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Maintenance of Neural Progenitor Cells in the Spinal Cord by Promyelocytic Leukemia Zinc Finger and Repressor Protein 58: Forerunners of a New Family of Neural Developmental Regulators

A dissertation submitted in partial satisfaction of the Requirements for the Degree Doctor of Philosophy in Molecular Biology

by

Zachary Beyer Gaber

2013

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ABSTRACT OF THE DISSERTATION

Maintenance of Neural Progenitor Cells in the Spinal Cord by
Promyelocytic Leukemia Zinc Finger and Repressor Protein 58:
Forerunners of a New Family of Neural Developmental Regulators

By

Zachary Beyer Gaber
Doctor of Philosophy in Molecular Biology
University of California, Los Angeles, 2013
Professor Bennett G. Novitch, Chair

One of the key challenges of neural development is achieving the precise balance between progenitor self-renewal and differentiation. The production of early born neurons must be controlled such that the necessary numbers are generated without depleting the pool of progenitors for subsequent glial differentiation. This work reports the identification of two transcription factors, PLZF and RP58, as pivotal factors in the preservation of neural progenitor cells in the developing spinal cord through the period of neurogenesis. PLZF has been previously shown to promote the maintenance of both hematopoietic and male germ-line stem cells. This work will demonstrate that PLZF is expressed within a central domain of the developing spinal cord of chick and mouse embryos, that it is sufficient to oppose neuronal differentiation, and that its loss results in compromised progenitor maintenance and excessive neuron formation. Furthermore, it will be shown that PLZF functions in part by

promoting the expression of FGF receptor 3 and thereby enhancing neural progenitor receptivity for the pro-progenitor FGF signaling pathway. Unlike PLZF, previous research has indicated that RP58 acts to promote cellular differentiation in the context of neocortical, hippocampal, and cerebellar development. However, in the context of the developing spinal cord, RP58 acts as a potent anti-differentiation factor. RP58 misexpression results in a greatly expanded neural progenitor pool whereas its reduction through RNAi knockdown results in rapid neuronal differentiation. This work will propose that this divergent activity of RP58 in the two fundamental regions of the CNS can be explained as different manifestations of the same gene regulatory module achieving different effects depending on the relative order in which its component parts are activated. Both PLZF and RP58 are members of the BTB-ZnF family of transcription factors, a family previously with only a limited known role in the development of the CNS. This work will close with a consideration of the prospects of PLZF and RP58 not only being important factors in maintaining neural progenitor cells, but as potential forerunners of a previously unappreciated family of spinal developmental regulators.

The dissertation of Zachary Beyer Gaber is approved.

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2013

DEDICATION

To my parents,

without whom this work would not have been possible.

To the members of the Novitch Lab, past and present,

without whom this work would not have been successful.

And to the insensate forces of evolution that forged PLZF,

without whom this work would not have been necessary.

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LIST OF ABBREVIATIONS

Abbrev.	Definition	Description
AKT	Ak Strain,Thymoma	Family of serine/threonine kinases that transducer many signaling pathways
ASP	Astrocyte Precursor	See AST
AST	Astrocyte	CNS glial cell type involved in many aspects of neuronal support and physiology
bHLH	basic-Helix-Loop-Helix	DNA binding and protein dimerization domain
BCL6	B-Cell Lymphoma 6	Protein family possessing an N-terminal BTB-ZnF family transcription factor
BTB	Bric-à-Brac, Tramtrack, Broad Complex	Dimerization and co-factor recruitment domain
BTB-ZnF	see BTB and ZnF	Protein family possessing an N-terminal BTB domain and a C-terminal ZnF domain
ERK	Extracellular signal-Regulated Kinases	Subclass of MAPK family of serine/threonine kinases
FAZF	Fanconi anemia zinc finger	BTB-ZnF family transcription factor
FGF	Fibroblast Growth Factor	Family of secreted protein ligands
FGFR	Fibroblast Growth Factor Receptor	FGF associated receptor tyrosine kinase
HD	Homeodomain	DNA binding protein domain
HDAC	Histone Deacetylase	Chromatin remodelling protein, associated with transcription repression
HMG	High Mobility Group	DNA binding protein domain
MAPK	Mitogen-Activated Protein Kinases	Family of serine/threonine kinases that transduce many signaling pathways
NEUN	Neuronal Nuclei	Nuclear antigen, highly expressed by neurons
NEUROG	Neurogenin	Proneural bHLH transcription factor
NPC	Neural Progenitor Cell	Self-renewing, multi-potent cells that give rise to subset of cell types in CNS
NSC	Neural Stem Cell	Self-renewing, multi-potent cells that give rise to all cell types of CNS
OL	Oligodendrocyte	Myelinating cell type of the CNS
OLP	Oligodendrocyte Precursor	See OL
PI3K	Phosphoinositide 3-Kinase	Signaling kinase that targets membrane associated phosphatidyl-inositols

Abbrev.	Definition	Description
PLCγ	Phospholipase C Gamma	Signal transducer that cleaves phosphatidyl-inositol into second messengers
PLZF	Promyelocytic Leukemia Zinc Finger	BTB-ZnF family transcription factor
POU	PIT, OCT, UNC	HD containing transcription factor family
RP58	Repressor Protein 58	BTB-ZnF family transcription factor
simiRP58	Similar to Repressor Protein 58	BTB-ZnF family transcription factor
SOX	Sry-related HMG Box	HMG containing transcription factor family
SOX2	Sry-related HMG Box 2	SOXB1 transcription factor, highly expressed by NPCs
SOXB1	Sry-related HMG Box B1	Subclass of SOX family, associated with progenitor maintenance
SOXB2	Sry-related HMG Box B2	Subclass of SOX family, associated with neuronal differentiation
SOXC	Sry-related HMG Box C	Subclass of SOX family, associated with neuronal differentiation
SOXD	Sry-related HMG Box D	Subclass of SOX family, associated with suppression of gliogenesis
SOXE	Sry-related HMG Box E	Subclass of SOX family, associated with neural crest formation and gliogenesis
STAT	Signal Transducer and Activator of Transcription	Transcription factor downstream of multiple signaling pathways
ZBTB39	Zinc Finger and BTB Domain Containing 39	BTB-ZnF family transcription factor
ZBTB7A	Zinc Finger and BTB Domain Containing 7A	BTB-ZnF family transcription factor
ZnF	Zinc Finger	DNA binding protein motif

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CHAPTER 1 - INTRODUCTION

1-1 An overview of spinal development

The mature spinal cord is a tissue of immense complexity. It is the conduit of communication between the brain and the body, one of the principle means of sensory input to the brain and the primary means of instructive output from the brain (Goshgarian, 2003). In addition to this, the spinal cord is also the location of many circuits that function semi-autonomously from the brain, such as reflex circuits and the central pattern generators that control much of rhythmic motion (Garcia-Campmany et al., 2010). However, at the root of this ultimate complexity lies the comparative simplicity of the embryonic neural tube. The neural tube consists of undifferentiated, proliferative neural stem and neural progenitor cells (NSCs and NPCs respectively) that will ultimately give rise to the multitude of cell types that compose the mature spinal cord (Briscoe and Novitch, 2008; Kintner, 2002). Understanding how the neural tube becomes the spinal cord raises some of the most fundamental question in developmental neurobiology. How are NSCs and NPCs directed towards the formation of the appropriate cell types, in the required numbers, at the proper positions, and at the proper times? How do NSC and NPCs achieve the optimal balance between self-renewal and differentiation? And how are all these processes encompassed within a self-perpetuating, self-correcting, interrelated network of regulatory genes and signaling factors?

Much progress has been made in recent years in characterizing the fundamental events of spinal development and in identifying the key genetic regulators of these events. From a cellular differentiation perspective, spinal development may be broadly divided into three

phases: amplification, neurogenesis, and gliogenesis. During amplification, NSCs undergo symmetric divisions to expand the pool of undifferentiated cells (Shitamukai and Matsuzaki, 2012). This is also a period during which spinal progenitors undergo extensive patterning along both dorsal-ventral and rostro-caudal axes to produce positionally distinct domains. During neurogenesis, NPCs begin dividing asymmetrically and producing daughter cells that exit the cell cycle, migrate laterally, and differentiate into neurons. Each NPC domain gives rise to spatially distinct neuronal subtypes (Briscoe and Novitch, 2008; Kintner, 2002). Following upon neurogenesis, spinal NPCs cease differentiating neurons and instead begin generating glial precursors, again in a positionally distinct manner (Briscoe and Novitch, 2008; Rowitch and Kriegstein, 2010). To maintain a pool of undifferentiated, proliferative NPCs through both neurogenesis and gliogenesis and still produce differentiated cells in requisite numbers demands that self-renewal be tightly balanced with terminal differentiation. In addition, this balance must be finely calibrated for each NPC domain, the rate of cell differentiation being enhanced or attenuated according to the number of cells each domain must generate during different periods of development.

A multitude of genes have been identified that control the balance between progenitor self-renewal and terminal differentiation (Bertrand et al., 2002; Briscoe and Novitch, 2008; Cayuso and Marti, 2005; Kageyama et al., 2008). Collectively these genes form a vast interconnected regulatory network that inexorably drives the process of development forward from the undifferentiated simplicity of the neural tube to the precisely specialized complexity of the mature spinal cord. Although there are few generalizations that will adequately embrace such a vast network, it is accurate to say that a frequently observed feature is that related genes often function in related processes. Many of the key regulators of spinal development are members of gene families with multiple members participating in

the control of a process such as neuronal differentiation or the neurogenesis/gliogenesis switch. As will be discussed more fully in Chapter 4, this trend derives from the manner in which new genes arise and are integrated into gene regulatory networks. Briefly, new genes are typically created by the duplication of existing genes and as such are created with many aspects of their regulation and functionality intact. Over the course of evolutionary history the duplicates diverge, gaining and losing associations and functions. However, because they began their existence with the same activities and situated at the same point in the developmental network they typically remain participants in a common developmental process for prolonged periods of time (Nowick and Stubbs, 2010). As such, the identification of a family of transcription factors as having a previously unappreciated role in neural development is potentially of great consequence. Firstly, the identification of a new family of developmental regulators immediately suggests many previously unknown candidates for further analysis. Secondly, the characterization of the first discovered members of the family provides a useful contexts and baseline for conducting studies of other members.

In Chapter 2 of this work, we report our identification of multiple members of the BTB-ZnF family as regulators of spinal development. We discovered that the BTB-ZnF gene PLZF acts to maintain a central domain of neural progenitors by enhancing the expression of FGFR3 and as a result neural progenitor receptivity to the FGF signaling. In addition, we discovered that FGF signaling during neurogenesis was mediated through the activation of the STAT pathway, a significant departure from how FGF signaling had previously been thought to act. We propose that this PLZF-FGFR3-STAT3 pathway is of central importance to the fine control of the balance between progenitor maintenance and neuronal differentiation in the central NPC domains of the spinal cord.

In the third chapter of this work, we report our discovery that a second BTB-ZnF gene, RP58, also acts in spinal development to restrain neuronal differentiation. This finding suggests that the role of RP58 in the developing spinal cord is fundamentally different from its previously characterized activities in the developing brain where it has been shown to promote neuronal maturation. However, we propose that our findings and the existing body of literature indicate RP58 participates in a common genetic circuit in both regions of the developing CNS and that this circuit can be deployed to either promote or hinder neuronal differentiation depending upon the precise gene expression context in which it is placed.

Lastly, we propose that PLZF, RP58, and an additional four BTB-ZnF genes that we have found to be expressed in the developing spinal cord are forerunners of a previously unappreciated family of neural developmental regulators. Although some work has been conducted on individual members of the BTB-ZnF family in the context of the forming CNS, to our knowledge it has never been appreciated that these genes are not functional isolates but rather the first identified individuals of a family with many members potentially involved in neural development. We propose that future research into other BTB-ZnF family members will yield great fruits for our understanding of genetic regulation of progenitor maintenance, differentiation, and potentially many other aspects of the formation of the spinal cord and entire central nervous system.

1-2 Principal regulators of neural progenitor maintenance and differentiation

Even within the comparatively narrow parameters of a discussion of the regulation of neural progenitor maintenance and differentiation (excluding as this does the vast fields of patterning, symmetric and asymmetric divisions, cellular morphogenesis, migration, axon guidance, synapse formation and modulation, and circuit formation to list but a sample) we are still confronted by a wondrous diversity of gene families and signaling systems that are integrally involved in this process. The ultimate “decision” of a cell on whether to persist as a neural progenitor cell or undergo terminal differentiation is the result of integrating a wide range of inputs. Although each family and pathway are treated separately below, it will be repeatedly stressed how these pathways do not work in isolation but in concert with and in opposition to each other. As such, the ultimate determinant of whether a cell will change its fate or behavior lies not just in the individual regulators but also emerges from a network that embraces all inputs.

SOXB1 transcription factors establish and preserve neural progenitors cells

The SRY-related HMG Box (SOX) family of transcription factors contains approximately twenty members in most vertebrates and is divisible into eight subfamilies. The defining characteristic of the SOX family is the HMG (high mobility group) DNA binding domain related to the HMG domain of SRY protein and which recognizes the consensus sequence (A/T)(A/T)CAA(A/T)G (Kondoh and Kamachi, 2010). Although *in vitro*, SOX proteins promiscuously bind DNA segments containing an HMG binding site, *in vivo* SOX proteins are

highly discriminatory in their selection of binding sites and, consequently, genetic targets due to their differential affinities for cofactors. HMG binding sites are typically situated adjacent to targets for SOX binding partners, frequently members of the POU or homeodomain families of transcription factors. Therefore, the specificity of action observed among different SOX proteins or by a particular SOX protein in different contexts is in no small part dependent upon co-factor affinity and availability (Kondoh and Kamachi, 2010; Kondoh et al., 2004).

The SOXB1 subfamily been found to be of central importance to the establishment and preservation of NSCs and NPCs. This subfamily consists of three members: SOX1, SOX2, and SOX3. These three proteins are all transcriptional activators and are thought to be broadly functionally equivalent, differing principally in the timing and location of their expression with SOX2 generally being the most widely expressed. Supporting this view are the repeated findings that these genes produce similar effects when misexpressed and that the loss of any one of these genes individually tends to result in significant phenotypes primarily in contexts where other family members have lower expression (Miyagi et al., 2009). The differential expression of SOXB1 genes may also reflect a requirement for the precise regulation of gene dosage. It has been argued that subtle differences in the relation of HMG binding sites to that of their cofactor binding sites can greatly impact binding synergy and thereby require greater gene dosage for stable occupancy (Kondoh and Kamachi, 2010; Remenyi et al., 2003). This dosage requirement may also be influenced by the two members of the closely related SOXB2 subfamily, SOX14 and SOX21. Members of the SOXB2 subfamily are highly similar to the SOXB1 but act as transcriptional repressors instead of activators. It has been proposed members of these two subfamilies compete for occupancy on an overlapping set of genetic targets. As a result, shifts in the balance of expression levels between SOXB1 and SOXB2

subfamilies are thought to greatly influence the equilibrium between progenitor maintenance and neuronal differentiation (Sandberg et al., 2005).

In the initial stages of embryonic development, SOX2 has been found to both be highly expressed in and necessary for the maintenance of pluripotent embryonic stem cells (ESCs). Loss of SOX2 expression from the early embryo results in rapid loss of ESC identity (Avilion et al., 2003) whereas misexpression of SOX2 along with OCT3/4 and KLF4, has been found able found sufficient to reprogram post-mitotic cells into induced pluripotent stem cells (Nakagawa et al., 2008). SOX2 is thought to promote ESC identity by binding, in complex with OCT3/4, and activating several targets including NANOG, FGF4, and LEFTY1. In addition, the SOX2 – OCT3/4 complex has been found to induce their own expression, forming a stable feedback loop opposing differentiation (Masui et al., 2007).

Members of the SOXB1 subfamily are expressed starting from the earliest stages of vertebrate neural development within the proliferating, undifferentiated stem and progenitor cells of the neural plate and remain active within neurogenic regions of the CNS into adulthood (Pevny and Nicolis, 2010). *In vitro*, $SOX2^{+}$ cells have been found to exhibit the two critical hallmarks of stem cells: self-renewal and have the potential to differentiate into any of the three principal lineages of the CNS: neurons, oligodendrocytes, and astrocytes (Graham et al., 2003; Zappone et al., 2000). Studies drawing from multiple regions of the CNS and at many stages of development have consistently tied SOX2 and the other members of the SOXB1 family to the maintenance of NSCs and NPCs (Pevny and Nicolis, 2010). Within the developing spinal cord, SOXB1 proteins have been found to be expressed by the proliferating progenitors of the ventricular zone and down-regulated as cells undergo terminal differentiation. Over-expression of SOX2 and SOX3 in chick spinal cords has been found to be

sufficient to inhibit neuronal differentiation. Conversely, antagonizing SOXB1 activity by over-expressing obligate repressor forms of SOX2 and SOX3 has been found to induce premature neuronal differentiation (Bylund et al., 2003; Graham et al., 2003). Although the precise molecular mechanism of SOXB1 action has not been fully determined, evidence suggests that SOXB1 proteins function at least in part by antagonizing the activity, but not the expression, of proneural bHLH proteins such as ASCL1, NEUROG1, and NEUROG2 (Bylund et al., 2003).

bHLH transcription factors

Proneural bHLH proteins constitute a vital regulatory nexus in the control of neurogenesis. Many of the pathways that regulate neurogenesis exert their control by supporting or opposing the expression and action of proneural bHLH proteins. The vertebrate proneural bHLH transcription factor family is broadly divisible into two classes named for their homology to Drosophila proneural bHLH proteins: the Achaete-Scute Homologous subfamily (e.g. ASCL1/MASH1) and the Atonal Homologous subfamily which can be further divided into the ATOH (e.g. ATOH1/MATH1), NEUROD, and NEUROG (e.g. NGN2/NEUROG2) groups. All bHLH proteins, of which the proneural bHLH proteins are a subset, are defined by their possession of two closely associated domains: a **b**asic DNA binding domain and a **H**elix-**L**oop-**H**elix dimerization domain. Proneural bHLH proteins typically form heterodimers with members of the E-protein family of bHLH proteins (E12 and E47). The proneural bHLH – E protein heterodimer then binds to a recognition sequence termed an E-box (CANNTG) and transactivates the expression of nearby genes (Powell and Jarman, 2008).

Proneural bHLH proteins are among the most critical factors for the promotion of neuronal terminal differentiation, orchestrating both the acquisition of pan-neuronal properties and many sub-type specific traits. Gain and loss of function studies in multiple contexts and in multiple model organisms have consistently found members of this family to be necessary and sufficient for directing neuronal differentiation and cell cycle exit (Powell and Jarman, 2008). Because of the typically overlapping nature of the expression of proneural bHLH proteins, the loss of any one may only result in a fate conversion between the neuron subtype directed by one protein to that directed by another. However, when multiple bHLH proteins are knocked out, both increased progenitor proliferation and increased glial differentiation have been observed, demonstrating the centrality of proneural bHLH proteins for promoting neurogenesis (Nieto et al., 2001; Tomita et al., 2000).

Many aspects of proneural bHLH activity appear to be mediated through modulation of multiple subfamilies of SOX proteins. In particular, proneural bHLH negatively regulate the pro-progenitor SOXB1 proteins. Within the developing spinal cord, ectopic NEUROG2 has been found to be sufficient to repress the SOXB1 protein SOX3 (Bylund et al., 2003). In addition, NEUROG2 has also been found to induce SOX21, a member of the SOXB2 family that competitively inhibit SOXB1 activity (Sandberg et al., 2005). Because, as discussed previously, SOXB1 proteins have also been found to oppose the action of proneural bHLH proteins, competition between these two protein families represents a central pivot in the balance between progenitor maintenance and neuronal differentiation. Conversely, proneural bHLH mediated activation of members of the SOXC subfamily appears to be important for promoting neuronal differentiation. NEUROG2 has been sufficient to activate the SOXC proteins SOX4 and SOX11 whose expression in turn has been found to be capable of directing neuronal differentiation. However, SOXC proteins have been shown to induce neuronal

differentiation without activating proneural bHLH proteins. In addition, NEUROG2 is unable to induce neuronal differentiation in the context of spinal cords where SOX4 and SOX11 activation is blocked with siRNA. Both of these observations strongly suggest that, at least in the context of the developing spinal cord, SOXC proteins are crucial downstream mediators of proneural bHLH activity (Bergsland et al., 2006).

One of the most striking aspects of the proneural bHLH family is specificity of neuronal induction observed among members despite the seeming simplicity of their common binding site, the E-box. Within the developing spinal cord, misexpression of ASCL1 directs the differentiation of $ISL1^+$ dI3 interneurons, whereas misexpression of ATOH1 promotes the formation of $LHX2/9^+$ dI1 interneurons, and the misexpression of NEUROG2 appears to promote increased formation of region appropriate cell types (Mizuguchi et al., 2001; Nakada et al., 2004). In addition to this specificity among different proneural bHLH proteins, specificity exists for each particular proneural bHLH protein, allowing it to direct the differentiation of distinct classes of neurons in distinct developmental contexts. One notable instance of this is ASCL1, which is associated with the formation of forebrain GABAergic neurons, midbrain dopaminergic neurons, dorsal spinal interneurons, and neural crest derived autonomic neurons (Powell and Jarman, 2008). As with SOXB1 proteins this striking specificity among transcription factors with seemingly identical binding sites is thought to derive from preferential binding of cofactors. For example, proneural bHLH protein induction of several genes has been found to be dependent upon synergism with the POU proteins BRN1 and BRN2 (Castro et al., 2006) or upon the formation of a complex with the HD proteins ISL1 and LHX3 (Lee and Pfaff, 2003). Similarly, NEUROG2 acts principally as a permissive factor when misexpressed in isolation in the spinal cord, inducing region appropriate neuronal differentiation, when co-transfected with the motor neuron specifying factor OLIG2,

NEUROG2 directs primarily motor neuronal differentiation, supporting the view that the neuronal cell type promoted by proneural bHLH proteins is dependent upon genetic context (Mizuguchi et al., 2001).

The four ID family proteins (ID1, ID2, ID3, and ID4) are important negative regulator of the proneural bHLH family. IDs have been found to be expressed in large, overlapping but non-identical regions of the ventricular zone during neurogenesis (Jen et al., 1997). The deletion of ID1 and ID3 has been found to result in premature neuronal differentiation and cell-cycle exit (Lyden et al., 1999) while misexpression of IDs is sufficient to block the differentiation of neurons (Norton, 2000). The ID proteins are atypical members of the bHLH family, lacking as they do the basic DNA binding domain. Deficient for a DNA binding domain, ID proteins are instead thought to function principally through their binding and sequestration of the E-proteins required by proneural bHLH proteins for their transactivation of pro-neuronal differentiation, pro-cell cycle exit targets (Norton, 2000; O'Toole et al., 2003). However, the full scope of ID's ability to block differentiation may also involve interaction other proteins, particularly the cell cycle inhibitor RB and members of the ETS and PAX families (Roberts et al., 2001; Yates et al., 1999). Lastly, ID proteins have also been linked to the repression of HES1 auto-inhibition, thereby linking this inhibitor of proneural bHLH proteins with Notch signaling, one of the most important anti-differentiation signaling pathways in the developing CNS (Bai et al., 2007).

The HES family and NOTCH signaling

The HES family of bHLH transcription factors is a critical regulator of proneural bHLH proteins and vital for the maintenance of neural stem and progenitor cells. Three HES

proteins are particularly broadly expressed in the developing CNS: HES1, HES3, and HES5. HES1 and HES3 are expressed throughout the early developing neural plate, although as development persists and the neuroepithelium matures into radial glia, complimentary domains of HES1 and HES5 expression become established throughout most of the ventricular zone (Hatakeyama et al., 2004). Deletion of individual HES genes in the mouse CNS results in significant up-regulation of proneural bHLH proteins and an increase in neuronal differentiation, although this phenotype is thought to moderated by compensatory increases in the expression of other HES genes (Ishibashi et al., 1995; Ohtsuka et al., 1999). The loss of HES1, HES3, and HES5 together results in the rapid and near complete differentiation of NPCs into neurons (Hatakeyama et al., 2004). Conversely, electroporation of either HES1 or HES5 into the developing telencephalon has been shown to be sufficient to promote NPC maintenance and to inhibit proneural bHLH genes (Ohtsuka et al., 2001). These observations and others have identified the HES family as among the most important preservers of NPCs against neuronal differentiation.

There are believed to be two principal modes of HES protein activity. Firstly, HES protein homodimers are thought to bind and repress the transcription of proneural proteins such as ASCL1 (Chen et al., 1997). The basic domain of HES proteins has been found to preferentially bind N boxes (CACNAG) and class C sites (CACG[A/C]G) (Kageyama et al., 2008). Repression by HES proteins is thought to be at least partially dependent on its ability to recruit members of the GROUCHO family of transcriptional co-repressors via a conserved WRPW motif near their C-termini (Paroush et al., 1994). The second mode of HES protein activity is thought to be somewhat analogous to that of members of the ID family, namely the sequestration of proneural bHLH proteins or E proteins away from DNA (Sasai et al., 1992).

NOTCH signaling is arguably the most important positive regulator of HES gene function. Although there is much potential for regulation of these transcription factors by other pathways, HES1 and HES3 in particular are known to be induced in the neural plate prior to the initiation of NOTCH signaling (Hatakeyama et al., 2004), in many developmental contexts HES gene induction is associated with the activation of the NOTCH pathway. Briefly, the NOTCH proteins are single-pass, transmembrane receptors that bind membrane associated ligands from the DELTA and JAGGED families. Upon ligand binding, the NOTCH intracellular domain (NICD) is cleaved, translocates to the nucleus, and associates with the bifunctional transcription regulator RBP-J and facilitates the recruitment of co-activators to RBP-J targets, particularly members of the HES family (Borggrefe and Oswald, 2009). Among the evidence demonstrating the centrality of HES genes to NOTCH signaling, it has been found that ectopic NICD is unable to block neuronal differentiation in cell lines where HES1 and HES5 have been deleted (Ohtsuka et al., 1999). Significantly, given that HES proteins are thought to function primarily through their inhibition and repression of proneural bHLH and E proteins, it has been found that misexpressing NEUROG2 and E47 are sufficient to rescue the neuronal differentiation block caused by ectopic NICD (Holmberg et al., 2008). Thus, although NOTCH signaling may have other targets than HES genes, it appears in the context of neurogenesis, HES mediated repression of proneural bHLH proteins is the principal mechanism of NOTCH signaling's pro-progenitor activity.

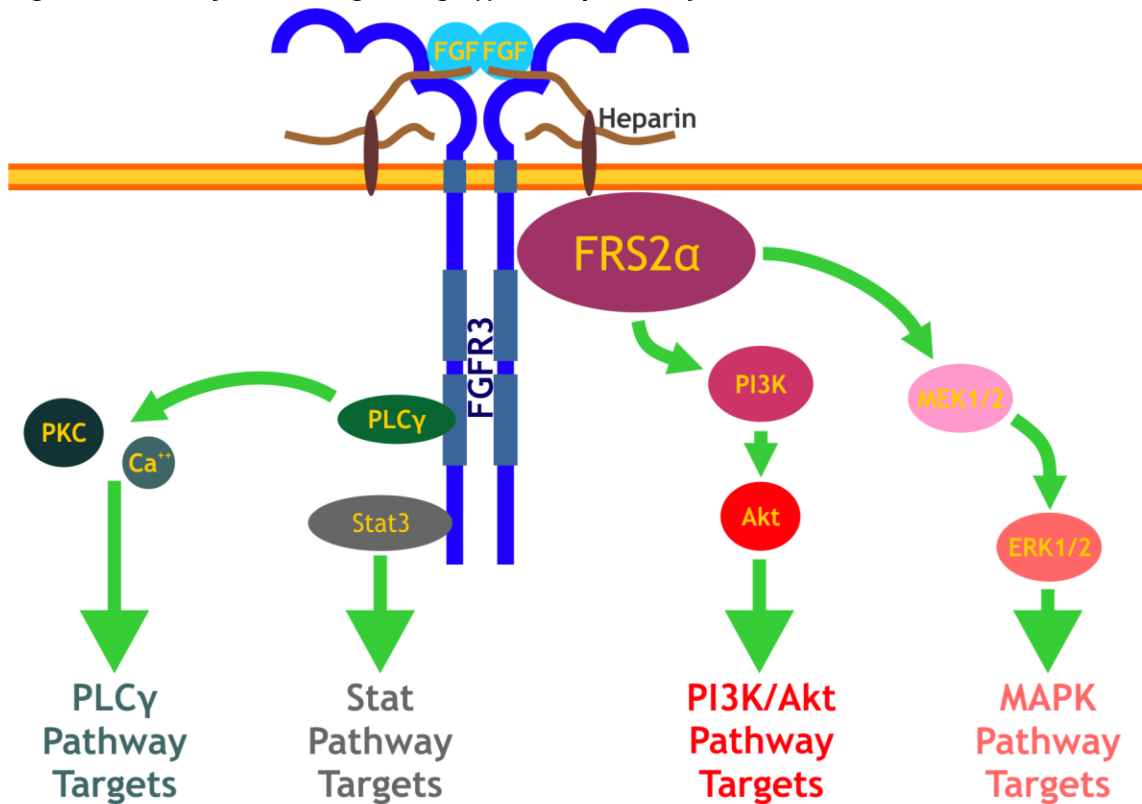
NOTCH signaling and cross-repression between HES proteins and proneural bHLH proteins has long been associated with the phenomenon of lateral-inhibition wherein the differentiation of one cell reduces the likelihood of the differentiation of neighboring cells. Proneural bHLH proteins have been found to induce increased expression of NOTCH ligand, which results in elevated NOTCH signaling in neighboring cells and subsequently elevated HES

expression and reduced proneural bHLH protein expression. Thus, the differentiation of one cell signals back to its neighbors to inhibit their differentiation and thereby prevent premature depletion of the progenitor pool. However, work in recent years has shown this to be a far more dynamic process than originally conceived (Kageyama et al., 2009). This has been best demonstrated in the context of HES1. The motor for this dynamism is HES1's ability to inhibit its own expression and the rapid degradation of HES1 transcript and protein (Hirata et al., 2002). NPCs receiving NOTCH signaling from neighboring cells elevate HES1 expression and thereby repress the expression of proneural bHLH proteins such as NEUROG1 and are as a result kept undifferentiated. However, because HES1 auto-inhibits and its gene products are rapidly degraded, HES1 expression cannot be maintained and proneural bHLH expression will rise until such time as HES1 levels are sufficiently low that it is no longer able to repress itself. At this time, HES1 expression will rise again and bHLH expression will fall. As a result, cells oscillate between a HES1 high state and a NEUROG1 high state. Further supporting this oscillation is the regulation of NOTCH ligands by proneural bHLH proteins like NEUROG1. When HES proteins silence NEUROG1, NOTCH ligand expression also falls, reducing the ability of NOTCH signaling to induce HES in neighboring cells and providing another means of promoting the dynamic oscillation of HES and proneural bHLH proteins (Kageyama et al., 2009; Shimojo et al., 2008).

This discovery of an oscilliation between high HES^{HIGH}/proneural $bHLH^{LOW}$ and HES^{LOW}/proneural $bHLH^{HIGH}$ phases grants a new perspective on how the balance between progenitor maintenance and neuronal differentiation is achieved. Developmental regulators might not always, or even often, be expressed at static levels but rather may be in continuous flux. As such alterations in cellular behavior may not require that a factor overcome what might be called an enormous "genetic inertia" wherein, for example, a pro-differentiation

factor would have to elevate its own expression and that of its targets in the face of continuous opposition from high levels of pro-progenitor factors. Rather, factors may instead function by altering the equilibrium of oscillatory networks such as HES/proneural bHLH. Thereby, a small amount of a newly-introduced factor, by favoring the activation of targets already present at one peak of the oscillation, might be enough to subtly shift the balance and direct a cell towards cell cycle exit or conversely towards renewed proliferation.

Figure 1-1 - Major FGF signaling effector pathways



1-3 FGF signaling: many pathways to many ends

Fibroblast growth factor (FGF) signaling constitutes one of the most critical and wide-ranging regulators of neural development, its influence extending into every stage from the broad regionalization of the CNS to the establishment of proper synapses (Guillemot and Zimmer, 2011). However, as progress is made in understanding the nature of FGF activity throughout this broad range of activities, it has become increasingly apparent that FGF signaling appears to be is achieving such divergent effects through seemingly similar if not identical signaling events. The following discussion on the nature of FGF signaling will focus on how FGF receptors mediate differing responses, particularly in the context of maintaining progenitor cells.

At the heart of FGF signaling are the 18 secreted[1] FGF ligands and the four FGF receptors (FGFRs)[2]. FGF pathway activation is initiated by the formation of a complex containing two FGFs, two FGFRs, and two molecules of the heparan sulfate glycosaminoglycan, a vital membrane-associated cofactor that stabilizes both ligand-receptor and receptor-receptor interactions. All FGF proteins have at their core a highly conserved trefoil arrangement of β-sheets with more flexible, variable regions at each terminus. It is the differential affinities of these variable regions for the extracellular domain of FGFR proteins that is the primary source of the specificity of FGF-ligand receptor interactions. In particular, there are extensive contacts between FGF ligand variable regions with the immunoglobulin-like motifs in the extracellular domain of FGFR receptors (Mohammadi et al., 2005). As such, differential splicing of the third immunoglobulin like motif (IgIII) between IgIIIb and IgIIIc isoforms is a crucial means of regulating receptor-ligand affinity (Zhang et al., 2006) (Mohammadi et al., 2005).

FGFR proteins are single-pass transmembrane receptor tyrosine kinases that are generally considered to function as homodimers, although some evidence does exist that FGFRs might be able to heterodimerize in some circumstances (Bellot et al., 1991; Ueno et al., 1992). However, the evidence for heterodimerization rests principally on studies conducted using over-expressed and often mutant proteins and may represent interactions that are not feasible at endogenous expression levels. Upon ligand-mediated dimerization, the kinase domain become active and autophosphorylate multiple conserved tyrosine residues

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[1] It is often reported in the literature that there are 22 FGF ligands. However, FGFs 11-14 have been found to neither be secreted nor capable of inducing FGFRs when artificially placed in the extracellular environment. They instead mediate their effects through intracellular interactions. For this reason, it has been proposed that FGF11-14 be reclassified as an evolutionarily related by distinct family of FGF Homologous Factors (FHFs), although this terminology is not yet universally embraced (Olsen et al., 2003).
[2] A fifth FGFR has been identified, variously identified as either FGFR5 or FGFRL1. Although it is localized to the cell membrane and has been found to bind FGF ligands and heparan, FGFR5 lacks an intracellular kinase domain and has been proposed to act instead as a decoy receptor to attenuate FGF signaling (Trueb et al., 2003).

on FGFR's intracellular domain. Some of these phosphorylations serve to enhance the kinase activity of the receptor. Other phosphorylated tyrosine constitute docking sites for signaling proteins, often possessing SH2 or PTB domains, and thereby permit the rapid formation of large signaling complexes on the receptor. Many of elements of these complexes are also subject to FGFR phosphorylation (Hart et al., 2001; Mohammadi et al., 1996).

The signaling cascades downstream of the activated FGFR has been best characterized for FGFR1, although it is believed to be similar for all FGFRs. Firstly, Phospholipase C γ (PLCγ), directly binds pY766 in FGFR1 and upon activation catalyzes the cleavage of phosphatidyl-inositol-4,5-bisphosphate within the cell membrane into inositol triphosphate (IP_3) and diacylglycerol (DAG). IP_3 and DAG are second messengers that promote calcium release from the endoplasmic reticulum and the activation of protein kinase C respectively (Mohammadi et al., 1991). Several of the other pathways downstream of FGFRs are indirect and involve the FRS2α/β adaptor proteins. FRS2α/β are membrane associated, myristylated proteins that constitutively bind FGFRs. Upon activation of the FGFR kinase domain, FRS2α/β become phosphorylated on multiple sites that permit the recruitment of GRB2, SHP2, and SOS proteins that in turn facilitate the recruitment and activation of RAS, which in turn ultimately culminating in the induction of MAP kinases (MAPK), the proteins for whom this pathway is generally named (Kouhara et al., 1997). The GRB2 protein is also capable of recruiting another signaling protein, GAB1, which mediates the activation of PI3 and AKT kinases (Ong et al., 2001). SRC kinase has also been found to be activated by FGFR in an FRS2α/β dependent manner. Lastly, FGF receptors have been found capable of inducing STAT signaling, although the mechanism is incompletely understood. STAT3 has been found able of directly binding to pY677 of FGFR1, a site conserved in all FGFRs, although it has been argued that the actual phosphorylation and activation of STAT3 is not direct but is instead catalyzed by an FGFR

associated Src and JAK2 kinases (Dudka et al., 2010). Upon phosphorylation, STAT proteins are thought to dimerize and translocate into the nucleus where they are typically associated with the activation of proliferation promoting genes such as C-MYC, CYCLIN D1, and $p21^{WAF/CIP1}$ (Brantley and Benveniste, 2008).

Despite the great existing knowledge of the events immediately downstream of FGFR activation, how these downstream pathways directly impact cellular behavior remains unclear. To date, FGF induced PI3K/AKT signaling has not been significantly linked to CNS development and the activation of the PLCγ pathway by FGFs has been primarily associated with neurite outgrowth (Guillemot and Zimmer, 2011). Similarly, the activation of STATs by FGFs has principally been studied in the context of gliogenesis. However, far too little research has been done in these areas to rule any of these pathways out from making significant contributions to FGF signaling in any area of CNS development. Most research, in the CNS and throughout development, has focused on characterizing FGF mediated activation of the MAPK/ERK pathway. This pathway has been strongly associated with FGF's mitogenic activity (Guillemot and Zimmer, 2011). In MAPK/ERK the pathway, the activation of a RAS family GTPase by FGFR begins a complex, multi-tier kinase cascade, the kinases within each tier potentially activating multiple kinases in the subsequent tier. The ERK cascade, activated by FGFs and several other growth factors, takes its name from in the activation of the Ser/Thr kinases ERK1 and ERK2. However, these kinases, which are three kinase tiers removed from the FGFR, are but two of many proteins phosphorylated by this pathway and themselves may potentially phosphorylate hundreds of additional targets within the cytoplasm and nucleus, many of which are themselves kinases (Rubinfeld and Seger, 2005). Given this enormous multiplication of effectors, it is extremely difficult to establish the direct molecular links between the activation of the MAPK/ERK pathway with the cellular

effects it induces or to explain how MAPK/ERK can mediate so many effects in so many different contexts.

Given the multitudinous and multifarious contexts to which FGF signaling has been linked, one of the major questions in the field is how is specificity of action achieved? How are the downstream consequences of activated FGF signaling regulated such that only the contextually appropriate effects are manifested? Although some progress has been made in recent years in this area, the matter remains largely unresolved. Here follows a brief discussion of current evidence for how context-appropriate responsiveness to FGF signaling is achieved, focusing on four primary areas: specificity of ligands, specificity of receptors, and contextual specificity.

Specificity of FGF ligands

As previously mentioned, there are 18 FGF and four FGFRs. Due to structural differences in the variable region of each ligand, they are only able to effectively bind particular receptors, with the exception of FGF1 (also known as acid or aFGF) which is able to bind any FGFR. Further enhancing this specificity is the differential splicing of FGFR1, FGFR2, and FGFR3 between b and c isoforms which have distinct ligand affinities (Zhang et al., 2006). This specificity is of immense importance for whether or not a cell is able to respond to an FGF ligand: activation of FGF signaling can only occur in regions where both the appropriate ligand and the appropriate receptor are present.

It is generally thought that all ligands induce comparable downstream events in direct proportion to their affinity for a given FGFR. Furthermore, it has a been proposed that the

binding of certain FGF ligands may induce subtly different spatial arrangements between the kinase domains of the FGFR dimers, potentially resulting in variable degrees of receptor activation (Olsen et al., 2006). However, there is some evidence that suggests different FGF ligands may induce significantly different developmental programs, such as in the developing telencephalon where FGF8 promotes progenitor maintenance and FGF15 promotes neuronal differentiation. Intriguingly, FGF8 and FGF15 appear to induce different signaling pathways in mouse cortex primary culture, with FGF8 promoting sustained phosphorylation and activation of ERK and AKT whereas FGF15 induces only transient ERK phosphorylation and fails to activate AKT (Borello et al., 2008). However, it is unclear at which level these two ligands are manifesting different effects as it has yet been demonstrated whether they are binding the same or different receptors.

Specificity of FGF receptors

The four FGFRs are thought to differ principally in their differential ligand affinities, discussed above, and are believed to activate the same pathways when bound by FGF ligands (Guillemot and Zimmer, 2011). However, there is some evidence that the receptors do not all activate each pathway to the same degree, although interpretations are somewhat complicated by the frequent use of mutant and chimeric proteins by investigators seeking to isolate specific FGFR signaling activities from their differential affinities for the ligands being used to induce them. Data suggest that among the FGFRs, FGFR1 possesses the most active kinase activity, typically phosphorylating targets in all known downstream pathways to a greater extent than the other receptors. There is some evidence that FGFR3 is comparatively poor at inducing the phosphorylation of PLCγ, FGFR4 relatively less able to phosphorylate FRS2, although the *in vivo* significances of these distinctions is unclear (Hart et al., 2000;

Raffioni et al., 1999). A possible example of different FGFRs promoting different developmental programs occurs in the developing telencephalon where it has been proposed that FGFR1 and FGFR3 promote the self-renewal of NSCs and the up-regulation of FGFR2 is associated with a shift towards maturation into a more restricted progenitor state capable of forming primarily radial glia and astrocytes in culture (Maric et al., 2007).

