *R26R^{YFP}* and *R26R^{LacZ}* mice were kindly provided by Drs. Capecchi and Fuhrmann laboratories, respectively. *Z/EG* conditional reporter mice were purchased from the Jackson Laboratory (Stock number: 003920). They express β-galactosidase (β-gal) before *cre* mediated excision, and EGFP after that (Novak et al., 2000). Since the genotyping protocol provided by the Jackson Laboratory could not be used in *Sbt1$^{eGFPCre/+}$; Z/EG* mice, I designed genotyping primers by amplifying the LacZ sequence in the *Z/EG* locus. Primers used for specific genotyping are listed in Table 3.1.

X-gal staining of embryos

For β-galactosidase (β-gal) staining, embryos were dissected from the uterus in PBS and fixed for 10-20 min (depending on the age of embryos) in 1% paraformaldyhyde (PFA) at room temperature. β-gal staining of embryos was carried out at 37^{o}C from 1 hour to overnight in 1mg/ml 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal), 5mM $K_3Fe(CN)_6$, 5mM $K_4Fe(CN)_6$,5mM $MgCl_2$ in PBN (PBS with 0.02% NP40). After staining, embryos were rinsed in PBS and post-fixed for overnight in 4% PFA at 4^{o}C. The whole-mount stained embryos were rinsed in 40% glycerol then 80% glycerol before taking photographs (Verma-Kurvari et al., 1996).

Immunohistochemistry

Deeply anesthetized mice aged 6-9 weeks old were transcardially perfused with saline solution followed by 4% PFA in PBS. After dissection, the brain and spinal cord were removed and postfixed for 1 hour at room temperature and rinsed in PBS. The

enucleated eyes of 3-4 weeks old mice were fixed in 4% PFA in PBS for 30 min at room temperature. After fixation, samples were cryopreserved with 15% then 30% sucrose in PBS, embedded in OCT compound, and cryosectioned at a thickness of 20 μm.

Primary antibodies used included goat anti-GFP (1:1000, Abcam), chicken anti-GFP (1:300, Aves), mouse anti-NeuN (1:500, Chemicon), goat anti-Sox10 (1:500, Invitrogen); mouse anti-s100 (1:500, Abcam), rabbit anti-Calbindin (1:500, Calbiochem), rabbit anti-phosphohistone H3 (1:500, Upstate Biotechnology), rabbit anti-β-gal antibody (1:300, 5 Prime-3 Prime), mouse anti-rhodopsin (1:500, Chemicon), rabbit anti-Prox1 (1:4000, Covance), rabbit anti-CRALBP (1:500; a gift from Dr J. Saari as described previously (Bunt-Milam and Saari, 1983)), and Hoechst (1:10,000, Invitrogen). 4D5 antibody to Isl1 (1:30, Development Hybridoma Bank) was provided by Drs. Chien in the University of Utah. Goat anti-brn3 (1:50, Santa Cruz Biotechnology), sheep anti-chx10 (1:500, Exalpha Biologicals), mouse anti-Tuj1 (1:1000, Covance) and rabbit anti-recoverin (1:500, Chemicon) antibodies were provided by Dr. Edward Levine in the University of Utah, Moran Eye Center. Secondary antibodies used were conjugated to Alexa Fluor488, Alexa Fluor568 or Alexa Fluor647 at 1:400 to 1:1000 dilution, from Invitrogen or Jackson Immunologicals.

Microscopy

Imaging was conducted by compound microscopy using an Olympus BX51 and confocal inverted microscopy using a Nikon A1. Cells that were labeled by different cell type markers were analyzed by NIS-Elements 3.1 software.

Results

To assess the embryonic viability of *Sbt1* mutants, we inter bred *Sbt1*$^{eGFPCre/+}$ mice with littermates. Offspring of all three genotypes were produced in the expected Mendelian ratio and had no obvious morphological or behavior defects. We used *Sbt1*$^{eGFPCre/+}$ mice as control to compare with *Sbt1*$^{eGFPCre/eGFPcre}$.

GFP expression in *Sbt1*$^{eGFPCre/+}$ mice faithfully recapitulates endogenous expression of *Sbt1* in the mouse cortex

To determine whether GFP expression mimics endogenous *Sbt1* expression, we analyzed GFP mRNA and protein expression in the cortex by reverse transcriptase-PCR (RT-PCR) and immunostaining, respectively. By performing RT-PCR, we detected GFP mRNA in the *Sbt1*$^{eGFPCre/+}$ and *Sbt1*$^{eGFPCre/eGFPCre}$ cortex, while *Sbt1* mRNA was detected in the *Sbt1*$^{+/+}$ and *Sbt1*$^{eGFPCre/+}$ cortex, but not in *Sbt1*$^{eGFPCre/eGFPCre}$ (Figure 3.1D). By generating cryosections of adult mouse brain and performing immunostaining with a goat anti-GFP antibody, GFP-positive cells were found in the cerebral cortex (Figure 3.2), as well as in hippocampus and brainstem (data not shown), which coincided with endogenous *Sbt1* mRNA expression (Figure 3.2). However, GFP expression in the upper cortical layers was weak, and we could not detect GFP-positive cells in the spinal cord and retina, which may be due to the low level of *Sbt1* expression in these two tissues, or different stability of mRNA.

To further investigate whether e*GFPCre* expression was controlled by *Sbt1* regulatory sequence, *Sbt1*$^{eGFPCre/+}$*; R26R*$^{LacZ/+}$ mice were generated by crossing *Sbt1*$^{eGFPCre/+}$ with Rosa26^{LacZ} reporter (*R26R*^{LacZ}) strain (Figure 3.3A). Embryos were collected at E11.5, followed by X-gal staining. LacZ expression was detected in the

midbrain, hindbrain and spinal cord, which was consistent with endogenous *Sbt1* mRNA expression domains (Figure 3.3 B and C).

The above results suggest that the *Sbt1* gene was successfully targeted in *Sbt1* mutants, and that the *eGFPCre* cassette was inserted into the *Sbt1* locus. In addition, GFP expression in $Sbt1^{eGFPCre/+}$ mice faithfully recapitulated the endogenous expression of *Sbt1* in the cortex.

Sbt1 is not required for neuronal versus glial balance in the spinal cord

One major function for proneural bHLH factors is promoting neurogenesis and inhibiting glial cell fates. Since GFP expression was not detectable in the spinal cord, it was unclear in which cell population *Sbt1* is expressed, so our analysis of spinal cord cell fate is restricted. However, based on *Sbt1* in situ hybridization analysis showing *Sbt1* mRNA expression in the spinal cord gray matter, with scattered expression in the white matter, we predicted that *Sbt1* is expressed in neurons and probably in oligodendrocytes. Moreover, *Sbt1* is expressed in the mouse embryonic spinal cord in a similar pattern to that of oligodendrocyte progenitors (Figure 2.1 G), indicating that it may be involved in oligodendrocyte development. To determine if *Sbt1* regulates neuronal versus oligodendrocyte generation, I examined neuronal and oligodendrocyte numbers by using the pan-neuronal marker, NeuN, and oligodendrocyte marker, Sox10, in the spinal cord of 6-9 week-old mice. NeuN labeled neurons in the gray matter, while Sox10 was uniformly expressed in oligodendrocytes both in the gray and white matter (Figure 3.4 A, B, D and E). No differences were detected between dorsal and ventral spinal cord with respect to NeuN and Sox10 expression, so we just focused on the ventral motor neuron

pool. To quantify these data, the percentage of NeuN and Sox10 positive cells was compared to the DAPI-positive cells in *Sbt1*$^{eGFPCre/+}$ vs. *Sbt1*$^{eGFPCre/eGFPCre}$ animals. Unexpectedly, no significant defects were found in *Sbt1* mutants (Figure 3.4 C and F), suggesting that deletion of *Sbt1* alone is not sufficient to alter neuronal versus glial balance in the spinal cord.

However, this study only provides a preliminary assessment of *Sbt1* function in spinal cord neurogenesis. Additional experiments will be required, analyzing more cell types at multiple stages, to conclude whether *Sbt1* is required for cell fate specification during spinal cord development.

Sbt1 is mainly expressed in cortical neurons, but is not essential for cortical neurogenesis

To address *Sbt1* function in mouse cortical development, we first examined GFP expression in the cortex of *Sbt1*$^{eGFPCre/+}$ mice. GFP was detected in the deep cortical layers, but was weakly expressed in upper layers (Figure 3.2). It was mainly expressed in neurons, which are labeled by the pan-neuronal marker NeuN (Figure 3.5 arrow). However, there were also a few GFP^{+}/NeuN^{-} cells present, indicating that *Sbt1* is not restricted to the neuronal lineage (Figure 3.5 arrowhead). We quantified the percentage of GFP-positive cells in the *Sbt1*$^{eGFPCre/+}$ cortex and found that they account for 24% of the total cell population (n=2). In the *Sbt1*$^{eGFPCre/eGFPCre}$ cortex, GFP was expressed from deep layer neurons to upper layer neurons, similar to its expression domain in the control cortex. However, the intensity of GFP in *Sbt1*$^{eGFPCre/eGFPCre}$ cells was significantly stronger than in the *Sbt1*$^{eGFPCre/+}$ cortex, especially in deep layer neurons. This brighter fluorescence may be due to the presence of two copies of GFP in the *Sbt1*$^{eGFPCre/eGFPCre}$

genome. Also we found that in *Sbt1* mutants, GFP-positive cells account for 30% of the total cell population, slightly higher than those in *Sbt1*$^{eGFPCre/+}$ mice, suggesting that more cells express GFP in the *Sbt1*$^{eGFPCre/eGFPCre}$ cortex than in control (n=2).

Next, we compared the different cell populations by immunostaining with the pan-neuronal marker NeuN and oligodendrocyte marker Sox10. Similar to what we found in the spinal cord, there were no significant differences in neuronal and oligodendrocyte cell numbers between the *Sbt1* mutant cortex and control (data not shown), suggesting that *Sbt1* is not essential for neuronal versus glial balance in the cortex.

No significant defects were found in the *Sbt1* mutant retina

Sbt1 has a strong effect on *Xenopus* retinal neurogenesis by promoting early generated neurons at the expense of later cell fates. Mouse *Sbt1* mRNA injection into *Xenopus* embryos had the similar effect to *Xenopus* mRNA injection, suggesting a conserved function among species (Logan, 2006). Therefore, we first analyzed cell fates in the *Sbt1*$^{eGFPCre/eGFPCre}$ retina. All major cell types were characterized by immunostaining with antibodies shown in Table 3.2, and compared between *Sbt1*$^{eGFPCre/+}$ and *Sbt1*$^{eGFPCre/eGFPCre}$ retinas at P28. Surprisingly, we did not found any significant differences between *Sbt1*$^{eGFPCre/+}$ and *Sbt1*$^{eGFPCre/eGFPCre}$ retinas (Figure 3.6). For example, there are no differences with respect to Brn3 labeled RGCs, Calbindin labeled horizontal and amacrine cells and CRALBP labeled Müller glia.

Since *Sbt1* is a downstream target of *Ngn2* (see Chapter 2), and *Ngn2* is required for the leading edge of neurogenesis in mouse retinal development (Hufnagel et al., 2010), we were interested to address whether neurogenesis propagation is normal in the

Sbt1 mutant retina. To answer this question, we examined the newly generated neurons with anti-Tuj1 antibody, combined with anti-pHH3 antibody to label proliferating progenitors at E15. No remarkable differences were detected between *Sbt1* homozygous with control embryos (Figure 3.7). In addition, P42 whole mount retinas were used to examine RGC and HZ cell numbers and distributions in the $Sbt1^{eGFPCre/eGFPCre}$ retina, and they were all identical to control retinas (Figure 3.8). The above results show that deletion of *Sbt1* alone is not sufficient to change major retinal neuron genesis, indicating that mouse *Sbt1* plays different roles than in *Xenopus*. It is possible that there are other factors compensating for *Sbt1* function in retinogenesis, or that it has a subtle effect on small retinal subtype formation that are beyond the scope of our investigation in this experiment.