Specificity of cellular context

Although there are some tantalizing signs that different FGFs and FGFRs have different developmental effects, it is probably accurate to say that the key factor in regulating the precise activity induced by FGFs is cellular context. For example, FGFR3 activation has been found to restrain the proliferation of chondrocytes within developing bone. Deletion of FGFR3 results in significant increases in the length of long bones in mice and constitutively activating mutations in FGFR3 are the leading cause of achondroplasia, thanatophoric dysplasia I and II, and SADDAN[3], three major forms of human dwarfism (Foldynova-Trantirkova et al., 2012). However, in the context of the bladder epithelium and in hematopoietic progenitors, FGFR3 has been found to promote proliferation. Indeed, the very same FGFR3 mutations associated with dwarfism in bone development are also often associated with oncogenic transformation in urothelial cell carcinoma (Knowles and Goebell, 2010). The importance of context has also been demonstrated for the activity of FGFR3 in neural development. Activation of FGFR3 within neuroepithelial cells expressing the HD protein IRX3 has been shown to induce midbrain identity whereas activation in cells expressiong the HD protein SIX3 promotes the expression of forebrain markers (Kobayashi et al., 2002).

[3] Severe Achondroplasia with Developmental Delay and Acanthosis Nigricans.

The manner by which FGF signaling is altered by cellular context is unclear and is likely to ultimately emerge many of the aforementioned distinctions. The two fundamental, but by no means mutually exclusive, possibilities are either that FGF signaling is promoting the action of different downstream pathways in different contexts or that the significance of activating FGF's downstream pathways differs from context to context. There is some evidence for both possibilities. Supporting the possibility that FGFs activate different pathways in different contexts, FGF signaling has been found to activate the STAT pathway when promoting chondrocyte arrest but not when promoting urothelial cells proliferation and transformation. Supporting the possibility that FGF's induced pathways have different significances in different contexts, it has been shown that FGF signaling activates MAPK/ERK both in the inhibition of proliferation in chondrocytes and with FGF-associated transformation in urothelial cell carcinoma (Foldynova-Trantirkova et al., 2012; Knowles and Goebell, 2010). However, it is currently impossible to make any definitive conclusions in this area, in part because in many contexts only one of the potential FGF-induced pathways has been characterized. A more full understanding of how FGF signaling functions will likely require more detailed analysis of downstream pathways and they differ among cell types. For example, the proteins available for phosphorylation by one of the many kinases downstream of FGFRs may vary radically cell type to cell type. In addition, the final activity of many of these proteins many not be solely dictated by FGF-induced events but many be equally dependent upon modifications induced by other pathways. Similarly, the set genes activated by FGF-induced transcription factors, such as the STAT proteins, is likely to depend greatly on the availability of co-factors and opposing repressors, an availability potentially regulated by non-FGF pathways.

FGF signaling and NPC maintenance

Among the most important roles of FGF signaling in the developing CNS is its role in NPC maintenance. FGFs has been found to be vital for promoting the initial specification of neural progenitors, both as an instructive cue in themselves (Delaune et al., 2005) and through their multilayered inhibition of the BMP signal that would convert the future neuroectoderm into epidermis (Londin et al., 2005; Pera et al., 2003). After neural induction, FGF signaling promotes progenitor proliferation. Mice possessing the K644E mutation in FGFR3, which renders it constitutively active, exhibit significantly increased proliferation and reduced apoptosis in the telencephalic ventricular zone, phenotypes that ultimately result in a greatly expanded ventricular zone (Inglis-Broadgate et al., 2005). Activated FGFR receptors have been linked to the symmetric division of NSCs (Maric et al., 2007) and to promoting NPC survival (Paek et al., 2009). FGF signaling has been associated with inducing the expression of SOXB1 family proteins (Stavridis et al., 2007) and with inhibiting the expression of proneural bHLH proteins and neuronal differentiation (Diez del Corral et al., 2002). In addition, loss of FGFR1, FGFR2, and FGFR3 from the developing telencephalon results in premature maturation of neuroepithelial cells into radial glia, suggesting an endogenous role for FGF signaling in inhibiting progenitor maturation (Kang et al., 2009).

As all these findings suggest, FGF signaling constitutes a potent block on neural development that while advantageous for maintaining proliferating progenitors must ultimately be overcome in order to permit neurogenesis. Within the spinal cord, this is achieved by the secretion of retinoic acids from the somitic mesoderm, which functions in part through the repression of FGF8 expression in the neural tube and surrounding mesenchyme. Conversely, FGF signaling has been found able to inhibit the expression of

RALDH2, an enzyme vital for the synthesis of retinoic acid, suggesting that the rate of neuronal differentiation is regulated through a balancing of FGF and retinoic acid inputs (Diez del Corral et al., 2003).

Thus, FGF signaling appears to promote many aspects of neural stem and progenitor cell maintenance. In many cases, it is currently unknown through which effector pathways FGF signaling is acting, although when this matter has been investigated, the MAPK/ERK pathway has frequently been implicated. FGF inhibition of BMP signaling during neuralization has been linked to MAPK/ERK phosphorylation of multiple sites on the SMAD1 protein (Pera et al., 2003). Furthermore, the ability of FGF5 to induce SOXB1 proteins in mouse embryonic stems cells can be blocked by overexpressing MKP3, a phosphatase that dephosphorylates targets of ERK1/2 signaling (Stavridis et al., 2007). Furthermore, during the early stages of development, from the formation of the neural plate to neural tube closure, the presence of phosphorylated ERK1/2 and other pathway elements within the developing CNS tracks closely with the presence of FGF ligands and receptors (Lunn et al., 2007). MAPK/ERK signaling is clearly important for many aspects of FGF signaling in the developing CNS, although the lack of evidence for the involvement of other pathways is partially attributable to the lack of experiments performed to detect the presence of other pathways.

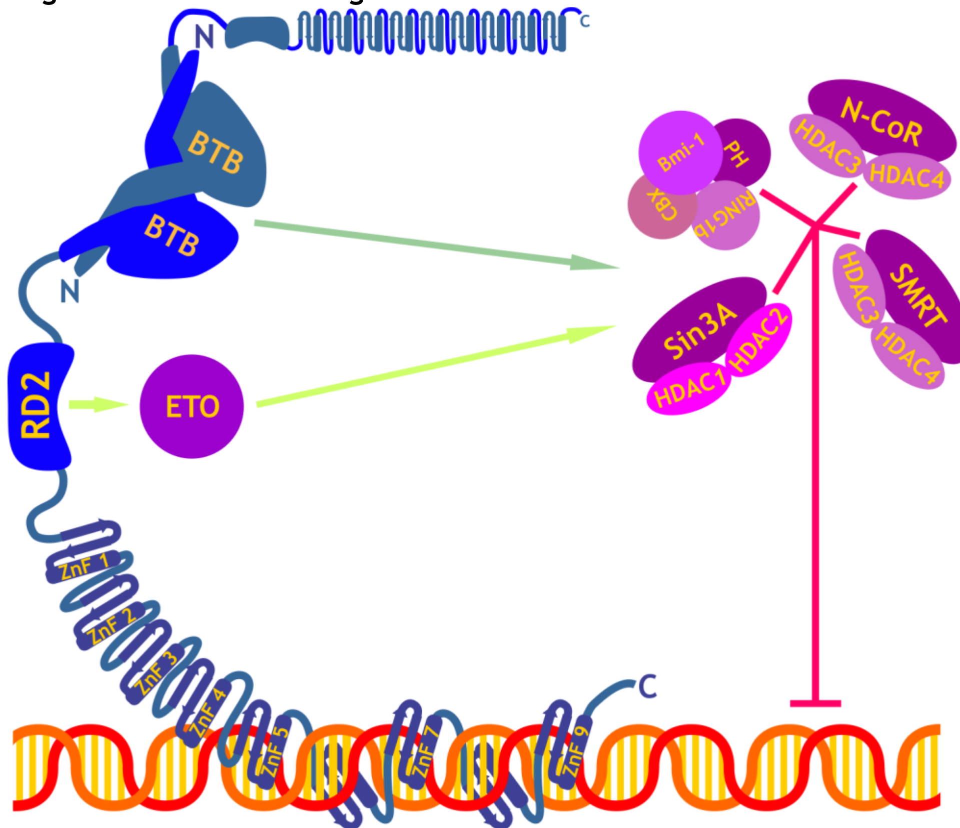
Summary

The proper balance between progenitor maintenance and differentiation during neural development is the end product of a vast interconnected genetic and signaling network. SOXB1, HES, and ID family promote progenitor maintenance and receive supporting inputs from both NOTCH and FGF signaling. Conversely, members of the proneural bHLH, SOXB2,

and SOXC families promote neuronal differentiation and receive negative inputs from NOTCH and FGF signaling and positive inputs from signals such as retinoic acid. As is clear from this summary, more is known about how extrinsic signals such as NOTCH and FGF induce changes in the expression of intrinsic factors than is known about how intrinsic factors regulate receptivity to extrinsic factors. In the following section, the transcription factor PLZF will be introduced. We have found it to be a critical participant in this regulatory network, inhibiting the up-regulation of proneural bHLH proteins and promoting the maintenance of SOXB1 expressing NPCs. Furthermore, we have found that PLZF promotes this pro-progenitor activity by means of promoting the expression of FGFR3, thereby providing a novel example of a mechanism by which an intrinsic factor may promote changes in cellular behavior by means of enhancing sensitivity to an extrinsic signal.

Figure 1-2 - The interrelation of principal regulators of neural progenitor maintenance during neurogenesis

Figure 1-3 – PLZF binding to DNA



1-4 The structure and activity of the PLZF transcription factor

The *Zbtb16* gene, hereafter referred to as PLZF[4], is a member of the BTB-ZnF[5] family of transcription factors, members of which have long been known to be vital regulators of many developmental processes. This family is defined by the possession of an N-terminal BTB

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[4] A note on nomenclature. The official gene symbol for PLZF in most vertebrate species is currently *Zbtb16*. However, this designation is infrequently used in the literature and often only in the limited sense of distinguishing the gene *Zbtb16* from the PLZF protein that it encodes. Although an occasional publication may prefer the label *Zbtb16* or the older designations *Znf145* and *Zfp145*, by far the most broadly recognized and utilized label for both gene and protein in the literature is PLZF. I have chosen for ease of use to conform to this practice. Happily, older terms for PLZF such as *Luxoid* or *Green's Luxoid* (not to be confused with *Strong's Luxoid* – an entirely different gene altogether) are now entirely moribund as designations for the gene and now refer exclusively to a particular murine nonsense allele of PLZF, described below.

[5] Because the BTB domain may also be referred to as a POZ domain, the BTB-ZnF family is also often referred to as the BTB/POZ family, the POZ family, or, less commonly, the POK family. BTB-ZnF is preferred here as it accurately refers to the two defining attributes of the family: the possession of both a BTB and a ZnF domain. Because the labels BTB/POZ and POZ refer solely to the BTB or POZ domain, the use of such terms lead to undesirable confusion as there are many proteins with BTB/POZ domains that are not BTB/POZ proteins due to their lack of ZnF domains. The terms POK – referring to the **PO**Z domain and the **K**rüppel-type zinc fingers of the ZnF domain – would also serve this purpose, but is seldom used in the literature.

domain and a C-terminal zinc finger (ZnF) domain. The BTB domain is the principle protein association for most BTB-ZnF family members, mediating both dimerization and binding of cofactors. The zinc finger domain consists of a series of zinc finger motifs (the number ranging between approximately three and thirteen) that are used for both DNA binding and to facilitate some protein-protein interactions (Costoya, 2007; Kelly and Daniel, 2006). The family is ancient, having been identified across much of the Eukaryotic clade, and is quite large in many species of vertebrates, with 43 BTB-ZnF genes currently known in the mouse genome and around 60 in the human genome. Within the chicken genome, there are currently only 26 known BTB-ZnF genes (Stubbs et al., 2011), although reduced number is may be partially attributable to the less complete state of the annotation of the chicken genome relative to other species.

The PLZF gene is conserved throughout the vertebrate subphylum. Homologues have been identified in multiple representatives of the mammalian, avian, reptilian, amphibian, jawed bony fish (osteichthyes), and in a species of jawless fish (cyclostomes), a clade believed to be the most basal of the vertebrates (Kuraku and Kuratani, 2006), suggesting the gene was present in the most recent common ancestor of vertebrates.[6] Claims have been made that the *C. elegans eor-1* gene is a PLZF homolog (Howard and Sundaram, 2002; Zhang et al., 1999). Although PLZF and *eor-1* do share some structural similarities, most notably both have nine ZnF motifs, there is no persuasive evidence for a 1:1 homology and a far more likely scenario is that PLZF is but one of many BTB-ZnFs descended from an ortholog of *eor-1*

[6] The precise evolutionary relationship between jawed vertebrates (gnathostomes) and jawless vertebrates (agnathostomes), the principal living examples of which are lampreys and hagfish, is currently undergoing dispute and revision. The traditional model, based primarily on comparative anatomy, groups lampreys with gnathostomes as vertebrates and considers the hagfish as the closest outgroup to the vertebrate clade. More recently, molecular analysis has favored linking hagfish and lampreys together as cyclostomes and that this clade constitutes the most basal group of vertebrates and it is this analysis of vertebrate evolution followed here (Kuraku and Kurutani, 2006).

that underwent extensive radiation. A stronger case can be made for the recently identified *dPLZF* to be the *Drosophila* ortholog of PLZF. In addition to sequence similarities, vertebrate PLZF and *dPLZF* are apparently functionally equivalent when over-expressed in Drosophila tissues (Maeng et al., 2012), although this cannot be considered a conclusive argument in a protein family with extensive functional redundancy. However, when the BTB domain of *dPLZF* was compared against 47 human BTB-ZnF proteins its sequence was found to be most similar to two human proteins: FAZF and PLZF (Z.B. Gaber, unpublished data). While a more elaborate phylogenetic analysis would be required for confidence, it seems plausible that dPLZF is orthologous to a gene that became duplicated in the vertebrate lineage into FAZF and PLZF, and that future research into *dPLZF* may shed highly relevant light onto the function of PLZF.

The PLZF BTB Domain

The BTB[7] domain was first discovered as a conserved fold present in three Drosophila transcription factors: ***b****ric-à-brac*, ***t****ramtrack*, and ***b****road complex*. The core BTB domain consists a sheet of three ß-strands overlying a surface composed of five α-helices. This domain is present in many gene families and used to mediate protein-protein interactions. BTB-ZnF family proteins, such as PLZF, possess an extended BTB domain, sometimes referred to as a long-form BTB domain, which includes an additional N-terminal α-helix and ß-strand. Functional studies and crystals structures have found that this extension renders long-form BTB domains particularly suitable for homodimerization and heterodimerzation with other BTB domains by providing a surface for additional contacts (Strogios et al., 2005).

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[7] The BTB domain is also sometimes referred to as the POZ domain, after the **Po**x Virus Zinc Finger protein which also contains this domain.

The BTB domain of PLZF is known to be essential for PLZF's ability to mediate transcription repression (Li et al., 1997). This activity is believed to stem from the PLZF BTB domains ability to bind chromatin remodeling complexes, particularly repressor complexes such as HDAC1, HDAC4, mSin3A, N-CoR, and SMRT (Chauchereau et al., 2004; David et al., 1998; Grignani et al., 1998; Lin et al., 1998; Wong and Privalsky, 1998). HDAC1 and HDAC4 are histone deacetylases that remove acetyl groups from the tails of histone proteins, a modification long associated with condensed chromatin and silenced transcription. SIN3A, SMRT, and N-CoR are proteins that have been found to associate with nuclear receptors and many transcription factors and assist in their recruitment of transcription repressors, such as HDACs (Watson et al., 2012). These interactions appear to be critical for PLZF function as HDAC inhibitors have been shown to greatly compromise PLZF mediated transcription repression (David et al., 1998). PLZF has also been found promote DNA methylation through the recruitment of DNMT1 (Guidez et al., 2007) and to bind the polycomb group protein BMI-1 (Barna et al., 2002), a core element of the PRC-1 complex implicated in mono-ubiquination and epigenetic silencing (Richly et al., 2011), although neither of these interactions have been definitively mapped to the BTB domain.

The long-form BTB domain of PLZF is particularly well characterized, both structurally and functionally, and is often used as a baseline to which the properties of other BTB domains are compared. The PLZF BTB domain has been found to be able to form homodimers (Ahmad et al., 1998) and heterodimers with the BTB-ZnF proteins BCL6 and FAZF (Dhordain et al., 2000; Hoatlin et al., 1999). However, PLZF is unable to form heterodimers with either the BTB-ZnF protein ZBTB7A or the BTB domain containing protein NAC1, suggesting a high degree of specificity to these interaction (Davies et al., 1999; Korutla et al., 2009). The crystal

structure of the human PLZF BTB domain (amino acids 1-132) has been solved in both monomeric and homodimeric states. PLZF's BTB domain was found to have a large hydrophobic surface, extending over approximately a quarter of the monomer's total surface area. The crystal structure suggests that dimerization between PLZF BTB domain monomers is stabilized by interactions across this entire surface (Ahmad et al., 1998) and point mutations that disrupt this interface destroy PLZF's ability to dimerize (Melnick et al., 2000a). Of particular interest, the crystal structure found that formation of a PLZF BTB dimer creates a charged pocket (Ahmad et al., 1998). Point mutations that neutralize this charged pocket, particularly residues D35 and R49, result in BTB domains that are able to dimerize but are unable to repress transcription (Melnick et al., 2000a) through their inability to recruit the cofactors N-CoR, SMRT, and HDAC1 (Melnick et al., 2002). Intriguingly, this charged pocket appears to be conserved among PLZF and its two known BTB-ZnF binding partners, FAZF and BCL-6, and mutating the charged pocket of one to more closely resemble another is sufficient to confer comparable protein association capabilities, underlining the importance of this pocket for cofactor specificity (Melnick et al., 2002).

Thus, the dominant mode of PLZF action is thought to be that PLZF first forms a homodimer or heterodimer via its BTB domain and that the dimeric BTB domain is able to recruit transcription repressing co-factors via its charged pocket.

The PLZF RD2 Domain

In roughly the middle of the PLZF protein exists a second domain associated with transcription repression, the logically named repressor domain two (RD2). After having been implicated in PLZF's associations with mSin3A and HDAC1 (David et al., 1998), this domain

was functionally mapped as lying between amino acids 217 and 387 and has been shown to be vital for PLZF's interaction with the ETO protein (Melnick et al., 2000b). Like PLZF itself through its BTB domain (see above), ETO has been found to independently bind the mSin3A, Nco-R, SMRT, and HDAC1 complexes. Thus, it is currently thought that the binding of ETO to RD2 allows PLZF a second, indirect mechanism for binding with these repressor complexes that supports PLZF's direct, BTB-mediated interactions (Melnick et al., 2000b).

Lysine 242 within RD2 has been shown to be a target for sumoylation by SUMO-1 and, at least in the context of HEK293T cells, this modification appears to be critical for PLZF repression. Intriguingly, while wild type PLZF was able to bind synthetic reporters and the endogenous cyclin A2 gene in HEK293T cells, the ability of PLZF(K242R) mutants to do either was severely compromised. The precise mechanism by which sumoylation of RD2 promotes PLZF repressor activity has yet to be fully explored, apart from the finding that sumoylated PLZF in cell extracts binds DNA targets sites with greater affinity (Kang et al., 2003). However, given its presence within a protein association domain, it is probable that sumoylation alters PLZF's binding with cofactors.

The PLZF ZnF Domain

The C-terminal half of PLZF consists of a set of nine Krüppel C2H2 zinc finger motifs (Chen et al., 1993) through which PLZF binds to specific DNA target sites (Li et al., 1997). Krüppel C2H2 zinc fingers, named after Drosophila Krüppel protein, each consist of a loop of 28 amino acids stabilized by interactions between a zinc ion and a pair of cystein (C) and a pair of histidine (H) residues. Zinc fingers (ZnFs) typically occur in arrays that wind along the major groove of DNA and interact in a sequence dependent manner with four base pairs via an

α-helix at the C-terminal end of each finger. There is not believed to be a 1:1 relationship between amino acids identity in a given ZnF and nucleotide identity in the target DNA; rather ZnF target specificity derives from the net effect of whether a sufficient proportion of amino acids among multiple ZnFs are able to form stable interactions across a segment of DNA (Stubbs et al., 2011).

Table 1-1 - Characterized PLZF Binding Sites

	A	G/T	C/G	T	A/C	A/C	A	G	T			Consensus after (Li et al., 1997)	
T	A	T	G	T	A	C	A	G	T	A	C	Consensus after (Sitterlin et al., 1997)	
				T	A	C	A/T	G	T	A	C	Consensus after (Ivins et al., 2003)	
T	A	G/T	C/G	T	A/C	A/C	A/T	G	T	A	C	Consensus of Consenses	
												Target Gene	Source
			A	T	A	C	A	G	T			*c-myc*	(McConnell et al., 2003)
G	A	G	C	T	A	A	A	G	G	C	T	*Cyclin A2*	(Yeyati et al., 1999)
G	A	C	G	T	C	A	A	G	G	C	C		
				T	A	C	T	G	T	A	C	*Hoxb2*	(Ivins et al., 2003)
	A	G	C	T	C	C	A					*Hoxd11*	(Barna et al., 2002)
	A	T	G	T	A	A	A						
	A	T	G	T	C	C	A	C					
	A	T	G	T	C	A	A	G					
T	T	G	C	A	A	C	T	G	T	A	C	*IL-3R*	(Ball et al., 1999a)
			A	T	C	C	A	G	T			*Pbx1*	(Shiraishi et al., 2007)
G	A	T	A	T	A	A	A	G	T	G	C	*TpoR*	(Labbaye et al., 2002)
		A	C	T	A	A	A	A	T	G	T	*Vla-4*	(Quaranta et al., 2006)

Core PLZF recognition site in blue, sites which diverge from consensus are depicted in red

PLZF has been shown repeatedly to bind to specific DNA targets. Although the consensus sequence has been variously reported, the observations of several groups converge on a core recognition sequence of T(A/C)(A/C)AGT that some groups extend to include either 5' or 3' accessory nucleotides to form an extended recognition sequence of TA(G/T)(G/C)T(A/C)(A/C)AGTAC (Ivins et al., 2003; Li et al., 1997; Sitterlin et al., 1997). It should be noted that most PLZF targets sites known in the literature do not appear to strictly

adhere to even this flexible consensus (see Table 1-1). The specificity of PLZF's interaction with DNA has been mapped by deletion studies to ZnF 5-9 (Li et al., 1997; Sitterlin et al., 1997), and plausibly modeled as ZnF 6-8 as binding the core PLZF consensus sequence and ZnF 5 and ZnF 9 binding to the extended recognition sequence (Guidez et al., 2005). PLZF's affinity for DNA, and possibly target specificity may in at least some contexts be influenced by acetylation. Multiple lysines within ZnF 6 and ZnF 9 have been found to be targets for the histone acetyltransferase p300 and their modification has been shown to promote PLZF's ability to bind DNA (Guidez et al., 2005).

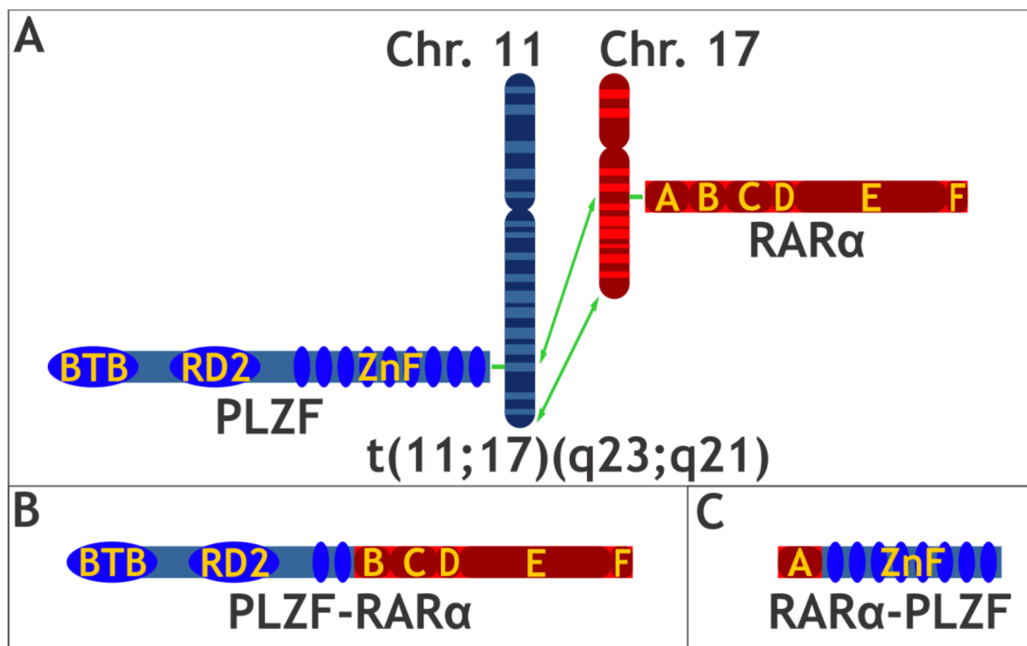
Genomic Organization and Alternative Splicing of PLZF

In humans, mice and chickens, the PLZF gene is distributed across 6 exons. For all three species, over half of the protein coding sequence is encoded on exon 1 (including the BTB and RD2 domains as well as the first zinc finger) while the remaining five exons encode one or two zinc finger motifs each, a common feature of ZnF arrays (Stubbs et al., 2011). Studies of the human PLZF transcript have found that the large exon 1 exists in at least four different alternatively-spliced forms (AS-I, AS-II, AS-III, and AS-IV), some of which truncate or even remove PLZF's BTB domain and many of which exhibit tissue-specific expression patterns. AS-I encodes the longest PLZF open-reading frame and appears to have the widest expression of all exon 1 splice forms (Zhang et al., 1999). Little is known about the functional consequence of the alternative splicing of exon 1 as only AS-I and the full-length PLZF protein it encodes has received significant attention in the literature. A truncated form of PLZF, lacking both the BTB domain and most of the RD2 domain, has been reported to be expressed in the liver alongside full length PLZF. This truncated protein associated with

ATP7B to promote ERK signaling, although since full length PLZF was found to be possess a comparable activity, the significance of the truncation is unclear (Ko et al., 2006).

1-5 PLZF in development and disease

Figure 1-4 – t(11;17) recombination associated with APL



Ever since PLZF was first identified as a critical factor in acute promyelocytic leukemia acute promyelocytic leukemia pathology just under twenty years ago, researchers have uncovered roles for PLZF in an ever expanding range of developmental and physiological contexts, most notably in hematopoiesis, limb and axial skeleton patterning, spermatogenesis, and an as yet only partially understood role in CNS development.

PLZF in leukemia and hematopoiesis

PLZF was first molecularly identified due to its linkage to cases of acute promyelocytic leukemia (APL) and the greatest focus of PLZF research has remained in this area and served as a point of departure for the understanding of PLZF in other contexts. APL (also known as AML-III) is a form of leukemia typified by a differentiation block in the granulocyte lineage at the promyelocyte stage, promyelocytes being a proliferative precursor that form that derives from myeloblasts and ultimately differentiate towards the cells of the basophilic, eosinophilc, and neutraphilic lineages. In APL, promyelocytes over-proliferate within the bone marrow and are sometimes found in the circulating blood. This over-proliferation not only compromises proper granulocyte production but also interferes with normal erythrocyte and platelet differentiation, resulting anemia and thrombocytopenia respectively. As a result, the APL mortality is often a consequence of uncontrolled bleeding (hemorrhagic diathesis) and disregulated clotting in circulating blood (disseminated intravascular coagulation) (Baljevic et al., 2011; Wang and Chen, 2007). To date, APL have been exclusively linked to reciprocal chromosomal translocations between the retinoic acid receptor-α (RARα) genes and one of four other loci. By far the most commonly observed translocation t(15;17)(q22;q21), estimated to occur in roughly 95% of APL cases, occurs between *RARa* and the *PML* gene (Rowley et al., 1977). However, APL-producing translocations have also been between RARα and NuMA, NPM, STAT5b, and PLZF (Wang and Chen, 2007).

The recombination between PLZF and RARα, t(11;17)(q23;q21) was first identified in a single patient in 1993 (Chen et al., 1993) although multiple studies over the next 14 years would identify an additional 17 cases, suggesting that the t(11;17) translocation is responsible for less than 1% of incidence of APL (McConnell and Licht, 2007). The exchange of chromosome arms in the t(11;17) translocation fuses the N-terminus of each gene to the C-terminus of the other and the generates two stable fusion proteins, designated PLZF-RARα

and RARα-PLZF (Chen et al., 1993). In the PLZF-RARα fusion protein, PLZF's BTB and RD2 domains linked to the DNA binding domain of RARα. As a result, in t(11;17) APL, the pro-differentiation genes normally activated by RARα are subject to PLZF-RARα mediated repression (Chen et al., 1994). In most forms of APL, the X-RARα fusion protein (X being any of the multiple genes with which RARα recombines) can be silenced by treatment with all-*trans* retinoic acid (ATRA) which associates with RARα's ligand binding domain and relieves most of the dominant negative effects of X-RARα. As a result, most forms of APL respond very well to treatment a ATRA and a broad cytostatic agent such as arsenic trioxide (Wang and Chen, 2007). However, in the case of t(11;17) APL, it has been found that ATRA is unable to relieve PLZF-RARα mediated transcription repression and the disease prognosis remains poor (Grignani et al., 1998; Licht et al., 1995; Lin et al., 1998).

Most APL research, even with regards to t(11;17) APL, has focused on the role of X-RARα and the disruption of RARα regulated pathways common to all forms of APL. Given the comparative infrequency of t(11;17) APL, this is perhaps understandable. However, it has been found that t(11;17) APL pathology stems is not solely a consequence of defective retinoic acid signaling, but also derives from the activity of RARα-PLZF and the oncogenic activation of genes normally repressed by PLZF. In the RARα-PLZF fusion protein, the RARα transactivation domain becomes linked to PLZF's ZnF DNA binding domain and thereby targets PLZF repressed genes for inappropriate expression (Grignani et al., 1998; Lin et al., 1998). Additionally, the ability of PLZF-RARα to heterodimerize with the cells functional PLZF proteins likely sequesters much of the healthy protein away from its appropriate targets (Dong et al., 1996).

After the activation of PLZF regulated pathways was found to have an oncogenic effect, research began to uncover the pathways regulated by PLZF in normal hematopoiesis. PLZF is found to be most highly expressed by undifferentiated progenitor cells but is progressively down-regulated by most lineages as cells differentiate (Labbaye et al., 2002; Reid et al., 1995). Ectopic PLZF is sufficient to cause cells to arrest in phase G1 of the cell cycle (McConnell et al., 2003; Shaknovich et al., 1998) and maintain cells in an immature state (Shaknovich et al., 1998). It is therefore modeled that PLZF's endogenous function in hematopoiesis is to maintain cells in an undifferentiated state and to inhibit over-proliferation by inhibiting cell cycle progression. PLZF's repression of the cell cycle has been found to be mediated in part through its ability to directly repress transcription of both cyclin A2 and c-myc (McConnell et al., 2003; Yeyati et al., 1999). Significantly, RARα-PLZF is able to induce cyclin A2 expression suggesting that this constitutes one of the underlying mechanisms of APL pathology (Yeyati et al., 1999).

PLZF in limb and axial skeleton patterning

The Luxoid mouse, possessing a null nonsense mutation in its PLZF gene (Buaas et al., 2004), was first discovered due to its severely malformed hindlimbs. The extent of malformation was observed to vary from animal to animal, but generally, hindlimbs exhibited an enlarged fibula, a reduced tibia, preaxial polydactylly (i.e. a partial or complete duplication or triplication of the hallux or "big toe"), and triphalangy of the hallux (i.e. formation of the hallux containing three bones instead of the normal two) (Forsthoefel, 1958; Green, 1955). Broadly, this phenotype may be described as an expansion of central and pre-axial limb structures, such as the increase in size of the fibula at the expense of the tibia, and incomplete fate-conversion of post-axial structures towards a more central identity, such

as the conversion of the hallux into a three-phallanged structure. Comparable forelimb defects do occur in PLZF deficient mice, but at reduced frequency and severity (Barna et al., 2000; Forsthoefel, 1958). Luxoid mice also frequently possessed an additional sacral vertebrae (Forsthoefel, 1958) and in some strains additional ribs and sternebrae (Green, 1955). This axial skeletal phenotype appears to stem from an expansion of rostral structures at the expense of caudal structures, with multiple vertebrae exhibiting homeotic transformations towards a more anterior identity (Barna et al., 2000).

The primary molecular basis for both limb and axial phenotypes was ultimately found to stem from misregulation of Hox genes in PLZF mutants. In the limb buds, all members of the AbdB HoxD cluster (Hox genes homologous to the Drosophila gene AbdB: Hoxd9-13) were observed to expand towards the posterior edge whereas in the axial skeleton Hoxc6 and Hoxc8 were observed to expand caudally, consistent with fate-conversion to more rostral identities (Barna et al., 2000). Research into the mechanism of PLZF repression of Hoxd11 found that the Hoxd11 locus contained five conserved PLZF bindings sites. Through these sites, PLZF recruit the polycomb group, chromatin-remodeling protein BMI-1 to silence Hoxd11 transcription. Intriguingly, PLZF appears unable to repress these sites in the context of forelimb development. Primary cell cultures taken from either forelimbs or hindlimbs found that PLZF was able to repress the expression of a luciferase reporter linked to the Hoxd11 promoter only in hindlimb derived cultures, suggesting that one explanation for the lack of a strong PLZF mutant phenotype in the forelimbs is a lack of PLZF sufficiency to regulate targets in the forelimbs (Barna et al., 2002). Another curious regional of PLZF's function is that while PLZF is expressed broadly throughout the entire limb bud PLZF mediated repression seems only to occur at the posterior end of the bud. Evidence suggests

that opposing inductive signals, such as retinoic acid, are able to overcome PLZF repression in the anterior limb bud (Barna et al., 2002).

PLZF in spermatogenesis

Shortly after the discovery of the Luxoid mutation it was observed that it was impossible to sustain the line as homozygotes (Green, 1955) and that this was attributable to male sterility (Forsthoefel, 1958). Studies have shown, using both the Luxoid mouse and a knock out line, that male sterility derives from a failure to maintain male germline stem cells. In wild type testes, PLZF is found to be expressed by $OCT4^{+}POU5F1^{+}$ cells, a quiescent population known to contain stem cells (Buaas et al., 2004), and is subsequently down-regulated as cells differentiate (Costoya et al., 2004). Although Luxoid testes were histologically normal as late as four weeks old (Buaas et al., 2004), as early as two weeks of age a reduction in the frequency of proliferative cells is observable (indicated by markers such as cyclinD1, phosphor-Histone H3, and BrdU) and this deficit worsens over time (Costoya et al., 2004). By eight months, PLZF deficient testes are atrophied, the frequency of GCNA+ germ line cells overall approximately halved, and seminiferous tubules contain few, if any maturing spermatids (Buaas et al., 2004). Significantly, transplants of germ-line cells from PLZF deficient males into germ-line deficient males failed to colonize, suggesting a severely reduced frequency of germ-line stem cells (Buaas et al., 2004; Costoya et al., 2004). Supporting these data is the finding that cultured PLZF-deficient spermatogonial progenitor cells (SPCs) have a greatly reduced ability to remain undifferentiated (Hobbs et al., 2010).

The underlying mechanism by which PLZF preserves male germ-line stem cells in an undifferentiated state is incompletely understood. PLZF has been linked to the suppression of

mTORC1, an important promoter of cell growth in response to many different growth factors and stimuli. PLZF is thought to suppress mTORC1 through a direct transcriptional activation of the REDD1 gene that in turn is an inhibitor of mTORC1. REDD1 is up-regulated in PLZF mutant cultured SPCs and PLZF. It has been found that PLZF is able to directly bind a site located within a 2kb element upstream of REDD1 and that when this element is linked to a luciferase reporter, co-transfection with PLZF is sufficient to induce reporter expression, providing an unusual example of PLZF acting as a direct transcriptional activator (Hobbs et al., 2010).

PLZF in the CNS

The role of PLZF is the either the development or mature physiology CNS is currently insufficiently understood. A survey of PLZF expression in the developing mouse embryo using ^{35}S-labelled *in situ* hybridization probes found that PLZF expression begins in the headfold of the neuroectoderm at e7.5 and then rapidly expands throughout the ventricular zone of the developing CNS until e10.5. From e10.5 to e12.5, PLZF expression becomes increasingly regionally restricted although it remains primarily localized to the ventricular zone. Lastly, by e16.5 when the survey concluded, PLZF expression was largely confined to dorsal or alar nuclei (Avantaggiato et al., 1995).

More recent research into PLZF in the CNS has almost exclusively focused on the hindbrain. Between e8.5 and e10, the developing mouse hindbrain (rhombencephalon) becomes segmented into units called rhombomeres. PLZF is first down-regulated in rhombomeres 3 and 5 and then, a day later in the other rhombomeres. By e10.5, PLZF expression is remains highest primarily at the boundaries between rhombomeres (Cook et al.,

1995) and along central strip of the dorsal-ventral axis of each rhombomere as well (Takahashi and Osumi, 2011), a position analogous to its expression in the spinal cord (Z. Gaber, unpublished observation). Intriguingly, PLZF has been found able to bind and repress the enhancer element that directs Hoxb2 expression within rhombomeres 3 and 5, thus the timing of Hoxb2 induction, and therefore segmental identity, in different rhombomeres may in part be regulated by the timing of PLZF down-regulation (Ivins et al., 2003).

In addition to its role in segmental identity, PLZF also appears to play an important role in hindbrain neurogenesis. In studies conducted in zebrafish, which possess two PLZF genes (PLZFA and PLZFB), over-expression of PLZFA was sufficient to reduce the expression of the proneural bHLH protein NEUROG1 and differentiation of $ISL1^{+}$ neurons. Morpholino knockdown of both PLZFA and PLZFB had no impact upon neurogenesis. However, in a context of reduced NOTCH activity, obtained with the NOTCH / γ-secretase inhibitor DAPT, the additional loss of PLZFA and PLZFB resulted in significant ectopic neurogenesis. These data suggest that while PLZF may play a role in inhibiting neuronal differentiation it is functionally redundant with other genes and pathways. Intriguingly, this study also identified a novel pathway for regulating PLZF through its association with the adaptor protein BTBD6A (BTBD6 in mammals). BTBD6A is up-regulated in the by NEUROG1 and appears to promote neurogenesis at least in part by targeting PLZF for ubiquitin mediated degradation (Sobieszczuk et al., 2010).

PLZF mutation in disease

Although there exists an extensive literature on the oncogenic pathology resulting from the presence of a dominant-activator PLZF allele in humans, only a single case of disease in

humans stemming from a loss of PLZF has been reported. A patient was discovered with an 8 Mbp deletion from chromosome 11q23, a region spanning 72 genes, including PLZF. The patient presented many symptoms associated with deletions in this region: microcephaly, severe mental developmental deficits, craniofacial dysmorphia, and short stature. However, this patient also exhibited atypical symptoms: preaxial polydactyly, reduced preaxial structures in the forelimbs, extra ribs, and testicular hypoplasia. Analysis found that the 11q23 deletion had uncovered a hypomorphic mutation in the patient's remaining PLZF allele, a mutation of a conserved site within ZnF 8 (M617V) that is predicted to destabilize ZnF 8 and therefore DNA binding. Excluding symptoms commonly attributable to the 11q23 deletion, the patient's symptoms strongly resembled the features of the Luxoid mouse, strongly suggesting a highly conserved role for PLZF between mice and humans (Fischer et al., 2008).

1-6 Functional relationships among BTB-ZnF proteins

It is a common phenomenon in development for related proteins to participate in related processes. Notable instances of this phenomenon in neural development include the aforementioned SOX and bHLH proteins (see Chapter 1-2). The SOXB1 genes maintain undifferentiated NPCs and later confer neuronal subtype identity, SOXB2 and SOXC genes proteins promote neuronal differentiation, and SOXE genes proteins induce neural crest and gliogenesis (Wegner and Stolt, 2005). Proneural bHLH proteins direct the cell cycle exit and terminal differentiation of neurons. Conversely, HES family bHLH proteins and ID family proteins, with modified bHLH domains, both promote progenitor maintenance, often through direct antagonism of proneural bHLH protein function. Lastly, members of the OLIG family of bHLH proteins have been implicated in neural patterning and neuronal and OL differentiation (Kageyama et al., 2005; Ligon et al., 2006).

In accordance with the evolutionary tendency of related genes to regulate different aspects of related processes (a tendency that will be considered more thoroughly upon in Chapter 4), BTB-ZnF family members have been linked to the control of many different aspects of hematopoiesis and to a lesser extent spermatogenesis. At present, at least eight members of the BTB-ZnF family have been linked with the regulation of hematopoiesis and in particular the development of the lymphatic lineage (Beaulieu and Sant'Angelo, 2011), the among most prominent being PLZF, FAZF, BCL6, and ZBTB7A.[8]

To review, PLZF is believed to act principally as a transcription repressor that recruits chromatin silencing HDACs to specific target genes. PLZF is expressed within the proliferative cells of the early hematopoietic lineage and acts as a cell cycle inhibitor to limit growth. Deregulation of PLZF targets has been associated with acute promyelocytic leukemia. As cells differentiate, PLZF is down-regulated within most lineages, although it is retained by and important for the differentiation and maturation of induced natural killer T-cells (see Chapter 1-5 for a more full treatment of these matters).

Far less well understood is FAZF, PLZF's closest paralog. PLZF and FAZF are able to heterodimerize and FAZF has been shown to be able to bind PLZF target sites (Hoatlin et al., 1999). Unlike PLZF deficient mice, FAZF knockout mice have no apparent defect in spermatogenesis, despite high expression within the seminiferous vesicles. However, mice deficient for FAZF do manifest several subtle hematopoietic phenotypes suggestive a role as a hematopoietic cell cycle inhibitor, similar to PLZF. FAZF is highly expressed among

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[8] The terminology for many BTB-ZnF genes is distressingly unstandardized. FAZF is often referred to as PLZP, ROG, TZFP, and ZBTB32. BCL6 is also known as LAZ3 and ZBTB27. ZBTB7A can also be found referenced as FBI-1, LRF, POKEMON, and as ZBTB7.

populations enriched for hematopoietic stem cells and loss of FAZF appears to result in a slight but significant increase in the fraction of cells exiting a quiescent state for entry into the cell cycle. In addition, FAZF mutant mice exhibit subtle defects such as increased proliferation and over-production by cytokines among T cells (Piazza et al., 2004), potentially due to FAZF's opposition to induction of cytokine genes by GATA3. Intriguingly, this inhibition of GATA3 targets, such as IL-4 and IL-5, does not appear to be dependent upon recruitment of HDACs, but rather on competition between FAZF and the transactivator GATA3 for common binding sites (Miaw et al., 2000).

BCL6 is another BTB-ZnF protein implicated in hematopoiesis. Like FAZF, it has been found capable of heterodimerizing with PLZF (Dhordain et al., 2000). Like PLZF, BCL6's BTB domain is also able to recruit NCoR, SMRT, SIN3A, and HDAC complexes to genes to inhibit their expression (Dhordain et al., 1998). Unlike PLZF, BCL6 seems to target cell-cycle inhibitors such as p53 (Phan and Dalla-Favera, 2004). Intriguingly, and again unlike PLZF which we have argued enhances FGFR3 mediated STAT signaling (see Chapter 2), BCL6 appears to suppress at least some forms of cytokine induced STAT signaling. BCL6 has been found to repress the expression of multiple cytokine receptors that activate STAT as well as and STAT1 and STAT3 themselves (Basso et al., 2010; Reljic et al., 2000; Shaffer et al., 2000). Additionally, the inhibitory BCL6 recognizes a target sequence similar to that of transactivating STAT6 and appears to compete for a subset of STAT6 binding sites in the genome (Harris et al., 1999). Unlike PLZF and FAZF, BCL6 is principally expressed in maturing B lymphocytes and in a small subset of T cells. BCL6's primary activity appears to be in germinal center formation. Within the germinal centers of the lymphatic system, immature B cells undergo clonal expansion and affinity maturation in response to T cell dependent antigens. In mice deficient for BCL6, germinal centers fail to properly form because, instead

of expanding, B cells prematurely differentiate, resulting in severely compromised immune systems (Shaffer et al., 2000). Unsurprisingly for a gene with such a potent, anti-differentiation activity, up-regulation of BCL6 within lymphocytes has been strongly linked with several varieties of non-Hodgkins Lymphoma, particularly Diffuse Large Cell Lymphoma (Cattoretti et al., 2005). BCL6, like PLZF and FAZF, is also expressed within the testes, and its loss results in a slight reduction in spermatocyte production that seems to stem from increased apoptosis, suggesting BCL6 is a pro-survival factor in spermatocyte development (Kojima et al., 2001).

Lastly, ZBTB7A has been found to be among the most critical BTB-ZnF family members involved in maintaining hematopoietic stem and progenitor cells. THE ZBTB7A BTB domain has been shown capable of recruiting HDAC complexes and has been shown capable of heterodimerizing with BCL6 but, curiously, incapable of binding the more closely related PLZF protein (Davies et al., 1999; Maeda et al., 2005). Loss of ZBTB7A results in embryonic lethality in mice due to severe anemia (Costoya, 2007). In cell lines, loss of ZBTB7A has been found to render cells resistant to transformation when transfected with known oncogenes. Correspondingly, ectopic ZBTB7A is sufficient to result in oncogenesis in transgenic mice. A significant portion of ZBTB7A's activities appear to be mediated through the direct inhibition of the tumor-suppressor gene $p19^{ARF}$ (Maeda et al., 2005) Intriguingly, PLZF has also been linked indirectly to the repression of $p19^{ARF}$ through its association with the polycomb group protein and $p19^{ARF}$ inhibitor BMI-1 (Barna et al., 2002).

In surveying these four members of the BTB-ZnF family and their roles in hematopoiesis and, to a lesser extent spermatogenesis, several broad trends are observable. Firstly, all four have similar functions: promoting progenitor maintenance albeit in somewhat different

contexts in the hematopoietic lineage. Secondly, all those with known cofactors appear to use the same basic mechanism: recruitment of HDAC containing repressor complexes to specific loci. Thirdly, although broadly similar, no two BTB-ZnF proteins had identical phenotypes nor seemingly identical mechanisms. BCL6 is associated with the suppression of many cytokine signaling and plasma cell differentiation pathways. FAZF has principally been linked to the regulation of GATA proteins, and ZBTB7A to repression of $p19^{ARF}$. PLZF has been linked with the repression of multiple pro-cell cycle progression genes, notably C-MYC and CYCLIN A2. It currently remains unclear the extent to which this represents a genuine divergence of regulatory targets and the extent to which this is simply due to each BTB-ZnF protein being tested against different targets. However, given the apparent uniqueness of each BTB-ZnF's DNA binding consensus sequence, except perhaps for PLZF and FAZF, it seems likely that these transcription factors regulate different subsets of genes. One trend observable in the literature is that BCL6, FAZF, and PLZF have all been linked to the repression of aspects of interleukin cytokine signaling. PLZF directly represses the interleukin receptor IL-3Rα, FAZF inhibits IL-3, IL-4, and IL-5, and BCL6 inhibits multiple STATs and interleukin and interferon cytokine receptors (Ball et al., 1999b; Basso et al., 2010; Miaw et al., 2000; Piazza et al., 2004; Reljic et al., 2000; Shaffer et al., 2000).

In conclusion, the study of BTB-ZnF proteins in hematopoiesis and spermatogenesis has found that many BTB-ZnF proteins exhibit complementary roles in maintaining progenitor cells, although potentially employing significantly different genetic mechanisms. Over the course of this work evidence will be put forward to demonstrate that there is a comparable role for many BTB-ZnF proteins in the maintenance of spinal NPCs.

1-7 Summary

The regulation of the balance between NPC self-renewal and differentiation is regulated by a vast, interconnected transcription factor network. One of the common features of this network is the participation of multiple members of gene families and many distinct aspects of development. Therefore, when a microarray screen for genes down-regulated during motor neuron differentiation identified PLZF, it rapidly became apparent that not only was this gene was an excellent target for further exploration within the context of spinal development but that the broader BTB-ZnF family might be relevant as well.

The BTB-ZnF transcription factor PLZF has been found to function principally as a sequence-specific transcription repressor, recruiting HDACs and similar chromatin remodeling complexes to its targets. Subsequent research has identified PLZF as playing key roles in several aspects of development and physiology. It has been linked with the maintenance of hematopoietic and male germ-line stem cells as well as neural progenitor cells. Disregulation of PLZF within the hematopoietic lineage is important for the pathogenesis of a form of chemotherapy resistant acute promyelocytic leukemia. It has been associated with proper patterning of the limbs and axial skeletons and has been found to regulate Hox gene expression with segments of the hindbrain.

However, in many areas, such as the developing CNS, much still remains insufficiently understood regarding PLZF. Within the CNS, it has been found that PLZF is expressed in a highly dynamic fashion by ventricular NPCs but the identity of these progenitors and the significance of this regional expression is entirely unexplored. The discovery that PLZF is capable of reducing the neurogenesis and the expression of the proneural bHLH transcription

factor NEUROG1 suggests that PLZF maintains NPCs in much the same way it preserves hematopoietic and germ-line stem cells. However, whether this is true in all regions of the CNS and whether this constitutes the sole or even the primary mode of PLZF activity are unknown.

As will be shown in Chapter 2, PLZF is situated at a vital nexus between FGF signaling and NPC maintenance during neurogenesis, and represents possibly the first and best characterized member of what has the potential to be an entirely new family of critical regulators of neural development. Having identified one member of the BTB-ZnF as being an important for maintaining NPCs in the developing of the spinal cord, we proceeded to investigate whether other members of this family might be playing complementary roles. Out of this survey, described in Chapter 3, we identified several BTB-ZnF proteins as being expressed in the developing spinal cord and conducted a preliminary investigation as to whether one of them, RP58, might also promote NPC maintenance. Lastly, in Chapter 4, we discuss prospects for the broader BTB-ZnF family as being a previously unappreciated set of neural developmental regulators.

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CHAPTER 2 – PLZF regulates Fibroblast Growth Factor Responsiveness and Maintenance of Neural Progenitors

Abstract

Distinct classes of neurons and glial cells in the developing spinal cord arise at specific times and in specific quantities from spatially discrete neural progenitor domains. Thus, adjacent domains can exhibit marked differences in their proliferative potential and timing of differentiation. However, remarkably little is known about the mechanisms that account for this regional control. Here, we show that the transcription factor Promyelocytic Leukemia Zinc Finger (PLZF) plays a critical role shaping patterns of neuronal differentiation by gating the expression of Fibroblast Growth Factor (FGF) Receptor 3 and responsiveness of progenitors to FGFs. PLZF elevation increases FGFR3 expression and STAT3 pathway activity, suppresses neurogenesis, and biases progenitors towards glial cell production. In contrast, PLZF loss reduces FGFR3 levels leading to premature neuronal differentiation. Together, these findings reveal a novel transcriptional strategy for spatially tuning the responsiveness of distinct neural progenitor groups to broadly distributed mitogenic signals in the embryonic environment.

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All experiments were performed by ZBG. Text was jointly written by BGN, SJB, and ZBG.

2-1 Introduction

The formation of neural circuits within the developing central nervous system (CNS) depends upon the spatially and temporally ordered generation of distinct classes of neurons and glia from multipotent neural stem and progenitor cells (NPCs). An essential feature of this progression is the ability of NPCs to self-renew in a manner that permits early-born cells such as neurons to form while maintaining a sufficient progenitor pool to generate later-born cell types such as glia. At the heart of this process is the interplay between mitogenic signals from the extracellular environment and cell intrinsic factors, which integrate this information to permit either progression through the cell cycle or the onset of terminal differentiation (Edlund and Jessell, 1999). At early stages of development, NPCs are broadly responsive to mitogenic stimulation. However, this responsiveness markedly changes over time and often becomes region-specific such that some groups of cells proliferate for protracted time periods while others rapidly differentiate (Lobjois et al., 2004; Ulloa and Briscoe, 2007). While important for determining the size and shape of the developing CNS, the mechanisms underlying these differences in mitogen sensitivity remain poorly defined.

These features of NPCs are exemplified in the developing spinal cord, where many extrinsic and intrinsic factors regulating progenitor maintenance and differentiation have been characterized. In the early neural plate and tube, NPCs are organized in a proliferative neuroepithelial sheet and sustained by the mitogenic actions of several growth factors, particularly Fibroblast Growth Factors (FGFs). FGFs are broadly present in neural tissues and the surrounding mesoderm and act through receptor tyrosine kinases (FGFRs) expressed by NPCs throughout the course of neural development (Delfino-Machin et al., 2005; Diez del Corral et al., 2003; Walshe and Mason, 2000). Ligand binding to FGFRs activates multiple

downstream signaling cascades such as the MAPK/ERK, PI3K/AKT, PLCγ, and STAT3 pathways to both promote cell division and inhibit neuronal differentiation (Guillemot and Zimmer, 2011). Among the many targets of FGF signaling are members of the SOXB1 family of transcription factors, which play key roles first, sustaining neuroepithelial progenitor properties and second, blocking the expression and activity of proneural basic helix-loop-helix (bHLH) proteins that promote cell cycle exit and neuronal differentiation (Bylund et al., 2003; Graham et al., 2003; Rousso et al., 2012; Saarimaki-Vire et al., 2007; Streit et al., 2000).

As development proceeds, NPCs become increasingly poised to undergo terminal differentiation through the actions of retinoids, which activate the expression of homeodomain and bHLH transcription factors such as PAX6 and OLIG2. These factors participate in the dorsoventral patterning of NPCs and promote the accumulation of proneural bHLH proteins needed to trigger cell cycle exit and neuronal differentiation (Briscoe and Novitch, 2008). These activities are counterbalanced by the mitogenic actions of FGFs acting in concert with NOTCH receptors and their downstream effectors, the HES proteins (Diez del Corral et al., 2003; Kageyama et al., 2009). Mutual inhibition between proneural bHLH and HES proteins sets up a dynamic equilibrium between self-renewal and terminal differentiation (Shimojo et al., 2008) that must be resolved in a progenitor domain-specific manner. The mechanism by which this resolution is achieved has remained unclear. One possibility is that further intrinsic or extrinsic factors regulate this equilibrium by regionally altering the sensitivity of NPCs to mitogens, such as the FGFs.

The period of neurogenesis in the spinal cord is relatively brief, lasting for only a few days in chick and mouse development, after which time undifferentiated NPCs upregulate expression of the pro-glial transcription factors SOX9 and NF1A, and begin to give rise to

astrocyte and oligodendrocyte precursors (Deneen et al., 2006; Kang et al., 2012). During this transition, NPC maintenance remains dependent on FGF signaling (Furusho et al., 2011; Kang and Song, 2010; Kilpatrick and Bartlett, 1995). Moreover, the expression of FGFR3 becomes particularly enriched in astrocyte progenitors (Pringle et al., 2003), suggesting that differential FGF signaling might play a role in the specification or expansion of astroglial cells over others. Despite its importance for progenitor maintenance and gliogenesis, very little is known about the mechanisms through which FGFR expression and activity is regulated in specific populations of NPCs.

To identify the regulatory factors that influence NPC maintenance in the spinal cord, we carried out expression profiling experiments to define the genes that are deregulated in the spinal cord of *Olig2* mutant mice. $Olig2^{+}$ NPCs exhibit limited capacity for self-renewal, suggesting that Olig2 represses genes that promote NPC proliferation (Mukouyama et al., 2006; Rousso et al., 2008; Skaggs et al., 2011). Through these studies, we identified the gene *Zbtb16*, which encodes the Promyelocytic Leukemia Zinc Finger (PLZF) transcription factor, as one of the most prominently elevated genes in the absence of *Olig2* function (Supplemental Fig. 2-S1 A,E). PLZF is a member of the BTB-POZ family of transcription factors known to regulate progenitor maintenance in multiple tissues (Kelly and Daniel, 2006). PLZF was initially identified as a protein whose functions are subverted through chromosomal rearrangements resulting in acute promyelocytic leukemia (Suliman et al., 2012). It has subsequently been found to be a key stem cell maintenance factor in both the hematopoietic system and male germline (Suliman et al., 2012). PLZF also exhibits a highly dynamic expression pattern in the developing rodent forebrain and hindbrain (Avantaggiato et al., 1995), and is associated with neural rosette formation in differentiated embryonic stem cell cultures (Elkabetz et al., 2008). More recently, PLZF was found to suppress the earliest steps

in neurogenesis in developing zebrafish (Sobieszczuk et al., 2010). However, it remained unclear whether PLZF plays a role sustaining neural progenitors at later stages of development, and its mechanism of action was unresolved.

In this study, we identify a novel role for PLZF preserving a population of NPCs in the central region of the spinal cord from early development through to the onset of astrogliogenesis. Loss of PLZF compromises progenitor maintenance, leading to premature neuronal differentiation. Conversely, its elevation is sufficient to repress neurogenesis and enhance glial cell production. These phenotypes result from the ability of PLZF to promote the expression of FGFR3 in NPCs, which then acts though the STAT3 pathway to gate the response of NPCs to FGF mitogens present in the neural tube. This mechanism permits PLZF-expressing progenitors in the central spinal cord to differentiate at a slower pace than neighboring cells and expand the population of cells available for astrocyte production. Together, these data indicate that PLZF provides a critical link between the transcriptional programs and mitogenic signals that regulate the balance between NPC proliferation and differentiation.

2-2 Materials and Methods

Plasmid Expression and shRNA Constructs

Plasmid expression vectors were generated by cloning cDNAs of interest into a Gateway cloning-compatible variant of the vector pCIG (Megason and McMahon, 2002; Skaggs et al., 2011). The following cDNAs were used: PLZF, full-length chick clone isolated by PCR from e4

chick cDNA; FGFR3, WT form of the human FGFR3 (Chen et al., 2005); caFGFR3, myristoylated and constitutively activated (K650E) form of the human FGFR3 cytoplasmic domain (aa 399-806) (Webster and Donoghue, 1997); STAT3-C, mouse STAT3 containing two activating mutations (A662C, N664C) (Bromberg et al., 1999) obtained from Addgene. Sustained misexpression vectors were created using the Tol2kit system (Kwan et al., 2007). Briefly, Multi-Site Gateway Technology (Invitrogen) was used to transfer the CMV enhancer/β-actin promoter, the gene of interest, and an IRES-GFP reporter into the pDestTol2pA2 vector, which contains recognition sites for Tol2 transposase that permits stable integration into the chick genome (Sato et al., 2007). The following expression vectors were also used in the experiments: RCAS-activated FGFR1 (Liu et al., 2001; Novitch et al., 2003), pCMX-FGF8 (Dasen et al., 2003; Novitch et al., 2003) were FGFR1, an RCAS virus plasmid, pCAGGS-T2P2 (Tol2 transposase) (Sato et al., 2007).

PLZF shRNA vectors were created by subcloning target sequences against the chick PLZF transcript (5'-cgcagctgagatcctagaaata-3' and 5'-ttcagcctgaagcaccagctgg-3') into the plasmid pCIG-shRNA (Rousso et al., 2012; Skaggs et al., 2011). STAT3 activity was measured by transfection of the reporter vector BGZA-4m67-STAT3 containing four STAT3 binding sites driving the expression of a LacZ reporter (Yan et al., 2004).

In ovo *electroporation, animal husbandry, and tissue preparation*

Fertilized chicken eggs were acquired from AA Lab Eggs, Inc. and McIntyre Poultry and Fertile Eggs. Eggs were incubated at 37˚C and 60% humidity, staged, and electroporated with plasmid vectors as previously described (Novitch et al., 2001; Rousso et al., 2012). The Luxoid mouse strain deficient for PLZF was rederived from cryopreserved embryos purchased from

the Jackson Laboratory (Strain Name B6.C4-Zbtb16Lu/J). Mice were maintained in accordance with the guidelines specified by the UCLA Chancellor's Animal Research Committee. Tissue was collected, fixed, and cryosectioned prior to immunohistochemical staining or in situ hybridization as described previously (Novitch et al., 2001; Novitch et al., 2003). Specific antibodies and in situ probes are described in Tables 2-1 and 2-2.

Table 2-1 - Antibodies used for Immunohistochemistry

Antigen	Host Species	Source and References
BHLHE22 (BHLHB5)	Guinea Pig	(Skaggs et al., 2011)
BrdU	Rat	Accurate Chemical (MAS250p)
cleaved-CASP3	Rabbit	Cell Signaling Technology (9661)
phospho-ERK1/2	Rabbit	Cell Signaling Technology (4695)
FOXP2	Rabbit	Abcam (ab16046)
GATA3	Goat	Santa Cruz Biotechnology (sc-1236)
GFP (RABBIT)	Rabbit	Invitrogen (A6455)
IRX3 (Chick Tissue)	Rabbit	(Novitch et al., 2003)
IRX3 (Mouse Tissue)	Guinea Pig	(Briscoe et al., 2000)
ISL1	Goat	R&D Systems (AF1837)
ISL1/2	Mouse	Developmental Studies Hybridoma Bank (4D5), (Tsuchida et al., 1994)
LHX1/5	Rabbit	Millipore (AB3200)
MSX1/2	Mouse	Developmental Studies Hybridoma Bank (4G1), see also (Liem et al 1995)
NEUN	Mouse	Millipore (MAB377B)
NEUROG2 (Chick Tissue)	Guinea Pig	(Skaggs et al., 2011)
NEUROG2 (Mouse Tissue)	Goat	Santa Cruz Biotechnology (sc-19233)
OLIG2 (Chick Tissue)	Guinea Pig	(Novitch et al., 2001)
OLIG2 (Mouse Tissue)	Guinea Pig	(Novitch et al., 2003; Wichterle et al., 2002)
PAX3	Goat	R&D Systems (AF2457)
PAX6	Mouse	Developmental Studies Hybridoma Bank, (Ericson et al., 1997; Kawakami et al., 1997)
PAX7	Mouse	Developmental Studies Hybridoma Bank, (Ericson et al., 1997; Kawakami et al., 1997)
PLZF (Chick Tissue)	Mouse	Millipore (OP128)
PLZF (Mouse Tissue)	Mouse	Active Motif (39987)
SOX2	Goat	Santa Cruz Biotechnology (sc-17320)
SOX9	Rabbit	Millipore (AB5535)
TUJ1	Rabbit	Covance (MRB-435P)
VSX2 (CHX10)	Rabbit	(Ericson et al., 1997)

Table 2-2 - PCR Primers used to create in situ *probes.*

Probe Target	Forward Primer (5' to 3')	Reverse Primer (5' to 3')
ASCL1	CCTCTCGGTGTGTAGACGTG	GAGATTAACCCTCACTAAAGGGA TTATACAGGGCCTGGTGAGC
FGF2	TGCAGCTTCAAGCAGAAGAA	GAGATTAACCCTCACTAAAGGGA TCAGCTTTTAGCAGACATTGGA
FGFR1	TGCATGGTTGACAGTTCTCG	GAGATTAACCCTCACTAAAGGGA CTTGCCGATCATCTTCATCA
FGFR2	GTGGCAGTGAAGATGCTGAA	GAGATTAACCCTCACTAAAGGGA GGTGCAGTTGGCAGGTTTAT
FGFR3	CATGAAACTGCTCGGTGATG	GAGATTAACCCTCACTAAAGGGA GCTGGGAAATAAGGTCACGA
GFAP	TCGAATGAGTCCCTGGAGAG	GAGATTAACCCTCACTAAAGGGA AGAGGTGAGGGTGGGTTTCT
HES5-2	GGGGAAGGCTTTGTTTTTCT	GAGATTAACCCTCACTAAAGGGA CCCACCCTCACCCAAGATA
ID2	TCGACAGGATTTGGGTTTTT	GAGATTAACCCTCACTAAAGGGA TCCTAGGCTTGGGTCAGAAA
SPRY1	GTGATTCAGCAGCCCTCTCT	GAGATTAACCCTCACTAAAGGGA ATCAACGACGACTGCTTGG
SPRY2	CACTGCTGCACTAGGTGGTC	GAGATTAACCCTCACTAAAGGGA CGATGTACTGCATCCCCTTT
STAT3	GACAGGCGACACATCCAAC	GAGATTAACCCTCACTAAAGGGA TACTCCATGGCTGACAGCAG

Underlined text indicates T3 polymerase binding site.

In situ probes to *HAIRY1* and *NEUROG1* were generated from plasmid DNA as previously described (Perez et al., 1999; Vasiliauskas et al., 2003).

X-GAL Staining of Tissue Sections

Dissected tissue was briefly fixed in 4% paraformaldehyde at 4°C, rinsed repeatedly in PBS containing 2mM $MgCl_2$, equilibrated overnight in 30% sucrose, frozen on crushed dry ice in OCT mounting media (Sakura Tissue-Tek), and cryosectioned. Prior to staining, slides were fixed in 4% paraformaldehyde for an additional 10 minutes at 4°C and then rinsed twice in PBS containing 2mM $MgCl_2$, for ten minutes per wash. Slides were overlaid with 1mL of X-Gal Staining Buffer (1 mg/mL X-GAL [5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside], 35mM potassium ferrocyanide, 35mM potassium ferricyanide, 0.02% NP-40, 2mM $MgCl_2$, in PBS) and placed in a humidified chamber at 37°C for several hours to overnight. Once signal had developed, slides were repeatedly rinsed in PBS with 2mM $MgCl_2$, coverslipped, and imaged using brightfield microscopy.

Imaging and analysis

All images were collected using either a Zeiss Observer D1 microscope equipped with an Apotome optical imaging system or a Zeiss LSM5 Exciter confocal imaging system. Images were processed using Zeiss Axiovision and LSM Exciter software suites and Adobe Photoshop. Pixel intensity analysis of mRNA and protein expression was performed using NIH ImageJ software. Cell counts were performed manually and in most cases represented as mean value of multiple tissue sections collected from several independent specimens. Unless stated otherwise, results are expressed as fractional change relative to normalized controls set to a value of 1.0. The statistical significance of differences observed between experimental and control groups were assessed using the Student's t test using Graphpad Prism 5.0 software.

2-3 Results

PLZF is broadly expressed by early neural progenitors and becomes restricted to a central domain associated with interneuron and astrocyte production

To explore the function of PLZF in neural development, we first mapped its expression relative to other markers of NPCs and differentiated neurons in the chick spinal cord. PLZF is first detected at e2 (Hamburger Hamilton (HH) stage 10) in a subset of $SOX2^+$ NPCs in the open neural plate and then becomes broadly expressed by NPCs in the neural tube through e3 (HH stage 17) (Fig. 2-1 A-D). Between e4 to e5 (HH stages 21-28), PLZF becomes restricted to pdI6-p2 progenitors in the intermediate spinal cord that express high levels of IRX3 and PAX6, and are bounded by progenitors expressing MSX1/2 dorsally and OLIG2 ventrally (Fig. 2-1 E,F,I,J,M; Supplemental Fig. 2-S2 C,D). These $PLZF^+$ progenitors are thus fated to give rise to interneurons early in development and astrocytes at later times (Goulding, 2009; Hochstim et al., 2008). A very similar pattern of expression is observed in the developing mouse spinal cord (Supplemental Fig. 2-S1), suggesting an evolutionarily conserved role for PLZF in spinal cord development.

Within the central progenitor domain, PLZF is prominently expressed by dividing $SOX2^+$ progenitors and is down-regulated as cells express the proneural transcription factor NEUROG2 and interneuron differentiation markers such as LHX1/5 (Fig. 2-1 F,K; data not shown). This pattern of PLZF expression in NPCs is distinct from that seen in the dorsal spinal cord, where PLZF is excluded from the ventricular zone (VZ) and instead expressed by differentiated interneurons throughout the course of development (Fig. 2-1 K,L; data not

shown). For the remainder of this study, we will focus solely on the actions of PLZF in the central progenitor populations.

From e5-e7 (HH stages 25-30), progenitors in the intermediate spinal cord upregulate the expression of the early glial fate determinants SOX9 and NF1A, and transform into astrocyte progenitors (Deneen et al., 2006; Kang et al., 2012). During this time, PLZF expression in the VZ overlaps with SOX9 and NF1A, but then declines by e9 (HH stage 35), the time at which astrocyte progenitors migrate into the grey matter and differentiate (Fig. 2-1 G,H,L,N) (Deneen et al., 2006; Kang et al., 2012). PLZF is not detectable within migratory astrocyte progenitor by e10 (HH stage 36) or later stages (data not shown). PLZF was also excluded from $SOX9^{+}$ $OLIG2^{+}$ oligodendrocyte progenitors, consistent with its downregulation from the $OLIG2^{+}$ motor neuron progenitors from which many oligodendrocyte progenitors emerge (Fig. 2-1 L). Together, these data indicate that PLZF is associated with the maintenance of a central population of spinal NPCs during the progression from neurogenesis to gliogenesis, and its extinction coincides with the onset of cellular differentiation (Fig. 2-1 M-N).

PLZF elevation promotes progenitor maintenance and reduces neuronal differentiation

Since PLZF is associated with stem and progenitor cell maintenance in other tissues, we set out to examine whether its function plays a comparable role in the developing spinal cord. We first investigated the consequences of elevated PLZF activity on NPC maintenance and neuronal differentiation. Expression vectors encoding PLZF and an IRES-nuclear EGFP reporter cassette under the control of the cytomegalovirus enhancer-chick beta actin promoter were electroporated into the chicken spinal cord, and embryos collected 2 days later to assess

changes in neuronal differentiation. In spinal cords electroporated with the empty vector, ~60% of transfected cells expressed NPC markers such as SOX2 and the remaining ~40% expressed neuronal markers such as NEUN (Fig. 2-2 A-C,F). When PLZF was misexpressed, the fraction of transfected cells expressing SOX2 increased by ~26% relative to empty vector controls and the proportion giving rise to neurons was reduced by ~39% (Fig. 2-2 C-F). Thus, ectopic PLZF expression is sufficient to restrict neuronal differentiation and sustain cells in a progenitor state.

To determine the basis of these changes, we examined the impact of PLZF misexpression on proneural bHLH transcription factors. In the spinal cord, three proteins, ASCL1, NEUROG1, and NEUROG2 play a critical role promoting cell cycle exit and neuronal differentiation in different regions (Guillemot, 2007). Where PLZF was elevated, we observed a ~14% reduction in the expression of both *ASCL1* and *NEUROG1* mRNA (Fig. 2-2 G,H,J,K,M,N) and a ~28% decrease in the number of NPCs expressing NEUROG2 protein relative to spinal cords transfected with the control vector (Fig. 2-2 I,L,O). We further investigated whether these changes resulted from increased expression of HES genes, which are well-described inhibitors of proneural bHLH gene expression (Guillemot, 2007; Kageyama et al., 2009). Despite clear changes in proneural gene expression, there was no apparent effect of PLZF misexpression on the two primary HES genes expressed in the chick spinal cord, *HAIRY1* and *HES5-2* (Supplemental Fig. 2-S2 A,B). PLZF thus appears to be capable of suppressing the expression of multiple proneural genes, blocking neuronal differentiation, and promoting NPC maintenance in a HES gene-independent manner.

PLZF loss compromises progenitor maintenance leading to premature neuronal differentiation

Since ectopic PLZF is sufficient to enhance NPC maintenance, we next investigated whether PLZF is required for continued progenitor proliferation. Towards this end, we generated a plasmid vector encoding two short-hairpin RNAs to target the chick *PLZF* transcript (shPLZF) along with an IRES-nEGFP reporter cassette to identify the transfected cells. Electroporation of this construct into the spinal cord reduced the PLZF levels to nearly background staining levels (Fig. 2-3 A,G; Supplemental Fig. 2-S3 A-C), and led to substantial changes in NPC maintenance. The overall area of the VZ decreased by ~20%, and the average expression level of SOX2 within the remaining transfected progenitors declined by ~11% (Fig. 2-3B,H,M,N). These changes were further accompanied by reduced expression of other genes associated with NPC maintenance including *HES5-2* and *ID2* (Fig. 2-3 C,D,I,J,O). As these progenitor features were lost, early differentiation markers such as NEUROG2 were correspondingly elevated (Fig. 2-3 E,K,P). Despite these changes, we did not observe significant changes in the total number of $NEUN^{+}$ or $TUJ1^{+}$ neurons formed after PLZF knockdown (data not shown). Perhaps accounting for this result, we found that PLZF loss was associated with a ~2-fold increase in the frequency of cells undergoing apoptotic cell death measured by activated CASPASE3 staining (Fig. 2-3 F,L,Q). Importantly, defects in progenitor maintenance observed when PLZF was knocked down were rescued by the inclusion of an expression plasmid encoding human PLZF that lacks the shRNA target sequences (Supplemental Fig. 2-S3 D-N), confirming the specificity of these manipulations.

To complement this analysis, we investigated the effect of PLZF loss on progenitor maintenance and interneuron differentiation in Green's Luxoid mice (*Zbtb16*$^{Lu/Lu}$), which possess a nonsense mutation in the PLZF coding sequence that ablates its DNA binding function (Buaas et al., 2004). Using a panel of lineage-restricted makers on the spinal cords

from *Zbtb16*$^{Lu/Lu}$ mutant and control littermates, we found that differentiation was significantly increased among interneurons whose progenitors normally express PLZF (pdI6, p0-p2; Fig. 2-2 I,J,M). Specifically, we observed a ~14 to 23% increase in the number of dI6, V1, V2a, and V2b interneurons distinguished by their expression of specific transcription factors including Bhlhe22 (Bhlhb5), Foxp2, Vsx2 (Chx10), and Gata3 (Fig. 2-3 R,U-X,AA-AE) (Goulding, 2009; Skaggs et al., 2011). In contrast, no alterations were observed in the numbers of either Isl1^{+} dI3 interneurons or Isl1^{+} motor neurons, both cell types deriving from progenitors that do not sustain PLZF expression (Fig. 2-3 R-T,Y,Z,AE). Together, these data demonstrate that PLZF function is required to maintain a population of progenitors within the intermediate spinal cord and restrict their differentiation into spinal interneurons.

Sustained PLZF expression promotes gliogenesis

We next sought to determine the long-term consequences of manipulating PLZF activity on cell fate. Do the observed reductions in neuronal differentiation and enhanced progenitor maintenance associated with elevated PLZF expression ultimately result in increased glial production or continued expansion of neuroepithelial progenitors? To discriminate between these outcomes, we used the Tol2 transposon-mediated gene transfer system (Sato et al., 2007) to stably transfect chick NPCs in ovo with either an IRES-*EGFP* or *PLZF*-IRES-*EGFP* expression cassette at e3, and analyzed the effects on neuronal and glial development 12 days later at e15. Since SOX2 is expressed by both NPCs and glial-restricted progenitors at this time, we used antibody staining for NESTIN as a marker for uncommitted neural progenitors along with NEUN and SOX9 to respectively distinguish differentiated neurons and glial progenitors. At this time point, the majority of transfected cells had initiated lineage-specific differentiation irrespective of PLZF misexpression, reflected in a low

frequency of NESTIN staining in both control (~7%) or PLZF-transfected (~5%) embryos (Fig. 2-4 L; data not shown). However, the differentiated fates of the transfected cells were markedly different. Whereas ~27% of control transfected cells expressed NEUN, PLZF expression reduced this frequency to ~9% (Fig. 2-4 A,F,L). Instead, the majority (~86%) of the PLZF-transfected cells expressed glial progenitor markers such as SOX9 compared to ~66% in the control population (Fig. 2-4 B,G L).

Despite its normal exclusion from OLIG2^{+} cells (Fig. 2-1 I), ectopic PLZF expression resulted in a ~2-fold increase in the number of SOX9^{+} OLIG2^{+} oligodendrocyte progenitors, as well as a comparable increase in the expression of *FGFR3,* which is commonly associated with astrocyte progenitors (Pringle et al., 2003), and a ~2 to 3-fold increase in the definitive astrocyte marker GFAP^{+} (Fig. 2-4 C-E,4H-J,4M). Interestingly, these ectopic glia were not uniformly distributed throughout the spinal cord but instead clustered adjacent to the VZ as if the cells were impaired in their differentiation or migration. Collectively, these data suggest that PLZF plays an important role preserving a pool of progenitors available for gliogenesis at the later stages of embryonic development, but its function must ultimately be silenced for glial cell maturation (Fig. 2-4 N).

PLZF promotes neural progenitor maintenance by increasing FGFR3 expression and STAT3 activity

We next set out to identify the mechanism by which PLZF maintains specific NPCs in an undifferentiated state. Since PLZF misexpression did not appear to elevate the expression of NOTCH-responsive HES genes (Supplemental Fig. 2-S2 A,B), we considered other pathways known to block neurogenesis. Several observations suggested that the effects of PLZF on

differentiation could be mediated by the FGF signaling pathway. First, the FGF pathway is crucial for the establishment, preservation, and proliferation of NPCs both in vivo and in vitro (Guillemot and Zimmer, 2011). Second, FGF signaling regulates the expression of SOX2 (Stavridis et al., 2007; Takemoto et al., 2006), which was consistently elevated after PLZF misexpression. Third, we observed a striking coincidence between the expression patterns of *PLZF* and *FGFR3*, one of the principal receptors that mediates FGF signaling during the period of neurogenesis under consideration in this study (Walshe and Mason, 2000) (Fig. 2-5 A-F; Supplemental Fig. 2-S4 A-F). Taken together, these findings raised the possibility that PLZF promotes NPC maintenance by upregulating *FGFR3* expression and thereby enhancing the responsiveness of NPCs to FGFs in the embryonic environment. Supporting this model, ectopic expression of PLZF expanded *FGFR3* expression into the dorsal spinal cord, whereas PLZF knockdown decreased *FGFR3* in the intermediate spinal cord (Fig. 2-5 G-K). These alterations in *FGFR3* occurred without significant changes in the homeodomain proteins associated with dorsoventral patterning such as IRX3, PAX3, PAX6, and PAX7 (Supplemental Fig. 2-S2 E-H), suggesting that these effects were not simply due to alterations in NPC identity. Moreover, the effects were specific to *FGFR3*, as PLZF manipulations did not alter the expression of either *FGFR1* or *FGFR2* (Supplemental Fig. 2-S4 G-I).

If changing the level of FGFR3 expression accounts for the actions of PLZF on NPC maintenance and differentiation, then directly elevating FGFR3 levels or blocking its receptor kinase activity should respectively recapitulate the effects of PLZF misexpression and knockdown. To test this prediction, we electroporated spinal cords with expression vectors encoding either full-length FGFR3, or a truncated, dominant-negative (dn) form of FGFR1 that forms non-functional heterodimers with FGFR3 and blocks its downstream signaling activity (Ueno et al., 1992). Embryos transfected with FGFR3 displayed a strikingly similar phenotype

to that observed after PLZF misexpression. In both cases, there was a ~23% increase in SOX2 expression and a corresponding reduction in the formation of $NEUN^+$ neurons within the transfected cells (Fig. 2-2 A-F; Fig. 2-5 L,M,P; data not shown). In contrast, when endogenous FGFR3 activity was disrupted by dnFGFR misexpression, the fraction of transfected cells expressing SOX2 dropped by ~25%, suggesting that the loss of FGF signaling, as with loss of PLZF expression, compromises progenitor maintenance as seen with PLZF knockdown (Fig. 2-3 H,M-N; Fig. 2-5 N-P).

If PLZF acts by promoting FGFR3 expression, then the activity of FGFR3 should be epistatic to that of PLZF. In this case, the pro-progenitor activity of ectopic PLZF would be dependent upon FGFR function while direct elevation of FGFR3 levels should, in turn, overcome the loss of NPCs seen after PLZF knockdown (Fig. 2-6 X). To examine this possibility, spinal cords were first concomitantly electroporated with expression vectors encoding both PLZF and dnFGFR. Supporting the hypothesis, the increases in progenitor maintenance associated with PLZF elevation were blocked, and cells instead differentiated precociously as observed with dnFGFR misexpression alone (Fig. 2-6 A-H,U,X). In the converse experiment, when FGFR3 was coelectroporated with shPLZF, FGFR3 rescued both the loss of SOX2 progenitors and increase in $NEUROG2^+$ cells observed after electroporation with shPLZF alone (Fig. 2-6 I-T,V-X; data not shown). Together, these experiments suggest that PLZF does indeed act upstream of FGFR3.

We next assessed how manipulations of PLZF and FGFR were reflected in the activity of the second messenger effectors of FGF signaling. Early in chick development, FGF stimulation is associated with increased phosphorylation of ERK1/2 and expression of the ETS domain transcription factor ETV1 (ER81) and ETV4 (PEA3), as well as feedback inhibitors of

the pathway such as *SPRY1* and *SPRY2* (Chambers and Mason, 2000; Lunn et al., 2007). Surprisingly, we were unable to detect changes in any of these effectors in the chicken spinal cord, even under conditions in which embryos had been electroporated with constructs encoding constitutively activated forms of FGFR1 and FGFR3 (Webster and Donoghue, 1997) that potently blocked neuronal differentiation and expanded the progenitor pool (Fig. 2-7 A-D; Supplemental Fig. 2-S4 J,K,M,N). These results indicate either that the available reagents are insufficient to report pathway activity at the stages of development examined, or that PLZF and FGFR3 act through an alternative signaling pathway.

STAT3 is a non-canonical effector of FGF signaling (Dudka et al., 2010; Hart et al., 2000) that has been implicated in blocking neurogenesis and promoting either NPC maintenance or astrogliogenesis in various systems (Bonni et al., 1997; Foshay and Gallicano, 2008; Gu et al., 2005). We also confirmed that STAT3 is expressed broadly throughout the VZ of the spinal cord at the time of our experiments (Fig. 2-7 E). To test whether PLZF and/or FGFR3 regulate STAT3 activity in the spinal cord, we co-expressed either PLZF or FGFR3 with a STAT3 transcriptional reporter construct capable of measuring pathway activity in the chick embryo (Yan et al., 2004). In both cases, the activity of the STAT3 reporter was elevated ~2 to 5-fold (Fig. 2-7 F-I). Consistent with this result, we found that electroporation with a plasmid encoding a constitutively activated form of STAT3 (Bromberg et al., 1999) promoted progenitor maintenance and blocked neuronal differentiation in a manner that was nearly identical to the results seen with PLZF and FGFR3 misexpression (Fig. 2-7 J-O). Thus, the actions of PLZF and FGFR3 appear to be mediated by the STAT3 arm of the FGF signaling pathway rather than the ERK/MAPK pathway typically associated with FGFR signaling activity. Together, these data demonstrate that PLZF plays a critical role both gating the abundance of

FGFR3 on NPCs and controlling the nature of the downstream signaling pathway used to sustain a population of NPCs in the spinal cord.