Taken together, the above results suggest that *Sbt1* function may be dispensable for cell fate specification in the mouse nervous system.

Discussion

Sbt1 is extensively expressed in the mouse nervous system, and plays essential roles in *Xenopus* neurogenesis. However, we found no obvious defects in *Sbt1* mutant mice, in contrast to what we found in *Xenopus*. There are several possible reasons for this, including the low level of homology between mouse and *Xenopus Sbt1*, the lack of important partners, or that its function may be redundant with other factors in mouse. In addition, the low level of *Sbt1* expression in the spinal cord and retina, and deficiency of data from detailed lineage analysis may also contribute to the difficulties in detecting a phenotype in *Sbt1* mutant mice.

Low level of homology between *Xenopus* and mouse *Sbt1*

Xenopus Sbt1 sequence has low identity to that of chick, mouse and human sequence, and there is only 25% amino acid identity between *Xenopus* and mouse SBT1 (Figure 1.3). Whether there is an RNA splicing difference between species is also uncharacterized. In addition, there are no known functional domains or motifs located in *Sbt1* sequence, so that we cannot compare its homology in functional domains. Although mouse *Sbt1* mRNA injection into *Xenopus* embryos promotes neural differentiation, similarly to *Xenopus* mRNA injection, there are no studies to analyze if it has the same effect in mammalian cells. In general, due to the low homology of *Sbt1* sequence between *Xenopus* and mouse, it is possible that mouse *Sbt1* plays a different role in mammals.

Sbt1 may need a partner to regulate neurogenesis

Sbt1 is expressed in early differentiating cells as well as in postnatal neurons in the mouse nervous system, suggesting that it may play different roles depending on timing and cellular context. Whether *Sbt1* coordinates with other factors to perform its function is unclear. For example, in cell fate determination, it may need to interact with components of Notch signaling pathway, and/or cell cycle regulators. In governing cell morphology and migration, it may need to collaborate with genes controlling cytoskeletal rearrangement. In performing neuronal functions, it may need to combine with factors in different signal transduction pathways. For example, in the retina, *Brn3b*, a downstream target of *Ath5*, controls RGC differentiation and function by activating genes encoding cytoskeletal and presynaptic molecules (Mu et al., 2004). In addition, *Sbt1* expression in *Xenopus* may shuttle between nucleus and membrane. Which proteins transport *Sbt1*

expression between different subcellular compartments, and whether *Sbt1* has different effects in different locations are interesting questions to be addressed.

Sbt1 function may be compensated by other factors in mouse

Proneural bHLH transcription factors and their downstream targets compose a complex network to govern neurogenesis. There are many reciprocal and redundant regulatory relationships between these genes. For example, *NeuroD* induces *Ebf2* and *Ebf3*, and *Ebf2* also activate *Ebf3* and *NeuroD* (Dubois et al., 1998; Pozzoli et al., 2001; Seo et al., 2007). *Sbt1* is one of the proneural bHLH targets, and its expression overlaps with numerous proneural genes and their downstream targets. In the retina, *Sbt1* expression domain overlaps with *Ath5*, *Ngn2*, *NeuroD*, *Ash1* and *Bhlhb5*, and in the cortex, *Sbt1* is expressed in the preplate, similar to *Ngn2* downstream targets (Chapter 2). It is highly possible that other factors in this regulatory hierarchy compensate for *Sbt1* function in mutant animals, suggesting that they are functionally redundant with *Sbt1*.

Finally, I removed most of the coding sequence from the *Sbt1* locus, there are still 45 amino acid residues (mostly from exon1 and 2) left in *Sbt1* mutant animals. What their effects are is unclear. It is unlikely but possible, that these 45 amino acid peptides contain essential functional motifs and can rescue *Sbt1* function in mouse neurogenesis. Careful domain analysis needs to be performed to rule out this possibility.

Low level of *Sbt1* expression in the spinal cord and retina limits GFP expression in *Sbt1-eGFPCre* mice

Comprehensive functional analysis depends on detailed expression data. Low levels of *Sbt1* expression in the spinal cord and retina made it difficult to analyze which cell populations express *Sbt1*, and that may be the main reason why there is no GFP

expression detectable in the *Sbt1*$^{eGFPCre/+}$ spinal cord and retina. Even though *eGFPCre* fusion protein can target to the nucleus and catalyze efficient DNA recombination in cultured cells and embryonic stem (ES) cells (Gagneten et al., 1997), its efficiency for fluorescent expression has not been examined extensively in transgenic animals. Recent evidence shows that discrepancy between GFP and endogenous gene expression exists in *eGFPCre* transgenic mice, especially in regions with weak or transient endogenous gene expression (Rivkin and Cordes, 2008). In this study, we detected GFP expression in the cortex, and its expression pattern mimicked endogenous *Sbt1* expression, because it is the region with the most robust endogenous *Sbt1* mRNA. However, even in the cortex, we could only detect a low level of GFP expression, and it was worse in the spinal cord and retina. It is possible that the activity of the *eGFPCre* fusion protein is weaker that GFP alone.

Collectively, these data suggest that *Sbt1* expression level is too low to drive detectable GFP in *Sbt1* mutant animals, so that restricts our analysis in nervous system development.

Sbt1 may regulate the generation of small subtype of neurons, which were beyond the scope of this study

All major cell types in the *Sbt1* mutant retina were examined by immunostaining, including RGC, AM, BP, HZ, photoreceptors and Müller glia, and they were all present in the *Sbt1* mutant retina with normal numbers and distributions (Figure 3.6), suggesting that *Sbt1* is not required for major cell type generation. However, it is more clear now that bHLH factors and their downstream targets not only control major cell fate genesis, but are also involved in the generation of small subclasses of neurons. For example, there

are more than 10 subtypes of RGCs, 29 subtypes of amacrines and 10 subtypes of bipolar cells in the mouse retina (Ghosh et al., 2004; MacNeil and Masland, 1998; Masland, 2001a; Masland, 2001b; Wassle and Boycott, 1991). *Bhlhb5*, a bHLH transcription factor of the *Olig* family, is tightly associated with the generation of selective GABAergic amacrine and type 2 OFF-cone bipolar subtypes (Feng et al., 2006), and misexpression of *Ebf* family genes, which are downstream of *NeuroD* and *Ath5*, bias retinal precursors toward the fates of non-AII glycinergic amacrine, type 2 OFF-cone bipolar and horizontal cells (Jin et al., 2010). Both of these are small subclasses of neurons in the mouse retina. *Sbt1* acts as a downstream target of proneural bHLH factors, but may only mediate a subset of effects of a single bHLH factor, so it may have a more subtle role in retinal neurogenesis. However, we do not know in which cell population *Sbt1* is expressed. Thus, it is extremely difficult to examine its function in small retinal subtypes.

Sbt1 has potential roles in neuronal maturation

Sbt1 is not only expressed in early differentiating cells but also expressed in postnatal neurons, and its expression persists to adulthood in the cortex and spinal cord. Interestingly, in the mouse postnatal cortex, *Sbt1* is dynamically expressed from deep layer neurons to upper layer neurons, coinciding with neuronal terminal differentiation (see Chapter 2). Recent evidence has shown that bHLH factors are also involved in neuronal maturation, morphology and migration. For example, *NeuroD* induces terminal differentiation in olfactory neurogenesis (Boutin et al., 2010), and its downstream target *Ebf2* is important for neuronal migration and nerve development (Corradi et al., 2003). Whether *Sbt1* plays a role in neuronal maturation is an interesting question to address.

In summary, we analyzed *Sbt1* function in vertebrate neurogenesis in two aspects, 1) comparing neuronal versus glial cell numbers in the spinal cord and cortex, 2) examining major neuronal cell types in the retina. Although we did not find any dramatic changes in *Sbt1* mutant mice, we cannot rule out the possibility that *Sbt1* may be involved in small subtype neuron genesis or that it may have potential roles in neuronal maturation, such as axon guidance or synaptic formation.

Appendix

Lineage analysis in *Sbt1-R26R* and *Sbt1-Z/EG* retinas

Since *Sbt1* is only expressed in the mouse retina from E11.5 to P14, it is not clear which kind of cells are coming from the *Sbt1* lineage in the adult retina. The *eGFPCre* cassette in the *Sbt1* locus provides a valuable tool to perform lineage analysis and compare if the lineage has shifted in *Sbt1* mutant retina. We crossed $Sbt1^{eGFPCre/+}$ mice with *Rosa26* reporter strains *(*$R26R^{LacZ}$ or $R26R^{YFP}$) and *Z/EG* reporter to generate *Sbt1-*$R26R^{LacZ}$, *Sbt1-* $R26R^{YFP}$ and *Sbt1-Z/EG* transgenic mice.

In *Sbt1-*$R26R^{LacZ}$, and *Sbt1-*$R26R^{YFP}$ retinas, the reporter genes, LacZ and YFP label progenies that are coming from *Sbt1*-expressing cells. First, we performed X-gal staining and β-gal immunostaining to analyze LacZ expression in the $Sbt1^{eGFPCre/+}$*;* $R26R^{LacZ}$ retina. Both X-gal and β-gal antibody strongly labeled the outer segment of photoreceptors and cells in the INL, weakly labeled the cell bodies of photoreceptors in the ONL, and the processes spanning throughout the retina with Müller glial morphology (Figure A.1). This expression pattern was further confirmed by immunostaining with an anti-GFP antibody, which also recognizes YFP in the $Sbt1^{eGFPCre/+}$*;* $R26R^{YFP}$ retina (data not shown). This result is not consistent with *Sbt1* mRNA expression, which shows RGC

expression by in situ hybridization from E15 to P0 (Chapter 2). So we switched to examine *Sbt1* lineage in the *Sbt1-Z/EG* retina.

Z/EG transgenic line is a double reporter strain that expresses EGFP upon *cre*-mediated excision (Novak et al., 2000). In this line, *Z/EG* transgene was inserted upstream of *Rasa4* (Ras 21 protein activator 4) gene, which is ubiquitously expressed (Lockyer et al., 2001). This reporter strain has been extensively used in retinal histogenesis analyses (Ding et al., 2009; Riesenberg et al., 2009).

Unexpectedly, there were only few cells labeled by anti-GFP antibody in the *Sbt1*$^{eGFPCre/+}$*; Z/EG* retina (less than 10 cells per section), and they were all located in the ONL and outer INL, with presumptive photoreceptor and bipolar cell localization (Figure A.2). We compared GFP-positive cells in the *Sbt1*$^{eGFPCre/+}$*; Z/EG* retinas with *Sbt1*$^{eGFPCre/eGFPCre}$; *Z/EG* retinas, and found that there were many more GFP-positive cells in the mutant retina, with the same locations as in the heterozygous retinas (Figure A.2). This result is similar to our finding of more GFP-positive cells and brighter fluorescent expression in the *Sbt1* mutant cortex. We conclude that *eGFPCre* may not be strong enough to induce reliable *cre* mediated excision in the *Sbt1* mutant retina, and the weak expression of *Sbt1* further limits the effectiveness of *eGFPCre*, which results in the deficiency of reporter gene expression in RGCs.

Conclusion

Sbt1 mutant mice were generated to analyze *Sbt1* function in the mouse nervous system. So far, we did not find any significant changes in the spinal cord, cortex and retinal cell fates and numbers, indicating that *Sbt1* function is dispensable for mouse neurogenesis in cell fate specification.

Acknowledgments

We thank Dr. Sen Wu for careful suggestions on targeting vector designing and construction, Drs. Mario Capecchi and Sabine Fuhrmann for providing *R26R*^{YFP} and *R26R*^{LacZ} reporter mice, Drs. Chi-Bin Chien and Edward Levine for antibodies. We also thank Dr. Alejandra Bosco for assistance on confocol analysis, Micheal Steele for Southern blot screening of ES cells, Joy Corley for technical help with genotyping and all Vetter lab members for discussion. This work was supported by NIH grants EY012274 to M.L.V.

References

Boutin, C., Hardt, O., de Chevigny, A., Core, N., Goebbels, S., Seidenfaden, R., Bosio, A., and Cremer, H. (2010). NeuroD1 induces terminal neuronal differentiation in olfactory neurogenesis. Proc Natl Acad Sci U S A *107*, 1201-1206.

Brunet, J. F., and Ghysen, A. (1999). Deconstructing cell determination: proneural genes and neuronal identity. Bioessays *21*, 313-318.

Bunt-Milam, A. H., and Saari, J. C. (1983). Immunocytochemical localization of two retinoid-binding proteins in vertebrate retina. J Cell Biol *97*, 703-712.

Corradi, A., Croci, L., Broccoli, V., Zecchini, S., Previtali, S., Wurst, W., Amadio, S., Maggi, R., Quattrini, A., and Consalez, G. G. (2003). Hypogonadotropic hypogonadism and peripheral neuropathy in Ebf2-null mice. Development *130*, 401-410.

Ding, Q., Chen, H., Xie, X., Libby, R. T., Tian, N., and Gan, L. (2009). BARHL2 differentially regulates the development of retinal amacrine and ganglion neurons. J Neurosci *29*, 3992-4003.

Dubois, L., Bally-Cuif, L., Crozatier, M., Moreau, J., Paquereau, L., and Vincent, A. (1998). XCoe2, a transcription factor of the Col/Olf-1/EBF family involved in the specification of primary neurons in Xenopus. Curr Biol *8*, 199-209.

Feng, L., Xie, X., Joshi, P. S., Yang, Z., Shibasaki, K., Chow, R. L., and Gan, L. (2006). Requirement for Bhlhb5 in the specification of amacrine and cone bipolar subtypes in mouse retina. Development *133*, 4815-4825.

Gagneten, S., Le, Y., Miller, J., and Sauer, B. (1997). Brief expression of a GFP cre fusion gene in embryonic stem cells allows rapid retrieval of site-specific genomic deletions. Nucleic Acids Res *25*, 3326-3331.

Ghosh, A., and Greenberg, M. E. (1995). Distinct roles for bFGF and NT-3 in the regulation of cortical neurogenesis. Neuron *15*, 89-103.

Ghosh, K. K., Bujan, S., Haverkamp, S., Feigenspan, A., and Wassle, H. (2004). Types of bipolar cells in the mouse retina. J Comp Neurol *469*, 70-82.

Gross, R. E., Mehler, M. F., Mabie, P. C., Zang, Z., Santschi, L., and Kessler, J. A. (1996). Bone morphogenetic proteins promote astroglial lineage commitment by mammalian subventricular zone progenitor cells. Neuron *17*, 595-606.

Guldenagel, M., Sohl, G., Plum, A., Traub, O., Teubner, B., Weiler, R., and Willecke, K. (2000). Expression patterns of connexin genes in mouse retina. J Comp Neurol *425*, 193-201.

Hufnagel, R. B., Le, T. T., Riesenberg, A. L., and Brown, N. L. (2010). Neurog2 controls the leading edge of neurogenesis in the mammalian retina. Dev Biol *340*, 490-503.

Jin, K., Jiang, H., Mo, Z., and Xiang, M. (2010). Early B-cell factors are required for specifying multiple retinal cell types and subtypes from postmitotic precursors. J Neurosci *30*, 11902-11916.

Lockyer, P. J., Kupzig, S., and Cullen, P. J. (2001). CAPRI regulates Ca(2+)-dependent inactivation of the Ras-MAPK pathway. Curr Biol *11*, 981-986.

Logan, M. A. (2006) Mechanisms of neuronal differentiation in Xenopus Laevis.

Logan, M. A., Steele, M. R., Van Raay, T. J., and Vetter, M. L. (2005). Identification of shared transcriptional targets for the proneural bHLH factors Xath5 and XNeuroD. Dev Biol *285*, 570-583.

MacNeil, M. A., and Masland, R. H. (1998). Extreme diversity among amacrine cells: implications for function. Neuron *20*, 971-982.

Masland, R. H. (2001a). The fundamental plan of the retina. Nat Neurosci *4*, 877-886.

Masland, R. H. (2001b). Neuronal diversity in the retina. Curr Opin Neurobiol *11*, 431-436.

Miller, R. H. (2002). Regulation of oligodendrocyte development in the vertebrate CNS. Prog Neurobiol *67*, 451-467.

Mu, X., Beremand, P. D., Zhao, S., Pershad, R., Sun, H., Scarpa, A., Liang, S., Thomas, T. L., and Klein, W. H. (2004). Discrete gene sets depend on POU domain transcription factor Brn3b/Brn-3.2/POU4f2 for their expression in the mouse embryonic retina. Development *131*, 1197-1210.

Novak, A., Guo, C., Yang, W., Nagy, A., and Lobe, C. G. (2000). Z/EG, a double reporter mouse line that expresses enhanced green fluorescent protein upon Cre-mediated excision. Genesis *28*, 147-155.

Perters A., P., Webster D. (1990). The fine structure of the nervous system: Saunders, Philadelphia.

Pozzoli, O., Bosetti, A., Croci, L., Consalez, G. G., and Vetter, M. L. (2001). Xebf3 is a regulator of neuronal differentiation during primary neurogenesis in Xenopus. Dev Biol *233*, 495-512.

Riesenberg, A. N., Liu, Z., Kopan, R., and Brown, N. L. (2009). Rbpj cell autonomous regulation of retinal ganglion cell and cone photoreceptor fates in the mouse retina. J Neurosci *29*, 12865-12877.

Rivkin, E., and Cordes, S. P. (2008). Generation of a transgenic mouse line expressing GFP-Cre protein from a Hoxb4 neural enhancer. Genesis *46*, 119-124.

Rowitch, D. H. (2004). Glial specification in the vertebrate neural tube. Nat Rev Neurosci *5*, 409-419.

Rowitch, D. H., and Kriegstein, A. R. (2010). Developmental genetics of vertebrate glial-cell specification. Nature *468*, 214-222.

Seo, S., Lim, J. W., Yellajoshyula, D., Chang, L. W., and Kroll, K. L. (2007). Neurogenin and NeuroD direct transcriptional targets and their regulatory enhancers. Embo J *26*, 5093-5108.

Verma-Kurvari, S., Savage, T., Gowan, K., and Johnson, J. E. (1996). Lineage-specific regulation of the neural differentiation gene MASH1. Dev Biol *180*, 605-617.

Wang, D. D., and Bordey, A. (2008). The astrocyte odyssey. Prog Neurobiol *86*, 342-367.

Wassle, H., and Boycott, B. B. (1991). Functional architecture of the mammalian retina. Physiol Rev *71*, 447-480.

Wu, S., Ying, G., Wu, Q., and Capecchi, M. R. (2008). A protocol for constructing gene targeting vectors: generating knockout mice for the cadherin family and beyond. Nat Protoc *3*, 1056-1076.

Table 3.1. PCR primers and product sizes used in genotyping.

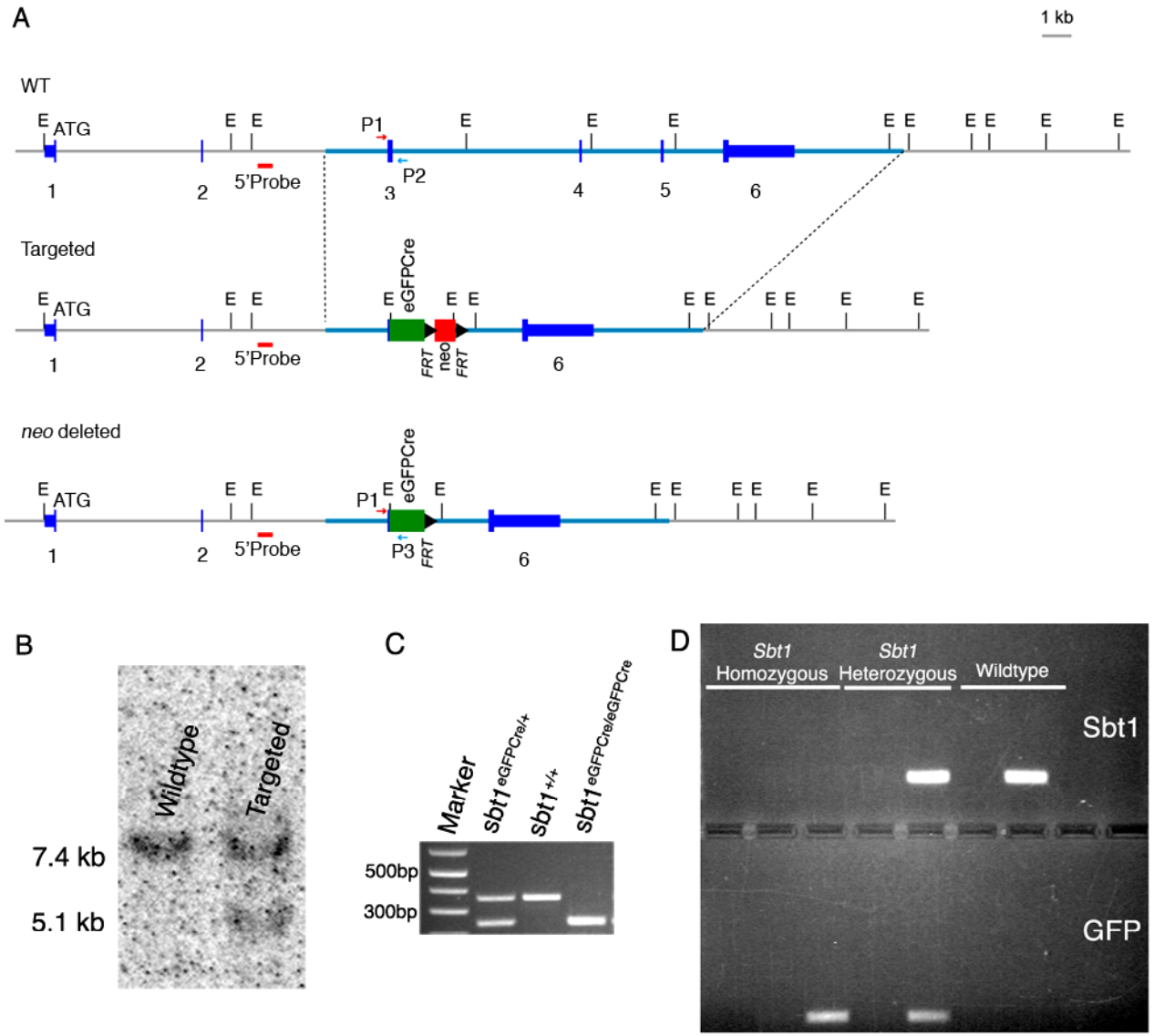
Genes	PCR primers	PCR product sizes
Sbt1	5'-GGTAA TGCAA CAACC ACCTT AGCC-3' 5'-AGATG AACTT CAGGG TCAGC TTGC-3' 5'-CACAG CGTTG AGGCA GTAGC TC-3'	WT allele = 247bp Mutant allele = 361bp
R26R ^{YFP}	5'-GTTATCAGTAAGGGAGCTGCAGTGG-3' 5'-AAGACCGCGAAGAGTTTGTCCTC-3' 5'-GGCGGATCACAAGCAATAATAACC-3'	WT allele = 415 bp YFP allele = 302 bp
R26R ^{LacZ}	5'-GGAGCGGGAGAAATGGATATG-3' 5'-GCGAAGAGTTTGTCCTCAACC-3' 5'-AAAGTCGCTCTGAGTTGTTAT-3'	WT allele = 584 bp LacZ allele= 350 bp
Z/EG	5'-CACCCGAGTGTGATCATCTG-3' 5'-CGGATAAACGGAACTGGAAA-3'	LacZ allele = 550 bp

Table 3.2. Cell type specific antibodies used for retinal neurogenesis and lineage analysis.