PLZF gates the response of neural progenitors to FGFs

The observations that FGFR3 elevation is alone sufficient to expand the progenitor pool and block differentiation suggest that NPC maintenance in the spinal cord might be principally constrained by the amount of FGFRs present on the cells rather than availability of FGF ligands in the environment. Indeed, previous studies have shown that FGF2 and FGF8, two of the preferred ligands for FGFR3, are broadly expressed throughout the VZ of the developing spinal cord and present in the cerebrospinal fluid, and thus unlikely to provide spatial control over NPC expansion (Figure S4L and Diez del Corral et al., 2003; Martin et al., 2006; Murphy et al., 1994; Novitch et al., 2003; Stolte et al., 2002). To test whether FGFR3 levels are limiting, we reasoned that ectopic expression of FGFs throughout the spinal cord should elicit progenitor maintenance responses in a regional manner, with stronger effects seen in the PLZF^{+} *FGFR3*^{high} intermediate region of the spinal cord compared to the PLZF^{-} *FGFR3*^{low} dorsal spinal cord. For this analysis, PAX6 was used to monitor NPCs in place of SOX2. The extent of PAX6 expression completely overlaps with SOX2, and the high vs. low levels of PAX6 in the intermediate and dorsal spinal cord served as a convenient proxy for assessing the presence or absence of PLZF (Supplemental Fig. 2-S2 C-D). Consistent with our prediction, transfection of the spinal cord with an expression vector for FGF8 led to a ~20% increase in the expression of PAX6 and the fraction of cells incorporating BrdU in the dorsal spinal cord, compared to a ~75% enhancement in the intermediate spinal cord (Fig. 2-8 A-N,V,W). Moreover, FGF8 misexpression did not significantly change NEUROG2 expression in the dorsal spinal cord, whereas it reduced NEUROG2 by >20% in the intermediate spinal cord (Fig. 2-8 A-

E,H-L,M,N,X). Thus, $PLZF^+$ progenitors appear to be more responsive to FGF ligand stimulation than adjacent $PLZF^-$ domains.

Based on these results, we tested whether PLZF misexpression could enhance the response of NPCs to ectopically expressed FGFs. In regions of the spinal cord where PLZF and FGF8 were cotransfected, the VZ became dramatically enlarged and disorganized, with a ~2-fold increase in the number of $PAX6^+$ and $BrdU^+$ cells, and a ~20-25% reduction in the proportion of those progenitors undergoing neurogenesis (Fig. 2-8 O-X; Supplemental Fig. 2-S5 A-O). These effects were distinct from the relatively mild expansion of NPCs seen after ectopic expression of PLZF or FGF8 alone, yet remarkably similar to the effects seen after electroporation with constitutively activated FGFR3 plasmids (Supplemental Fig. 2-S5 P-R). Collectively, these experiments reveal regional differences in the sensitivity of spinal cord NPCs to FGF mitogen stimulation that correlates with their relative expression of PLZF and FGFR3. Moreover, PLZF elevation has the capacity to render cells hyper-responsive to FGF stimulation.

2-4 Figures

Figure 2-1

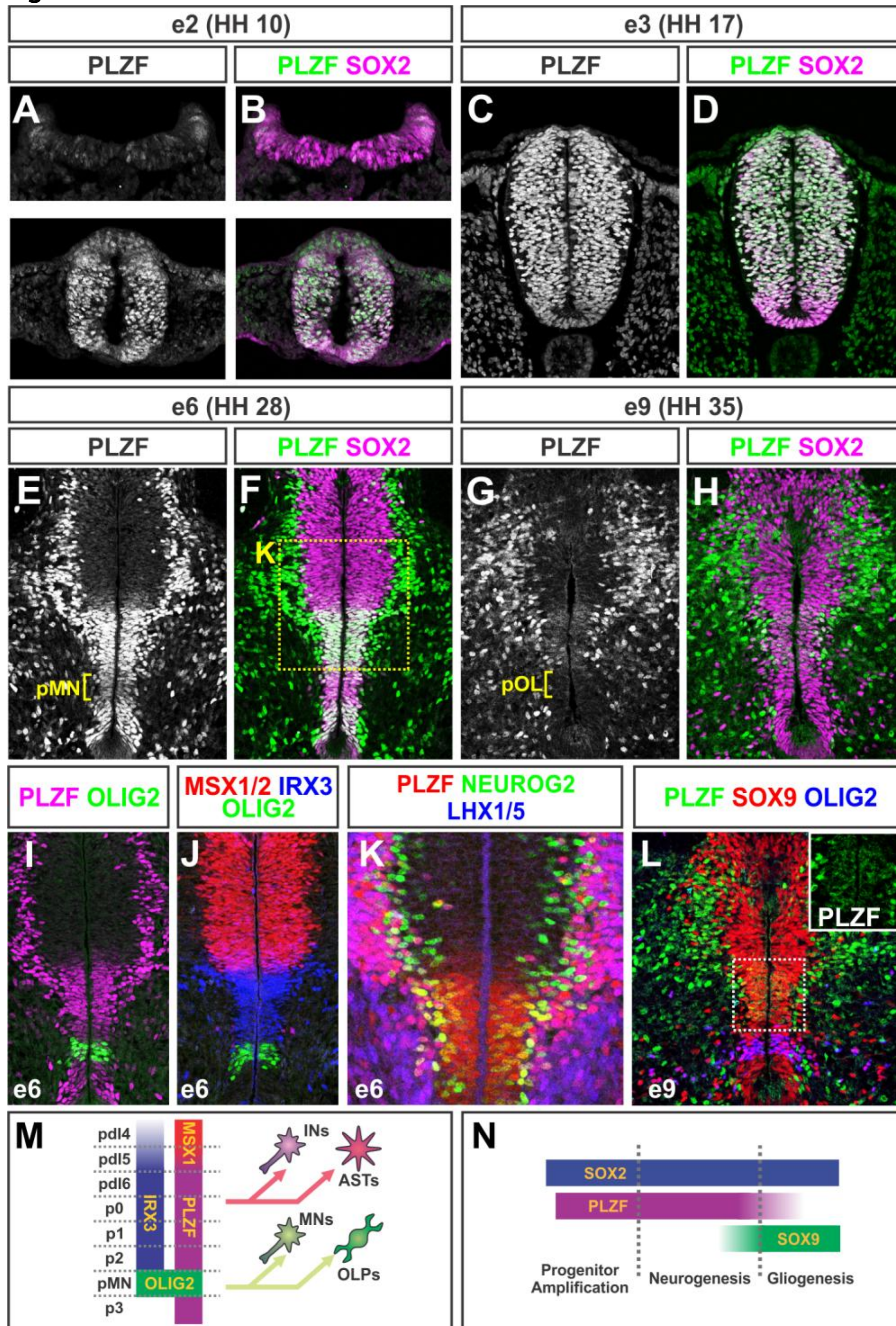


Figure 2-1 - PLZF is broadly expressed by early neural progenitors and then becomes restricted to a central domain committed to ventral interneuron and astrocyte production.

(A-H, K, N) Antibody costaining analysis shows that PLZF is initially expressed by a subset of $SOX2^+$ progenitors in the open neural plate at e2, and then becomes broadly expressed by most NPCs. From e4-e6, PLZF becomes confined to a central domain of NPCs in the intermediate spinal cord that persists throughout the course of neurogenesis and early stages of gliogenesis. PLZF is also expressed by many differentiated $LHX1/5^+$ neurons in the dorsal spinal cord. pMN, motor neuron progenitor domain; pOL, oligodendrocyte progenitor domain.

(I, J, M) Mapping of PLZF expression relative to the homeodomain proteins that pattern the spinal cord reveals that the progenitor expression of PLZF is associated with the pdI6, p0, p1, p2, and p3 domains known to give rise to interneurons early in development followed by astrocytes.

(L) During early gliogenesis, PLZF is expressed by $SOX9^+$ astroglial progenitors in the VZ, but absent from migratory $SOX9^+$ $OLIG2^-$ astrocyte progenitors and $SOX9^+$ $OLIG2^+$ oligodendrocyte progenitors. All of the $PLZF^+$ $SOX9^-$ cells at these later stages correspond to subsets of differentiated interneurons (data not shown).

Figure 2-2

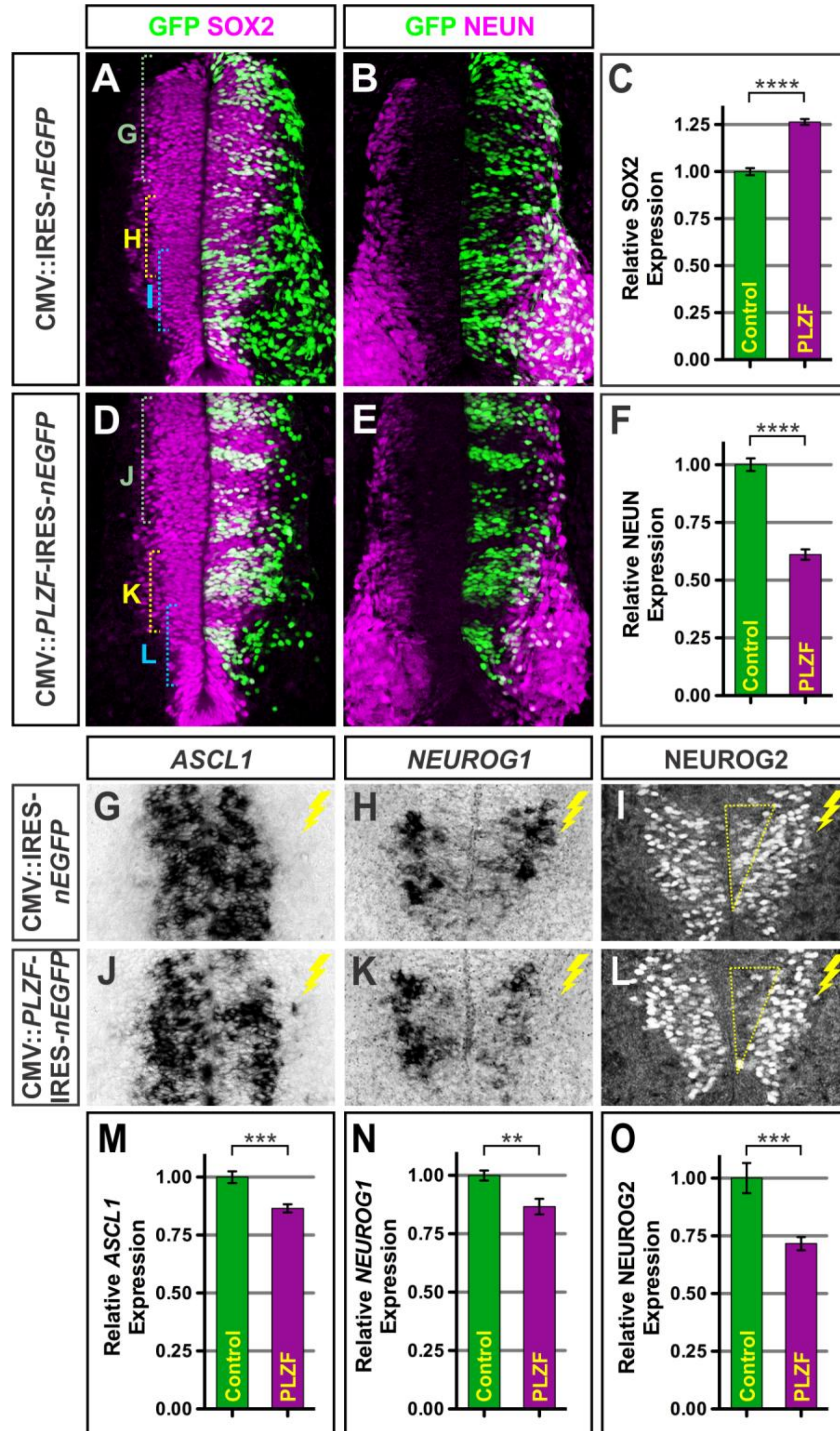


Figure 2-2 - PLZF misexpression promotes progenitor maintenance and reduces neuronal differentiation.

(A-F) NPCs were transfected with control IRES-*nEGFP* or *PLZF*-IRES-*nEGFP* vectors at e3 and analyzed at e5. PLZF-transfected cells display an increased expression of the progenitor marker SOX2 and reduced expression of the neuronal marker NEUN. Charts display the mean proportion of PLZF-transfected cells expressing the indicated markers ± SEM relative to empty vector controls. Data are representative of multiple sections taken from > 8 embryos for each condition.

(G-L) PLZF misexpression reduces the expression of *ASCL1*, *NEUROG1* mRNA and NEUROG2 protein. Particularly notable changes in NEUROG2 expression are indicated by the boxes in (I) and (L).

(M-N) Charts display the mean level of *ASCL1* and *NEUROG1* mRNA ± SEM in control and PLZF-electroporated spinal cords, relative to the contralateral control sides.

(O) Quantification of the mean number of transfected NPCs expressing NEUROG2 protein ± SEM relative to empty vector controls.

In all panels, ** = $p < 0.01$, *** = $p < 0.001$, and **** = $p < 0.0001$.

Figure 2-3

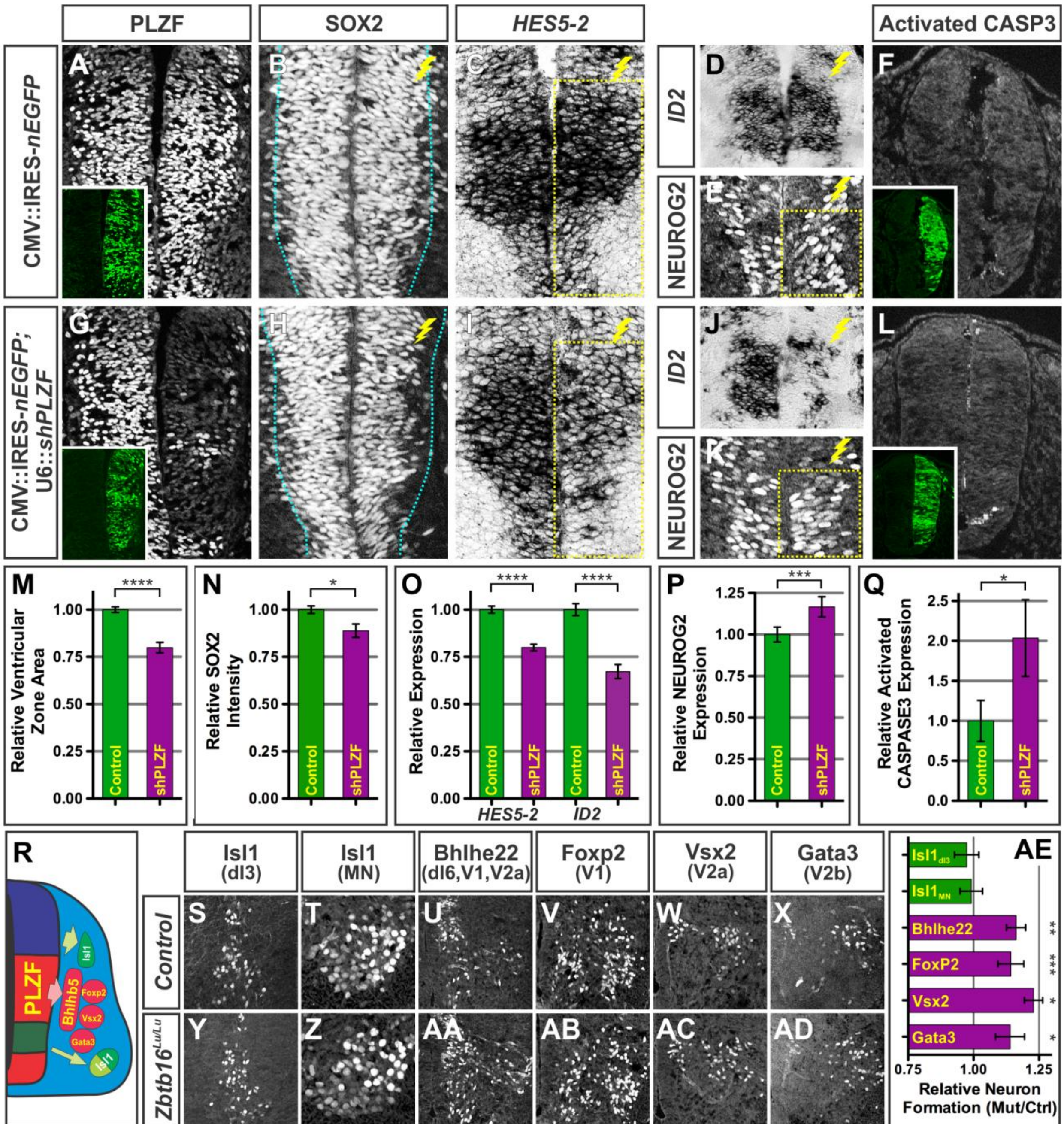


Figure 2-3 - Reduced PLZF activity compromises progenitor maintenance and promotes neuronal differentiation.

(A, G) Electroporation of chick embryos with shRNA vectors against *PLZF* (U6::*shPLZF*) at e2 dramatically reduces the expression of PLZF protein in the developing spinal cord upon collection at e4. Insets show the extent of electroporation marked by nEGFP fluorescence.

(B, H, M) PLZF knockdown reduces the area of the VZ. Chart indicates the mean VZ area ± SEM for both control and shPLZF-electroporated embryos relative to the untransfected contralateral sides of the spinal cord. Blue dotted lines demarcate the border of the contralateral VZ in each image

(B-D, H-J) PLZF knockdown reduces the expression of multiple genes and proteins associated with progenitor maintenance including SOX2, *HES5-2*, and *ID2*.

(E, K) PLZF loss coincides with an increase in the number and density of cells expressing the proneural transcription factor NEUROG2 within the VZ

(F, L, Q) PLZF knockdown also increases the frequency of apoptotic cell death.

(N) Chart displays the mean pixel intensity of SOX2 staining ± SEM in shPLZF-transfected cells relative to empty vector controls.

(O) Chart displays the level of *HES5-2* and *ID2* mRNA in control and shPLZF-electroporated spinal cords, relative to the contralateral control sides.

(P-Q) Charts display the mean number of shPLZF-transfected cells ± SEM expressing the indicated markers relative to empty vector controls.

Data are representative of at least 10 images taken from ≥ 8 embryos electroporated in the same experiment.

(R-AD) The number of dI6, V1, V2a, and V2b neurons, which are normally derived from PLZF$^+$ progenitors (Fig. 2-1), are increased in e13.5 PLZF mutant (*Zbtb16$^{lu/lu}$*) mice. However, neurons that are not associated with PLZF$^+$ progenitors, such as dI3 interneurons and motor neurons, are not changed.

(AE) Chart displays the mean number of cells expressing the indicated neuronal markers ± SEM relative to WT and *Zbtb16$^{lu/+}$* littermate controls. Results are representative of > 10 images collected from at least 5 embryos of each genotype.

In all panels, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, and **** = $p < 0.0001$.

Figure 2-4

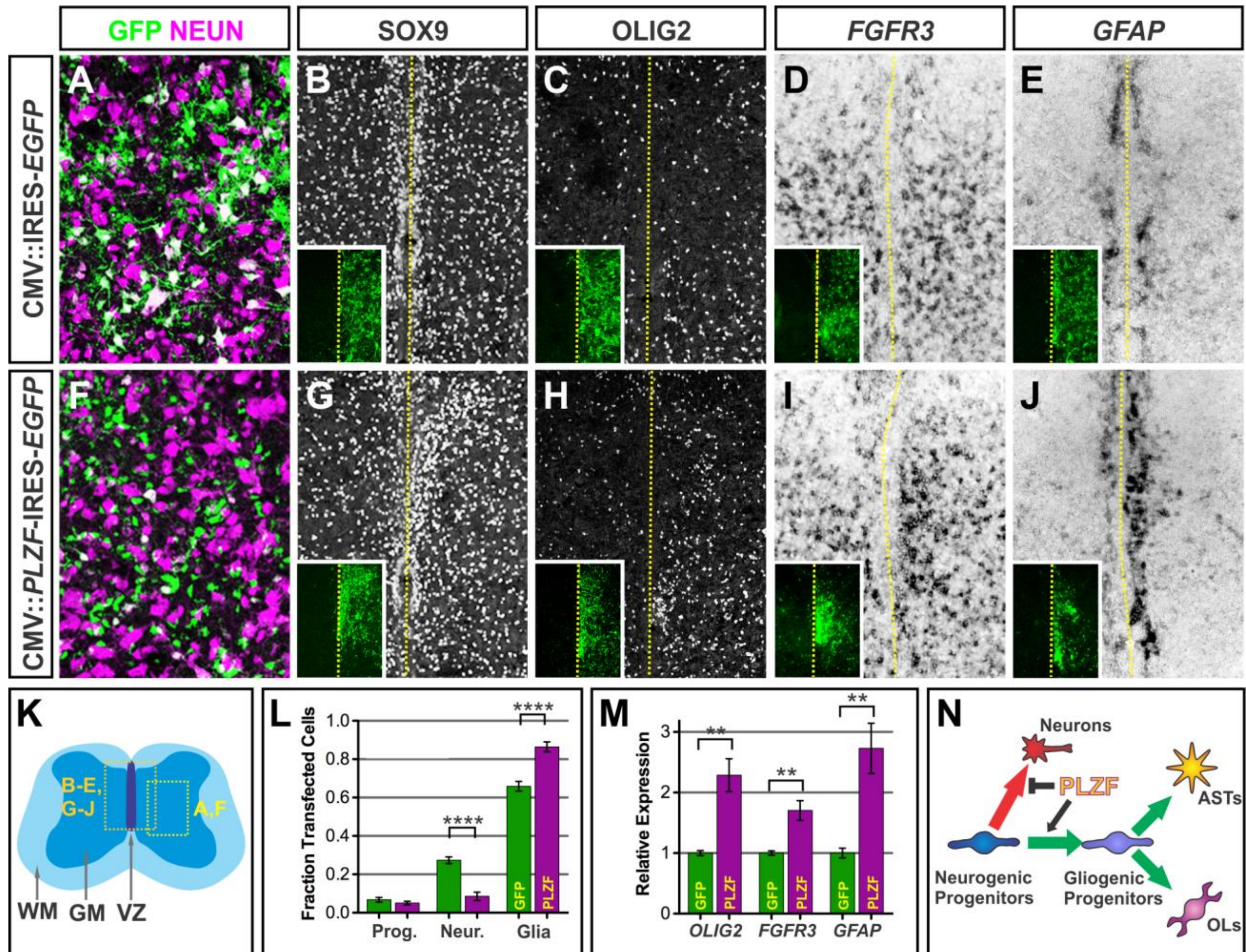


Figure 2-4 - Sustained PLZF misexpression promotes gliogenesis.

(A, B, F, G) Stable transfection of NPCs with PLZF expression plasmids at e3 directs most cells to form $SOX9^{+}$ glial progenitors when analyzed at e15 instead of $NEUN^{+}$ neurons. The frequency of cells expressing the undifferentiated NPC marker NESTIN is unchanged. Yellow lines indicate the midline of the spinal cord.

(L) Chart displays the mean fraction of control and PLZF-transfected cells expressing these markers ± SEM.

(C-E, H-J) Sustained PLZF expression enhances the formation of $OLIG2^{+}$ oligodendrocyte progenitors, $FGFR3^{+}$ astrocyte progenitors, and $GFAP^{+}$ astrocytes.

(M) Chart displays the mean number of PLZF-transfected cells expressing the indicated markers ± SEM relative to cells electroporated with the empty control vector.

Transfected cell counts were based on at least 10 images taken from ≥ 8 electroporated embryos. In all panels, ** = $p < 0.01$ and **** = $p < 0.0001$.

(K) Location of panels A-J within the e15 spinal cord. WM, white matter; GM, grey matter; VZ ventricular zone.

(N) Schematic model depicting the suppressive effects of PLZF on neurogenesis and enhancement of gliogenesis.

Figure 2-5

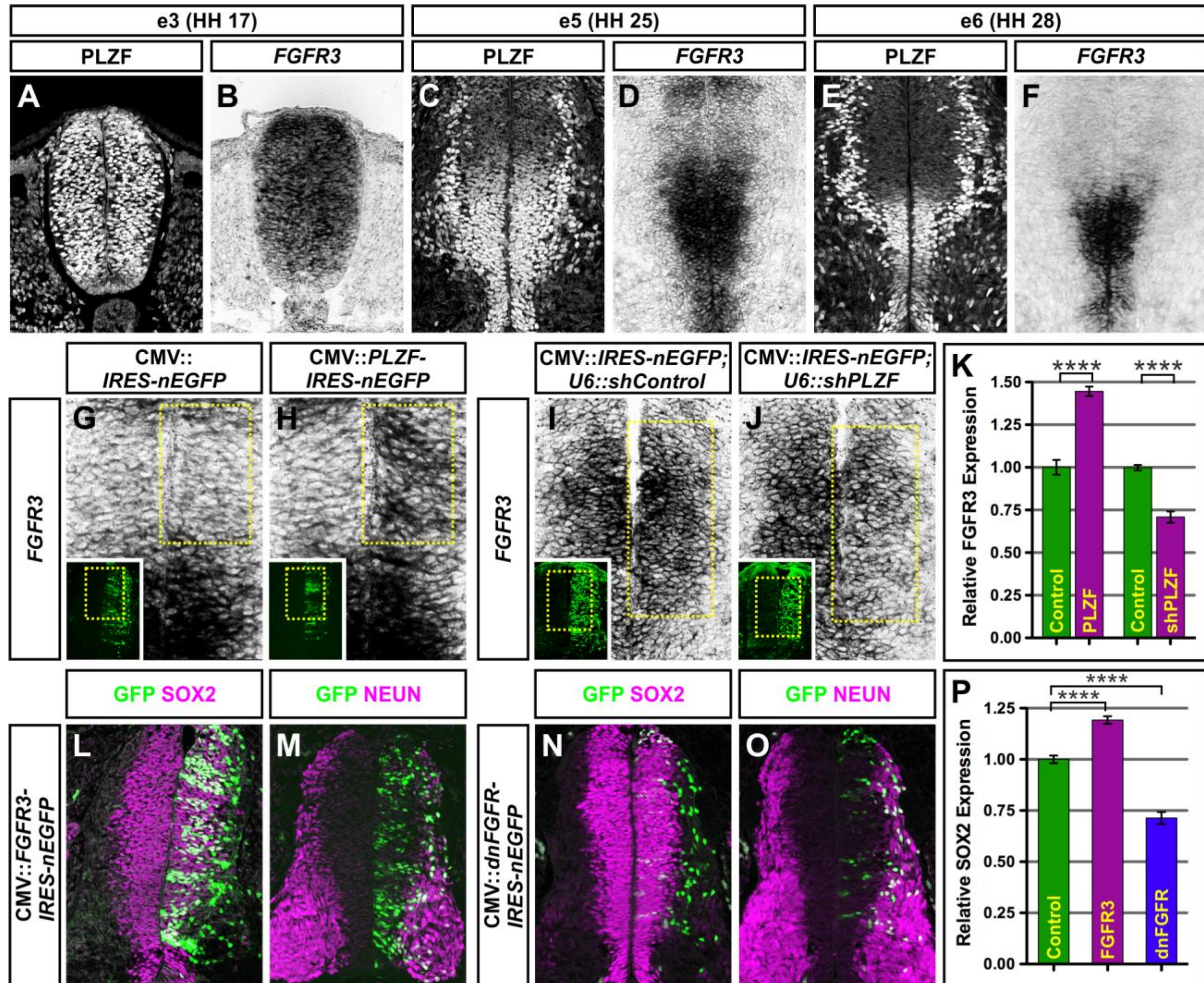


Figure 2-5 - PLZF gates the abundance of FGFR3, which is critical for neural progenitor maintenance.

(A-F) The pattern of PLZF expression closely matches that of *FGFR3* in the developing spinal cord.

(G-J) PLZF misexpression is sufficient to induce the ectopic expression of *FGFR3* in the dorsal spinal cord, while PLZF knockdown reduces FGFR3 expression in the intermediate spinal cord.

(K) Chart displays the mean level of *FGFR3* mRNA ± SEM in spinal cords electroporated with the indicated constructs relative to the contralateral control sides.

(L, M) NPCs transfected with FGFR3 expression plasmids display an increased propensity for SOX2 expression and reduced expression of NEUN.

(N, O) Disruption of endogenous FGFR3 function through the expression of a dominant negative FGFR promotes the formation of $NEUN^+$ neurons.

(P) Chart displays the mean number of cells expressing SOX2 ± SEM among the indicated experimental conditions, relative to empty vector controls.

Counts were based on at least 12 images taken from ≥ 8 electroporated embryos. **** = $p < 0.0001$.

Figure 2-6

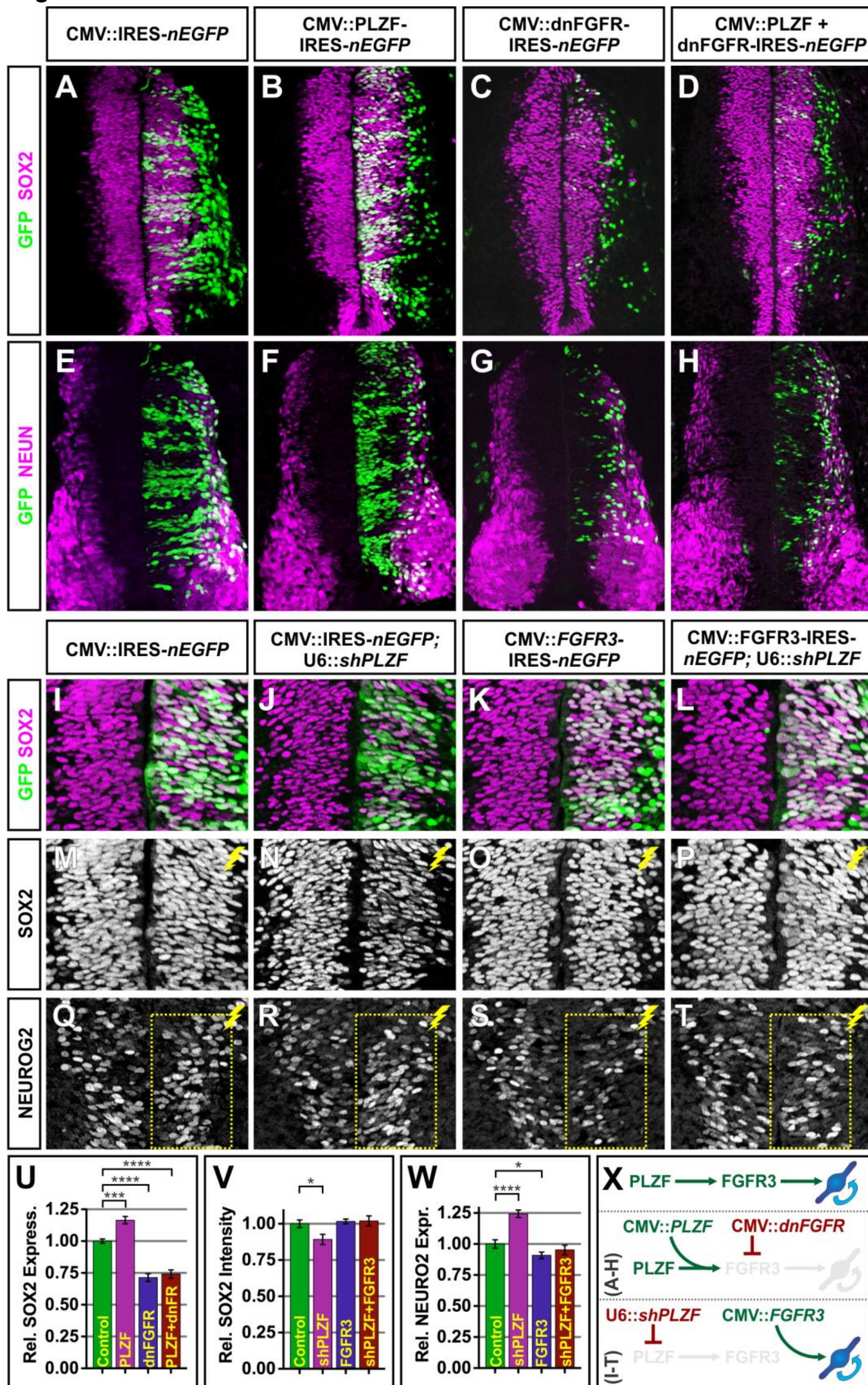


Figure 2-6 - FGFR3 expression and activity is epistatic to PLZF.

(A-H) The ability of ectopic PLZF to hold cells in a $SOX2^{+}$ progenitor state and suppress neurogenesis is blocked by the coexpression of dnFGFR.

(I-T) The reduced intensity of SOX2 expression and increased numbers of cells expressing NEUROG2 following PLZF knockdown are restored by the coexpression of FGFR3.

(U, W) Charts display the mean number of cells expressing SOX2 or NEUROG2 ± SEM between the indicated experimental conditions relative to empty vector controls.

(V) Chart displays the mean pixel intensity of SOX2 staining ± SEM relative to empty vector controls.

Counts were based on at least 12 images taken from ≥ 8 electroporated embryos. * = $p < 0.05$, *** = $p < 0.001$, and **** = $p < 0.0001$.

(X) Summary of the epistasis tests used to show that FGFR3 acts downstream of PLZF.

Figure 2-7

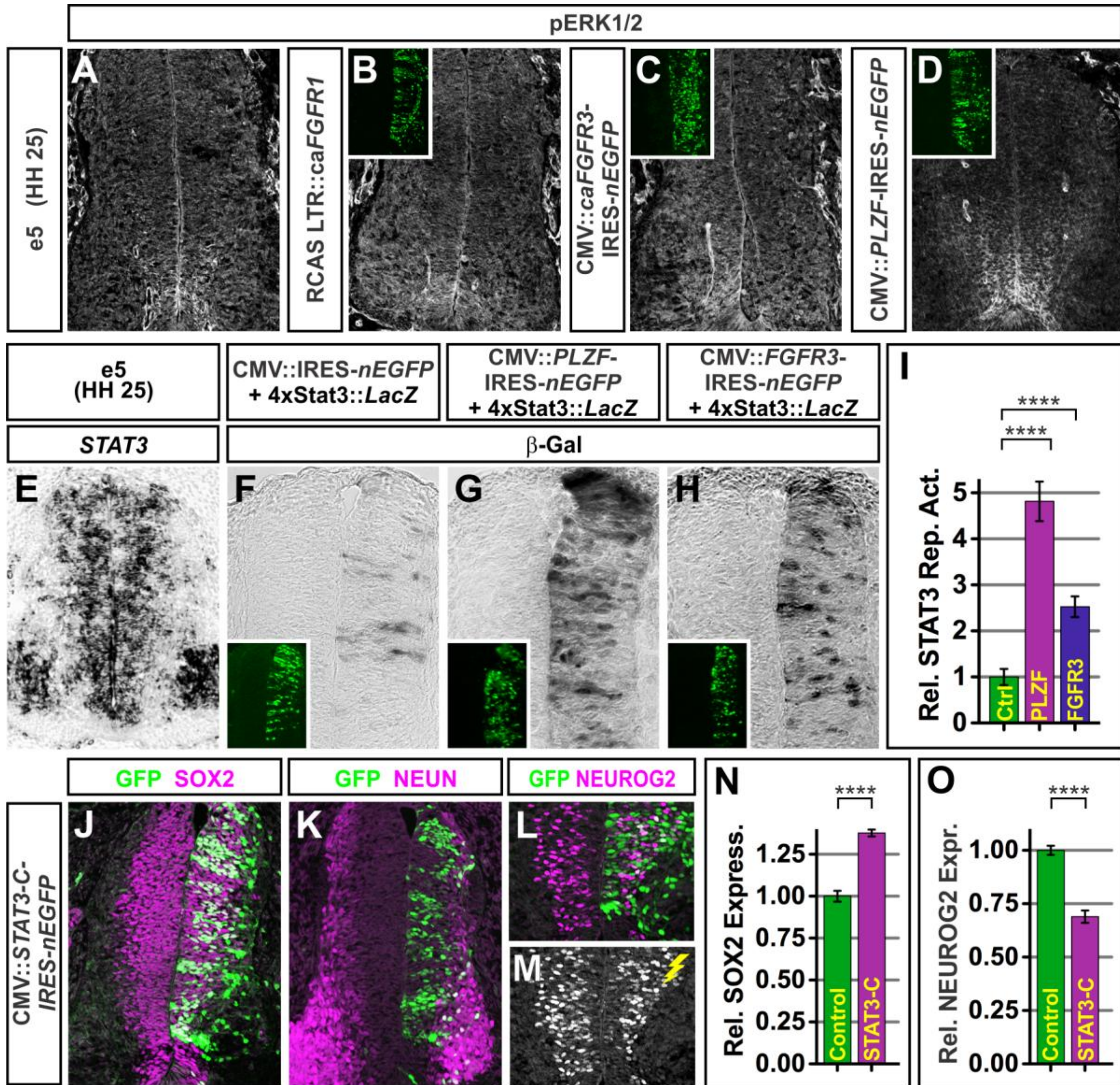


Figure 2-7 - PLZF and FGFR3 promote NPC maintenance through the STAT3 pathway.

(A-D) ERK1/2 phosphorylation is not observed in the central spinal cord of wild type embryos or those electroporated with expression constructs producing constitutively active (ca) FGFR1, caFGFR3, or PLZF.

(E) *STAT3* is expressed throughout the VZ of the e5 chick spinal cord.

(F-I) Both PLZF and FGFR3 misexpression increase the activity of a cotransfected STAT3 responsive-LacZ reporter construct, suggesting that elevated FGF signaling can stimulate the activity of the STAT3 pathway. Results in (I) are represented as the mean activity of the STAT3-LacZ reporter ± SEM seen following PLZF or FGFR3 misexpression, relative to the activity of the reporter transfected with control plasmids.

(J-O) The ectopic expression of constitutively active STAT3 (STAT3-C) produces a block in differentiation comparable to that seen with PLZF and FGFR3. Charts display the mean expression of SOX2 and NEUROG2 ± SEM in STAT3-C transfected spinal cord relative to empty vector controls.

Counts were based on at least 10 images taken from 8-10 electroporated embryos. **** = $p < 0.0001$.

Figure 2-8

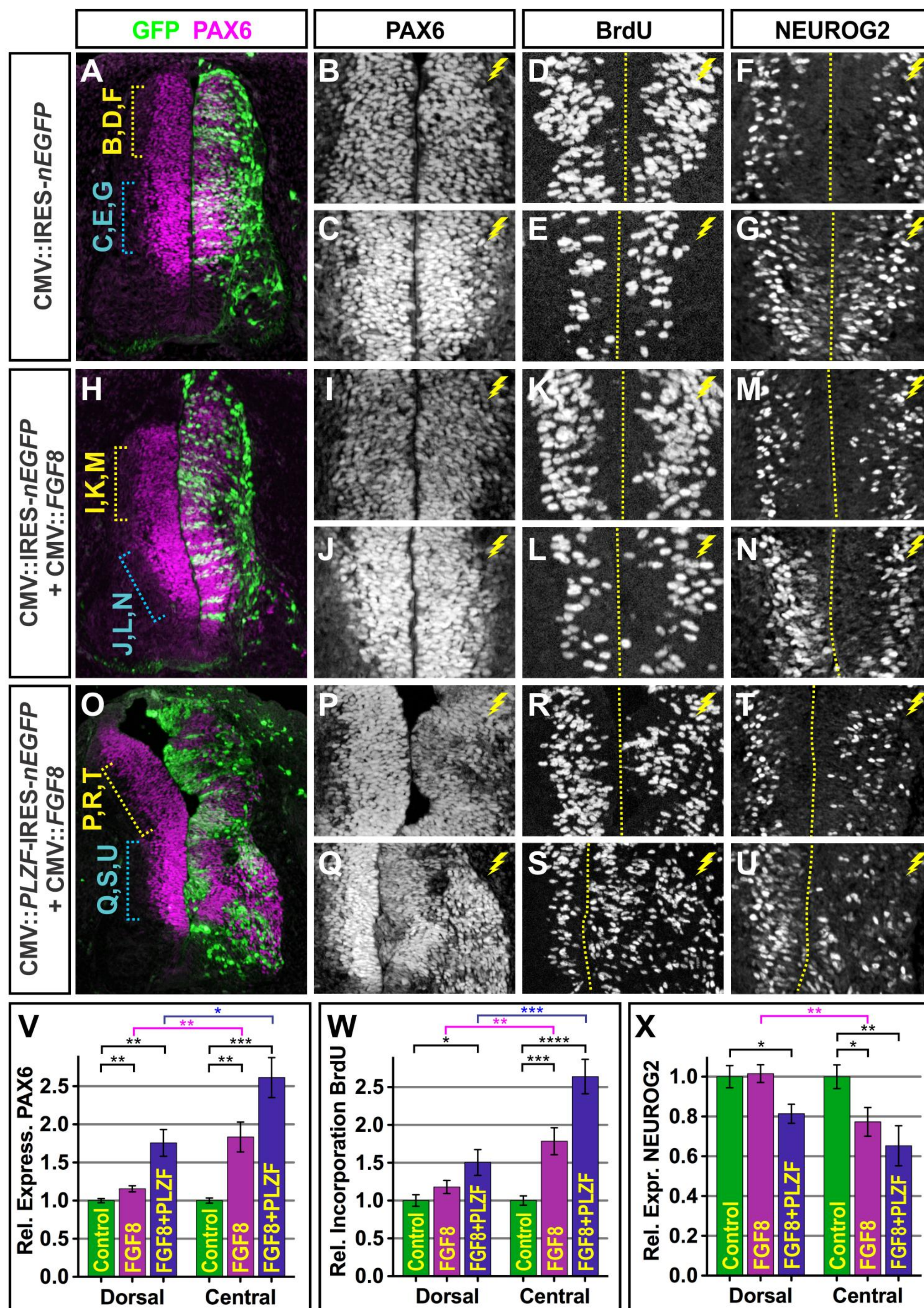


Figure 2-8 - The PLZF-positive central domain of the spinal cord exhibits heightened sensitivity to FGFs.

(A-N) Electroporation of FGF8 expression plasmids elicits a heightened progenitor proliferation response and reduced neurogenesis in the $PLZF^{+}$ $FGFR3^{+}$ $PAX6^{high}$ central region of the spinal cord (yellow brackets) relative to the $PLZF^{-}$ $FGFR3^{-}$ $PAX6^{low}$ dorsal spinal cord (blue brackets).