Cell types	Subtypes	Antibodies
RGC		Brn3; Isl1
AM	Pan AM Cholinergic AM	Calbindin Isl1
BP	Pan BP On BP	Chx10 Isl1
HZ		Calbindin, Prox1
Photoreceptors	Early Mature	Recoverin Rhodopsin
Müller glia		CRALBP

RGC: retinal ganglion cell; AM: amacrine cell; BP: bipolar cell; HZ, horizontal cell.

Figure 3.1 Generation of *Sbt1-eGFPCre* mice.
(A) Structure of *Sbt1-eGFPCre* targeting vector. There are 6 exons located in the mouse *Sbt1* locus. The start codon is in exon 1. Exons 3, 4, and 5 were removed by homologous recombination, exon 6 was frame-shifted and *eGFPCre* cassette was inserted into *Sbt1* locus. Primer pair P1 and P2 are used to screen the wild type allele, producing a 247-bp DNA fragment. Primer pair P1 and P3 are used to screen the targeted allele, producing a 361-bp DNA fragment. (B) Southern blot hybridization analysis to screen target ES cell clones. Mouse ES cells were electroporated with *Sbt1-eGFPCre* targeting vector. DNAs from ES cells were subtracted and digested by EcoRI. After Southern blot analysis, the WT ES cell showed one band at 7.4kb, while target allele showed two bands at 7.4kb and 5.1kb. (C) PCR genotyping on *Sbt1-eGFPCre* mouse showed WT and knockin alleles. (D) RT-PCR on RNAs subtracted from the *Sbt1-eGFPCre* cortex showed that *Sbt1* is expressed in the $Sbt1^{+/+}$ and $Sbt1^{eGFPCre/+}$ cortex, and GFP is expressed in $Sbt1^{eGFPCre/+}$ and $Sbt1^{eGFPCre/eGFPCre}$, consistent with genotyping result. E: EcoRI cleavage site.


A
1 kb
WT
ATG
5'Probe
P1
P2
1
2
3
4
5
6
Targeted
eGFPCre
FRT
neo
FRT
neo deleted
P3
B
Wildtype
Targeted
7.4 kb
5.1 kb
C
Marker
sbt1eGFPCre/+
sbt1+/+
sbt1eGFPCre/eGFPCre
500bp
300bp
D
Sbt1
Homozygous
Heterozygous
Wildtype
Sbt1
GFP

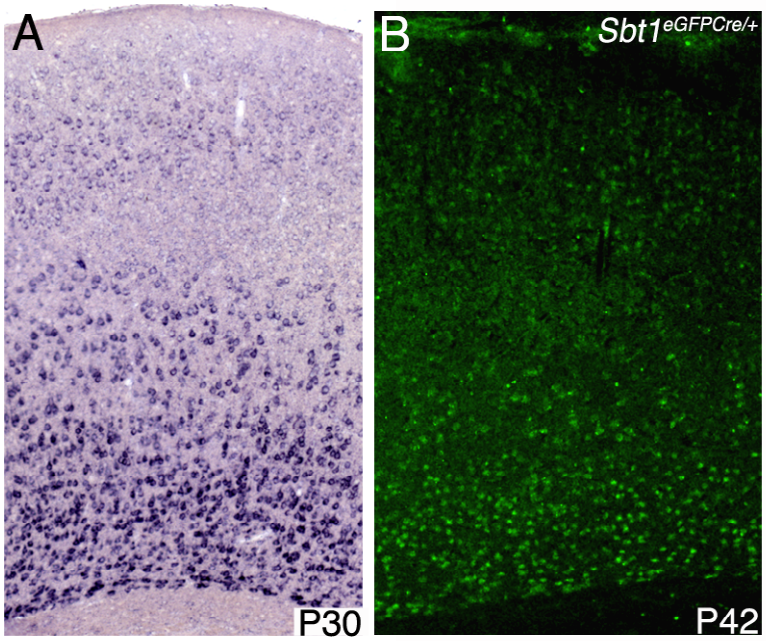


Figure 3.2 GFP expression follows endogenous *Sbt1* expression in the *Sbt1*$^{eGFPCre/+}$ cortex.
(A) In situ hybridization on P30 wild type mouse cortex. (B) GFP expression in the *Sbt1*$^{eGFPCre/+}$ cortex mimics endogenous *Sbt1* mRNA expression, which is shown in (A).

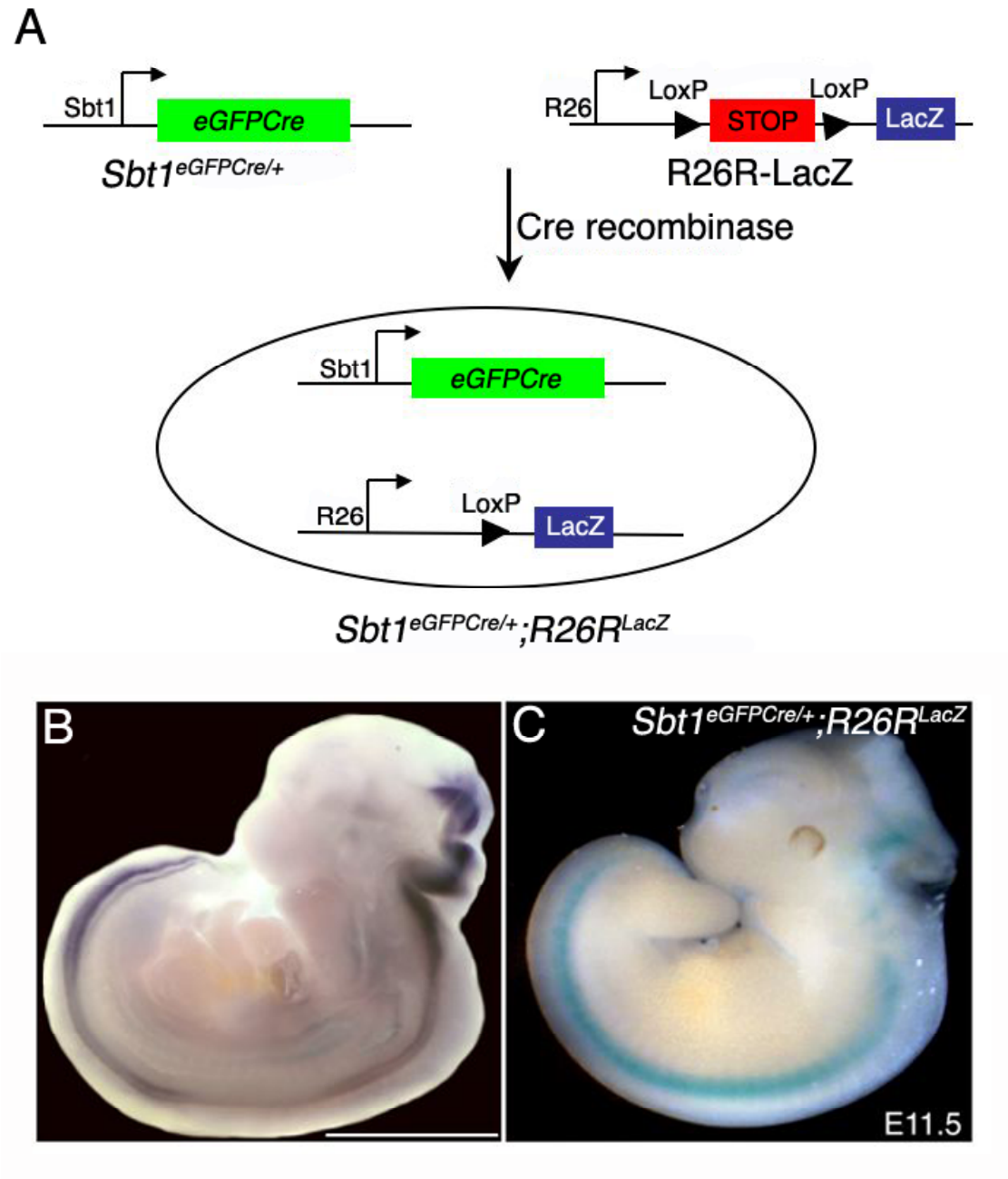
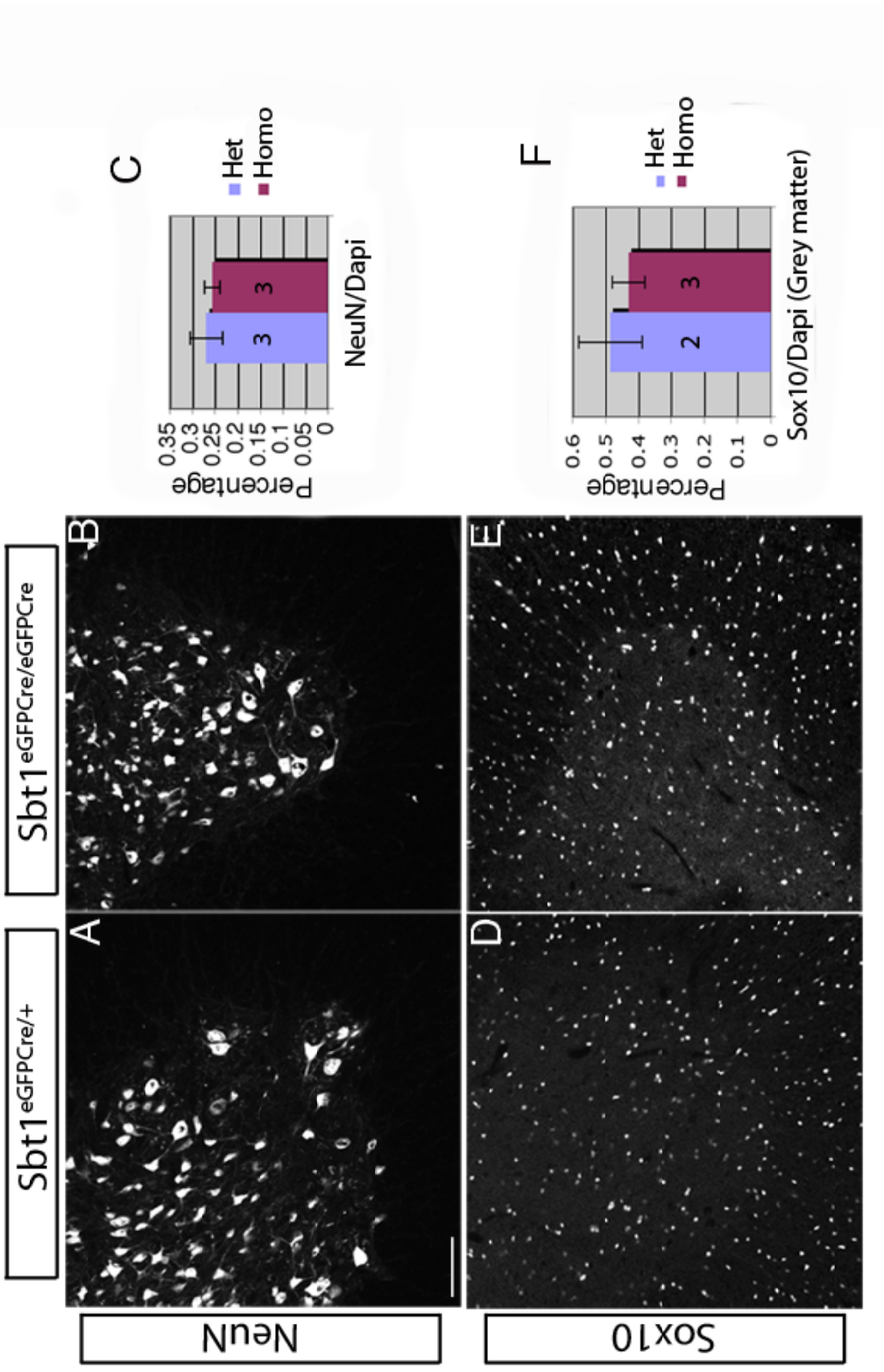


Figure 3.3 *eGFPCre* induces reliable recombination activity in *Sbt1* mutants.
(A) Generation of *Sbt1*$^{eGFPCre/+}$*; R26R*^{LacZ} mice by crossing *Sbt1*$^{eGFPCre/+}$ with R26R-LacZ reporters. (B) *Sbt1* in situ hybridization on E11.5 mouse embryos. (C) LacZ expression pattern is similar to endogenous *Sbt1* expression in *Sbt1*$^{eGFPCre/+}$*; R26R*^{LacZ} embryos. Scale bar 2.0 mm.

Figure 3.4 There are no changes in spinal cord neuron and oligodendrocyte numbers in *Sbt1* mutant mice.
(A, B and C) Pan-neuronal marker NeuN was used to label spinal cord ventral horn neurons (A, B). The percentage of NeuN-positive cells to DAPI-positive cells is compared in C, and there are no significant differences between the *Sbt1* heterozygous with *Sbt1* homozygous spinal cords. (D, E and F) Oligodendrocyte marker Sox10 was used to label spinal cord ventral oligodendrocytes (C, D). The percentage of Sox10-positive cells to DAPI-positive cells is compared in F, and there are no significant differences between *Sbt1* heterozygous with *Sbt1* homozygous spinal cords. Scale bar 100 μm.


Sbt1eGFPCre/+
Sbt1eGFPCre/eGFPCre
A
B
C
D
E
F
NeuN
Sox10
Het
Homo
NeuN/Dapi
Percentage
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
3
3
Sox10/Dapi (Grey matter)
0.6
0.5
0.4
0.3
0.2
0.1
0
2
3

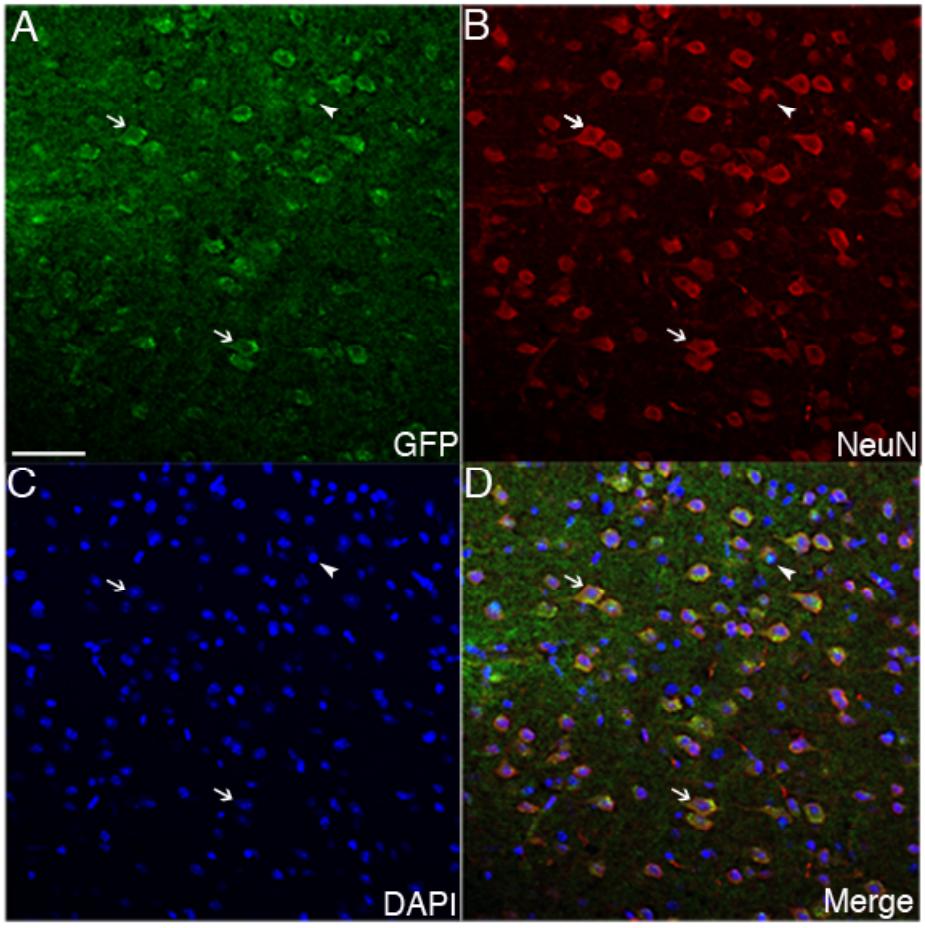


Figure 3.5 *Sbt1* is expressed in cortical neurons and other cell types in the cortex.
(A, B, C and D) GFP staining (A) overlaps with pan-neuronal marker NeuN (B) in 6 weeks *Sbt1*$^{eGFPCre/+}$ cortex, suggesting that *Sbt1* is expressed in cortical neurons (arrow). There are few scattered cells, which are GFP^{+} but NeuN^{-} (arrowhead), indicating that *Sbt1* is not restricted to the neuronal lineage. Scale bar 100 μm.

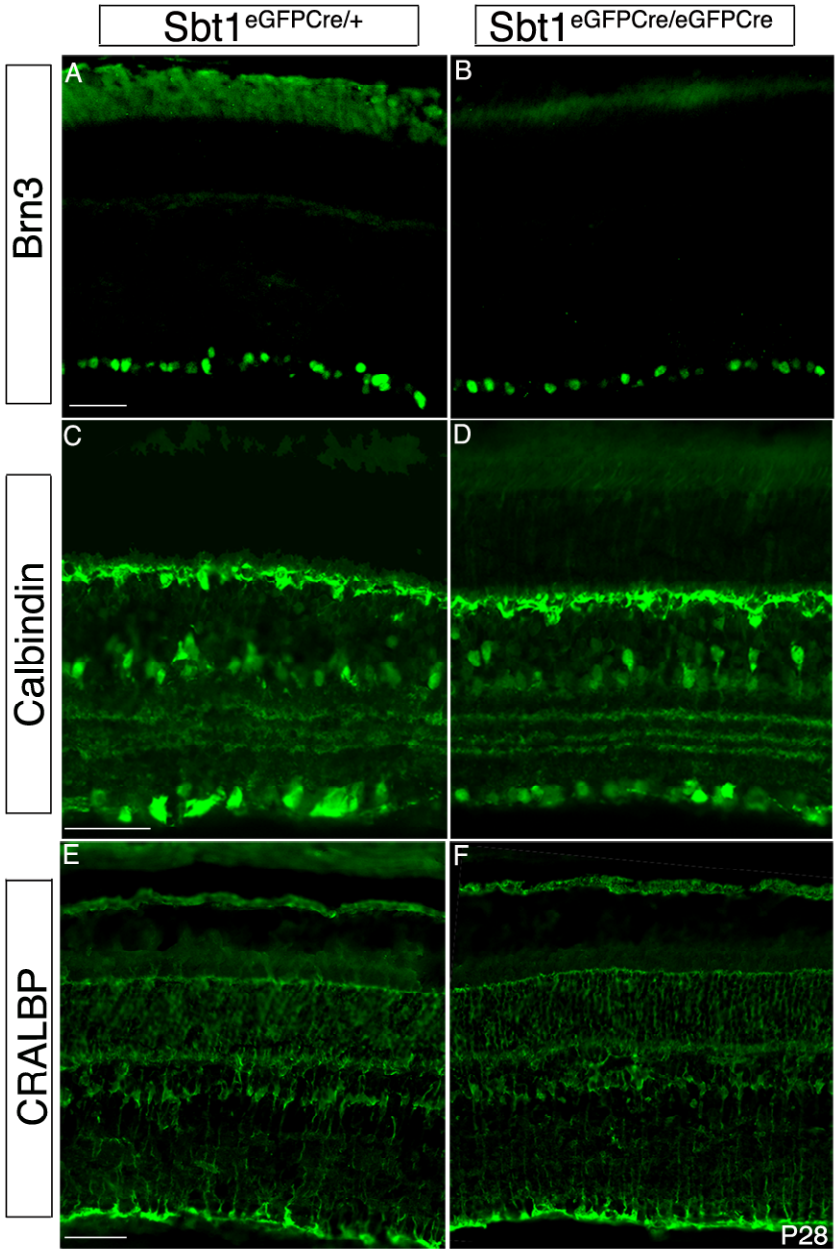
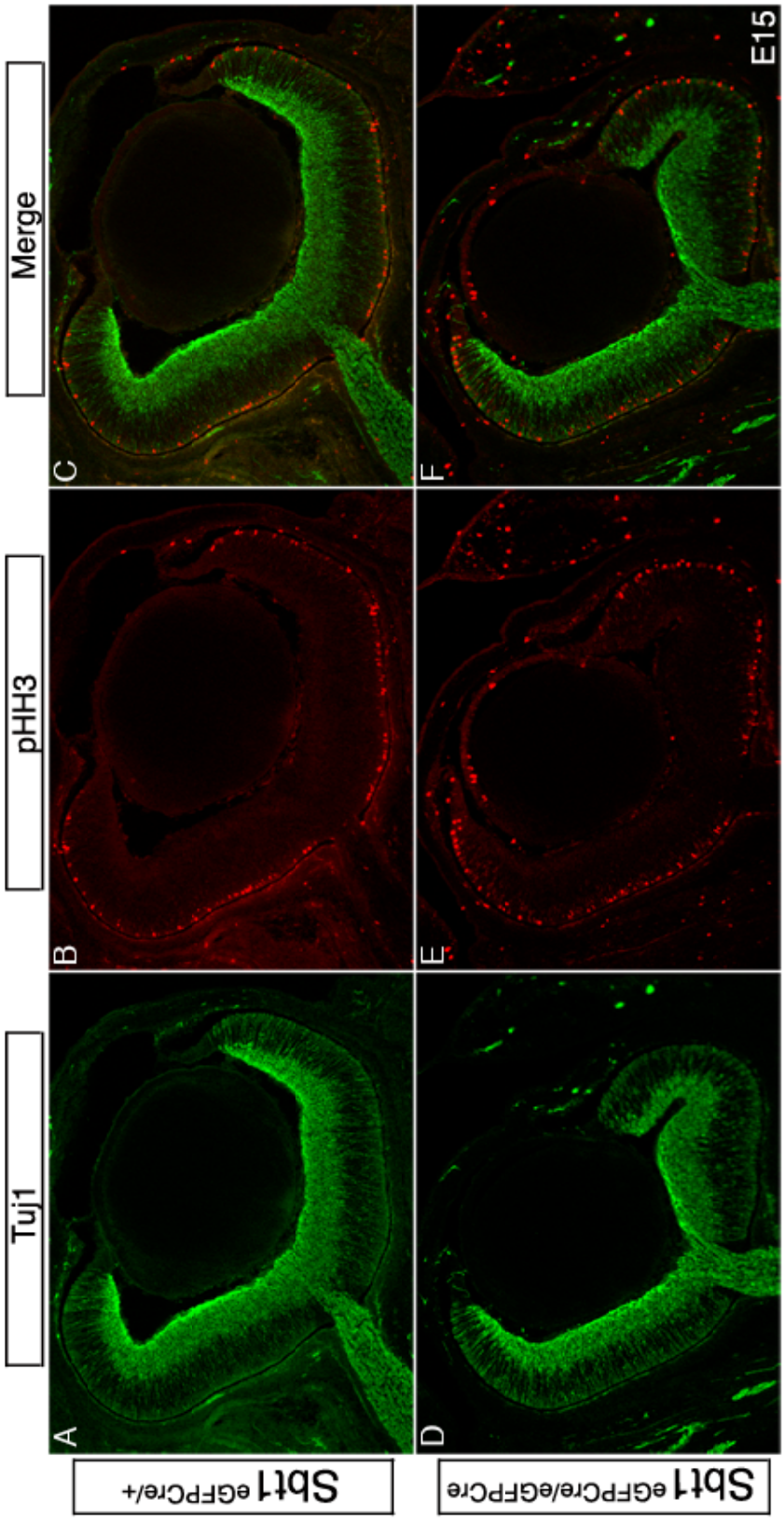


Figure 3.6 All major cell types are present in the *Sbt1* mutant retina with proper numbers and localizations.
(A, B, C, D, E and F) Immunostaining of *Sbt1*$^{eGFPCre/+}$ and *Sbt1*$^{eGFPCre/eGFPCre}$ retinas with anti-Brn3, anti-Calbindin and anti-CRALBP antibodies reveals that there are no defects on RGCs (A, B), horizontal cells (C, D), amacrines (C, D), and Müller glia cell (E, F) numbers and localization at P28. Scale bar 50 μm.