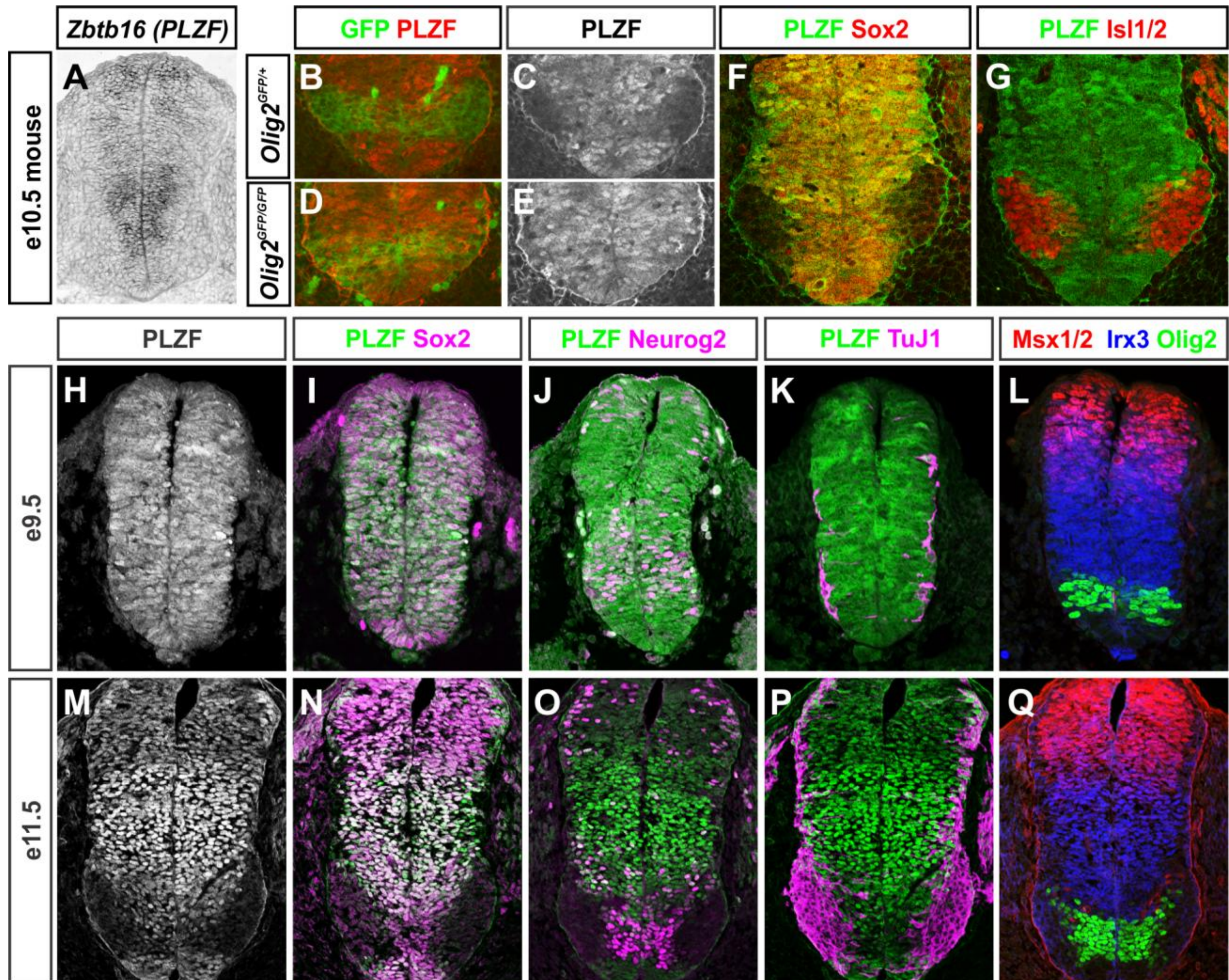
(O-U) Coexpression of FGF8 with PLZF further increases progenitor proliferation and decreases neuronal differentiation.

(V-X) Charts indicate the mean number of cells ± SEM expressing the indicated markers following transfection with FGF8 plasmids alone or in combination with PLZF, relative to empty vector controls.

Counts were based on at least 10 images taken from ≥8 electroporated embryos. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, and **** = $p < 0.0001$.

(Y) Summary model depicting the regional differences between $PLZF^{+}$ $\mathit{FGFR3}^{high}$ neural progenitors in the central spinal cord which exhibit a heightened responsiveness to FGF stimulation compared to $PLZF^{-}$ $\mathit{FGFR3}^{low}$ progenitors in the ventral and dorsal spinal cord.

Supplemental Figure 2-S1

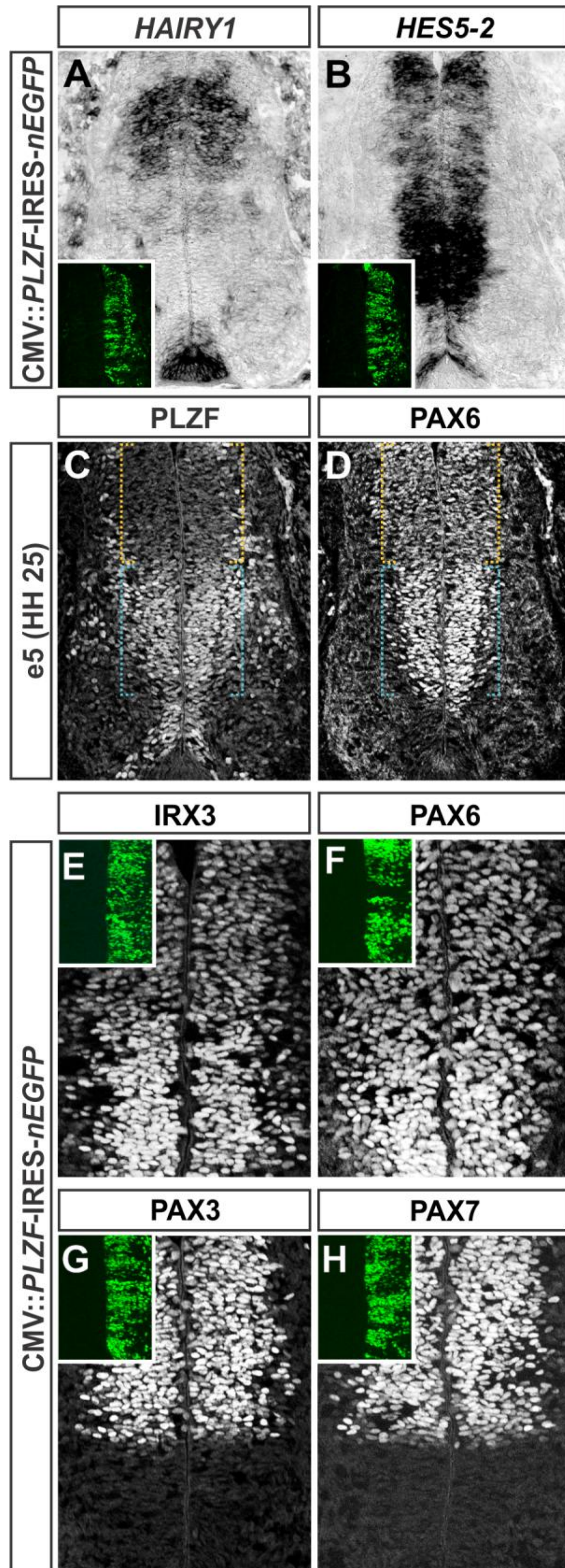


Supplemental Figure 2-S1 - PLZF is increased in Olig2 mutant mice and demarcates neural progenitors in the developing mouse spinal cord.

(A, F, G) Expression of *Zbtb16* mRNA and PLZF protein in wild-type e10.5 mice. PLZF is broadly expressed by $Sox2^{+}$ progenitors and absent from differentiated $Isl1/2^{+}$ motor neurons.

(B-E) PLZF expression is elevated in the ventral spinal cord of e10.5 *Olig2*$^{GFP/GFP}$ mice.

(H-Q) Analysis of wild-type mouse embryos at e9.5 and e11.5 shows that the pattern of PLZF expression is similar to that observed in chicken embryos. PLZF is initially expressed by all $Sox2^{+}$ progenitors and then becomes restricted to a central domain bordered by Msx1 and Olig2 expression. PLZF is subsequently down-regulated as cells differentiate into $TuJ1^{+}$ neurons.



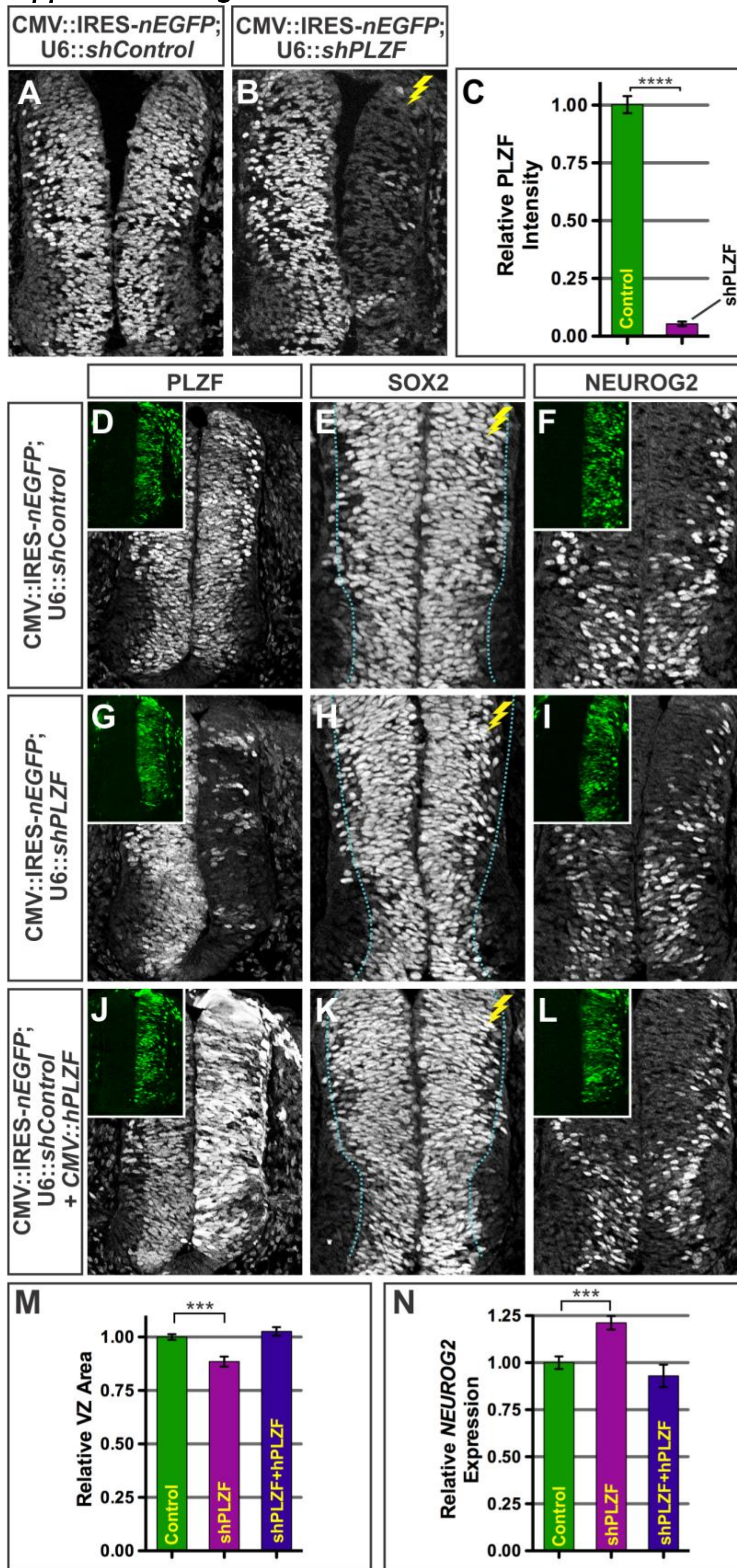
Supplemental Figure 2-S2 - PLZF misexpression does not lead to changes in HES gene expression or dorsoventral pattern.

(A-B) Spinal cords transfected with PLZF did not exhibit any significant alteration in the mRNA expression of two of the principal Notch effector genes, *HAIRY1* and *HES5-2*. Insets show the extent of transfection in the corresponding sections marked by the presence of nEGFP protein.

(C-D) PLZF^{+} cells in the intermediate spinal cord of e5 chick embryos express high levels of PAX6 protein (blue brackets). However, PLZF is largely absent from dorsal progenitors that express low levels of PAX6 protein (yellow brackets).

(E-H) PLZF misexpression does not alter the expression of the homeodomain proteins IRX3, PAX6, PAX3, or PAX7 that demarcate the boundaries of progenitor domains in the developing spinal cord. Electroporations were carried out at e3 and collected for analysis on e5.

Supplemental Figure 2-S3



Supplemental Figure 2-S3 - PLZF knockdown can be rescued by the coexpression of human PLZF.

(A-C) Electroporation of e3 chick spinal cords with a vector encoding PLZF shRNAs and an IRES-nEGFP transfection marker reduced endogenous PLZF protein expression at e5 by 93.7 ± 1.29%. Chart displays the mean pixel intensity of PLZF antibody staining ± SEM for spinal cords electroporated with the control or PLZF shRNA constructs, relative to PLZF expression on the non-transfected contralateral control sides.

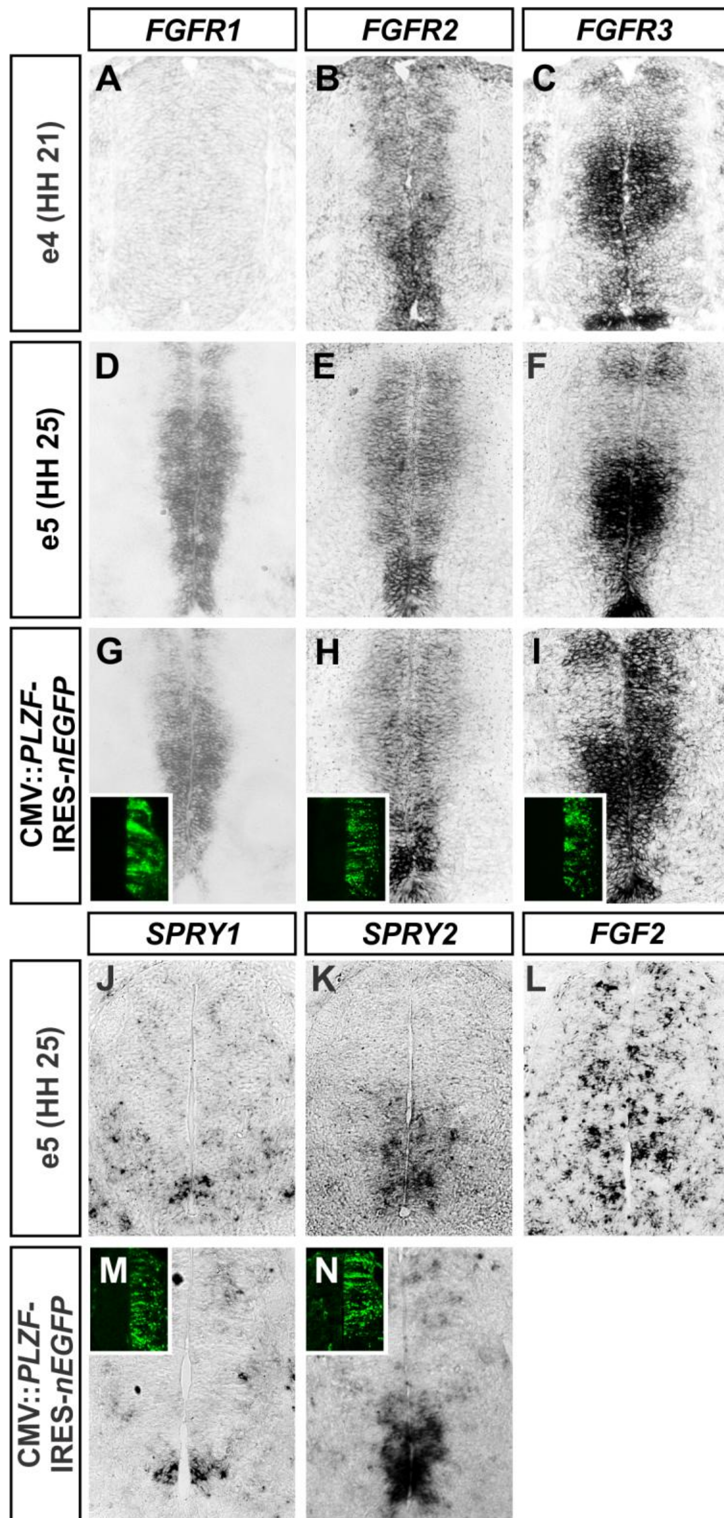
(D-F) Electroporation with a vector producing a non-targeting control shRNA does not alter PLZF, SOX2, or NEUROG2 expression.

(G-L) The effects of PLZF knockdown on SOX2 and NEUROG2 expression are rescued by coelectroporation with an expression construct encoding the human PLZF (*Zbtb16*) gene, which lacks the sites targeted by the shPLZF construct.

(M) Chart displays the mean ventricular zone area ± SEM for embryos electroporated with the indicated plasmids relative to the untransfected contralateral sides of the spinal cord. Blue dotted lines demarcate the border of the contralateral VZ in each image.

(N) Chart displays the mean number of transfected NPCs expressing NEUROG2 ± SEM, relative to empty vector controls.

In all panels, *** = $p < 0.001$ and **** = $p < 0.0001$. Counts were based on at least 12 images taken from ≥ 8 electroporated embryos.



Supplemental Figure 2-S4 - Expression of FGFR and Sprouty genes in the wild type and PLZF-electroporated spinal cord.

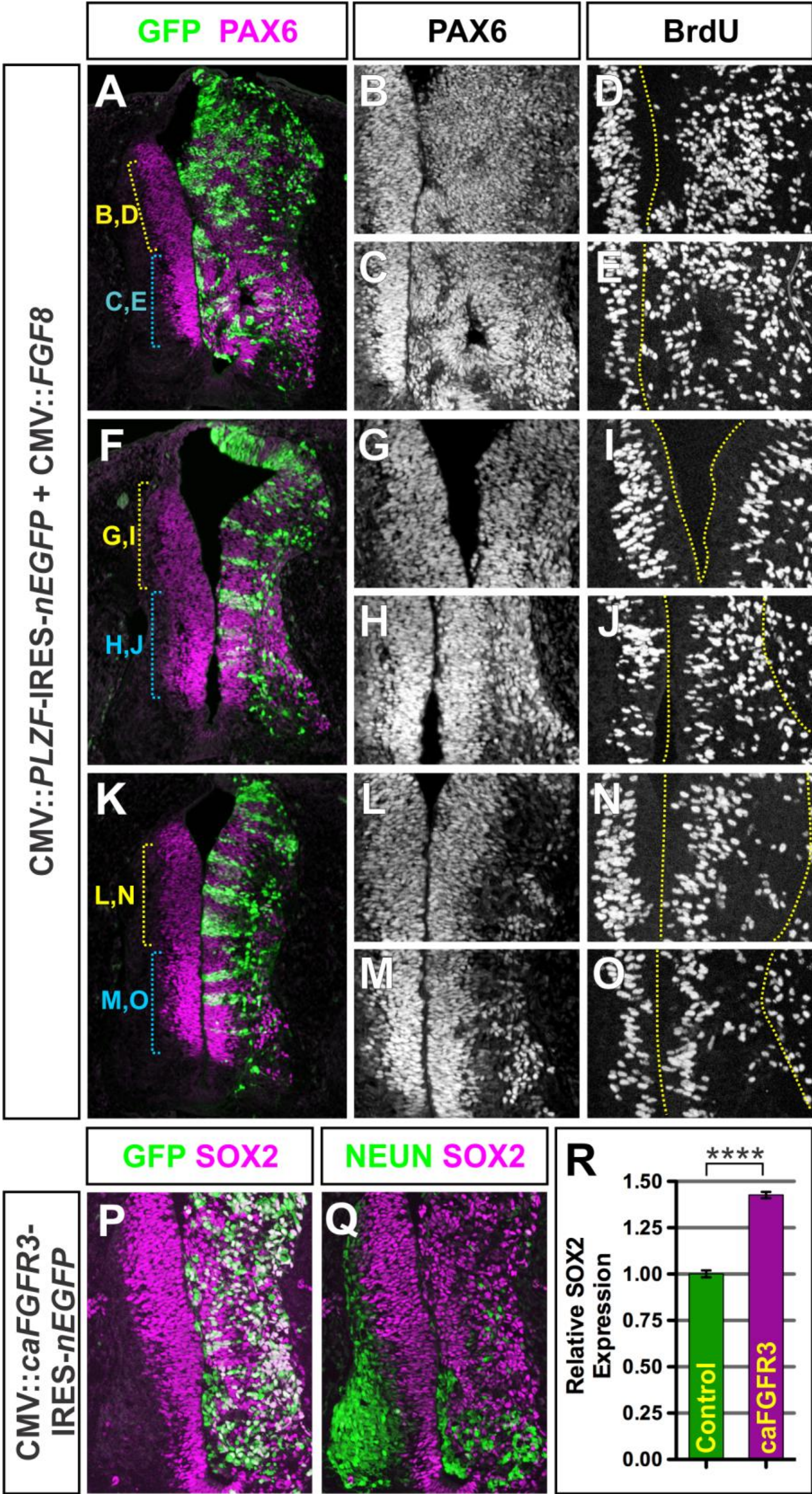
(A-F) Analysis of *FGFR1*, *FGFR2*, and *FGFR3* mRNA expression in e4 and e5 chick spinal cords. FGFR4 was not present in the spinal cord at any stage examined (data not shown).

(G-I) PLZF misexpression at e3 increases *FGFR3* expression in the e5 dorsal spinal cord, but does not alter the expression of either *FGFR1* or *FGFR2*.

(J, K, M, N) At e5, neither *SPRY1* nor *SPRY2* are expressed in the intermediate spinal cord where *FGFR3* levels are normally high (C), nor were they elevated following PLZF misexpression.

(L) *FGF2* mRNA is expressed by scattered cells throughout the e5 chick spinal cord.

Supplemental Figure 2-S5



Supplemental Figure 2-S5 - Coexpression of PLZF and FGF8 disrupts neuronal differentiation in a manner that recapitulates the expression of a constitutively activated form of FGFR3

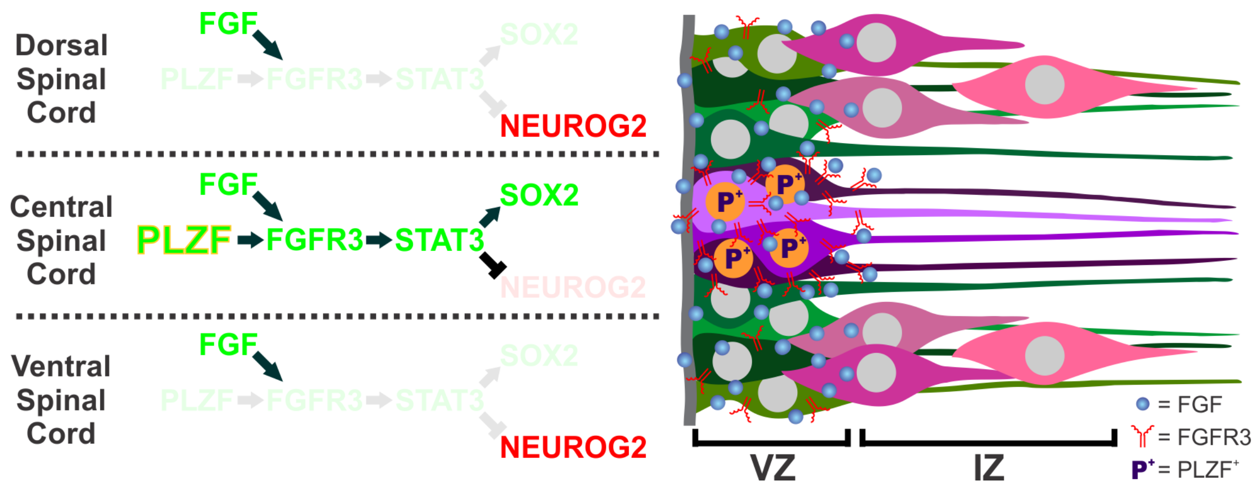
(A-O) The coexpression of PLZF with FGF8 leads to a significant expansion in the VZ marked by PAX6 expression. Effects were seen in both the dorsal spinal cord (yellow brackets and associated panels) and intermediate spinal cord (blue brackets and associated panels). This phenotype was fully penetrant and ranged from moderate (K-O) to extremely severe (A-E).

(P, Q) Misexpression of caFGFR3 increases the proportion of transfected cells expressing SOX2, similar to the effects seen with the concomitant misexpression of PLZF and FGF8 (A).

(R) Chart displays the mean number of caFGFR3-transfected cells expressing SOX2 ± SEM, relative to transfection with a empty control vector. Counts were based on at least 10 images taken from ≥8 embryos. **** = $p < 0.0001$.

2-5 Discussion

Figure 2-9 – Enhancement of FGF receptivity by PLZF in the central spinal cord



A notable feature of spinal cord development is that neurons, and later glia, arise from distinct progenitors domains along the dorsoventral axis of the VZ (Briscoe and Novitch, 2008; Hochstim et al., 2008). As well as having particular spatial characteristics, these progenitor domains have specific rates of proliferation and differentiate on different time schedules. For instance, the proliferative period for motor neuron progenitors is more restricted than that for interneuron progenitors in the intermediate spinal cord. Since the mechanisms that control the schedule on which NPC divide and differentiate are not well understood, we screened for genes that are repressed in pMN, predicting that they might confer the ability of interneuron progenitors to divide differently from motor neuron progenitors. We thereby identified *Zbtb16*, which encodes the BTB-Zinc finger transcription factor, PLZF. PLZF was first identified 20 years ago by its association with leukemia (Chen et al., 1993), and subsequent studies have shown that PLZF plays a critical role in progenitor homeostasis in a variety of tissues (Suliman et al., 2012). Our study identifies a novel activity

for PLZF in the CNS, regulating FGFR3 expression to heighten the responsiveness of NPCs to FGF mitogens present in the embryonic environment (Fig. 2-8Y). PLZF thereby provides a means of regionally tuning the proliferative potential and maintenance of particular progenitor populations to influence the size and shape of the developing nervous system. Moreover, PLZF plays a key role slowing the rate of neurogenesis in the intermediate regions of the spinal cord, thereby sparing a population of NPCs to subsequently differentiate into astrocytes.

PLZF and the transition from neurogenesis to gliogenesis

PLZF is first expressed throughout the VZ during the early phase of NPC expansion, but then becomes strikingly restricted to a central domain of progenitors fated to give rise to ventral interneurons early in development and astrocytes at later times. By manipulating PLZF expression in both chicken and mouse embryos, we found that its function is both necessary and sufficient to suppress neuronal differentiation and permit the emergence of glial progenitors. Although PLZF exhibits pro-glial activity, our data suggest that this function is most likely indirect and related to its effects on progenitor maintenance. For instance, neither misexpression nor knockout of PLZF function appeared to significantly alter the onset of expression for the early glial fate determinants SOX9 and NF1A (Z.B.G. and B.G.N. unpublished observations). Moreover, PLZF misexpression led to a marked increase in the numbers of both astrocyte and oligodendrocyte progenitors, even though PLZF is not normally present in oligodendrocyte progenitors. Lastly, PLZF levels notably decline as astrocyte progenitors begin to differentiate, and the sustained expression of PLZF impedes glial cell maturation. Taken together, these data indicate that the primary role for PLZF is to preserve

the progenitor pool over the course of neurogenesis such that it can acquire competence to give rise to glial cells at later stages of development.

It is notable that manipulation of PLZF activity resulted in consistent, but partial phenotypes. This lack of an absolute necessity for PLZF may stem from functional redundancy among genes of the BTB/POZ family. To date, we have identified five additional family members with expression in the developing spinal cord, suggesting there may be complementary functions with PLZF (Z.B.G. and B.G.N. unpublished observations). A comparable situation exists within the hematopoietic lineage where the lack of a prominent phenotype in either PLZF-null mice or in cell lines transfected with PLZF targeting shRNA is attributed to the presence of other BTB/POZ proteins such as the closely related FAZF (Kelly and Daniel, 2006). The FGF pathway also receives inputs from other signaling networks and has extensive feedback regulatory mechanisms (Guillemot and Zimmer, 2011; Tsang and Dawid, 2004). Thus, the absence of PLZF and reduced expression of FGFR3 could be compensated over time by changes in these modulatory components. Alternatively, the rather mild loss of function phenotypes seen in the nervous system may reflect the subtlety by which PLZF and FGFR3 act to keep cells in a proliferative state. Rather than constituting a simple on/off switch for progenitor maintenance, PLZF and FGFR3 finely sculpt the timing of neuronal differentiation and proportions of neurons formed to shape the functionality of neural circuits.

PLZF heightens the response of neural progenitors to FGFs

The growth and morphogenesis of the nervous system depends upon the ability of the FGFs to promote the rapid proliferation of NPCs and block neuronal differentiation (Akai et

al., 2005; Delfino-Machin et al., 2005). FGF8 is initially expressed throughout the neural plate but then becomes progressively restricted to the adjacent paraxial mesoderm and notochord (Diez del Corral et al., 2003; Novitch et al., 2003; Stolte et al., 2002). FGF2 is also expressed first in low levels by the notochord, but is ultimately present throughout the VZ of the spinal cord, and within the embryonic cerebrospinal fluid (Martin et al., 2006; Murphy et al., 1994; Stolte et al., 2002). Despite the broad distribution of these FGF mitogens, NPCs in the spinal cord exhibit spatially distinct proliferative responses (Lobjois et al., 2004; Peco et al., 2012). Our findings suggest the differential effects of FGFs may stem, in part, from the regional control of FGFR expression by PLZF.

When FGFR3 levels were increased by misexpression of either PLZF or FGFR3, NPCs continued to proliferate and neuronal differentiation was accordingly blocked. These findings strongly suggest that receptor availability is a limiting factor in NPC proliferation and maintenance (Fig. 2-8Y). This conclusion is further supported by the observation that the *FGFR3*^{high} NPCs in the intermediate spinal cord display a heightened response to ectopically expressed FGF8 compared to their *FGFR3*^{low} dorsal counterparts (Fig. 2-8Y). Regional restriction of FGFR3 expression may also be relevant for ventral progenitors. OLIG2^{+} motor neuron progenitors express low levels of PLZF and FGFR3, and perhaps as a consequence, differentiate earlier than many other progenitor populations in the spinal cord (Figure 8Y; Altman and Bayer, 1984; Novitch et al., 2001). The limited expression of FGFR3 within OLIG2^{+} cells may also explain why these cells exhibit limited stem cell capacities when grown in vitro (Agalliu et al., 2009; Mukouyama et al., 2006), since the culture conditions used for NPC expansion typically rely upon FGFs as the primary mitogenic signal.

While PLZF exhibits a positive effect on *FGFR3* expression, the nature of this interaction

remains unclear. In most studies, the actions of PLZF have been attributed to its ability to bind and silence transcription of target genes by recruiting co-repressor proteins through its amino-terminal BTB domain (Suliman et al., 2012). Nevertheless, recent work has suggested that PLZF can also serve as a transcriptional activator in certain contexts (Doulatov et al., 2009; Hobbs et al., 2010). The mechanism behind this duality in function is currently unknown. The identification of the direct targets of PLZF and the cofactors that it associates with in the developing CNS will thus be an important area for future investigation.

PLZF and FGFR3 activities are mediated through the STAT3 signaling pathway

Within the CNS, FGF signaling is implicated in many steps in neuronal development including neural induction, regional patterning, progenitor expansion, axon outgrowth and guidance, and synaptogenesis (Guillemot and Zimmer, 2011). This broad range of activities raises the question of how such distinct outcomes may be achieved from a common signal? In vertebrates, some of the diversity in response stems from the varying affinities of the 22 FGF ligands for 4 FGFRs, which exist in multiple splice isoforms, as well as interactions between FGFs and FGFRs with particular heparin sulfate proteoglycans present in the extracellular matrix (Guillemot and Zimmer, 2011). By selectively promoting the expression of FGFR3, PLZF could render the central spinal cord particularly sensitive to particular ligands or bias the selection of downstream signaling effectors. Upon ligand binding, FGFRs dimerize and phosphorylate a number of secondary messengers that feed into the ERK/MAPK, AKT/PI3K, PLCγ/PKC, and/or STAT pathways (Guillemot and Zimmer, 2011). It is currently unclear whether the diversity in cellular responses to FGF exposure can be explained simply by the differential activation of one or more of these signaling pathways. Nevertheless, it is clear that cellular responses to FGF are strongly influenced by the presence of particular intrinsic

factors and most likely crosstalk with other environmental signals. For example, in the developing brain, FGF8 exposure can drive cells to adopt a forebrain or midbrain identity depending on whether the cells express the homeodomain transcription factors SIX3 or IRX3 (Kobayashi et al., 2002). The situation in the spinal cord is likely similar, with transcription factors such as IRX3 and PLZF not only influencing levels of FGFR expression, but also the manner in which FGF signals are interpreted.

Our data, together with previous studies, further suggest that FGF signaling may utilize distinct transduction pathways at different times in development. During the processes of neural induction, neural tube formation, and early progenitor patterning, FGFs are associated with robust activation of the ERK/MAPK pathway (Akai et al., 2005; Lunn et al., 2007; Stavridis et al., 2007; Z.B.G. unpublished observations). However, during the peak period of neurogenesis in the spinal cord and transition towards gliogenesis, we were unable to detect signs of ERK/MAPK activity even under conditions where constitutively activated FGFR1 or FGFR3 were expressed. Rather, FGFR activation appeared to stimulate the STAT3 pathway. STAT3 forms a prominent node in multiple receptor tyrosine kinase and cytokine signaling pathways, and its activation can result in wide range of effects including NPC maintenance and gliogenesis (Rajan, 2011). During early CNS development, STAT3 promotes SOX2 expression, and disruption of its activity can impair the emergence of NESTIN^{+} NPCs from embryonic stem cells differentiated in vitro (Foshay and Gallicano, 2008). Additionally, deletion of STAT3 from the developing mouse neocortex results in premature and excessive neuronal differentiation (Yoshimatsu et al., 2006). Our data suggest that the ability of STAT3 to regulate SOX2 might be similarly utilized by PLZF and FGFR3 in the spinal cord. Later in development, STAT3 activity falls under the control of additional inputs, most notably the CNTF signaling pathway, and its function plays a critical role in regulating the onset of

astrocyte differentiation (Bonni et al., 1997). It is notable that the PLZF-expressing progenitors in the intermediate spinal cord are ultimately fated to give rise to astrocytes, raising the possibility that the early employment of STAT3 for progenitor maintenance may predispose those progenitors to assume an astroglial fate at later time through the continued activation of the STAT3 pathway.

Thus, PLZF regulates a downstream response to FGFs signaling distinct from the earlier role of FGFs promoting the rapid proliferation of the neural tube. This result suggests more nuance in the pathways downstream from FGF signaling than previously appreciated. However, it remains an open question as to how the shift in principal mode of FGF output between is effected. The genetic and signaling context in which FGF acts changes significantly between the neural plate and neural fold where the MAPK/ERK pathway predominates and the neurogenic neural tube that appears to rely more on STAT signaling. Of particular interest is the onset of retinoic acid signaling from the somites, a signal that opposes the FGF block on the expression of the Class I and Class II homeodomain proteins that pattern the neural tube (Novitch et al., 2003). Retinoic acid also inhibits the expression of FGF8, while other factors begin inducing the expression of FGF2 at this time (Diez del Corral et al., 2003; Murphy et al., 1994). Another change that occurs during this stage is a major shift in the expression of FGFRs. In the neural plate and neural fold stages, FGFR1 is the most highly and broadly expressed FGFR (Lunn et al., 2007), whereas during spinal neurogenesis, FGFR3 is the predominant receptor (Fig. 2-5 A-F, 2S4 A-I).

Any one of the aforementioned processes might be sufficient to alter how cells respond to FGF signaling. Firstly, the activation of homeodomain proteins may dramatically alter FGF activity. It has been shown at the forebrain/midbrain boundary that the differentiation

programs induced by FGFs was determined by the presence of homeodomain proteins such as IRX3 (Kobayashi et al., 2002), one of the Class II proteins induced in the neural tube. Secondly, the shift from FGF8 to FGF2 ligand or FGFR1 to FGFR3 receptor might potentially influence pathway choice. Although it is generally thought that ligands and receptors differ primarily in their affinities for one another and the relative strengths of their kinase domains, as discussed in Chapter 1-5, there are many suggestions in the literature that different ligands and receptors may induce significantly different effects.

An intriguing third possibility is that there is a change in the subcellular localization of FGF signaling. Although the FGFR signaling complex is generally assumed to form at the cell membrane, there is increasing evidence that in some contexts, receptor tyrosine kinases such as FGFR3 are internalized and trafficked to intracellular compartments. This appears to be particularly in cases where the induction of STAT signaling is at comparatively low levels. In the context of the HGF/C-MET receptor tyrosine kinase pathway, the translocation of phosphorylated STAT3 protein to the nucleus was found to be dependent upon the endocytosis of activated C-MET receptor (Kermorgant and Parker, 2008). Activating mutations in the receptor tyrosine kinase FLT3 have been linked with localization to the endoplasmic reticulum and a resulting decreased ability to signal through the MAPK and PI3K pathways but an increased ability to activate STAT5 (Choudhary et al., 2009). Similar findings have been made for oncogenic mutations in FGFR3 being localized to the endoplasmic reticulum with a corresponding increase in STAT activation (Lievens et al., 2004). In another study, a GFP protein was fused to the C-terminus of FGFR3. It was found in HEK293T cells in the absence of ligand, FGFR3-GFP localized to the plasma membrane. However, when cells were treated with FGF, FGFR3-GFP was rapidly internalized (Ben-Zvi et al., 2006). These

findings suggest that the distinction between modes of FGF signaling may be partially mediated by localization of activated FGFR to different cellular compartments.

In summary, PLZF and FGFR3 work in parallel with other FGFR, mitogen signaling pathways, and, most likely, other members of the BTB/POZ family, to modulate proliferation in the spinal cord and thereby permit NPCs to differentiate at characteristic rates and times in development. PLZF focuses the mitogenic activity of the FGFs in a STAT3-dependent manner to maintain a specific population of NPCs in a proliferative state and ensure that the necessary number of progenitors is available for the transition from neurogenesis to gliogenesis. Aberrant activation of FGFR3 and STAT3 has been observed in a multitude of human cancers (Frank, 2007; Hart et al., 2000; L'Hote and Knowles, 2005). The identification of PLZF as a critical regulator of FGFR3 and STAT3 activity thus provides important new insights into the mechanisms by which such tumors could arise and offers a novel therapeutic target.

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CHAPTER 3 – The Pro-Neuronal Differentiation Transcription Factor, RP58, Promotes Neural Progenitor Maintenance in the Context of Spinal Development

ABSTRACT

Although there are approximately 60 BTB-ZnF proteins in the mammalian genome, only a relative handful have undergone extensive characterization in a limited number of processes such as hematopoiesis and spermatogenesis. In the context of both tissues it is now apparent that their development is regulated by the overlapping and complementary activities of multiple members of the BTB-ZnF family. This propensity for overlapping activity and the identification of a single BTB-ZnF protein, PLZF, as being critical for the proper maintenance of a central domain of spinal neural progenitor cells led us to investigate whether other members of this gene family might also be involved in regulating spinal development. During the course of this study, we identified an additional five BTB-ZnF transcription factors as being expressed during spinal development and went on to characterize the activity of one of the, RP58. We found that RP58, like PLZF, acts to preserve spinal neural progenitor cells against premature differentiation during neurogenesis. However, this finding disagrees with much of the established literature regarding RP58 as being a pro-differentiation factor during the development of multiple regions of the brain. We therefore propose a model of RP58 activity that seeks to explain how a single transcription factor, regulating a common set of genes in both contexts, can still manifest radically divergent developmental activities.

3-1 Introduction

RP58 Structure and Binding Partners

In many ways, the state of knowledge of the BTB-ZnF transcription factor RP58 is the inverse to that of PLZF: limited understanding of the molecular action of the RP58 protein but a burgeoning literature on its function within the central nervous system. RP58[9] was first discovered in a yeast-two-hybrid assay for binding partners for the RNA binding protein translin and was subsequently identified as a novel BTB-ZnF transcription factor. RP58's structure is typical for members of the BTB-ZnF family with an N-terminal BTB domain and four C-terminal zinc finger motifs that have been found to recognize and bind the consensus sequence (A/C)ACATCTG(G/T)(A/C) (Aoki et al., 1998). This sequence is sufficiently distinct from PLZF that it is unlikely for the two proteins to act upon the same DNA targets. RP58 contains a putative nuclear localization signal immediately C-terminal of its BTB domain (Aoki et al., 1998) and has been shown to localize to the nucleus both *in vivo* and in several cell lines (Aoki et al., 1998; Okado et al., 2009; Takahashi et al., 2008). To date, all studies of RP58 activity have found it to act as a transcriptional repressor (Aoki et al., 1998; Fuks et al., 2001; Yokoyama et al., 2009), although context and co-factor dependent changes in activity cannot be ruled out.

At present, only three binding partners have been identified for RP58. Firstly, RP58 was identified due to its ability to bind the RNA trafficking protein translin (Aoki et al., 1998).

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[9] A note on nomenclature. The official gene symbols for RP58 in most species is Znf238, the notable exceptions being in mice and rats where the symbol is Zfp238. These labels are frequently used in the literature, although RP58 remains the preferred term used in most publications investigating this gene in the CNS. The term RP58 derives from the proteins repressor activity and its 58kDa mass. This gene is also occasionally identified as TAZ-1 or ZBTB18.