Figure 3.7 Neurogenesis is propagated accurately in the *Sbt1* mutant retina.
(A, B and C) At E15, Tuj1 labeled early differentiating neurons (A) and pHH3 labeled proliferating progenitors (B) are located in the *Sbt1*$^{eGFPCre/+}$ retina. (D, E and F) There are no dramatic changes in Tuj1 (D) and pHH3 (E) expression in the *Sbt1*$^{eGFPCre/eGFPCre}$ retina.


Tuj1
pHH3
Merge
Sbt1 eGFPCre/+
Sbt1 eGFPCre/eGFPCre
A
B
C
D
E
F
E15

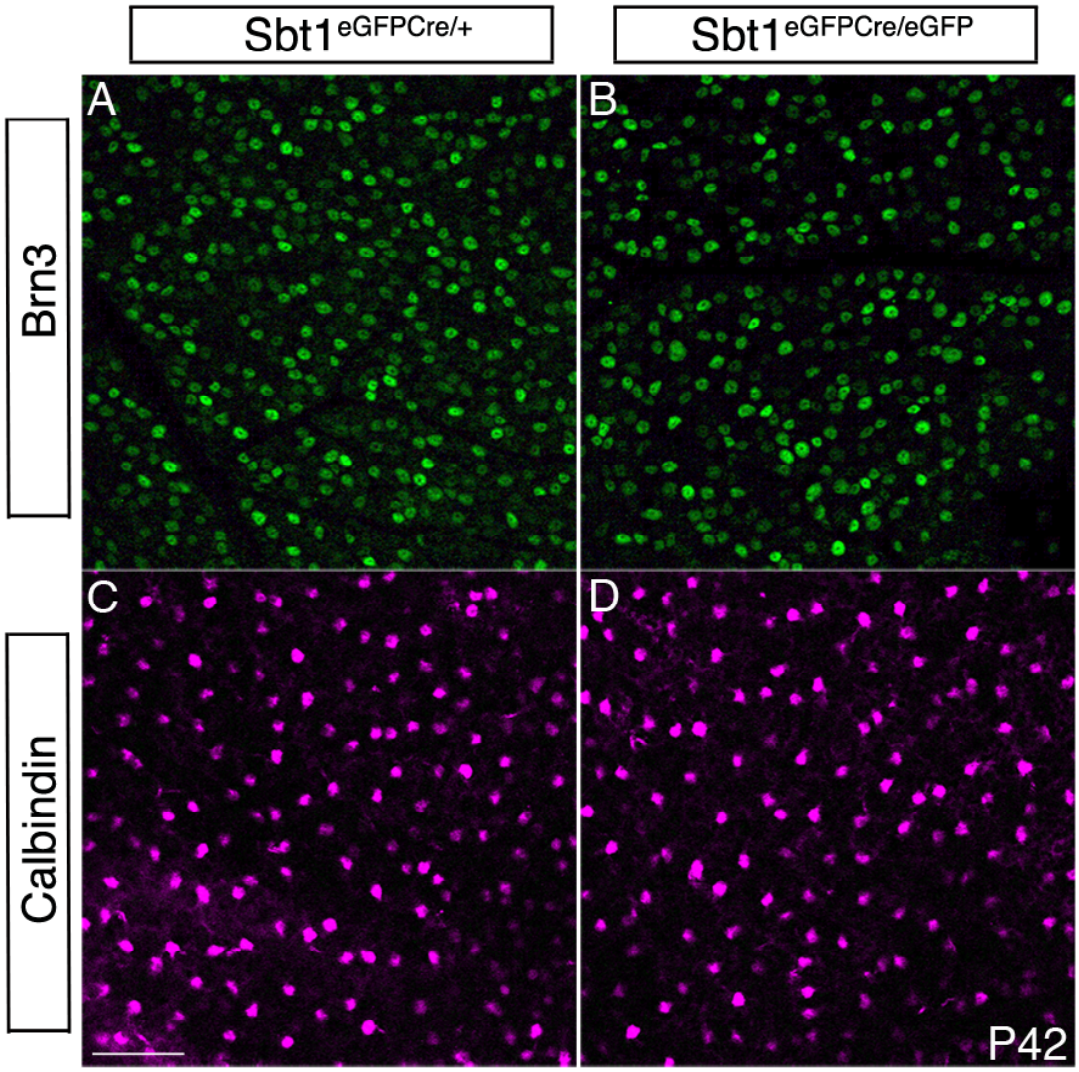


Figure 3.8 RGC and horizontal cell numbers and distributions are not changed in the *Sbt1* mutant retina.
(A, B, C and D) Brn3 labeled RGCs (A, B) and Calbindin labeled horizontal cells (C, D) display normal numbers and distributions in the *Sbt1*$^{eGFPCre/eGFPCre}$ retina at P42. Scale bar 20 μm.

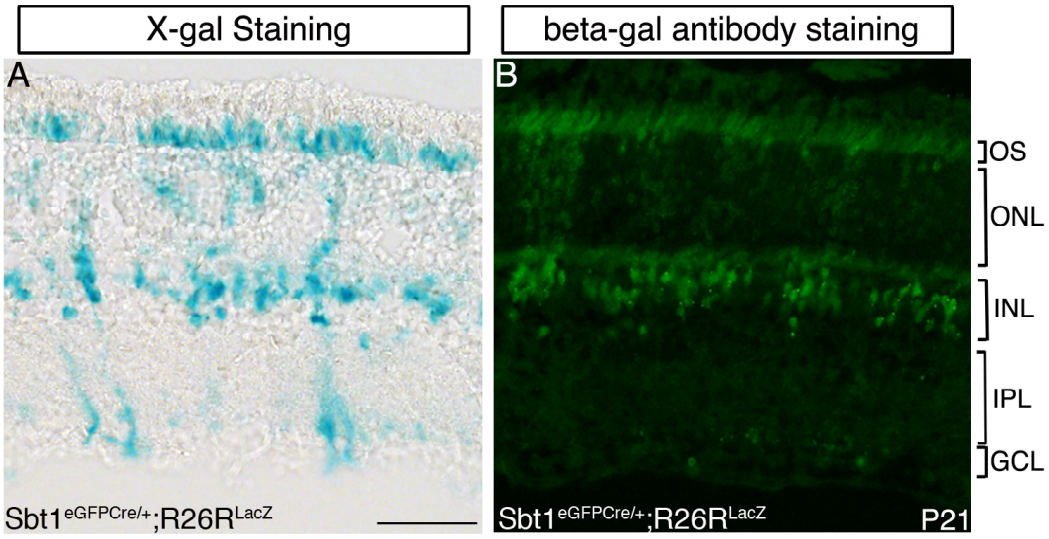
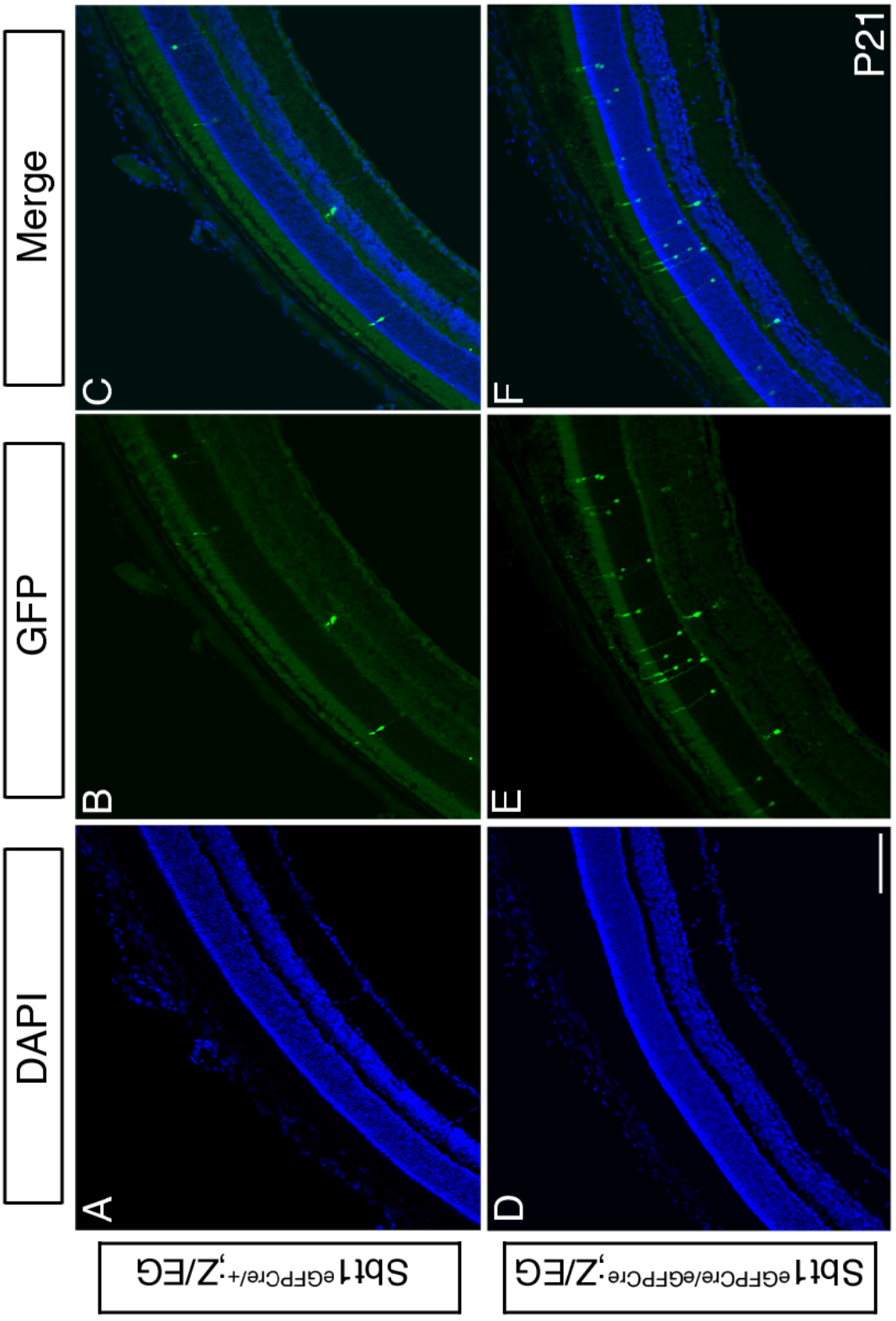


Figure A.1 Lineage analysis on the *Sbt1*$^{eGFPCre/+}$*; R26R*^{LacZ} retina is not consistent with endogenous *Sbt1* expression.

(A, B) X-gal staining (A) and β-gal antibody staining (B) on *Sbt1*$^{eGFPCre/+}$*; R26R*^{LacZ} retinas label photoreceptor outer segments, photoreceptors cell bodies in the ONL, cells in the INL and Müller glial processes spanning through out retina. However, there are no RGCs labeled in the GCL. It is not consistent with *Sbt1* endogenous mRNA expression, which shows *Sbt1* is expressed in the GCL from E15 to P0 (see Chapter 2). Scale bar 50 μm.OS: outer segments of photoreceptors; ONL: outer nuclear layer; INL: inner nuclear layer; IPL: inner plexiform layer; GCL: ganglion cell layer.

Figure A.2 Cre activity in the *Sbt1*$^{eGFPCre/+}$*; Z/EG* retina is too weak to induce reliable reporter gene expression.
(A, B and C) There are only few GFP-positive cells in the *Sbt1*$^{eGFPCre/+}$*; Z/EG* retina after anti-GFP antibody staining (B). (D, E and F). There are more GFP-positive cells (E) in the *Sbt1*$^{eGFPCre/eGFPCre}$*; Z/EG* retinas since there are two copies of *cre* in the genome. Scale bar 100 μm.


DAPI
GFP
Merge
A
B
C
D
E
F
P21
Sbt1eGFPCre/+;Z/EG
Sbt1eGFPCre/eGFPCre;Z/EG

CHAPTER 4

CONCLUSION

Sbt1 mRNA expression is regulated by *Ngn2* or other bHLH factors

Sbt1 mRNA is expressed in the mouse developing cortex, spinal cord, brain stem and retina, and its expression in the cortex and spinal cord persists to adulthood, coinciding with the timing of mouse neurogenesis and neuronal maturation. In contrast to proneural bHLH factors, which are mostly expressed in progenitors, *Sbt1* is mainly expressed in postmitotic cells, suggesting it is involved in cell differentiation after commitment. In the adult cortex, it is not only expressed in neurons, but also expressed in other cell types, indicating that its expression is not restricted to the neuronal lineage. *Sbt1* is expressed in multiple neural tissues and cell lineages, suggesting that it may mediate the core neuronal differentiation program instead of restricted to a specific cell fate, and indicating that it may also act as a shared downstream target of bHLH factors in mouse

More importantly, I showed that *Sbt1* expression in the developing midbrain and retina is downregulated in *Ngn2* mutants at E11.5 (Figure 2.5 and 4.1), and *Sbt1* expression is not completely absent in the *Ngn2* mutant retina, indicating other factors may also contribute to control *Sbt1* expression. However, it is very difficult to examine which bHLH factors, other than *Ngn2*, regulate *Sbt1* expression, because bHLH proteins are redundantly expressed in all analyzed tissues, including cortex, brainstem, spinal cord and retina. Whether other bHLH proteins regulate *Sbt1* expression in mouse will be determined in the future studies.

Similar to what we observed in the retina, *Ngn2* is the major bHLH factor that is expressed in the midline of the ventral midbrain, where we found reduced expression of *Sbt1* (Figure 2.5 and 4.1 B). These results consistently support the conclusion that *Sbt1*

expression is regulated by proneural bHLH factors and may medicate their functions in the nervous system development. However, bHLH factors regulate a huge network of target genes and each target may only mediate a subset of effect. So *Sbt1* function may be subtle or difficult to be detected.

Sbt1 function is dispensable for mouse neurogenesis

I analyzed *Sbt1* function in mouse neurogenesis by comparing retinal cell fates, numbers, distribution, as well as neuronal versus glial genesis in the spinal cord and cortex, between *Sbt1* mutant ($sbt1^{eGFPCre/eGFPCre}$) and littermates ($sbt1^{eGFPCre/+}$). Unexpectedly, we found no evidence showing that *Sbt1* is involved in controlling the above events. Even though it is possible that *Sbt1* has effects on small subclasses of neurons, we conclude that its function on major cell type generation is dispensable in mouse.

Mouse *Sbt1* has different functions than *Xenopus Sbt1*

Although *Sbt1* is a conserved gene among vertebrate species, the *Xenopus* protein sequence has low identity to those of chick, mouse and human sequences. Only 25% of the amino acid is identical between *Xenopus* and mouse, indicating it may have a different function among species. Previous reports have been shown that numerous factors have specific effects in certain species but not in others. For example, *p27/Xic1* is both necessary and sufficient to promote Müller glial cell fate in the *Xenopus* retina (Ohnuma et al., 1999), but its homolog in mouse, *p27/Kip1*, is not required for glial cell fate generation. (Dyer and Cepko, 2000; Levine et al., 2000). In addition, *Frizzled 5 (Fz5)* signaling governs the neural potential of progenitors in the developing *Xenopus* retina

(Van Raay et al., 2005). However, it plays a cell nonautonomous role in mouse, regulating hyaloid vitreous vasculature development (Liu and Nathans, 2008; Zhang et al., 2008). Moreover, overexpression of *Xath5* in *Xenopus* retinal progenitors promotes RGC generation, while overexpression of *Math5* in the same context increases bipolar cell numbers, indicating that not all aspects of *Xath5* function are conserved in *Math5* (Brown et al., 1998). Furthermore, *Sbt1* expression in *Xenopus* is restricted to transiently differentiating neurons. However, in mouse, it is expressed in early differentiating neurons as well as in mature cortical neurons, suggesting that its function is not restricted to early differentiating events.

S*bt1* function in the mouse nervous system maybe functionally redundant with other genes

Proneural bHLH factors and their downstream targets compose a complex network controlling neural differentiation. There are many reciprocal and redundant regulatory relationships between bHLH factors and their transcription targets. For example, *NeuroD* induces *Ebf2*, *Ebf3*, *Myt1*, and *NeuroD4* in different species, but *Ebf2* also activates *Ebf3* and *NeuroD* (Dubois et al., 1998; Pozzoli et al., 2001). Moreover, *NeuroD4* induces *Ebf2*, *Myt1* and *NeuroD* (Perron et al., 1999). These date indicate that *NeuroD* downstream targets (*Ebf2* and *NeuroD4*) act reciprocally and potentially redundantly to perform *NeuroD* effects, and that may account for lack of gross deficiencies in *NeuroD* mutant (Seo et al., 2007). *Sbt1* expression is overlapping with numerous bHLH factors and their targets (Chapter 2). Furthermore, *Sbt1* expression in the developing retina and brainstem is regulated by *Ngn2*. However, we do not know what S*bt1* position is in this complex network, and whether it can reciprocally regulate

bHLH expression. Thus, it is highly possible that other factors compensate for *Sbt1* function in different cellular context, since we did not observe any dramatic changes in *Sbt1* mutant mice.

S*bt1* may need critical cofactors to perform its function

The function of bHLH factors is highly influenced by the spatial and temporal context in which they are expressed. One explanation of the diverse function of bHLH factors is that they interact with context specific cofactors (Powell and Jarman, 2008). It has been shown that proneural bHLH factors regulate neurogenesis through combinatorial interaction with homeodomain genes, components of Notch/Delta signaling pathway, cell cycle regulators, proneural targets and/or other genes. For example, in the mouse ventral spinal cord, patterning factors *Olig2*, *Pax6* and *Nkx2.2* and proneural bHLH factors *Ngn* and *Mash1* coordinately regulate neuron, astrocyte and oligodendrocyte production at different times and locations (Sugimori et al., 2007). Whether *Sbt1* interacts with cofactors to exert its function is unknown. A yeast two-hybrid analysis has been performed to screen for *Sbt1* partners. The preliminary result suggests that *Sbt1* might interact with molecules that are involved in cytoskeleton arrangement, cell cycle modification or others. In addition, in the *Xenopus* retina, overexpression of *Sbt1* or *Ath5* can moderately induce RGC generation (Kanekar et al., 1997; Logan, 2006; Moore et al., 2002). However, when *Sbt1* and *Ath5* are overexpressed together, almost all labeled cells adopt the RGC cell fate, suggesting that *Sbt1* and *Ath5* coordinately induce RGC generation (Moore unpublished data). How these two factors interact and enhance each other's function is not clear. Whether *Sbt1* cooperates with

other factors or what possible cofactors could be in mouse nervous system development are interesting questions in the future.

S*bt1* has potential functions in neuronal terminal differentiation

Proneural bHLH factors and their targets are not only required for cell fate specification, but also involved in neuronal terminal differentiation, morphology and migration. For example, *NeuroD* induces neuronal terminal differentiation in olfactory neurogenesis (Boutin et al., 2010), and *NeuroD2* plays a critical role in regulating synaptic maturation and the patterning of thalamocortical connections (Ince-Dunn et al., 2006). In addition, the *NeuroD* downstream target *Ebf2* is important for neuronal migration and nerve development (Corradi et al., 2003). *Sbt1* is expressed in postnatal neurons and its expression persists to adulthood in the cortex and spinal cord. Notably, in the mouse postnatal cortex, it is dynamically expressed from deep layer neurons to upper layer neurons, coinciding with neuronal maturation occurring in this tissue (see Chapter 2). Moreover, *Sbt1* expression shuttles between the nucleus and membrane in the *Xenopus* animal cap ectoderm. It has been shown that *Xenopus* p21-activated kinase3 (PAK3) is activated by recruitment to the cell membrane and this activity enhances neuronal differentiation and cell cycle withdrawal in the open neural plate (Souopgui et al., 2002). In mouse, PAK genes (PAK1/2/3) are also expressed in the brain and have essential roles in axonal guidance, neuronal polarization, migration and synaptic plasticity (Boda et al., 2004; Hayashi et al., 2004; Kreis and Barnier, 2009). Whether s*bt1* interacts with PAK genes to influence neuronal terminal differentiation will be addressed in the future.

Collectively, *Sbt1*, as a downstream target of proneural bHLH factors, is expressed during late stages of neurogenesis, indicating it may be involved in neuronal terminal differentiation programs (Figure 4.2).

Conclusion

Sbt1 is expressed in the developing cortex, brainstem, spinal cord and retina, and its expression in the spinal cord and cortex is maintained through adulthood. Moreover, its expression in the early embryonic brainstem and retina is regulated by *Ngn2*, suggesting it acts as a downstream target of proneural bHLH factors. In contrast to *Sbt1* extensive expression within the nervous system, *Sbt1* mutant mice display no significant defects in neurogenesis, suggesting *Sbt1* activity in neural differentiation may be redundant with other factors in mouse, or that it regulates more subtle aspects of neuronal terminal differentiation, or that it regulates the differentiation of small subclasses of neurons, which is beyond the scope of this study.

References

Boda, B., Alberi, S., Nikonenko, I., Node-Langlois, R., Jourdain, P., Moosmayer, M., Parisi-Jourdain, L., and Muller, D. (2004). The mental retardation protein PAK3 contributes to synapse formation and plasticity in hippocampus. J Neurosci *24*, 10816-10825.

Boutin, C., Hardt, O., de Chevigny, A., Core, N., Goebbels, S., Seidenfaden, R., Bosio, A., and Cremer, H. (2010). NeuroD1 induces terminal neuronal differentiation in olfactory neurogenesis. Proc Natl Acad Sci U S A *107*, 1201-1206.

Brown, N. L., Kanekar, S., Vetter, M. L., Tucker, P. K., Gemza, D. L., and Glaser, T. (1998). Math5 encodes a murine basic helix-loop-helix transcription factor expressed during early stages of retinal neurogenesis. Development *125*, 4821-4833.

Corradi, A., Croci, L., Broccoli, V., Zecchini, S., Previtali, S., Wurst, W., Amadio, S., Maggi, R., Quattrini, A., and Consalez, G. G. (2003). Hypogonadotropic hypogonadism and peripheral neuropathy in Ebf2-null mice. Development *130*, 401-410.

Dubois, L., Bally-Cuif, L., Crozatier, M., Moreau, J., Paquereau, L., and Vincent, A. (1998). XCoe2, a transcription factor of the Col/Olf-1/EBF family involved in the specification of primary neurons in Xenopus. Curr Biol *8*, 199-209.

Dyer, M. A., and Cepko, C. L. (2000). Control of Muller glial cell proliferation and activation following retinal injury. Nat Neurosci *3*, 873-880.

Hayashi, M. L., Choi, S. Y., Rao, B. S., Jung, H. Y., Lee, H. K., Zhang, D., Chattarji, S., Kirkwood, A., and Tonegawa, S. (2004). Altered cortical synaptic morphology and impaired memory consolidation in forebrain- specific dominant-negative PAK transgenic mice. Neuron *42*, 773-787.