Both RP58 and translin are highly expressed in many populations of neurons. Translin has been found to assist in the trafficking of RNA towards the dendritic arbors (Finkenstadt et al., 2000), but the significance of its interaction with RP58 is unclear for either protein. Secondly, RP58 has been found able to bind and to have its capacity for repression enhanced by the chromatin silencing DNA methyltransferases DNMT3a and DNMT3b. This interaction involves a region immediately N-terminal of RP58's DNA binding domain and encompassing RP58's first zinc finger (Fuks et al., 2001), a region notably not significantly conserved with PLZF and which a BLAST analysis indicates may potentially be unique to RP58 (Z. Gaber, unpublished data). Thirdly, RP58 has been argued to bind a highly similar BTB-ZnF protein dubbed simiRP58. simiRP58's BTB domain is closely conserved with that of RP58 save for the absence of the putative nuclear localization signal. The interaction between these proteins is inferred from the movement of simiRP58 from its normal location in the cytoplasm in the absence of RP58 to the nucleus in the presence of RP58 (Takahashi et al., 2008). While this presumed dimerization is highly probable given and the known potential for BTB proteins to heterodimerize, this binding has not been directly tested and its biological significance remains undemonstrated. Regardless, it is intriguing to speculate that switching between RP58 homodimers and RP58-simiRP58 heterodimers may represent an important mechanism for directing distinct modes of RP58 activity.

RP58 and bHLH proteins

RP58 has been found to be intimately connected with bHLH family transcription factors in both muscle and neural tissues. In a survey where 6,049 cDNA clones were co-misexpressed with a luciferase reporter linked to a 1.6kb region of the RP58 reporter, 4 clones were found to be capable of inducing reporter expression: the bHLH transcription factors MYOD,

NEUROD1, NEUROG1, and NEUROG2 (Yokoyama et al., 2009). RP58 has been also identified in a screen for potential targets of the proneural bHLH protein ATOH1 in the cerebellum, although this has yet to be directly tested (Klisch et al., 2011). Furthermore, analysis of evolutionarily conserved elements upstream of the RP58 locus have identified multiple potential bHLH binding sites (CANNTG and designated E-boxes), many of which either the pro-myogenesis factor MYOD or the pro-neurogenesis factors NEUROD1 and NEUROG2 have been found capable of binding to and inducing expression from *in vitro* (Ohtaka-Maruyama et al., 2012; Seo et al., 2007; Yokoyama et al., 2009). In the developing mouse telencephalon, *in utero* transfection with NEUROG2 has been found to be sufficient to upregulate RP58 expression whereas RP58 expression is correspondingly reduced in *Neurog2*$^{-/-}$ animals (Gohlke et al., 2008).

There is also significant evidence that the interaction between RP58 and bHLH proteins is not unidirectional. RP58 has also been found to directly bind and repress conserved enhancers within the NEUROD1 and NEUROG2 loci (Xiang et al., 2012). Conversely, RP58 has been found capable of directly inhibiting all four members of the ID family, proteins that disrupt the activity of pro-differentiation bHLH proteins by sequestering their E-protein cofactors (Hirai et al., 2012; Yokoyama et al., 2009). Lastly, given the intimate connection between RP58 and the bHLH family, it is striking to note that RP58's own DNA binding consensus sequence – (A/C)ACATCTG(G/T)(A/C) – contains in its core an E-box (Aoki et al., 1998). While it has yet to be demonstrated, this may potentially suggest that competition between RP58 and bHLH proteins for common binding sites could constitute an additional layer of interaction.

RP58 in muscle development

The significance of these regulatory interactions between RP58 and bHLH proteins becomes readily apparent when reviewing what is known about the function of RP58 in development. RP58 knockout animals have been generated (Okado et al., 2009) and assessed with regards to both skeletal muscle and brain development. RP58 deficient mice die at birth and exhibit severe defects in skeletal muscle formation. Muscle tissue is generated but fails to differentiate into multinucleated myofibers, persisting instead as immature mononucleated fibrils (Yokoyama et al., 2009). The severe reduction in muscle-mass, particularly in the case of the diaphragm, is potentially the proximal cause of death of these animals. In this context, it has been convincingly demonstrated that RP58 constitutes an important pro-myogenesis factor, immediately downstream of the crucial muscle determinant MYOD. In this model, MYOD induces the expression of RP58 in early myogenic precursors. RP58 then inhibits the expression of the anti-differentiation factors ID2 and ID3, thereby removing a potent block on the activity of both MYOD and other downstream, pro-differentiation bHLH proteins such as MYOG and MRF4. Strongly supporting this model was the finding that MYOD's ability to convert 10T1/2 fibroblasts into muscle cells was severely reduced in cells deficient for RP58 and that this inability could be compensated for by knocking down ID2 and ID3 (Yokoyama et al., 2009).

RP58 in cortex development

Significant data suggest a similar mode of RP58 activity in the developing brain. RP58 is highly expressed in multiple regions of the mouse brain from e10 onwards, regions including the amygdala, hippocampus, dorsal thalamus, and hypothalamus (Ohtaka-Maruyama et al.,

2007). However, its functions have been best characterized in the cerebral cortex and the cerebellum and this summary will concentrate on these regions.

Briefly, the developing cortex is a multilayered structure. Proliferative radial glia (RG) are situated within the medial-most layer, the ventricular zone (VZ). The RG have a significant capacity for self-renewal and the ability to differentiate either neurons or a transitly amplifying population of intermediate progenitors (IMs). The IM's migrate laterally, along the RGs' cortex-spanning processes and take up positions within the subventricular zone (SVZ). Eventually, the IM's undergo terminal neuronal differentiation and proceed to migrate further latterly along the process of the RG, through the intermediate zone (IZ), until they take up their ultimate position within one of the sequentially generated layers of the cortical plate (CP) (Lui et al., 2011).

In situ hybridization (Ohtaka-Maruyama et al., 2007) and qPCR analysis of sorted cell populations (Xiang et al., 2012) reveal a gradient of increasing of RP58 expression as cortical neuronal differentiation proceeds. RP58 is first expressed at low levels among a subset of cells within the VZ (Okado et al., 2009). These cells exhibit a reduced expression of the undifferentiated NPC marker SOX2, a "pin-shaped" rather than radial morphology (Ohtaka-Maruyama et al., 2012), and frequently express TBR2, all of which indicate that RP58 in the VZ labels cells differentiating into IPs (Okado et al., 2009). High RP58 expression is first detected within the SVZ, where RP58 appears to be expressed by most if not all IPs. Expression increases as cells undergo neuronal differentiation and migrate laterally (Ohtaka-Maruyama et al., 2007; Okado et al., 2009; Xiang et al., 2012). Within the cortical plate, RP58 does not appear to be expressed by either GABAergic neurons or later-born astrocytes

but rather solely by glutamatergic neurons (Hirai et al., 2012; Ohtaka-Maruyama et al., 2007; Okado et al., 2009).

Complete knockouts and conditional neural knockouts for RP58 have been generated and cortical development has been found to be comparably perturbed in both mouse lines. Heterozygous mice are indistinguishable from wild type littermates, but mutant cortices exhibit severe hypoplasia that appears derive from reduced neuronal differentiation (Okado et al., 2009; Xiang et al., 2012). The VZ and SVZ are both observed to be greatly expanded while there is a broad reduction in numbers among all populations of neuronal progeny assessed. Strikingly, not only are cells failing to differentiate as frequently, those that do often fail to down-regulate genes characteristic of more immature identities. Markers of RG identity such as PAX6, HES5 (Okado et al., 2009), and SOX2 (Hirai et al., 2012) are found expressed by cells within the SVZ or even among supposedly differentiated neurons. In addition, markers of early neuronal differentiation, such as NEUROG2 and NEUROD1, are ectopically expressed by differentiated cells within the cortical plate (Xiang et al., 2012). Collectively, these data suggest that in the absence of RP58 functionality, there are severe defects in neuronal differentiation.

When RP58's expression pattern and mutant phenotype are considered in the context of what is known about regulation of and by RP58, particularly with regards to NEUROG2, much of these data become genetically explicable. RP58 has been found to be inducible by NEUROG2 but also to suppress NEUROG2 expression. Thus, it may be postulated that NEUROG2, acting within cells of the VZ that are maturing into IPs, induces RP58 in order to promote at least some aspects of subsequent neuronal differentiation and that RP58 then silences NEUROG2 to facilitate eventual maturation. When RP58 is lost, NEUROG2's ability to

initiate neurogenesis is severely compromised and even when it occurs, without RP58 there is a reduced ability to silence genes that expressed by differentiating cells but not by mature neurons, such as NEUROG2 itself (Ohtaka-Maruyama et al., 2012; Okado et al., 2009; Xiang et al., 2012).

The broader nature of this genetic circuit is poorly understood. There is significant evidence that, as in muscle, RP58 repression of ID proteins may play a critical role. RP58 mutants have been found to exhibit a premature and excessive degree of astroglial differentiation. This has been attributed to RP58's repression of ID proteins which become upregulated in RP58 mutants. Furthermore, it has been shown that that knocking down ID proteins largely rescues this excess astrogliogenesis whereas misexpressing ID proteins through *in utero* electroporation phenocopies the loss of RP58 (Hirai et al., 2012). However, the relevance of RP58 regulation of ID proteins in the context of neuronal differentiation has not yet been addressed with sufficient directness.

RP58 and cerebellum development

Although there are many significant differences between the developing cortex and cerebellum, the role of RP58 in both is quite similar. Briefly, during cerebellar development, there are two sources of neurons. GABAergic neurons, such as the Purkinje neurons, are derived from the ventricular zone whereas glutamatergic neurons derive from the upper rhombic lip (UPL). In particular, a population of mitotic granule neuron precursors (GNPs) migrates into the cerebellar anlage and undergoes a period of amplification. This layer of proliferating GNPs is called the outer external granule layer (oEGL). Post mitotic, differentiating GNPs migrate medially, to form the inner EGL (iEGL) before transiting further

medially through the molecular and Purkinje layers to take their places as mature granule neurons in the inner granule layer (IGL).

Loss of RP58 from the mouse cerebellum results in severe hypoplasia and a reduction in both VZ derived glutamatergic and URL derived GABAergic neuron formation. The deep projection neurons fail to differentiate within the UPL and instead an expanded pool of $PAX6^{+}$ progenitors is observed (Baubet et al., 2012). In the context of the GNPs that migrate in from the UPL, a developmental gradient of RP58 expression has been observed similar to what has been found in the cortex. RP58 is absent from the outer EGL where mitotic cells are located, is expressed at moderate levels by the post-mitotic cells of the iEGL, and is highly expressed by the mature neurons of the IGL. In primary culture of GNPs derived from *RP58*$^{-/-}$ mice, there is a dramatic reduction in the frequency of neuronal differentiation (Tatard et al., 2010) and in RP58 knockouts the IGL is significantly reduced (Baubet et al., 2012). As in the cortex, loss of RP58 results in persistence of proneural bHLH expression in a significant subset of differentiated neurons, again suggesting that RP58 functions in large part to repress the expression of proneural bHLH proteins during neuronal differentiation (Baubet et al., 2012).

Thus, RP58 has consistently and repeatedly identified as a pro-neuronal differentiation transcription factor in multiple regions of the brain. In addition, RP58 has been shown to have a comparable function during muscle development. In this study we report our investigation of the role of RP58 in the developing spinal cord. Unlike what has been previously shown in multiple regions of the brain, we found that RP58 in the spinal cord functions as a potent inhibitor of neurogenesis, an activity similar to that which we previously described for PLZF. However, although RP58 acts to achieve opposing ends in brain and

spinal development, we propose that both aims are achieved in large part through a single mechanism: the repression of proneural bHLH proteins.

3-2 Methods and Materials

All experiments were performed as described in ***Chapter 2-2 Materials and Methods*** except for the additional reagents listed below.

Table 3-1 - Antibodies used for Immunohistochemistry

Antigen	Host Species	Source and References
NKX2.2	Mouse	Developmental Studies Hybridoma Bank (74.5A5), see also (Ericson et al., 1997)
NKX6.1	Mouse	Developmental Studies Hybridoma Bank (F55A10), see also (Pedersen et al., 2006)

Table 3-2 - PCR Primers used to create* in situ *probes.

Probe Target	Forward Primer (5' to 3')	Reverse Primer (5' to 3')
BTBD15	AACGAAAGTGGGGAGATTTTC	GAGATTAACCCTCACTAAAGGGA ACGCATCCTGTTGTTTGTCA
RP58	TGCACTTTAAGGCAGCATGA	GAGATTAACCCTCACTAAAGGGA TCCAGTGGGAACTGTCAGAA
simiRP58	GAAAGTCTGCAAGGGCAAGT	GAGATTAACCCTCACTAAAGGGA GGGCTTGAAAGACAAATCCA
ZBTB7A	CCTCACAGTCAGCACTTCCA	GAGATTAACCCTCACTAAAGGGA CTGAGAAGGCGAGAACAAGG
ZBTB39	CTTCCCCGACCTGGAGAG	GAGATTAACCCTCACTAAAGGGA TGCTCTGCACCTTGTACTGC

Underlined text indicates T3 polymerase binding site.

3-3 Results

Expression of RP58 in the developing spinal cord

To determine whether BTB-ZnF family transcription factors other than PLZF were expressed during spinal development we performed an *in situ* hybridization screen. This screen identified five additional members of the BTB-ZnF transcripts as being present in the embryonic chick spinal cord at e6 (HH 28): ZBTB7A, ZBTB39, BTBD15, RP58, and simiRP58 (Fig. 3-1 A-D). ZBTB7A and ZBTB39 were both ubiquitously expressed within the spinal cord, although ZBTB7A appeared to be more highly expressed within the ventricular zone. BTBD15, RP58, and simiRP58 instead exhibited more regional expression patterns within ventricular progenitors. Strikingly, both RP58 and BTBD15 exhibited patterns of expression reciprocal to that of PLZF. Whereas PLZF is highly expressed within the central region of the ventricular zone and restricted to a subset of differentiating and differentiated neurons flanking the dorsal ventricular zone, RP58 and BTBD15 were expressed in the dorsal ventricular zone and among cells flanking the ventricular zone of the central spinal cord.

Because RP58 had previously been established to play a significant role in neurogenesis in the developing brain, it was decided to explore the function of this gene in more detail. At e3 (HH St 18), both RP58 and PLZF were expressed by all $SOX2^+$ NPCs (Fig. 3-1 E-G). However, RP58 differed from PLZF in that it already exhibited distinctly regional modes of expression, being highly expressed ventrally and moderately expressed dorsally. The region of highest RP58 expression appeared to also highly express NEUROG2 (Fig. 3-1 H), a gene known to induce RP58 expression (Ohtaka-Maruyama et al., 2012; Seo et al., 2007; Yokoyama et al., 2009).

By e4, PLZF also adopts regional modes of expression, being maintained at highest levels primarily in the central $SOX2^+$ NPC domains of the spinal cord (Fig. 3-1 I,J). RP58 remains expressed by all $SOX2^+$ NPCs, but is observed to be enriched ventrally and along the boundary between ventricular and mantle zones where differentiating neurons are often located (Fig. 3-1 I,K). Again, the regions of highest RP58 expression appear to closely match that of the regions of highest NEUROG2 expression (Fig. 3-1 L). Intriguingly, expression along the boundary between NPCs and neurons is also exhibited by PLZF in the dorsal spinal cord and BTBD15 in the central spinal cord (Fig. 3-1 A,J,N) and is apparently characteristic of many members of the BTB-ZnF. Lastly, starting by e5 (HH St 25) and culminating by e6 (HH St 28), RP58 becomes highly expressed by all dorsal progenitors as well as in ventral domains, being expressed at moderate levels only in the $PLZF^+$ central spinal cord (Fig. 3-1 M-O). It is also at this time that RP58 expression seems to significantly break from its close tracking with NEUROG2 (Fig. 3-1 P) as now the region of highest NEUROG2 expression shifts dorsally into the central spinal cord where RP58 is expressed only at moderate levels.

RP58, like PLZF, exhibits a highly dynamic mode of expression within NPCs. However, unlike PLZF, which exhibits a more, binary, on or off pattern of expression, changes in RP58 ventricular expression appear to primarily consist in shifts between high and low levels. At both e3 and e4, the regions of highest RP58 expression were also regions of high NEUROG2 expression, although this was no longer at e6. It should be stressed that at no point during the period of studied was RP58 observed to be expressed among the neurons of the mantle zone; all RP58 expression occurred within the $SOX2^+$ progenitors of the ventricular zone and in or near the NEUROG2 expressing cells at the boundary between the mantle and ventricular zone. This restriction to NPCs is markedly different from the expression pattern for RP58

observed during brain development where RP58 is absent from proliferating progenitors and is upregulated as cells undergo neuronal differentiation (Okado et al., 2009; Xiang et al., 2012). This difference in expression, where RP58 is absent from the very cell-type it is thought to regulate in the brain, strongly suggested to us the possibility that the role of RP58 in the developing spinal cord might be radically different from its function in the brain. The expression of RP58 in SOX2^{+} NPCs and its relation to PLZF instead suggested that it might have the opposite activity of inhibiting neuronal differentiation. To test this possibility, we undertook to both misexpress and knockdown RP58 in the developing chick spinal cord

Ectopic RP58 is a potent inhibitor of neuronal differentiation

cDNA encoding chick RP58 was cloned into a vector containing a cytomegalovirus enhancer and a chick ß-actin promoter to drive expression in chick. This vector also contains an nlsEGFP reporter linked to RP58 expression on the same transcript via an internal ribosomal entry sequence. The spinal cords of developing chick embryos were transfected with either RP58 or the empty misexpression vector at e2 (HH 11), prior to the onset of neuronal differentiation. Embryos then were allowed to develop for 72 hours and before being collected, fixed, and assessed for the impact of ectopic RP58 on the balance between progenitor maintenance and neuronal differentiation. When the empty control vector were misexpressed (Fig. 3-2, A-F), it was observed that ~60% of transfected cells remained SOX2^{+} NPCs after 72 hours. The remaining remaining ~40% were found to differentiate into NEUN^{+} neurons. Misexpression of the control vector had a negligible impact upon the area of the transfected ventricular zone relative to the contralateral ventricular zone. Similarly, the expression of the proneural bHLH transcription factor NEUROG2 was not significantly altered. However, the transfection of developing NPCs with RP58 was found to strongly promote

continued progenitor identity over neuronal differentiation (Fig. 3-2 G-L). The frequency of RP58 transfected cells remaining SOX2$^+$ NPCs increased to ~90%, an increase of 150% relative to controls. Additionally, the area of RP58-electroporated SOX2$^+$ ventricular zone was found to expand by a factor of ~1.6 when contrasted with the unelectroproated contralateral ventricular zone. Lastly, ectopic RP58 was found to be sufficient to severely repress the expression of NEUROG2 in the ventricular zone.

Cumulatively, these observations indicate that RP58 in the developing spinal cord, like its fellow BTB-ZnF protein PLZF, strongly promotes progenitor maintenance over neuronal differentiation.

Reduced RP58 expression promotes neuronal differentiation

To further test whether RP58 might promote NPC maintenance in the spinal cord, vectors expressing an shRNA that targets RP58 were created (Fig. 3-3 A-D). This vector, termed shRP58, expresses a single RP58-targeting shRNA under the control of a ubiquitin U6 promoter. The vector also expresses an nlsEGFP reporter under the control of the cytomegalovirus enhancer and a chick ß-actin promoter. Transfection with shRP58 prior to the onset of neuronal differentiaton at e2 (HH 11) resulted in widespread cell death (data not shown), possibly suggesting a role for RP58 in cell survival early in neural development. However, when embryos were transfected with either the empty vector or shRP58 early in neurogenesis at e3 (HH 18), the cell death phenotype was largely avoided and instead the impact of loss of RP58 on neuronal differentiation could be observed.

24 hours post-transfection, cells transfected with the empty control vector were broadly distributed throughout the ventricular and mantle zones with ~70% of transfected cells remaining $SOX2^+$ NPCs and ~30% differentiating into $TUJ1^+$ neurons (Fig. 3-3 E-J). However, when NPCs were electroporated with RP58-targeting shRNA (Fig. 3-3 K-P), transfected cells were observed to cluster within the ventricular zone, often with a slightly lateral concentration. The observed frequency of terminal differentiation into $TUJ1^+$ neurons was reduced to only ~10%. Strikingly, of the remainin transfected cells, only ~35% were $SOX2^+$ NPCs, despite their physical location within the bounds of the ventricular zone. Instead, the majority of cells with reduced RP58 expression, ~55%, neither expressed SOX2 nor TUJ1 neurons nor the proneural factor NEUROG2. Indeed, the identity of these cells remains indeterminate at present as thus far no marker has been observed to be upregulated by them although many widely expressed markers of progenitor and neuronal identity have been attempted (e.g. PAX6, IRX3, BRN2, NEUN, LIM1/2, ISL1/2).

This population of unlabeled cells however proved to be transitory, constituting only ~6% of transfected cells a day later. At 48 hours post-transfection, the majority of cells transfected with shRP58, ~60%, had migrated laterally into the mantle zone and begun expressing neuronal markers such as TUJ1 (Fig. 3-3 W-AB). This was a significant increase over controls where after 48 hours only ~40% of transfected cells differentiated into $TUJ1^+$ neurons (Fig. 3-3 Q-V). In addition, there was a clearly observable trend wherein the majority of cells that remained $SOX2^+$ NPCs expressed only low levels of GFP and therefore presumably low levels of shRNA. This observation appears to suggest that loss of RP58 ultimately results in an increase in neuronal differentiation, although apparently involving an as yet uncharacterized intermediate state.

Intriguingly, a subset of cells within the mantle zone appear to be expressing the proneural bHLH protein NEUROG2 (Fig. 3-3 Y-Z), a finding reminiscent of the sustained expression of early neurogenesis markers among the differentiated neurons of *RP58*$^{-/-}$ cortices (Xiang et al., 2012), although the possibility that this is an experimental artifact cannot be ruled out.[10] However, despite this potential parallel with the RP58 mutant phenotype in the brain, RP58 knockdown in the spinal cord overall appears to result in a significant increase in the frequency transfected cells undergoing neuronal differentiation, indicating a role for RP58 in opposing, not promoting neuronal differentiation.

Mechanistic distinctions between PLZF and RP58

Given their common membership within the BTB-ZnF family and shared anti-neuronal differentiation activities, we decided to next explore whether or not PLZF and RP58 act through common pathways. We have previously shown (see Chapter 2) that PLZF activity is mediated in large part by its ability to up-regulate the expression and function of FGFR3. However, when developing chick spinal cords were transfected with RP58, no comparable induction of FGFR3 was observed. Instead, ectopic RP58 resulted in a significant ventral shift and a slight reduction in FGFR3 expression. We next considered whether RP58, which is not highly expressed in the same region of the developing spinal cord as FGFR3, might be acting through another member of the FGFR family. RP58's enrichment in the ventral spinal cord suggested that it might instead regulate the action of FGFR2, which is also most highly expressed in the ventral spinal cord. However, this was found to not be the case. As with

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[10] A common experimental artifact observed during *in ovo* electroporation is the detachment and migration into the mantle zone of small clusters containing a few NPCs. It is possible that this accounts for some of the mantle zone NEUROG2 observed. However, it should be noted that these artifactual clusters are typically GFP^{-} whereas many of the ectopic NEUROG2^{+} cells observed are GFP^{+}.

FGFR3, ectopic RP58 resulted in a slight reduction in the intensity of FGFR2 expression. These data suggest that although PLZF and RP58 are both members of the BTB-ZnF family and have related roles in maintaining NPCs, they appear to achieve this effect through distinct mechanisms.

The expression of both FGFR2 and FGFR3 within the spinal cord is influenced greatly by the homeodomain and bHLH transcription factors that partition the spinal cord into a series of domains arranged along the dorsal-ventral axis. To determine whether ectopic RP58's ability to ventrally shift FGFR3 expression could be explained by an alteration in the arrangement of these patterning proteins, we investigated the effect of RP58 misexpression upon markers of ventral identity. We found that ectopic RP58 was sufficient to inhibit the expression of many ventral markers such as NKX2.2, OLIG2, and NKX6.1 whereas markers of more dorsal identities, such as PAX6, expanded ventrally (Fig. 3-4 D-I). These data suggest that the ventral shift in FGFR3 expression is due to RP58's inhibition of markers of ventral identity. This finding was surprising given that RP58's endogenous expression overlaps with these ventral markers. Additionally, previous work in our lab and by collaborators has found that the misexpression of other pro-progenitor factors, such ID1, NICD, and SOX3, have the opposite phenotype of promoting the dorsal extension of ventral identity markers (Fig. 3-4 J-U). Potential explanations for this phenotype will be addressed in the discussion.

3-4 Figures

Figure 3-1

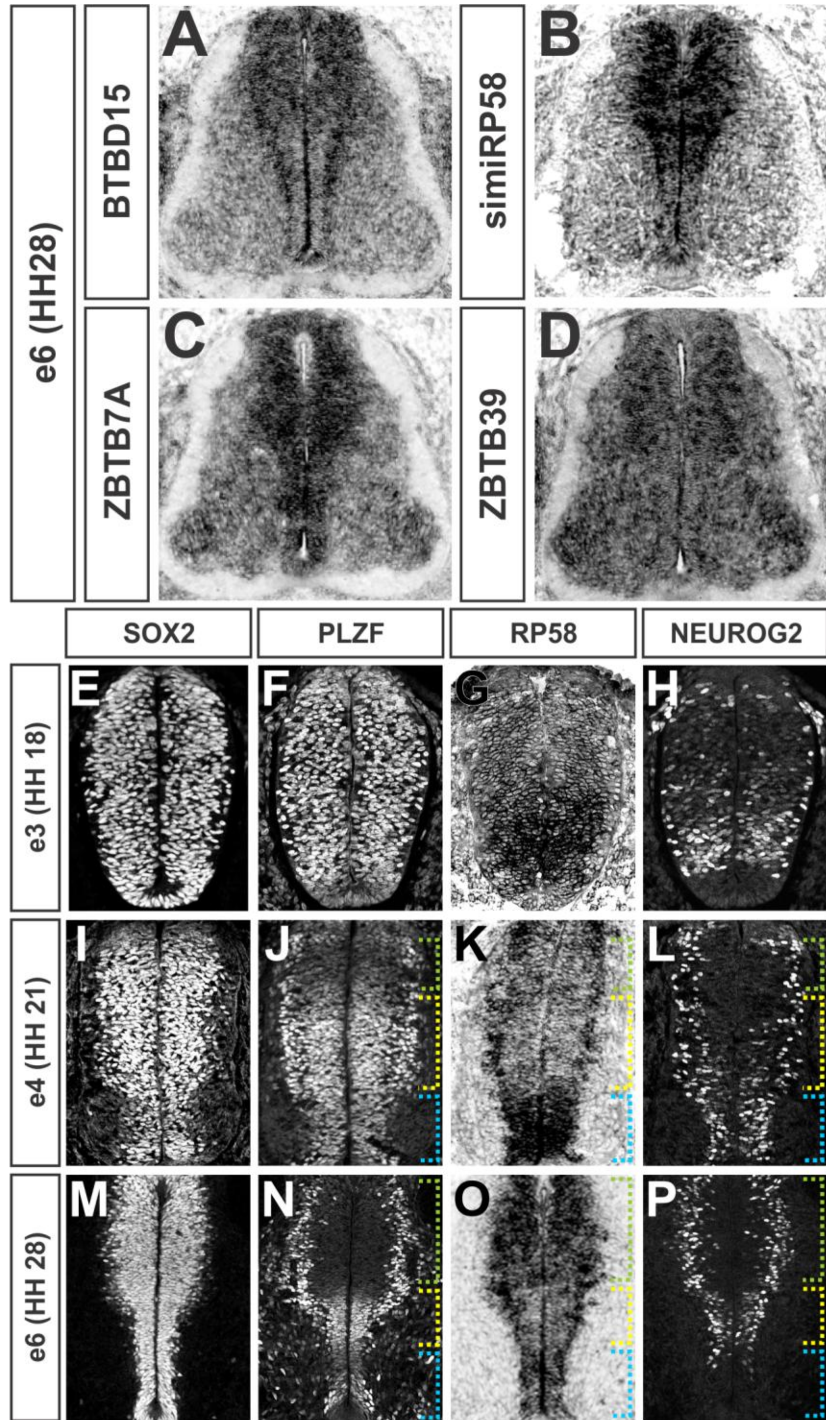

Figure 3-1 – Wild type expression pattern of RP58 and other BTB-ZnF proteins in the developing spinal cord

(A-D) An *in situ* hybridization screen was performed to identify additional members of the BTB-ZnF family expressed during spinal development. The screen identified five additional genes: BTBD15, RP58, simiRP58, ZBTB7A, and ZBTB39.

(E-P) The expression pattern of RP58 characterized was at key times in the early development of the chick spinal cord and compared against the expression of the NPC marker SOX2 and the BTB-ZnF protein PLZF.

(E-H) At e3 (HH St 18), at the onset of neurogenesis, RP58 was expressed throughout the $SOX2^{+}$ ventricular zone, but was most highly expressed in the ventral-most domains which are also enriched for NEURO2 expression.

(I-L) At e4 (HH St 21), as neurogenesis proceeds, RP58 maintains a moderate level of expression throughout the spinal cord but remains most highly expressed in the ventral spinal cord, a region where NEUROG2 expression is also high. Like PLZF and BTBD15, RP58 has by this time also begun to be expressed highly at the lateral edge of the ventricular zone, where proneural transcription factors such as NEUROG2 are highly expressed.

(M-P) By e6 (HH St 28) RP58 expression is elevated in the dorsal spinal cord as well, resulting in an expression pattern reciprocal to that of PLZF: highly expressed in dorsal and ventral progenitors but only moderately expressed in the PLZF expressing central spinal cord.

Figure 3-2

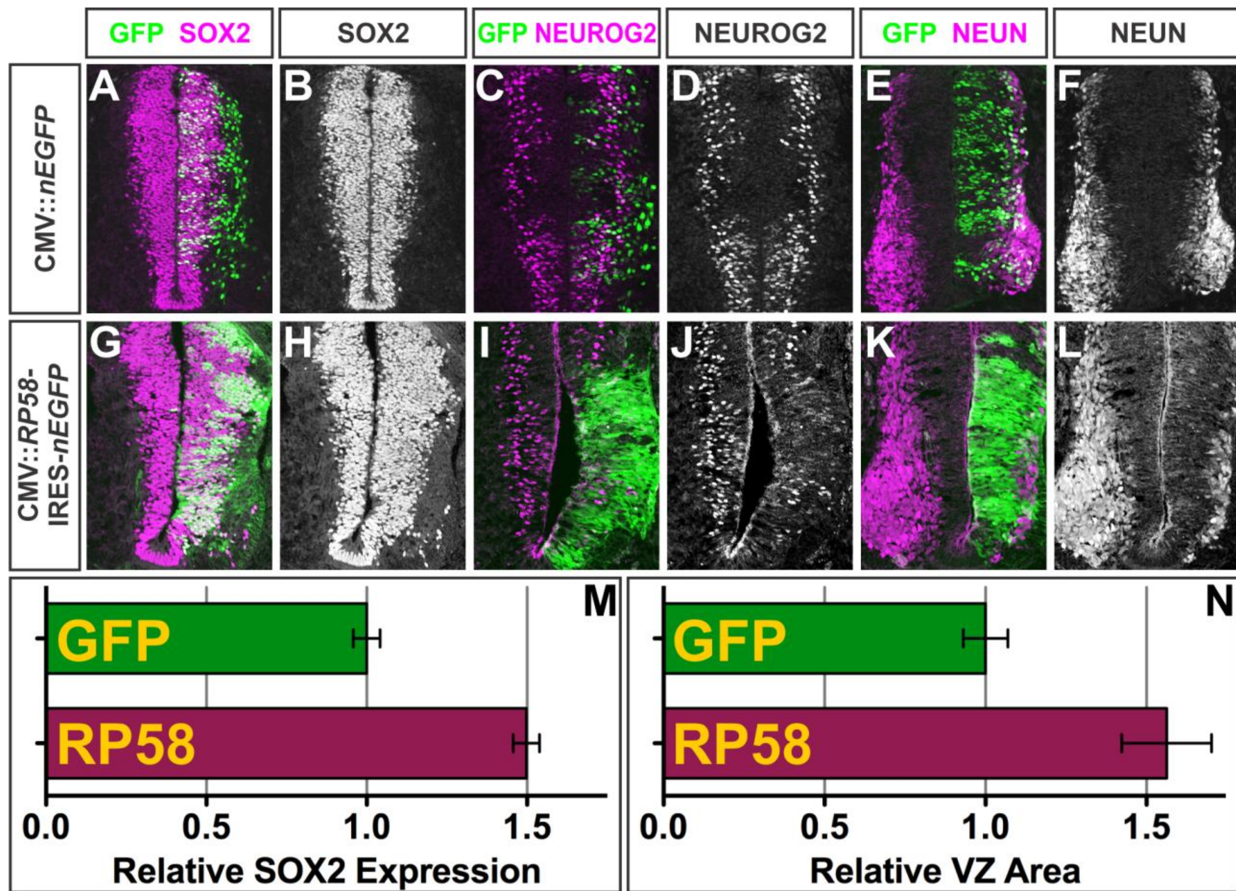

Figure 3-2 – RP58 misexpression suppresses neuronal differentiation and expands the NPC pool

NPCs were transfected with control IRES-*nEGFP* or *RP58*-IRES-*nEGFP* vectors at e2 / HH St 18 and analyzed at e5 / HH St 25. RP58-transfected cells exhibited a strong bias against undergoing neuronal differentiation.

(A-B,E-F) In control electroporations, ~60% of transfected cells remained SOX2$^+$ progenitors and ~40 differentiated into NEUN$^+$ neurons.

(G-H,K-L,M-N) However, when electroporated with RP58, the proportion of GFP$^+$ cells that remained SOX2-expressing increased by approximately half to 90%, with a corresponding drop in the proportion of transfected cells expressing NEUN. Similarly, the area of the SOX2$^+$ ventricular zone, which was not significantly affected by control electroporations, expanded by ~60% when RP58 was misexpressed within it.

(C-D,I-J) RP58 misexpression was found to greatly reduce the expression of the proneural bHLH protein NEUROG2 whereas control electroporations had only a minimal impact.

Figure 3-3

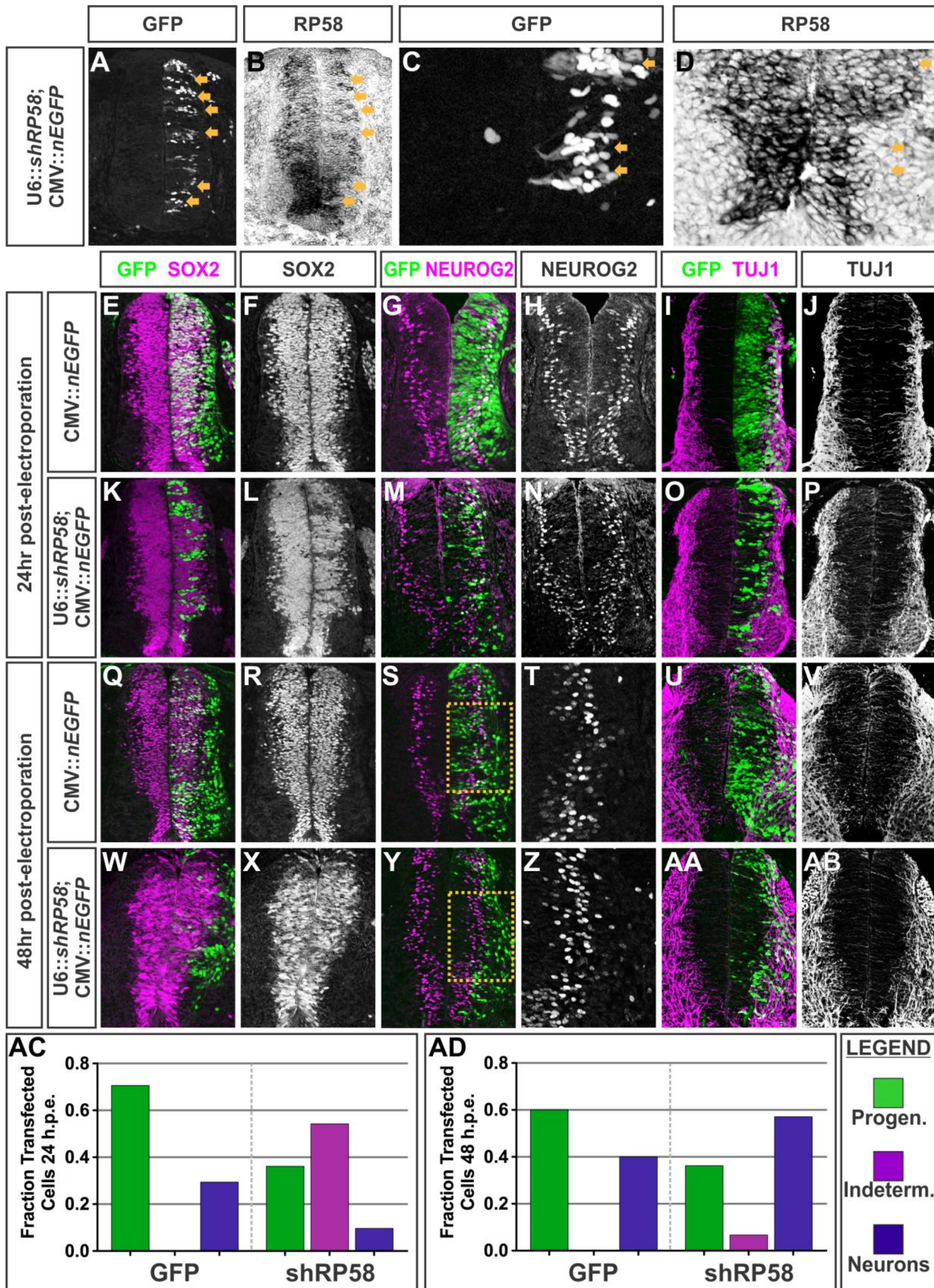

Figure 3-3 – RP58 knockdown compromises progenitor maintenance resulting in enhanced neuronal differentiation

(A-D) Developing chick spinal cords were transfected with vectors misexpressing shRNA targeting RP58 under the control of the ubiquitous U6 promoter. It was observed that within 24 hour of electroporation, RP58 mRNA levels were significantly reduced in regions of the spinal cord expressing the vector's GFP reporter.

(E-J) Embryos were transfected at e3 / HH St 21 and allowed to develop for 24 hours. In controls, ~70% of GFP^+ cells remained $SOX2^+$ NPCs while ~30% differentiated into $TUJ1^+$ neurons. It was observed that virtually all transfected cells expressed either SOX2 or TUJ1.

(K-P) Within 24 hours of transfection with RP58 targeting shRNAs, the proportion of electroporated cells expressing SOX2 cells dropped to ~35%, roughly half the proportion observed in control electroporations. However, there was no corresponding increase in differentiation of $TUJ1^+$ neurons, the fraction of which was observed to be reduced to ~10% of transfected cells. Instead, the majority of transfected cells, ~55%, assumed an indeterminate identity, expressing neither SOX2 nor TUJ1 nor any other marker as yet identified, including the proneural bHLH protein NEUROG2.

(Q-V) By 48 hours post-electroporation, the fraction of transfected cells that remained $SOX2^+$ NPCs fell to ~60% whereas the proportion expressing the neuronal marker TUJ1 increased to ~40%. Again, virtually all cells were observed to express either SOX2 or TUJ1.

(W-AB) 48 hours after electroporation, the frequency of cells with an indeterminate identity dropped to only ~5% of those expressing GFP. Although some of the cells may have undergone apoptosis, the majority appear to have differentiated into neurons, with ~60% of transfected cells expressing TUJ1 and only 35% expressing SOX2.

Figure 3-4

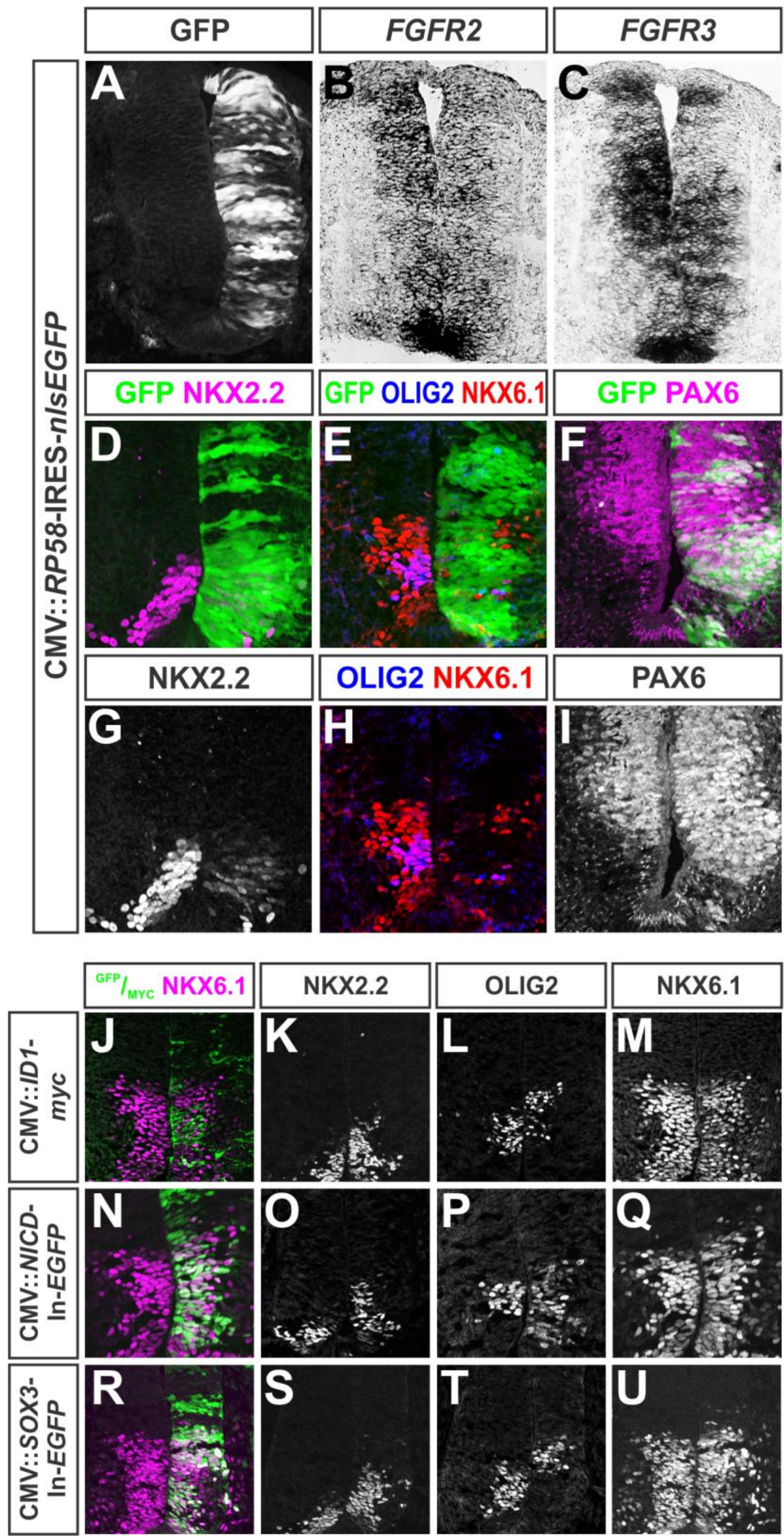

Figure 3-4 – RP58 misexpression inhibits the expression of ventral identity markers

(A-C) As discussed in Chapter 2, the BTB-ZnF protein PLZF promotes progenitor maintenance in part through the induction of FGFR3, thereby conferring upon cells an increased sensitivity to the pro-progenitor FGF signaling pathway. However, despite its similar, enhancement of progenitor maintenance, RP58 misexpression does not increase the expression of either FGFR2 or FGFR3. Instead, the expression of FGFR3 was observed to become ventrally displaced. In addition, expression levels for both FGFR2 and FGFR3 were observed to be slightly reduced.

(D-I) RP58 misexpression was then found to repress multiple markers of ventral progenitor identity including NKX2.2, NKX6.1, and OLIG2. However, markers of a more dorsal identity, such as PAX6, were observed to expand ventrally. Together these observations suggest that RP58 misexpression suppresses ventral identity.

(J-U) This inhibition of ventral identity markers by RP58 contrasts with other genes associated with progenitor maintenance. When such pro-progenitor factors such as ID1, NICD, and SOX3 were misexpressed in the developing spinal cord, all three promoted the expansions of ventral identity markers. This suggests that RP58's suppression of ventral identity may be a relatively unique feature of this gene and indicative of a previously unrecognized role in controlling the patterning of the spinal cord.

Figure 3-5

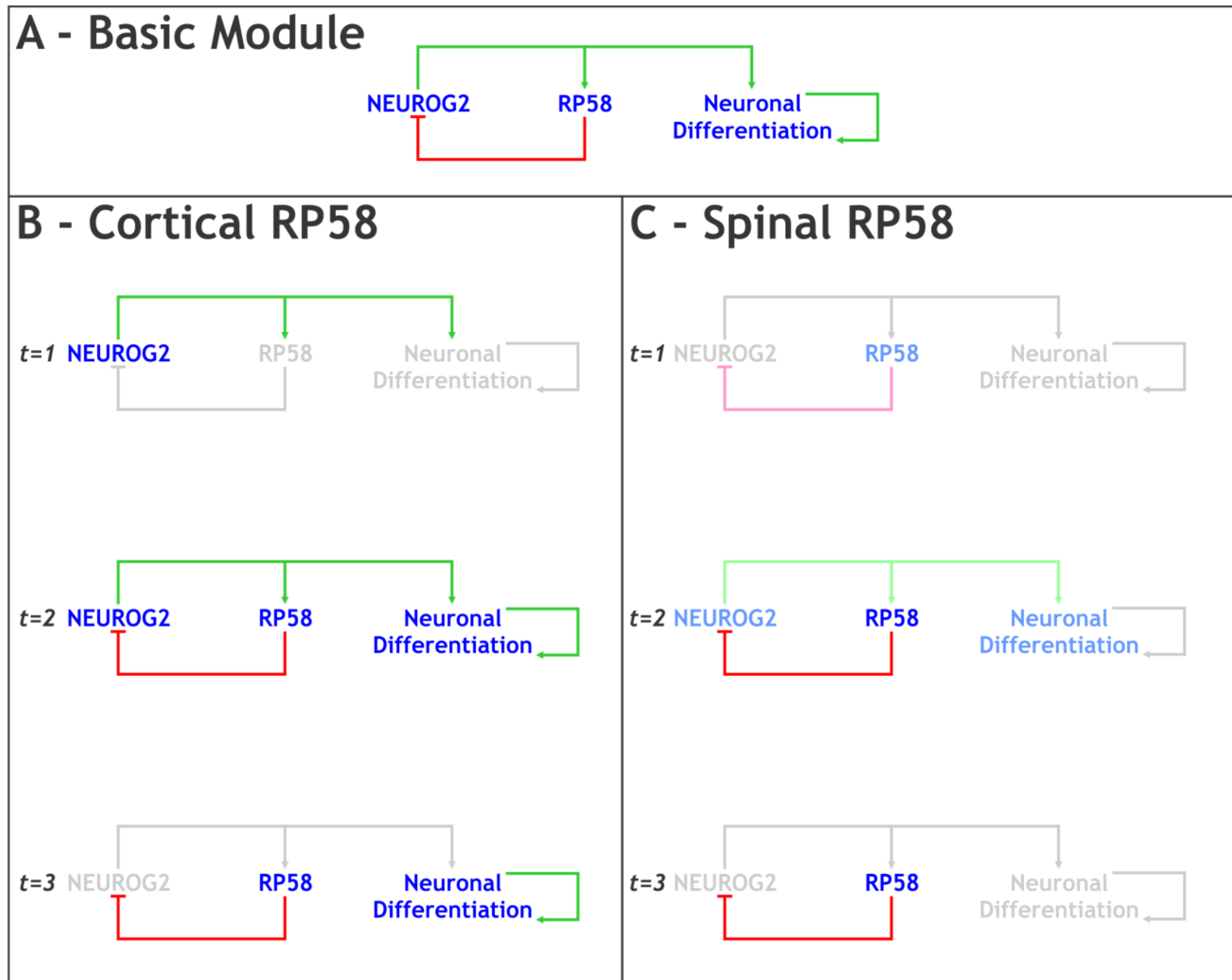

Figure 3-5 –Proneural Feedback Model of RP58 Activity

(A) In some neural contexts, RP58 has been found to promote neurogenesis, in others progenitor maintenance. We propose that in both sets of contexts, RP58 participates in a common genetic module and that the key distinction is the context and relative order of gene induction.

(B) When NEUROG2 is induced first, it activates both RP58 and pro-differentiation targets. By the time RP58 is activated and able to represses NEUROG2 expression, cells are committed to neuronal differentiation by self-sustaining proneural pathways. Therefore, the repression of NEUROG2 by RP58 serves to promote continued neuronal maturation by removing a transcription factor specifying an early stage in differentiation.

(C) When RP58 is activated first, initially at low levels, it acts to prevent the onset of NEUROG2 expression. However, should NEUROG2 become moderately activated by some other inputs, its induction of RP58 will result in NEUROG2 levels falling again prior to being able to activate any self-sustaining pro-differentiation pathways. Only sustained, high activation of NEUROG2 will permit it to overcome RP58 repression and induce neuronal differentiation programs. Therefore, in this context, RP58 repression of NEUROG2 acts to promote NPC maintenance by opposing the activation of NEUROG2.

3-5 Discussion

A proneural bHLH feedback model for RP58 activity

Previous work by others has identified RP58 as a pivotal regulator of neuronal differentiation within several regions of the developing brain although this has perhaps been best demonstrated in the neocortex (Okado et al., 2009; Xiang et al., 2012). The expression of RP58 has been shown to begin within intermediate progenitors as they begin to delaminate from the cortical ventricular zone and to increase as cells terminally differentiate into neurons. However, in the spinal cord, we found RP58 to be expressed within the ventricular zone and to be down-regulated as cells undergo neuronal differentiation. Within the neocortex, deletion of the RP58 gene results in a failure of neuronal differentiation, resulting in an expansion of both the ventricular and subventricular zones and in a reduction and disorganization in the layers of cortical neurons. Contrastingly, in the spinal cord we found that knocking-down RP58 with shRNA ultimately resulted in a significant increase in the differentiation frequency of transfected cells relative to controls. Furthermore, we found that the misexpression of RP58 within the developing spinal cord severely reduced the production of neurons and resulted instead in a significant expansion in the population of ventricular progenitors.

There are many potential explanations for this striking difference in activity of RP58 between these two regions of the CNS. Differential splicing of transcript, post-translational modifications by proteins present in one region but not the other, differential expression of co-factors, all these mechanisms and more might potentially permit a transcription factor to take on distinct activities in specific contexts. However, to date there are no known isoforms

of either the RP58 transcript or protein and no post-translational modifications have been described that could explain this shift in function, although lack of evidence is by no means proof of lack of existence. It is intriguing to consider whether RP58's potential ability to form in either homodimers or heterodimers might result in a shift between pro-neural and pro-progenitor activities. Within the spinal cord, we have found that the expression of simiRP58, a potential partner for heterodimerization, only partially overlaps with that of RP58. This raises the possibility that at different locations within the spinal cord, RP58 might exist in distinct dimeric arrangements. However, all that can be said with any certainty on these matters is that when the full-length protein is highly over-expressed and therefore presumably present primarily in a homodimeric state, it promotes progenitor maintenance. Thus, if differential splicing or changes in dimerization lie at the root of the distinction between the roles of RP58 in the brain and RP58 in the spinal cord, it is likeliest that it is some non-full length and or heterodimeric form that is associated with promoting neurogenesis in the brain.

However, it may not be necessary to invoke unknown binding partners and potentially nonexistent isoforms for RP58. It is quite possible that RP58 is acting in largely the same manner in both regions of the CNS and that the key distinction is the relative timing of its expression. As such, rather than considering RP58 to be a pro-differentiation gene or a pro-progenitor gene, it may be more useful to consider RP58 as a participant in a feedback inhibitory circuit with proneural bHLH proteins, a circuit that can be deployed in multiple contexts. That RP58 inhibits proneural bHLH proteins is one of its most consistent features within the literature. Using different methods and with varying degrees of directness, RP58 has been found to repress proneural bHLH proteins, in the context of muscle, brain, and now spinal tissue. Furthermore, it has been found that RP58 expression is activated by the

expression of proneural bHLH proteins, although this has not yet been demonstrated within the spinal cord. To adopt a reductionist approach, we might consider a simple genetic circuit between RP58 and the proneural bHLH gene NEUROG2. Data exist that suggest NEUROG2 activates RP58 and NEUROG2 is in turn repressed by RP58 (see Chapter 3-1). In addition, we observed that although RP58 is broadly expressed throughout the spinal cord at moderate levels, it is most highly expressed throughout most of neurogenesis in regions highly expressing NEUROG2 (Fig. 3-1 G,H,K,L,O,P). In this proposed circuit (Fig, 3-5 A), NEUROG2 induces both RP58 and other non-specified genes that promote neuronal differentiation whereas RP58 represses NEUROG2. In addition, it must be assumed that once these non-specified proneural genes are sufficiently-activated by NEUROG2, they are able to sustain their expression independently of NEUROG2, a reasonable assumption given the transient nature of NEUROG2 expression during differentiation. Let us consider how this circuit might function in differently when deployed in the developing cortex versus the developing spinal cord.

Within the developing cortex (Fig. 3-5 B), it has been found RP58 is expressed only as cells begin to delaminate from the ventricular zone. Furthermore, the expression of NEUROG2 expression appears to precede that of RP58. Therefore, NEUROG2 is potentially able to be activated and induce multiple non-specified target genes necessary for neuronal differentiation before its induction of RP58 enables is able to repress NEUROG2 expression. Therefore, in the context of cortical development, RP58 would function to promote neuronal maturation by silencing the expression of genes that specify an early stage of neuronal differentiation. Without RP58 activity, NEUROG2 would not be silenced and persistence of expression of gene characteristic of early neuronal differentiation would be expected, a

phenotype which has been observed in the cortex of *RP58*$^{-/-}$ mice itself (Ohtaka-Maruyama et al., 2012; Okado et al., 2009; Xiang et al., 2012).

However, within the developing spinal cord, we have found that RP58 is present at moderate levels among SOX2^{+} NPCs prior to their up-regulation of NEUROG2 expression and the onset of neuronal differentiation. In the context of our proposed genetic circuit (Fig. 3-4 C), if moderate RP58 expression precedes that of NEUROG2, it would serve to oppose the onset of NEUROG2 expression and thereby prevent the induction of NEUROG2's other, proneural targets. Furthermore, should regulatory events outside this circuit moderately activate NEUROG2, RP58's expression would become elevated by this induction of NEUROG2, thereby increasing its ability to oppose NEUROG2's activation of neuronal differentiation. Only in circumstances where NEUROG2 was highly activated for an extended period would it be able to overcome the RP58 block on its expression and induce neurogenesis. Thus, in the context of the spinal cord, RP58 repression of NEUROG2 serves to prevent neuronal differentiation, whereas in the context of the brain that very same repression might serve to promote continued differentiation.

This model of RP58 activity presents several testable hypotheses. Firstly, it would predict that ectopic NEUROG2 should entirely rescue the potent block on neurogenesis created by misexpressing RP58. Secondly, it would predict that forced misexpression of RP58 within the NPCs of the developing telencephalon, prior to the onset NEUROG2 expression and neurogenesis, should phenocopy the pro-progenitor activity of RP58 observed in the spinal cord. This phenotype should also be rescuable through the co-electroporation of NEUROG2. Thirdly, this model would also predict that misexpression of RP58 within spinal neurons would expedite their differentiation. Fourthly, the model predicts that loss of RP58 from the spinal

cord should result in a rapid up-regulation of NEUROG2. Notably, this fourth prediction has not yet been born out by our current shRNA data. However, at the 24 hour time point, the earliest stage at which we analyzed our knockdown embryos, SOX2 expression had already been lost. Because the great majority of NEUROG2^{+} cells are also SOX2^{+} it is likely that the 24 hour time point is too late and the developmental window during which the increase in NEUROG2 could be observed was missed. An alternative hypothesis is that NEUROG2 is not the appropriate proneural bHLH for this model and as such, in addition to looking earlier, it would also be advisable to determine whether other known proneural bHLH proteins linked with RP58 in brain and muscle development.

RP58 and ventral fate

We found that RP58 was a potent inhibitor of several markers of ventral identity: NKX2.2, OLIG2, and NKX6.1. Correspondingly, markers most highly expressed in the central neural tube, such as PAX6 and IRX3, expanded ventrally. Phenotypically, this pattern resembles a disruption of a ventralizing signal, most likely Sonic Hedgehog (SHH). SHH, secreted from the notochord and floorplate, ventralizes the developing neural tube by inducing the expression of what are known as the Class I genes (which includes NKX2.2, OLIG2, and NKX6.1) and repressing a set referred to as Class II genes (which includes PAX6). It has been repeatedly demonstrated that a reduction in SHH signaling leads to a loss of Class I gene expression and the expansion of Class II genes (Briscoe et al., 2000; Ribes and Briscoe, 2009), as is observed when RP58 is misexpressed.

The repression of ventral identity by RP58 is particularly unusual given that misexpression of other potent anti-neuronal differentiation factors tends to expand ventral

identity. This has been argued to be a consequence of the ability of NPC's to integrate SHH signal over time, adopting progressively more ventral identities the longer they are exposed to SHH. Thus, any factor that preserves NPCs in an undifferentiated, SHH-receptive state, would be predicted to also promote a ventral-shift in identity (Ribes and Briscoe, 2009). It is therefore highly significant that RP58 appears to deviate from the observed trend of pro-NPC maintenance factors also being pro-ventral identity factors and suggests that this may reflect a distinct aspect of RP58 activity.

Given that the region of highest RP58 expression closely corresponds to the region of the spinal cord that normally expresses the Class II genes NKX2.2, OLIG2, and NKX6.1, it is may seem counterintuitive to find that RP58 misexpression is so antagonistic to markers of ventral identity. However, this is not unprecedented. Hedgehog Interacting Protein (HHIP), a decoy receptor for the SHH ligand, has been found to induced by cells receiving high levels of SHH signal, including the ventral spinal cord (Aglyamova and Agarwala, 2007; Chuang and McMahon, 1999). Deletion of HHIP from mouse spinal cords results in a subtle but significant expansion of ventral markers such as OLIG2 and NKX2.2. It has been argued that HHIP functions as a means of ligand dependent antagonism, limiting the domain of expansion of SHH induced domains by reducing the sensitivity of cells receiving high levels of SHH to additional signaling (Jeong and McMahon, 2004).

It is possible that RP58 has a similar function to HHIP but at the level of transcriptional control, being expressed ventrally to attenuate the expansion of ventral identity markers. In this model, RP58 at endogenous expression levels would provide a moderate repression on some element of SHH pathway or on multiple members of the Class I genes set such as NKX2.2, OLIG2, and NKX6.1. Given this basal level of repression in the ventral neural tube,

the activation and maintenance of these Class I proteins might require higher levels of SHH signal and GLI-activator protein to be present than would otherwise be the case, giving RP58 a potentially important role in the fine tuning of ventral patterning. To date this model has proven difficult to test. It would predict that loss of RP58 activity through shRNA knockdown should result in a dorsal expansion of ventral progenitor markers. However, loss of RP58 instead results in rapid loss of all progenitor markers, ventral or otherwise, as cells differentiate into neurons. In addition, the ectopic neurons ultimately generated by knocking-down RP58 appear to be regionally appropriate, providing no evidence for an expanded ventral identity when RP58 is lost. Although looking at an earlier time points may help, it is possible that the dynamics of repatterning are slower than that of differentiation and it may be more effective to identify and test potential RP58 binding sites within the locus of Class I genes or modulators of SHH signaling.

BTB-ZnF diversity in the spinal cord

In this study, we identified an additional five members of the BTB-ZnF family as being expressed in the developing spinal cord: BTBD15, RP58, simiRP58, ZBTB7A, and ZBTB39. RP58 will be discussed below. Currently, little is known about simiRP58 apart from its probable ability to dimerize with RP58 (Takahashi et al., 2008). Furthermore, to date the literature on BTBD15 and ZBTB39 is limited to appearances within analyses of whole genome expression profiles and similar broad studies. Apart from RP58, ZBTB7A is the only member of this set of BTB-ZnF genes that has been well characterized, having been found to be important for maintaining hematopoietic stem and progenitor cells through its direct repression of $p19^{ARF}$ (Maeda et al., 2005). As such, the enrichment of ZBTB7A in NPCs is highly

suggestive that it, like PLZF and RP58, fulfills an important role in spinal progenitor maintenance during neurogenesis.

ZBTB39 is particularly intriguing for two reasons. Firstly, its expression is seemingly ubiquitous throughout the spinal cord, among both NPCs and differentiated neurons, making it to our knowledge among the most broadly expressed transcription factors in neural development. Secondly, ZBTB39 is among the closest known orthologs of PLZF. Although to date no extensive phylogenetic analysis has been performed for the BTB-ZnF family, when the BTB domains from 47 members of the family were aligned, it was found that PLZF, FAZF, ZBTB39, and GZF1 grouped together as a distinct clade (Z.B. Gaber, unpublished data). It is therefore conceivable that the apparent tolerance of the developing CNS to PLZF mutation and knockdown may be due to the presence of this closely related homolog. Additionally, given PLZF's known ability to heterodimerize with another member of its local clade, FAZF (Hoatlin et al., 1999), it is possible that PLZF and ZBTB39 heterodimerization may influence some aspects of the activity of both transcription factors.

Another interesting finding from this simple phylogenetic analysis is that there is no apparent concentration of the BTB-ZnF genes found to be expressed in the spinal cord within a particular region of the phylogeny. Although there does appear to be high sequence conservation between RP58 and simiRP58 and between PLZF and ZBTB39, the BTB-ZnF genes are otherwise found positioned all throughout the tree. RP58 and PLZF, despite their seeming similar functions during spinal development, have only minimal sequence similarity in their BTB domains. This may suggest both that the evolutionary kinship between PLZF and RP58 is very distant and, in more practical terms, there a high probability for their mediating their common pro-progenitor activity via significantly different mechanisms. This is further

supported by the known divergence of PLZF and RP58 DNA binding sites (see Table 4-1) and the differential effect of PLZF and RP58 upon FGFR3 expression.

Regional expression of BTB-ZnF proteins

Apart from ZBTB7A and ZBTB39, which were both broadly expressed throughout the entire ventricular zone, it was striking how most BTB-ZnF proteins exhibit a distinctly regional expression pattern. BTBD15 and simiRP58 were most highly expressed dorsally, PLZF centrally, and RP58 initially high ventrally, but then switching to being highly expressed by the entire ventricular zone except the central spinal cord. Given that the two best characterized BTB-ZnF proteins in spinal development, PLZF and RP58, both exhibit highly dynamic expression patterns, it is likely that a more detailed analysis of the others may show comparable dynamism. The significance of the overlapping and dynamic expression of these transcription factors is as yet unclear, although there are several intriguing possibilities.

Firstly, given the aforementioned prospects for heterodimerization, it is conceivable that overlapping fields of BTB-ZnF expression may result in the formation of distinct complexes in different regions of the CNS. For example, it is conceivable that at e6, simiRP58 and RP58 will form heterodimers in the dorsal spinal cord where their expression overlaps while RP58 forms homodimers in the ventral spinal cord where it is expressed alone.

Secondly, this pattern is somewhat reminiscent of regional expression patterns of other families of transcription factors during spinal development such as HES, ID, and proneural bHLH. In each case, members of each family are expressed in regionally distinct, sometimes overlapping, sometimes reciprocal patterns. Data suggest that although each family member

is broadly similar in function they each may possess some distinct activities that have evolved for the precise context of their expression. For example, although all proneural bHLH proteins promote neuronal differentiation, specific proneural proteins facilitate the differentiation of distinct neuronal subtypes (see Chapter 1-2). It is plausible something similar is being achieved through the regional expression of BTB-ZnF transcription factors. In this model, ZBTB7A might be a broad but generic pro-progenitor transcription factor whereas PLZF might be expressed within the central spinal cord so as to give this particular region extra protection against differentiation through its promotion of FGF signaling (see Chapter 2). Intriguingly, given the broad expression of ZBTB39 in both neurons and progenitors, it may not have any regional activity but may rather be a cofactor for other BTB-ZnF proteins in much the same way E proteins are for proneural bHLH proteins. Future experiments will be needed to test all these possibilities and to determine whether additional members of the BTB-ZnF family are expressed in the developing spinal cord.

Summary and conclusions

We have now identified a total of six BTB-ZnF transcription factors as being expressed during spinal development. Two of these, PLZF and RP58, we have characterized as having roles in maintaining NPCs during neurogenesis. In this study, we found that RP58 was expressed throughout the ventricular zone of the developing spinal cord, but most highly during the ventral spinal cord in regions closely matching that of NEUROG2 during peak neurogenesis. We found that this ventral expression may be implicated in the fine-tuning of ventral progenitor identity. We also established that ectopic RP58 was sufficient to block most neuronal differentiation and to promote significant expansion of the ventricular zone whereas reduction of RP58 through shRNA demonstrated that RP58 is necessary for preserving

NPCs. In addition, we found evidence that RP58 achieves these effects through a mechanism distinct from the induction of FGFR3 utilized by PLZF. Lastly, we proposed a model for RP58 where RP58 is not strictly a pro-progenitor nor a pro-differentiation factor but rather a participant in a proneural bHLH protein feedback inhibition circuit that can be deployed to divergent ends in different contexts.

This theory is by no means novel; indeed far more elaborate circuits have been found to be redeployed over evolutionary history. One notable example is the gene regulatory network directing skeletogenesis in sea urchins. Sea urchins are unique among echinoderms in that they have two distinct periods of skeletogenesis. The later period, which occurs during the larval stage and which generates the spicules of the adult organism, is shared by all echinoderms. However, sea urchins also undergo an embryonic period of skeletogenesis, a feature that uniquely evolved in the ancestors of modern sea urchins. It has been found that this additional period of skeletogenesis evolved through the near complete redeployment of the ancestral, adult skeletogenesis gene regulatory network into embryogenesis (Gao and Davidson, 2008). Similar redeployments of whole circuits, sometimes referred to as plug-in modules, is a commonly observed phenomenon. These plug-ins modules often represent molecular switches that can be integrated into widely divergent processes. Examples of this phenomenon described previously in this work include the frequent employment of signaling systems such as FGFs or NOTCH throughout development (Erwin and Davidson, 2009). Thus, we propose that the distinct functions observed for RP58 in the brain and in the spinal cord are explicable as different applications of a common RP58-mediated feedback repression circuit for proneural bHLH proteins such as NEUROG2.

Having shown that PLZF, RP58, simiRP58, ZBTB7A, and ZBTB39 are all expressed in spinal development, we will conclude with a discussion of the prospects for the broader BTB-ZnF as critical regulators of spinal development.

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CHAPTER 4 – Conclusions and Prospects for a New Important Family of Neural Developmental Regulators

Over the course of these studies, six BTB-ZnF family members were identified as being expressed during spinal development. Of these two, PLZF and RP58, were extensively characterized and both were found to promote NPC maintenance during spinal neurogenesis. Despite this ultimate functional similarity, it is possible that PLZF and RP58 achieve their activities through non-overlapping mechanisms as discussed below.

PLZF and RP58

Fundamental to the activity of any transcription factor is the ability to bind DNA targets. Consensus sequences have been proposed for three of the BTB-ZnF genes that were found to be expressed within the spinal cord. Unlike members of the SOX or bHLH family, there appears to be little commonality between the consensus sequences of PLZF, RP58, and ZBTB7A (Table 4-1). Furthermore, although the crystal structure of PLZF bound to DNA has not been solved, PLZF's association with its core consensus sequence has been modeled based on general knowledge of ZnF structure. From this critical amino acid residues have been identified within PLZF's ZnFs 6, 7, and 8 (Guidez et al., 2005). When these three ZnFs were aligned with their most similar counterparts in RP58 (ZnFs 2, 3, and 4), little sequence similarity was observed (Table 4-2). Significantly, of the 11 amino acids in the PLZF ZnF domain that are modeled to directly contact the DNA target (Table 4-2, blue residues) only 2 are conserved in RP58.

Table 4-1 – BTB-ZnF protein DNA binding consensus sequences

T A G/T C/G T A/C A/C A/T G T A C	PLZF	See Table 1-1
A/C A C A T C T G G/T A/C	RP58	(Aoki et al., 1998)

Table 4-2 – Alignment of critical region of PLZF and RP58 DNA binding domains

PLZF Zinc Finger 6	Linker	PLZF Zinc Finger 7	Linker	PLZF Zinc Finger 8
YECEFCGSCFRDESTLKSHKRIH	TGEKP	YECNGCGKKFSLKHQLETHYRVH	TGEKP	FECKLCHQRSRDYSAMIKHLRT-H
PTCSLCGKTFSCMYTLKRHERTH	SGEKP	YTCTQCGKSFQYSHNLSRHAVVH	TREKP	HACKWCERRFTQSGDLYRHIRKFH
RP58 Zinc Finger 2	Linker	RP58 Zinc Finger 3	Linker	RP58 Zinc Finger 4

These observations suggest that PLZF and RP58 likely recognize very distinct target sequences and therefore different sets of genes. This is supported in the literature (see Chapter 3-1) where few of the known targets of BCL6, FAZF, PLZF, RP58, or ZBTB7A were observed to overlap, although this may potentially be an example of different sets of genes being studied in the context of different BTB-ZnF transcription factors. In addition, this does not rule out the possibility that PLZF and RP58 also have the potential to regulate the same genes. Firstly, a particular target enhancer element might have both PLZF and RP58 binding sites. Secondly, given the potential for heterodimerization, it is very possible that PLZF or RP58 could be recruited to an enhancer it could not otherwise bind by associating with a BTB-ZnF protein that is able to bind the element.

It is a curious feature of PLZF that despite it functioning as a dimer, the majority of known binding sites are monomeric (see Table 1-1). Intriguingly however, the majority of characterized RP58 binding sites are dimeric, although most of these sites are sufficiently distant from one another that DNA looping would be required (Hirai et al., 2012; Hoatlin et al., 1999b; Xiang et al., 2012; Yokoyama et al., 2009). Although the number of characterized RP58 and PLZF binding sites is currently too small to be certain that this is genuine distinction, it does raise the possibility that RP58 may typically bind DNA as a homodimer and

that PLZF might more frequently bind DNA either as a heterodimer or using the ZnF domain of only one of the constituent monomers to contact DNA.

The ability of PLZF and RP58 to form these hypothetical homo- and heterodimers is of course determined by properties of their BTB domains. The sequence of the BTB domains of PLZF and RP58 share only a 31.3% sequence identity. However, by the standards of the BTB-ZnF family, this not a particularly great degree of sequence divergence. For example, the BTB sequences of PLZF's two closest known homologs, ZBTB39 and FAZF, are respectively only 35.9% and 35.1% conserved with PLZF. Furthermore, despite this divergence, FAZF is still known to associate with many of the same protein complexes as PLZF, including N-CoR, mSIN3A, HDAC1 and PLZF itself (Hoatlin et al., 1999b). The even more divergent BCL6, only 27.4% conserved with PLZF, has also been found to bind most of the same repressor complexes, albeit with different affinities (Dhordain et al., 1998). Thus far RP58 has not been linked to any of these protein complexes. However, given RP58's known repressor activity (Aoki et al., 1998) and the strong association between these repressor complexes and many members of the BTB-ZnF family, despite the apparent sequence divergence, it is likely that RP58 also recruits these proteins to its DNA targets.

In summary, we have shown that PLZF and RP58 are both BTB-ZnF transcription factors that preserve NPCs during spinal neurogenesis. Given the structure of the two proteins and drawing from the existing literature about them, it seems likely that both PLZF and RP58 recruit similar regulatory proteins to their targets but that the sets of genes they regulate may differ extensively due to their differential affinity for DNA targets. This observation is supported by the existing literature (see Chapter 3-1) where the sets of known targets for members of the BTB-ZnF family in hematopoiesis are largely non-overlapping. It is also

supported by our own findings that PLZF and RP58 have the unique regulatory activities such as PLZF promoting FGFR3 expression and RP58 repressing ventral identity markers. Furthermore, given the regionally restricted expression pattern observed by many of the BTB-ZnF family during spinal development (see Fig. 3-1) it is likely that many members of this family have acquired distinct activities suited to their precise domains of expression.

Prospects for a New Family of CNS Developmental Regulators

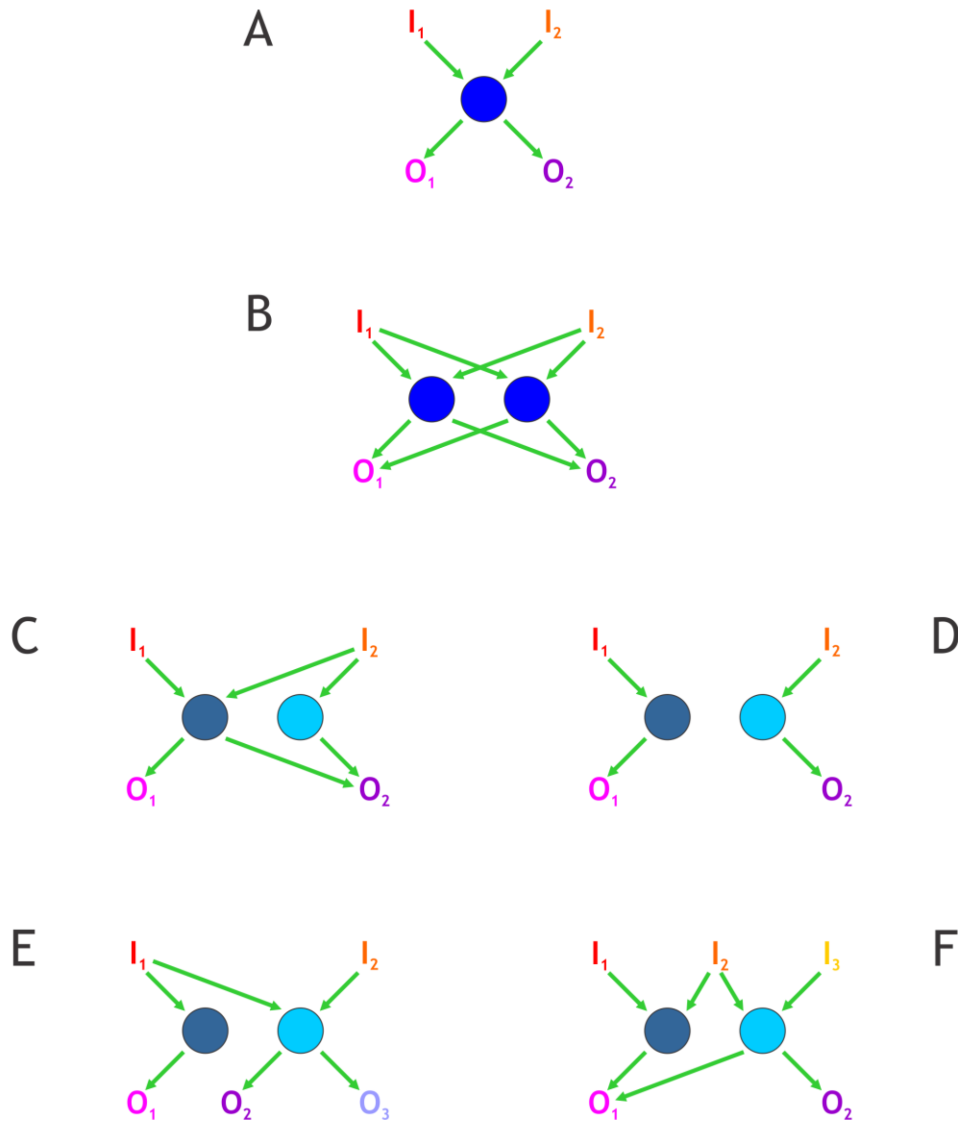
The development of the CNS is replete with examples of transcription factor families where multiple members are deeply involved in vital aspects of control (see Chapter 1-2). The most notable aspects of this phenomenon are members of the SOX and bHLH proteins whose influence can be readily discerned throughout CNS development. Notably for both families, closely related family members often have highly similar genetic targets and activities. For example, multiple members of the SOXB1 and HES subfamilies have implicated in the maintenance of NPCs. However, it is also clear that more distant members of these families can be involved in unrelated or even opposing processes. For example, while SOXB1 proteins preserve NPCs, members of the SOXC subfamily promote neuronal differentiation (see Chapter 1-2). This propensity of gene family members to be involved in related activities has long been a point of entry for the characterization of newly discovered genes. To pick just one prominent example, the discovery of proneural bHLH in Drosophila would provide concepts and principals that would later be applied to these families in mammals (Bertrand et al., 2002; Kageyama et al., 2008). Similarly, in the study of the BTB-ZnF family, the discovery of PLZF's and BCL6's roles in maintaining hematopoietic progenitors would lead directly towards efforts to characterize other family members and would also provide the interpretive framework for their initial study (Chen et al., 1993; Hoatlin et al., 1999a; Hoatlin

et al., 1999b; Melnick et al., 2002; Ye et al., 1993). As such, the identification of a new gene family with a role neural development is potentially of enormous significance.

But why should it be that members of gene families are often involved in related regulatory networks and developmental processes? This is currently thought to be a consequence of the manner in which new genes evolve. Over the evolutionary history of life, individual genes, segments of chromosomes, and even the entire genome have been duplicated and reduplicated through errors in chromosome replication, segregation, and repair. The creation of duplicate genes, or paralogs, generates functional redundancies and consequently a lessening of the negative selective pressure against the fixing of mutations. Commonly, this lessened selective pressure results in deleterious alterations and one of the paralogs may ultimately undergo nonfunctionalization (the degeneration of a paralog into a pseudogene). However, of more significance for the development of gene families and their involvement in developmental circuits, are the cases when the accumulated mutations direct paralogs towards complementary activities (subfunctionalization) or towards novel activities (neofunctionalization) (Conrad and Antonarakis, 2007; El-Mabrouk and Sankoff, 2012).

The multiplication of transcription factors has been particularly important in the history of evolution because they are the genes that control the activities of other genes and constitute the nodes of regulatory networks. When a transcription factor is initially duplicated, it is thought to be duplicated with all or most of its molecular associations intact. Firstly, this is because the duplicate gene encodes what is at first an identical protein and therefore one possessed of identical activities and affinities for cofactors and binding partners. Secondly, this is because the duplicate gene is duplicated *in situ* and retains some

Figure 4-1 – Development of Gene Networks Through Duplication with Inheritance



(A) The ancestral state, where the single transcription factor receives inputs (I_1 and I_2) and regulates outputs (O_1 and O_2).

(B) The state immediately upon gene duplication wherein both paralogs inherit the same sets of inputs and outputs from the ancestral gene.

(C-D) Four of many potential outcomes after mutation and selection cause the two paralogs to lose previous connections and gain connections to new inputs (I_3) and new outputs (O_3).

or all of the surrounding *cis*-regulatory elements that controlled its expression. However, such a perfect duplication tends to be unstable over evolutionary time. Eventually, random and unique mutations will accumulate in each paralog and both the coding sequence and regulatory sequences will diverge. For the protein, the mutations may alter DNA and protein binding specificities or alter its intrinsic transcriptional regulatory activity. For the *cis*-regulatory elements surrounding each paralog, mutations may change, destroy, or create regulatory motifs and thereby confer upon the two paralogs increasingly distinct modes of expression. The result of these mutations will ultimately be the creation of new transcription factors. However, because these new transcription factors were not created *ex nihilo* but rather already encoding functional proteins and already integrated into pre-existing gene regulatory networks, the two transcription factors will share many regulatory features and activities both with their ancestral gene and with each other (Nowick and Stubbs, 2010).

This study has identified six members of the BTB-ZnF family as being expressed during spinal development and characterized two of them. Given the current knowledge of the manner by which new genes arise from duplication and inherit pre-existing connections, it is highly likely that many additional BTB-ZnF family members will also be involved in the regulation of neural development. Given the twin cases of PLZF and RP58, it is likely that many members of this family will promote neural progenitor maintenance, particularly those closely related to PLZF and RP58. However, by analogy with other known families that regulate neural development, it is also likely that many will not. Indeed, one of the most parsimonious means of evolving a negative regulator of a transcription factor is the modification of a paralog. Mutations within a transcriptional regulatory domain may make it a competitor for a common binding site, such as the SOXB1 and SOXB2 proteins, while mutations in a DNA binding domain can convert a binding partner into a protein that

sequesters transcription factors away from their DNA targets, such as in the case of E and ID proteins. Furthermore, the BTB-ZnF family is ancient, being found throughout Eukaryotes (Stubbs et al., 2011), and has been associated with aspects neural development since prior to the divergence of the last common ancestor of *C. elegans* and vertebrates (see below). As such, sufficient time has passed for enormous functional divergence to evolve. For example, over a comparable evolutionary time period, members of the SOX family have become inextricably linked to both promoting and inhibiting the processes of neural progenitor maintenance, neurogenesis, and gliogenesis. It is therefore quite likely that such diversity will also be a feature of the BTB-ZnF family in neural development.

What might this diversity look like? Drawing from examples in the existing BTB-ZnF literature in *C. elegans* and Drosophila may provide some insight. *eor-1*, although its claim to being the *C. elegans* homolog of PLZF is disputable, is one of the few members of BTB-ZnF family possessed by *C. elegans*. *eor-1* has been linked with appropriate migration of some neuronal subtypes and the programmed cell death of others (Hoeppner et al., 2004). In Drosophila, BTB-ZnF have been linked to many aspects of neural development. The gene *Broad Complex* exists in four isoforms that are differentially expressed throughout the larval CNS, all of which have been implicated in different aspects of the morphological changes associated with metamorphosis into the adult CNS (Spokony and Restifo, 2009). One isoform, *Broad-Z3*, has been linked with promoting dendritic outgrowth in the dorsal bipolar dendrite sensory neuron (Scott et al., 2011) and whereas the BTB-ZnF gene *abrupt* has been associated with the repression of dendrite outgrowth in other populations (Li et al., 2004). *Chinmo*, another Drosophila BTB-ZnF gene, is expressed in a decreasing temporal gradient by neurons differentiating from mushroom body progenitors, a gradient that is thought to influence their ultimate cell type (Zhu et al., 2006). Mutations in the *fruitless* gene are associated with

defasciculation and improper axonal outgrowth (Song et al., 2002). Lastly, in the differentiation of the Drosophila sensillum, the BTB-ZnF protein *tramtrack* has been identified as being induced in the glial lineage to suppress neuronal identity, in part through the repression of proneural bHLH expression (Badenhorst et al., 2002).

Given that such diversity exists among the roughly dozen members of the Drosophila set of BTB-ZnF proteins, it is likely that even greater layers of complexity are to be found among the approximately 60 members of the mammalian array of BTB-ZnF proteins (Stubbs et al., 2011), with potential roles for BTB-ZnF protein in neurogenesis, gliogenesis, morphogenesis, guidance, and migration.