Ince-Dunn, G., Hall, B. J., Hu, S. C., Ripley, B., Huganir, R. L., Olson, J. M., Tapscott, S. J., and Ghosh, A. (2006). Regulation of thalamocortical patterning and synaptic maturation by NeuroD2. Neuron *49*, 683-695.

Kanekar, S., Perron, M., Dorsky, R., Harris, W. A., Jan, L. Y., Jan, Y. N., and Vetter, M. L. (1997). Xath5 participates in a network of bHLH genes in the developing Xenopus retina. Neuron *19*, 981-994.

Kreis, P., and Barnier, J. V. (2009). PAK signalling in neuronal physiology. Cell Signal *21*, 384-393.

Levine, E. M., Close, J., Fero, M., Ostrovsky, A., and Reh, T. A. (2000). p27(Kip1) regulates cell cycle withdrawal of late multipotent progenitor cells in the mammalian retina. Dev Biol *219*, 299-314.

Liu, C., and Nathans, J. (2008). An essential role for frizzled 5 in mammalian ocular development. Development *135*, 3567-3576.

Logan, M. A. (2006) Mechanisms of neuronal differentiation in Xenopus Laevis, Ph.D, The University of Utah.

Moore, K. B., Schneider, M. L., and Vetter, M. L. (2002). Posttranslational mechanisms control the timing of bHLH function and regulate retinal cell fate. Neuron *34*, 183-195.

Ohnuma, S., Philpott, A., Wang, K., Holt, C. E., and Harris, W. A. (1999). p27Xic1, a Cdk inhibitor, promotes the determination of glial cells in Xenopus retina. Cell *99*, 499-510.

Perron, M., Opdecamp, K., Butler, K., Harris, W. A., and Bellefroid, E. J. (1999). X-ngnr-1 and Xath3 promote ectopic expression of sensory neuron markers in the neurula

ectoderm and have distinct inducing properties in the retina. Proc Natl Acad Sci U S A *96*, 14996-15001.

Powell, L. M., and Jarman, A. P. (2008). Context dependence of proneural bHLH proteins. Curr Opin Genet Dev *18*, 411-417.

Pozzoli, O., Bosetti, A., Croci, L., Consalez, G. G., and Vetter, M. L. (2001). Xebf3 is a regulator of neuronal differentiation during primary neurogenesis in Xenopus. Dev Biol *233*, 495-512.

Seo, S., Lim, J. W., Yellajoshyula, D., Chang, L. W., and Kroll, K. L. (2007). Neurogenin and NeuroD direct transcriptional targets and their regulatory enhancers. Embo J *26*, 5093-5108.

Souopgui, J., Solter, M., and Pieler, T. (2002). XPak3 promotes cell cycle withdrawal during primary neurogenesis in Xenopus laevis. Embo J *21*, 6429-6439.

Sugimori, M., Nagao, M., Bertrand, N., Parras, C. M., Guillemot, F., and Nakafuku, M. (2007). Combinatorial actions of patterning and HLH transcription factors in the spatiotemporal control of neurogenesis and gliogenesis in the developing spinal cord. Development *134*, 1617-1629.

Van Raay, T. J., Moore, K. B., Iordanova, I., Steele, M., Jamrich, M., Harris, W. A., and Vetter, M. L. (2005). Frizzled 5 signaling governs the neural potential of progenitors in the developing Xenopus retina. Neuron *46*, 23-36.

Zhang, J., Fuhrmann, S., and Vetter, M. L. (2008). A nonautonomous role for retinal frizzled-5 in regulating hyaloid vitreous vasculature development. Invest Ophthalmol Vis Sci *49*, 5561-5567.

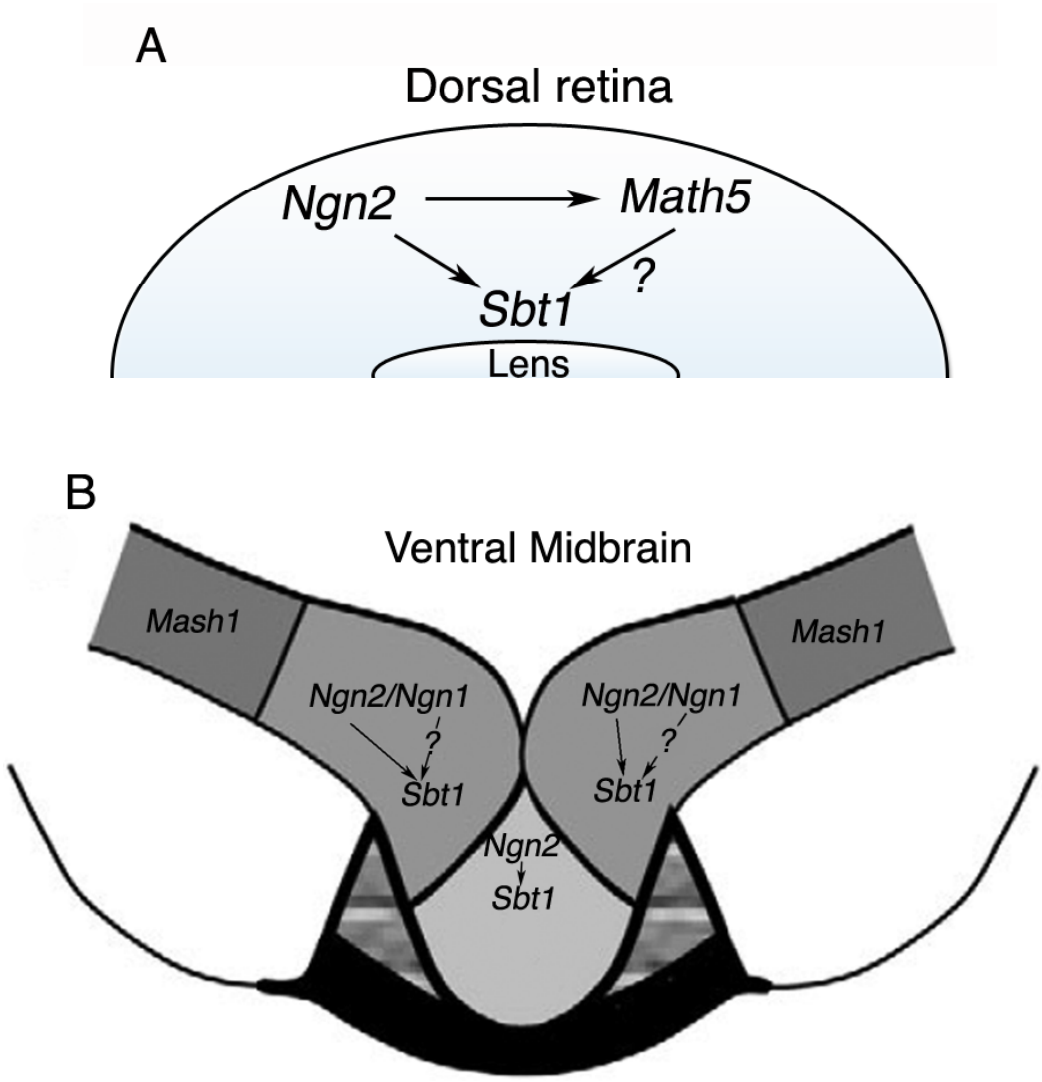
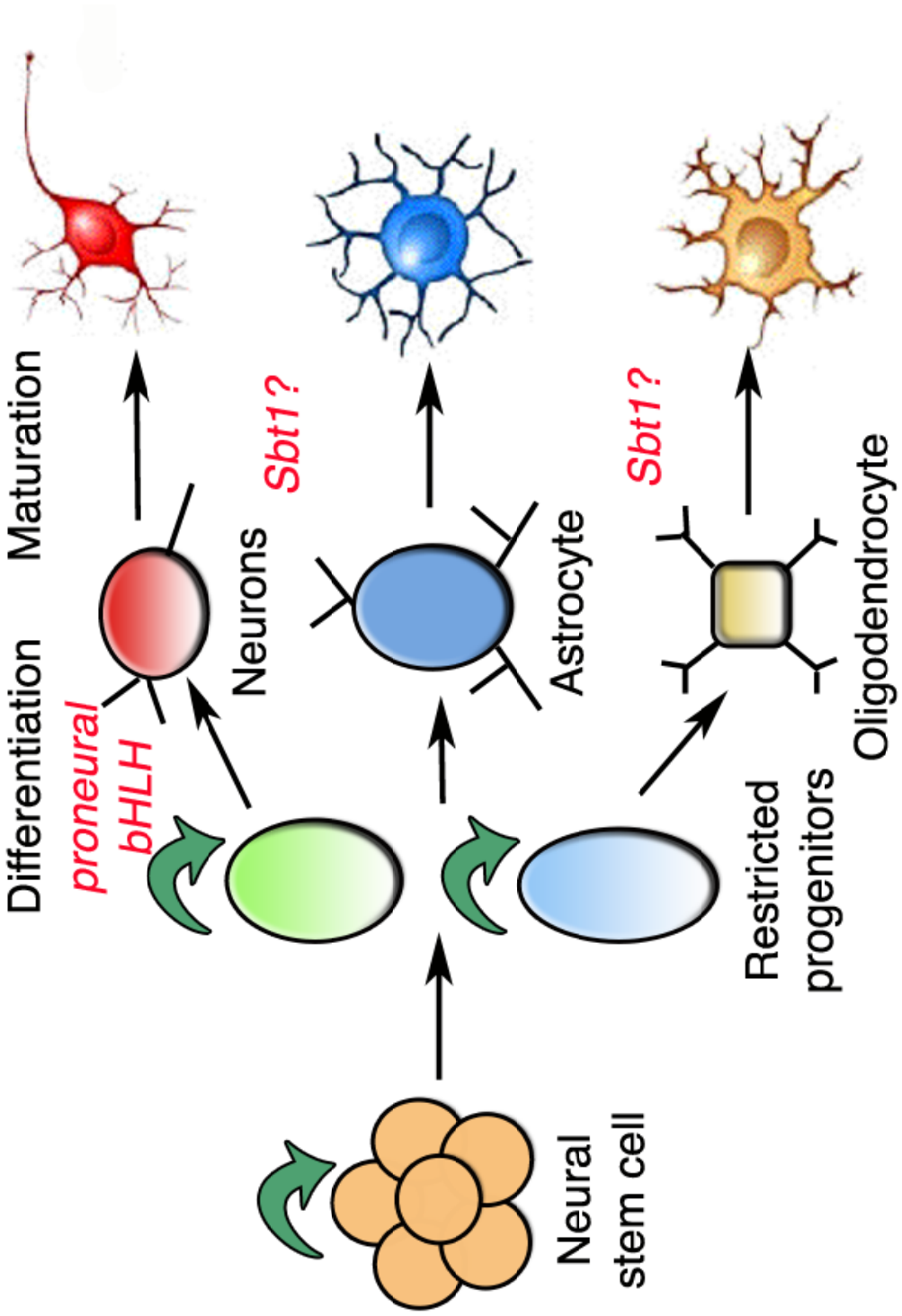


Figure 4.1 Model of bHLH factors regulate *Sbt1* expression.
(A) At E11.5 mouse retina, both *Ngn2* and *Math5* are expressed dorsally. And *Ngn2* specifically regulates *Sbt1* expression in this domain. (B) At E11.5 mouse ventral midbrain, proneural bHLH factors are expressed in different domains. *Ngn2* is the major factor that is expressed in the middle of ventral midbrain and regulates *Sbt1* expression (B: adapted and reprinted with permission from Kele 2006).

Figure 4.2 *Sbt1* may be not essential for early differentiation events, but may be involved in the late neurogenesis processes, such as neuronal morphology and synaptic formation.


Differentiation
Maturation
proneural
bHLH
Neurons
Sbt1?
Astrocyte
Sbt1?
Restricted
progenitors
Oligodendrocyte
Neural
stem cell