Similar diversity is to be expected in the hierarchical levels at which BTB-ZnF genes have become integrated into the neural developmental gene regulatory network. It has been proposed that such regulatory networks are made up of smaller modules that in turn are composed of circuits of inter-regulating genes, most of which are transcription factors or signaling factors. Each module fulfills one of a number of functions. For example, so-called kernel modules are highly conserved circuits that establish fundamental structural identities and initiate complete developmental programs. At the other extreme are the differentiation batteries that direct the acquisition of cell-type specific properties and induce the terminal selector circuits that sustain the gene expression profiles of differentiated cells (Erwin and Davidson, 2009; Nowick and Stubbs, 2010). Over the evolutionary history of the BTB-ZnF family, it is likely that they have integrated themselves into many levels of the gene regulatory network, much as members of the SOX family have. The SOXB1 family proteins appear to be vital components of a kernel module establishing NPCs, whereas the SOXC family can be considered components of a differentiation battery module promoting neuron

formation (see Chapter 1-2). Similarly, a case could be made that the SOXE and SOXD proteins represent a plug-in module, deployed in both neural crest formation and in gliogenesis, where SOX9 promotes specification, SOX10 initiates differentiation, and SOX5 and SOX6 inhibit the activities of SOX9 and SOX10 to control the rate of differentiation (see Chapter A-1). As previously discussed, we have proposed that RP58 is a participant in a plug-in module that is involved in the feedback inhibition of proneural bHLH proteins, and it is likely that other BTB-ZnFs participate in similar circuits. PLZF appears to participate in a FGF plug-in signaling module, although a complete understanding of PLZF's role will require the identification of its genetic targets.

Conclusion

Previous to the studies described in this work, the only BTB-ZnF family member with an extensive literature in the vertebrate CNS was RP58. RP58 had been identified as being important in promoting neuronal differentiation in several regions of the developing brain (Baubet et al., 2012; Okado et al., 2009; Xiang et al., 2012). PLZF had been identified as an anti-neuronal differentiation transcription factor (Sobieszczuk et al., 2010). We have now identified an additional four family members, BTBD15, simiRP58, ZBTB7A, and ZBTB39 as being expressed during the development of the spinal cord. With now six members found to be expressed during neural development, many seemingly only distantly related (Z.B. Gaber, unpublished observation), there now seems to be a high probability that these genes are but the forerunners of a previously unidentified transcription factor family regulating neural development. It is too soon to say what functions the members of this family might fulfill, but given the diversity found to exist among other key gene families in vertebrate neural development and within the BTB-ZnF family in invertebrates, the roles are likely to be

remarkably varied. Although it is probable that many BTB-ZnF proteins will perform a common function of recruiting HDACs and other elements of the cellular repression machinery to gene targets, divergence among the DNA binding domains of each protein are likely to target a distinct set of targets.

It has now been shown that PLZF promotes neural progenitor maintenance through the novel mechanism of enhancing FGFR3 expression and thereby FGF sensitivity and STAT signaling. It has been found that RP58's function in the brain and spinal cord are remarkably distinct but potentially share a common genetic circuit. It is currently unknown where other members of the BTB-ZnF family might be situated within the great puzzle that is the gene regulatory network that controls spinal development. However, now that the centrality of multiple members of the BTB-ZnF family has been established, we now know that the BTB-ZnF family is an excellent place to look for fascinating pieces.

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CHAPTER 5 – SOX9 Induces Precocious Differentiation of Oligodendrocytes

Abstract

The neuroepithelial progenitors cells (NPCs) of the developing spinal cord are arrayed along the dorsal-ventral axis in a series of domains with distinct competencies. Once established, each domain sequentially undergoes two periods of cellular differentiation. In the first period, the NPCs domains each produce specific neuronal subtypes. In the second period, the competence of the NPCs becomes altered and the cells within each domain begin giving rise to a domain-appropriate class of glia. This switch in cellular production between neurogenesis and gliogenesis is one of the most fundamental transitions in neural development. One of the most important regulators of this process is the transcription factor SOX9, the loss of which is associated with a failure of spinal progenitors to switch from neuron to glial production. However, the molecular pathways through which SOX9 acts are poorly understood. To elucidate events downstream of SOX9 in promoting the induction of gliogenesis, we misexpressed SOX9 in the develop chick spinal cord and observed changes in the expression of genes known to be involved in both neurogenesis and gliogenesis over a period of 48 hours. We found that ectopic SOX9 was sufficient to direct NPC differentiation and induce OLIG2 expression throughout the spinal cord. However, SOX9 was only competent to initiate the complete oligodendrocytes differentiation program when present in the ventral spinal cord. We further identified multiple genes as being misregulated within 10 hours of SOX9 misexpression, genes that potentially represent either direct targets of SOX9 regulation or important genes for the early phases of the initiation of gliogenesis. This work concludes with a brief discussion of a subsequent study conducted by collaborators that went on to identify additional SOX9 targets.

5-1 Introduction

The three fundamental cell types of the mature CNS are neurons, oligodendrocytes (OLs), and astrocytes (ASTs). Neurons form the electrochemical excitable cells whose connections form the neural network of the nervous system. OLs are the insulating, myelinating cells of the CNS that ensheath neuronal axons (Soldan and Pirko, 2012). The functions of ASTs are more diverse, entailing many distinct aspects of physiological, biochemical, and structural support. In addition, at least a subset of ASTs play vital roles in CNS injury repair through their capacity regeneration and glial scar formation (Sofroniew and Vinters, 2010). All three cell types are form from the neural progenitor cells (NPCs) of the neuroepithelium. However, not all cell types are produced at the same time nor are they produced at the same location. Temporally, NPCs undergo a period of neurogenic differentiation before switching over to the gliogenic differentiation of OLs and ASTs (Rowitch and Kriegstein, 2010). Spatially, different classes of neurons and glia are generated from precise domains situated along the dorsal-ventral axis of the neuroepithelium (Briscoe and Novitch, 2008; Briscoe et al., 2000; Hochstim et al., 2008). The majority of spinal OLs are generated from oligodendrocytes precursors (OLPs) that in turn derive from a single, OLIG2^{+} domain situated within the ventral spinal cord, termed the pOL, a domain that during neurogenesis produces motor neurons (Lu et al., 2000; Novitch et al., 2001; Rowitch and Kriegstein, 2010). Contrastingly, ASTs arise from astrocytes precursors (ASPs) whose production is more broadly distributed throughout many domains of the ventricular zone that during neurogenesis generated interneurons, although there is beginning to be some evidence that each domain may be generating distinct classes of astrocytes (Hochstim et al., 2008; Pringle et al., 2003).

Much is known about the spatial organization of gliogenesis, employing as it does the same combinatorial code of homeodomain and bHLH transcription factors that establish the domains for neurogenesis (Hochstim et al., 2008). Less well understood is the temporal control of the switch between neurogenesis and gliogenesis. Work over the past several years has identified two gene groups that are central to this switch: the nuclear factor I genes NFIA and NFIB, discussed later, and the SOXE genes, discussed here.

The three members of the SOXE subfamily of transcription factors (SOX8, SOX9, and SOX10) have been found to play critical roles in two distinct areas of neural development: neural crest formation and gliogenesis. In both areas, SOXE proteins have been found to be vital for both specification and maturation, with SOX9 being deeply involved in the progenitor specification whereas SOX10 is up-regulated later in development and is involved in maturation and differentiation. SOX8 expression typically overlaps with both SOX9 and SOX10 and many of its contributions are redundant with theirs (Stolt and Wegner, 2010).

SOXE proteins in neural crest formation

The neural crest is a transient population of cells that form along the dorsal ridge of the neural fold and neural tube. These cells subsequently delaminate and migrate out into the body before ultimately differentiating into a wide variety of cell types including the neurons and glia of the peripheral nervous system, pigment generating melanocytes, and many of the tissues of the craniofacial region. Although in Xenopus, SOX8 is crucial to the formation of neural crest, in avians and in mammals it is SOX9 that is both first expressed and most vital for neural crest induction (Stolt and Wegner, 2010). SOX9 is activated in the dorsal neural fold and its expression is retained as neural crest cells begin delaminating and migrating.

Misexpression of SOX9 has been found sufficient to convert the neuroepithelial cells of the early neural tube to a neural crest identity and to induce the expression genes associated with neural crest identity such as FOXD2, SLUG, HNK1, and SOX10. Correspondingly, loss of SOX9 through conditional deletion results in a dramatic reduction in the formation of the neural crest lineage. Intriguingly, although able to induce many neural crest markers throughout the neuroepithelium, SOX9 was only able to induce neural crest delamination effectively in the dorsal spinal cord. This appears to be due to the presence of RhoB in the dorsal spinal cord as co-transfection of SOX9 and RhoB facilitates delamination at all dorsal-ventral levels. Significantly, in light of SOX9's role in inducing gliogenesis in the CNS, sustained ectopic SOX9 expression in the neural crest lineage strongly biased cells against differentiating as neurons but rather favored maturation into peripheral nervous system glial cell types such as Schwann and satellite cells (Cheung and Briscoe, 2003; Cheung et al., 2005). This may suggest that the induction of PNS and CNS glia many involve the activation of common, SOX9 mediated pathways.

SOX10 expression begins after SOX9, starting in late-premigratory neural crest. However, SOX10 expression also persists longer in the glia of the PNS. As with SOX9, SOX10 also appears to direct cells neural crest cells towards a glial identity, its deletion resulting in a failure of both Schwann cell and satellite cell differentiation while PNS neuron formation is unaffected (Britsch et al., 2001).

SOXE proteins in gliogenesis

SOX9 has been found to play a vital role in the neurogenesis-gliogenesis switch. In the developing spinal cord, SOX9 is not expressed by either neurons or the neuroepithelium

throughout most of neurogenesis. However, at e4 in chick and e11.5 in mice, immediately prior to the onset of gliogenesis, SOX9 begins to be expressed throughout the ventricular zone (Kang et al., 2012; Stolt et al., 2003). In addition, SOX9 is also expressed at most stages of ASP and AST development but is down-regulated in OLPs and absent from differentiated OLs. When SOX9 is conditionally ablated from the CNS, the early phases of neural development, patterning and neurogenesis, are unaffected. However, the switchover to gliogenesis is severely impaired. Only small numbers of OLPs and ASPs are generated (Stolt et al., 2003), and it is likely that many of these only form due to the late deletion of SOX9 in the conditional mutants used in this study and due to functional redundancy between SOX9 and SOX8 (Stolt et al., 2005). Instead, of switching over to glial production, spinal NPCs continue to generate neurons (Stolt et al., 2003). This finding established SOX9 as one of the most important known regulators of the neurogenesis-gliogenesis switch.

As in neural crest, SOX10's activity during gliogenesis is situated at a later point the developmental pathway. Unlike in the PNS where SOX10 is expressed by all types of glia, in the CNS SOX10 expression is limited to the OL lineage and has been linked with their terminal differentiation from OLPs. Mice deficient for SOX10 successfully generate OLIG2^{+}, PDGFRα^{+} OLPs. However, these OLPs fail to up-regulate mature OL markers such as myelin proteins. In particular, SOX10 has been found able to bind and induce the expression of myelin basic protein (MBP), suggesting a direct role in regulating the expression of OL specific genes (Stolt et al., 2002).

Little is known about the factors that regulate and influence the activity of SOXE proteins in this process. Perhaps the most notable are the two the SOXD proteins, SOX5 and SOX6 which have been found to oppose the activity of SOX9 during gliogenesis. Both SOX5

and SOX6 expression patterns overlap with that of SOX9 in the ventricular zone and the glial lineage. In particular, SOX5 and SOX6 expressed by OLPs and, like SOX9, are subsequently down-regulated as they differentiate. This expression may be dependent upon SOXE activity as in *Sox8*$^{-/-}$;*Sox9*$^{-/-}$ double mouse mutants, none of the few remaining OLPs express SOXD proteins (Stolt et al., 2006). Evidence suggests that SOXD proteins function by antagonizing the activity rather than the expression of the SOXE proteins, an activity that occurs both in gliogenesis and some aspects of neural crest formation, particularly melanocytes (Stolt et al., 2008). In *Sox5*$^{-/-}$;*Sox6*$^{-/-}$ double mutants, precocious formation of OLPs and precocious differentiation of OLs are observed, suggesting that their endogenous activity is to oppose the development of the OL lineage induced by SOX9 and SOX10 (Stolt et al., 2006). Supporting this model, it was found that SOXD's were able to bind some known SOX9 and SOX10 binding sites and compete with these proteins for occupancy. Additionally, evidence has been found that at least in some contexts, SOXD proteins may recruit inhibitory factors such as CtBP2 and HDACs to targets of SOXE transactivation (Stolt et al., 2008; Stolt et al., 2006). It has therefore been proposed that the rate of gliogenesis is determined in part by the balance between pro-gliogenesis SOXE proteins and anti-gliogenesis SOXD proteins.

Because of its centrality to the neurogenesis-gliogenesis switch, we set out to further explore the mechanism of SOX9 induction of gliogenesis. We first established the experimental conditions under which SOX9 was competent to induce gliogenesis, finding that SOX9 was only able initiate glial differentiation after e4/St21 in chick. We next characterized how known markers of glial differentiation changed in response to ectopic SOX9, identifying several potential direct targets and allowing us to further develop existing models of how these genes interact.

5-2 Materials and Methods

All experiments were performed as described in ***Chapter 2-2 Materials and Methods*** except for the additional reagents listed below.

Table 5-1 - Antibodies used for Immunohistochemistry

Antigen	Host Species	Source and References
HNK-1	Mouse	BD Pharmingen CD57

***Table 5-2 - PCR Primers used to create* in situ *probes*.**

Probe Target	Forward Primer (5' to 3')	Reverse Primer (5' to 3')
PLP1	TGTTTGGGAAAGTGGCTAGG	GAG<u>ATTAACCCTCACTAAAGGGA</u> CTTCGCCGTCCTCAAGCTGA
SOX6	TGGGGCTGATTTTCTTGTGT	GAG<u>ATTAACCCTCACTAAAGGGA</u> GTGTCCACCACATCTGCAAG
SOX10	ACCATGGCCAACTCTTTGTC	GAG<u>ATTAACCCTCACTAAAGGGA</u> ACAGATGGGACAGGGGGAAG

Underlined text indicates T3 polymerase binding site.

5-3 Results

SOX9 activity dependent upon timing of transfection

SOX9 is known to be involved in the specification of neural crest as well as in promoting the neurogenesis/gliogenesis switch. It had previously been demonstrated that when ectopic SOX9 was introduced to the spinal cord at e2 / HH St 10-11, many of the transfected cells adopted a neural crest fate. This finding is consistent with the known role of SOX9 at this stage in development where it is expressed among the neural crest precursors (Cheung and Briscoe, 2003). However, SOX9's broad expression within the ventricular zone, the mode of expression associated with its role in the neurogenesis/gliogenesis switch, does not begin until e4 / HH St 21 in chick (Kang et al., 2012). We therefore decided to transfect the developing spinal cord at later time points to determine whether over time there is a shift in manner in which NPC's respond to ectopic SOX9.

To do this, we transfected developing chick embryos with SOX9 at e3 / HH St 18 and e4 / HH St 21 through *in ovo* electroporation. We utilized the pCIG misexpression vector (Megason and McMahon, 2002) which possess a cytomegalovirus (CMV) enhancer and chick β-actin promoter to drive the expression of SOX9. The pCIG vector also possesses an nlsEGFP reporter whose expression is linked to that of SOX9 by an internal ribosomal entry sequence. When embryos were transfected with SOX9 at e3 / HH St 18 and allowed to develop until e5 / HH St 25, significant production of ectopic neural crest was observed (Fig. 5-1 A,B) by their expression of the early migratory neural crest marker HNK-1(Bronner-Fraser, 1986). In addition, many of the GFP^+ transfected cells has delaminated from the neuroepithelium and migrated out into the surrounding mesenchyme. However, when SOX9 was electroporated

into the spinal cord at e4 / HH St 21 and embryos were then allowed to develop until e5 / HH St 25, GFP did not migrate out of the spinal cord but rather remained medial. Furthermore, no significant ectopic induction of HNK-1 was observed (Fig. 5-1 C,D). These data indicate that after e4 / HH St 21, spinal progenitors are no longer competent to become directed towards a neural crest fate by SOX9.

Having concluded that after e4 / HH St 21 SOX9 transfections were not inducing neural crest, we next investigated what cell types were being generated at this later time. We found that cells transfected with SOX9 down-regulated their expression of SOX2 (Fig. 5-1 E,F), a marker of undifferentiated NPC identity (Bylund et al., 2003). In addition, the great majority of SOX9 transfected cells did not undergo terminal neuronal differentiation, indicated by their failure to either migrate laterally into the mantle zone or to express neuronal markers such as TUJ1 (Fig. 5-1 G,H) (Lee et al., 1990). The discovery that SOX9 transfections were directing ventricular NPCs cells differentiate from a SOX2$^+$ state without becoming TUJ1$^+$ neurons, we next set out to determine whether differentiating into one or both of the other two principal cell types of the spinal cord: OLs and ASTs.

SOX9 is sufficient to induce the formation of oligodendrocyte precursors

To test the hypothesis that starting at e4 / St 21, ectopic SOX9 was sufficient to induce the differentiation of glial cells, we examined changes in the expression of multiple markers of progenitor and glial identity at three time points following electroporating SOX9 into the spinal cord: 10 hours post electroporation (~HH St 22), 24 hours post electroporation (~HH St 25), and 48 hours post electroporation (~HH St 28). At none of these time points were any markers associated with an AST fate (such as FGFR3, GLAST, or GFAP) observed to be

significantly up-regulated (Fig. 5-2 C,M,W and data not shown). However, ectopic SOX9 was found to induce the expression of multiple markers of the OL identity.

At 10 hours post electroporation (h.p.e.), nearly all transfected cells remained located within the ventricular zone and continued to express the NPC marker SOX2 (Fig. 5-2 A,E). This, and the lack of observable up-regulation among most of the OL markers examined (Fig. 5-2 G-J) indicates that at 10 h.p.e., SOX9 transfected cells remained undifferentiated NPCs. The only exception to this trend was that the expression of SOX6 transcript was found to be greatly increased (Fig. 5-2 F). SOX6 is normally absent from the most of the ventricular zone at this time but is present in NPCs after the onset of SOX9 expression and is also highly expressed among OLPs (Stolt et al., 2006). This rapid induction of SOX6, the first gliogenesis marker observed to be up-regulated, may potentially indicate that SOX6 is an early effecter of SOX9 activity or possibly even a direct target of SOX9 regulation (Fig. 5-2 F).

Although at 10 h.p.e. there were few indications of glial differentiation and virtually all transfected cells remained $SOX2^{+}$ NPCs, there were signs that progenitor maintenance was beginning to be compromised. The expression of the pro-progenitor transcription factor and NOTCH-signaling effector HES5-2 was greatly inhibited by SOX9 transfection. Additionally, the expression of FGFR3, the most broadly and highly expressed receptor for the pro-progenitor FGF signaling pathway, was also found to be dramatically reduced within 10 hours of the introduction of ectopic SOX9 (Fig. 5-2 B,C). Although the loss of either NOTCH or FGF signaling at this stage of spinal development is normally associated with increased neuronal differentiation (Z.B. Gaber, unpublished observations), this was found to not be the case here. Instead, SOX9 transfection appeared to slightly reduce the expression markers of neurogenesis such as the proneural bHLH protein NEUROG2 (Fig. 5-2 D).

By 24 h.p.e. many signs of OLP differentiation were evident. SOX9 transfected cells, although still medially located, had significantly down-regulated their expression of the NPC marker SOX2 without any corresponding increase in the expression of neurogenesis markers such as NEUROG2 (Fig. 5-2 M). Additionally, FGFR3, which in addition to its role in maintaining NPCs is also a marker of ASPs (Pringle et al., 2003), also remained down-regulated (Fig. 5-2 O). Instead, many SOX9 transfected cells were found to highly express multiple markers of OLP identity. SOX6 became highly expressed by nearly all transfected cells, while the broad OL lineage marker OLIG2 (Lu et al., 2000) became up-regulated by a large portion of GFP^+ cells (Fig. 5-2 P,Q). NKX2.2 and SOX10, markers normally expressed slightly later during OLP formation than OLIG2 (Stolt et al., 2002; Zhou et al., 2001), also began to be induced in a few scattered cells, primarily within the ventral spinal cord (Fig. 5-2 R,S). However, no expression of markers of mature OL identity, such as PLP1 or MBP, were observed (Fig. 5-2 T, data not shown). Collectively, these observations suggest that by 24 h.p.e. SOX9 transfected cells were beginning to differentiate into early stage OLPs.

OLP development was observed to progress further at 48 h.p.e. There was a further loss of $SOX2^+$ NPCs and as well as of neurogenesis markers such as NEUROG2, and there was a further increase in the number of cells expressing the OL lineage marker OLIG2 (Fig. 5-2 X-AA). It was observed that although SOX9 is sufficient to induce OLIG2 at all dorsal-ventral levels, markers of a more mature OLP identity, such as NKX2.2 and SOX10, and, for the first time, markers of an OL identity such as PLP1, appear to be principally be induced in the ventral neural tube near the endogenous pOL domain (Fig. 5-2, AB-AD). This finding may indicate that competency to initiate the complete OL differentiation program is regionally restricted to the ventral neural tube.

5-4 Figures

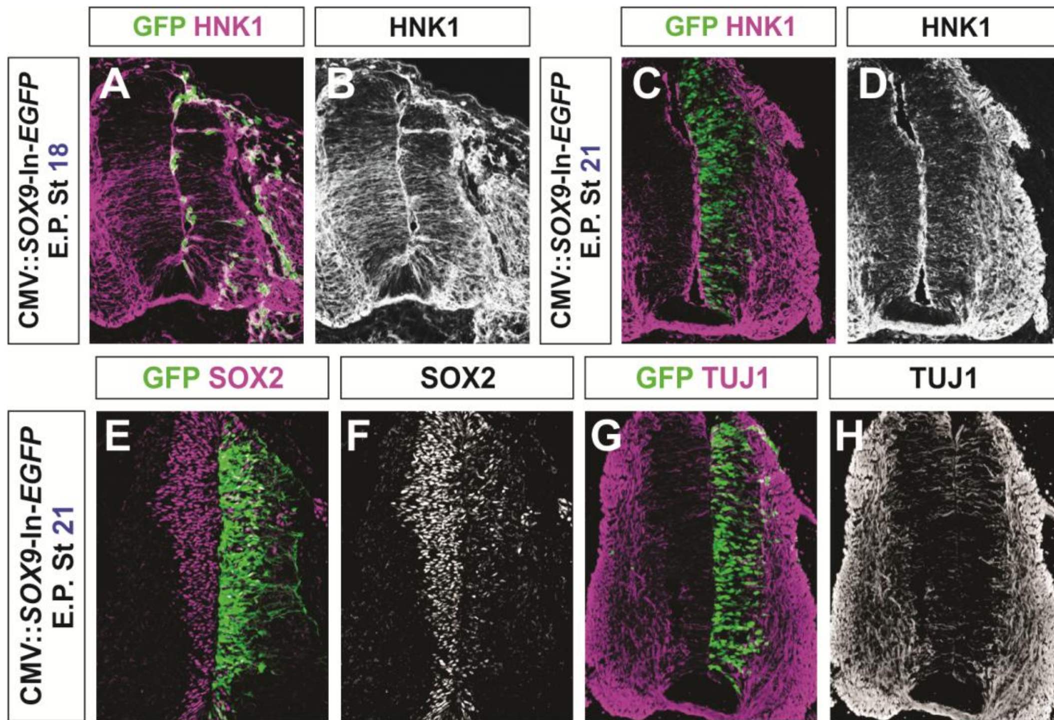

Figure 5-1 – The nature of NPC response to ectopic SOX9 changes over time

(5-D) NPCs were transfected with *SOX9*-IRES-*nlsEGFP* vectors at either e3 / HH St 18 or e4 / HH St 21 and analyzed at e5 / HH St 25. When electroporated at e3, SOX9-transfected cells displayed an increased expression of the neural crest marker HNK-1 and frequently exhibited a neural crest-like phenotype of delaminating and migrating outside the confines of the spinal cord. However, when electroporated at e4, SOX9-transfected cells neither up-regulated HNK-1 nor migrated from the spinal cord, indicating that by e4, SOX9 is no longer sufficient to induce the differentiation of neural crest from spinal NPCs.

(E-G) NPCs transfected with SOX9 at e4 are observed within 24 hours down-regulate the expression of the NPC marker SOX2. However, they did not up-regulate the broad neuronal marker TUJ1. This suggests that ectopic SOX9 is promoting a loss of $SOX2^{+}$ progenitor identity without directing cells towards neuronal differentiation.

Figure 5-2

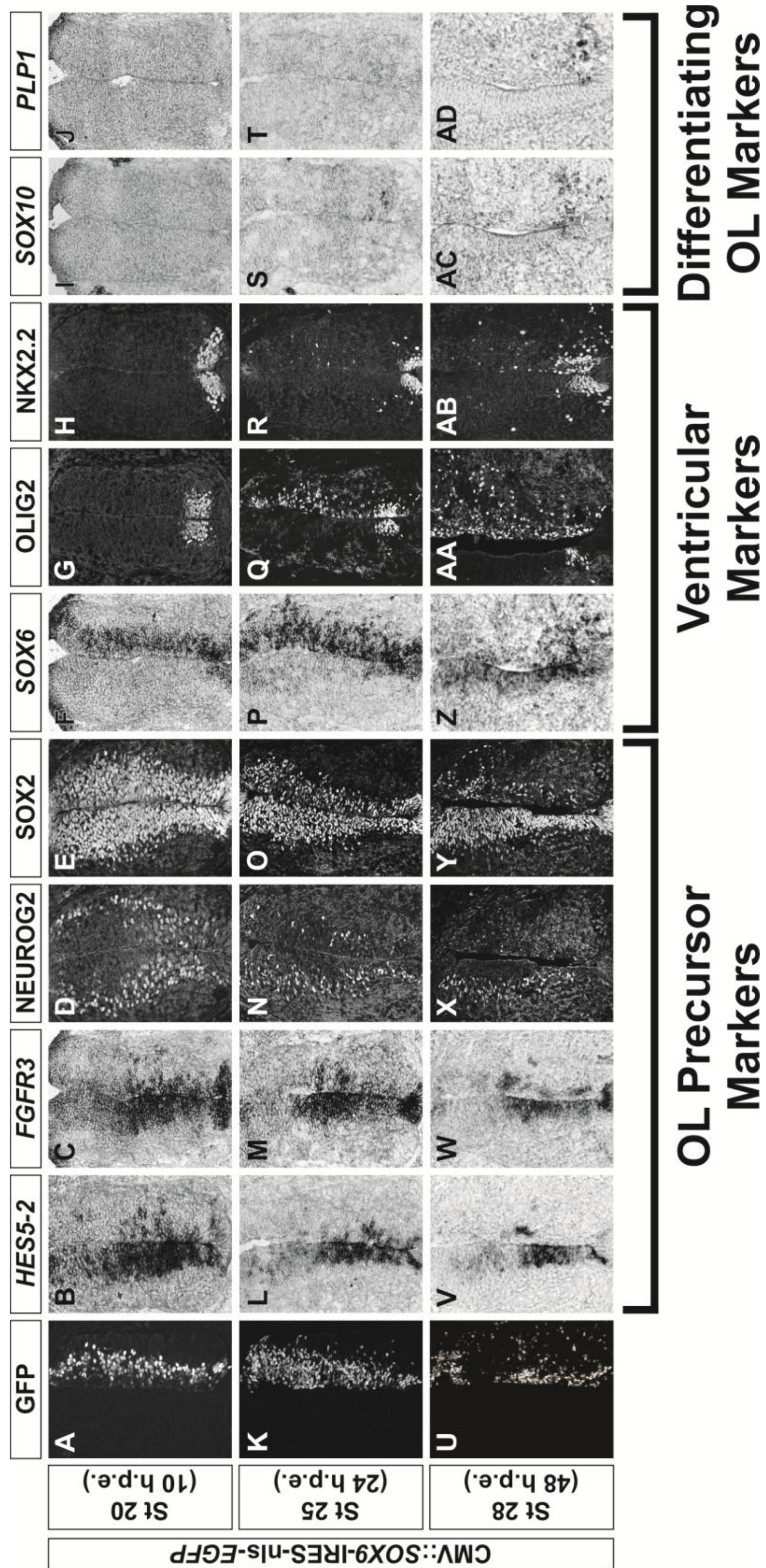

Figure 5-2 – Ectopic SOX9 induces the differentiation of OLPs

(A,K,U) Developing spinal cords were electroporated with *SOX9*-IRES-*nEGFP* vectors at e4 / HH St 21 and at 10, 24, and 48 hours post electroporation (h.p.e.).

(B-E,L-O,V-Y) The expression of the NOTCH effecter HES5-2 and the principal FGF receptor in the developing spinal cord, FGFR3, were both found to be down-regulated within 10 hours of transfection with SOX9, indicating a rapid inhibition of pro-progenitor signaling systems by SOX9. In addition, this loss of factors that promote progenitor maintenance was not accompanied by an increase in markers of neuronal differentiation such as NEUROG2, suggesting that transfected cells were ceasing to be progenitors without becoming neurons instead.

(F,P,Z) The first gene observed to be up-regulated by ectopic SOX9 was SOX6, a gene expressed within the ventricular zone shortly after the onset of SOX9 expression and which is subsequently highly expressed within the OLP lineage until their differentiation into OLs.

(G-J,Q-T,AA-AD) Starting 24 h.p.e. SOX9 transfected cells began to up-regulate multiple markers of the OL lineage. Notably, expression OLIG2, which is activated early in the OL lineage, was highly expressed starting at 24 h.p.e., while markers whose expression begins later in the OL development were expressed at lower levels. At 48 h.p.e., most transfected cells continued to express OLP markers, although the expression of differentiating OL cells such as PLP1 began to be detected. It was observed that SOX9 was most effective in inducing markers of later OLP and OL identity in the ventral neural tube, near the pOL where most OLPs normally form, potentially NPC competency for making OLs is regionally restricted.

5-5 Discussion

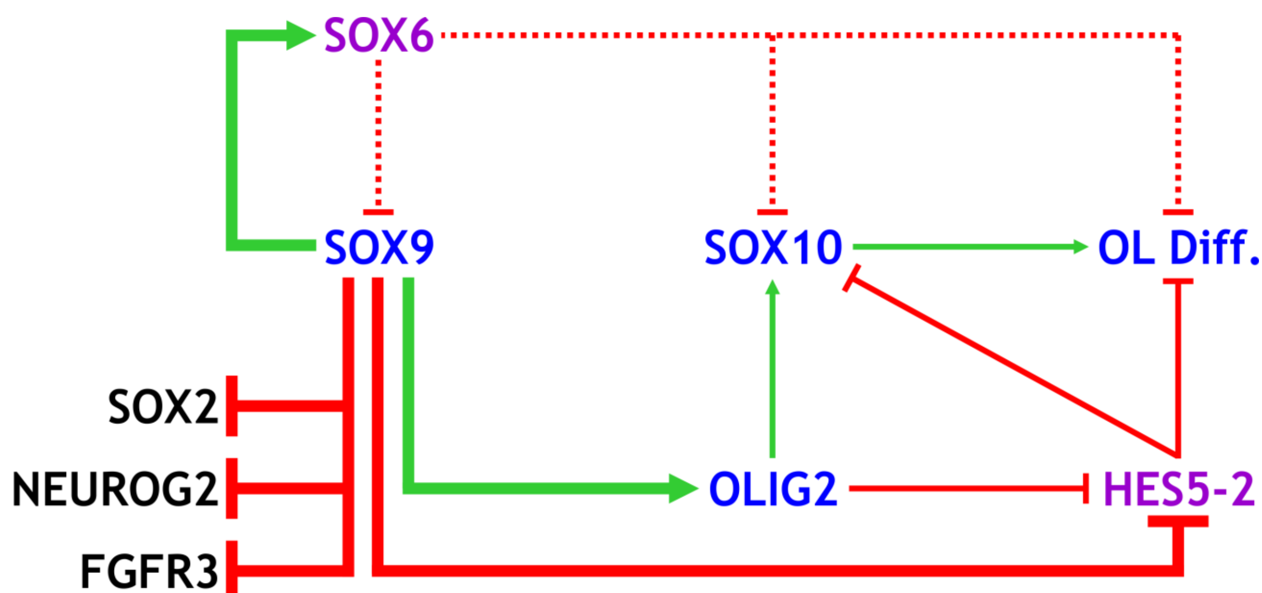

Figure 5-3 - Consolidated model of pathways regulated by SOX9 during OL differentiation

It has previously been established that SOX9 expression in the ventricular zone begins shortly prior to the onset of gliogenesis and that the conditional deletion of SOX9 from the mouse CNS results in a failure of NPCs to switch from neuronal to glial differentiation (Kang et al., 2012; Stolt et al., 2003). Furthermore, it has been found that SOX9 expression persists in developing ASPs but becomes down-regulated in developing OLPs (Stolt et al., 2003). In this study, for the first time we have demonstrated that ectopic SOX9 is sufficient to induce the precocious differentiation of OLPs but not cells of AST lineage. In addition, we discovered that this induction is associated with a rapid induction of SOX6 and repression of important elements of both NOTCH and FGF signaling, identifying potential candidates elements directly or indirectly regulated by SOX9. These findings raise two critical questions. Firstly, why was ectopic SOX9 sufficient for the differentiation of OLPs but not ASPs? Secondly, what are the implications of this study for our broader understanding of the genetic pathways that regulate gliogenesis and, in particular, the formation of OLPs?

Ectopic SOX9 and astrocytes

At no point following electroporation with SOX9 were markers of AST identity such as FGFR3, GLAST, or GFAP observed to be up-regulated. Indeed, FGFR3 was strongly inhibited by ectopic SOX9. This finding that SOX9 preferentially induces OLP development over ASP development is striking given SOX9's endogenous expression pattern and known developmental functions. Firstly, SOX9 is expressed by all NPCs during gliogenesis, including those that give rise to ASPs, and its expression is maintained in the AST lineage far longer than in the OL lineage. Secondly, loss of SOX9 in the mouse CNS is associated with a severe reduction in ASP production. These observations would suggest that SOX9 is necessary for ASP development. However, our data indicate at the experimental conditions we employed, SOX9 is not sufficient to induce astrogliogenesis.

There are several potential explanations for SOX9s apparent insufficiency in this matter. Firstly, as will be discussed in more detail below in the context of OLP differentiation, SOX9 may have been unable to induce ASPs due to a lack of necessary cofactors. In particular, it is possible that the developing spinal cord is not yet temporally competent for ASP production. As we shown, the competence of the spinal cord to respond to SOX9 shifts with time. At e3, electroporation with SOX9 induces primarily neural crest whereas at e4 electroporation with SOX9 induces primarily OLs. Given that ASP formation begins after the window of this experiment, it is possible that competency of cells to generate ASPs in response to SOX9 also may not develop until later. As such, when SOX9 is introduced prior to the spinal cord acquiring this competency, SOX9's induction of a gliogenic program may generate primarily OLs by default.

An alternative explanation is that SOX9 seeming insufficiency for generating ASPs may partially stem from the means used to detect ASPs. GFAP is a very late, mature AST marker, potentially not up-regulated until significantly later than the 48 hour experimental window. FGFR3 is a marker of many astrocytes, but not all, and its loss does not result in a failure of AST differentiation but rather a shift in AST subtypes where many protoplasmic, grey matter ASTs acquire at least some of the qualities of fibrous, white matter ASTs (Pringle et al., 2003). Thus, it is possible that SOX9 could be inducing FGFR3^{-} astrocytes, although it unclear whether this would reflect an aspect of SOX9's endogenous role in promoting the formation a particular subtype of AST or whether this is an artifact of a normally moderate SOX9 repression of FGFR3 being over-initiated due to the high levels of gene misexpression.

Potentially supporting both explanations is the finding by collaborators, using different conditions, that ectopic SOX9 could induce only a small number of GLAST expressing ASTs (Kang et al., 2012). As such, it does appear that SOX9's competence for inducing AST formation is highly limited at this stage of development and potentially very difficult to detect with some reagents.

SOX9 within the OLP differentiation network

Several key players in the differentiation of the OL lineage from NPCs have been identified, including OLIG2, SOX10, NKX2.2, SOX6, and HES5, each of which will be discussed in turn. Although all these factors have been situated in a common process SOX9, our findings shed significant light on interactions among these factors and their relative positions in a pro-OL development network.

OLIG2 is perhaps the most broadly expressed gene within the OL lineage and is absolutely indispensible for both OLP specification and differentiation (Ligon et al., 2006). Prior to neurogenesis, OLIG2 expression is established in a region of the ventral spinal cord termed the pMN due its being the site of motor neuron production (Novitch et al., 2001). During neurogenesis, OLIG2 expression overlaps with that of NEUROG2 and together these two proteins are thought to direct MN differentiation. However, prior to gliogenesis, NEUROG2 is cleared from the pMN and as oligogenesis begins, the pMN becomes the predominant site of OLP differentiation and is re-identified as the pOL (Zhou et al., 2001). In this study, we found that ectopic SOX9 was sufficient to both repress the expression of NEUROG2 and to induce the expression of the pOL marker OLIG2. It is likely that both actions are vital to SOX9's ability to promote precocious OLP formation. Firstly, by prematurely clearing NEUROG2 from the pMN, ectopic SOX9 is blocking neurogenesis and artificially generating the conditions normally present in the pOL. Secondly, SOX9 activation of OLIG2 is vital for many subsequent steps in OLP formation. OLIG2 has been found to promote the expression of many genes important for subsequent OLP maturation including SOX10, NKX2.2, and PDGFRα (Kuspert et al., 2011; Liu et al., 2007; Zhou et al., 2001). Intriguingly, SOX9 was able to induce OLIG2 throughout all dorsal and ventral levels of the neuroepithelium. Although OLIG2 expression in the pMN is absolutely dependent upon Sonic Hedgehog and the proper combination of ventral homeodomain proteins, OLIG2 expression in differentiating OLPs has been found to be Sonic Hedgehog independent (Chandran et al., 2003). Although these data are not conclusive, it is possible that OLIG2's acquisition of Sonic Hedgehog independence during gliogenesis stems from the ability of SOX9 to sustain its expression during gliogenesis.

After 48 h.p.e., SOX10 and NKX2.2 were only up-regulated in a small subset of SOX9 transfected cells, suggesting both that these genes are not direct targets of SOX9 activation

and possibly that some necessary co-factor was not present. However, as previously discussed, both SOX10 and NKX2.2 are activated by OLIG2 and promote subsequent maturation of OLPs (Liu et al., 2007; Stolt et al., 2002). It was therefore intriguing that SOX9's induction of OLIG2 did not subsequently result in more than small amounts of either SOX10 or NKX2.2. Furthermore, the majority of SOX10, NKX2.2, and PLP1 expression observed was induced ventrally. There are several potential explanations for this finding. Firstly, other, unknown factors are only present within the ventral neural tube, without which SOX9 is unable to initiate OL formation. Secondly, there may potentially be unknown factors present within the dorsal neural tube that repress the formation of OLPs. In either case, these factors presumably confer spatial specificity to the type of glia induced by the broadly expressed SOX9, particularly with regards to the selection between OL and AST lineages. Thirdly, the problem may actually stem from a failure to down-regulate SOX9. SOX9 expression is normally lost following OLP specification and delamination and it may be that some of its activities conflict with proper OLP maturation. A fourth possibility is that the limitation lies not with SOX9 activity but rather in a limited ability of OLIG2 to induce subsequent steps in OL differentiation. Some evidence for this exists within the literature. Earlier in development, during neurogenesis, it was found that although ectopic OLIG2 was sufficient to induce MN throughout the ventral neural tube, its capability to do this became significantly reduced dorsally (Novitch et al., 2001). Similarly, others studies have found that ectopic OLIG2 on its own was only able to induce moderate amounts of OLP formation whereas co-misexpression of OLIG2 and NKX2.2 were far more potent (Zhou et al., 2001). Furthermore, it has been argued that while OLIG2 acts as a homodimeric repressor during motor neurogenesis, OLIG2 potentially heterodimerizes with co-activators during OL formation, co-activators potentially not present in the dorsal neural tube (Li et al., 2011). Thus it may be that SOX9, while capable of inducing OLIG2, is incapable of activating a vital

factor without which OLIG2 is unable to efficiently induce OLP formation outside the ventral neural tube.

SOX6 is thought to act as a competitive and direct inhibitor of SOX9 and SOX10 targets during glial development, potentially as a means of feed-forward inhibition to slow the rate of OLP and OL differentiation. Supporting this model are the observations that SOX6 expression pattern closely shadows that of SOX9 and that SOX6 is expression is lost in *Sox8*$^{-/-}$*;Sox9*$^{-/-}$ double knockout mice (Stolt et al., 2008; Stolt et al., 2006). Our finding that SOX6 expression highly up-regulated within 10 hours of SOX9 misexpression suggests SOX6 is an excellent candidate to be a direct target of SOX9 transactivation. The over-activation of SOX6 by ectopic SOX9 may also present another potential explanation for the failure of OLPs induced by SOX9 to further differentiate. *In vivo*, expression of the SOXE proteins SOX9 and SOX10 are thought to exist in careful balance with SOX6 wherein SOX6 is activated such that it slows but does not stop OL differentiation from OLPs. However, when SOX9 is over-expressed, SOX6 becomes activated both prematurely and to levels far higher than observed *in vivo*. This over-induction of SOX6 may disrupt its balance with SOXE proteins and result in an arrest in OLP maturation.

Lastly, ectopic SOX9 was found to rapidly down-regulate the expression of FGFR3, the most highly expressed FGF receptor at this stage of development, and to inhibit the expression of HES5-2, one of most important effectors of the NOTCH signaling pathway. Both FGF and NOTCH signaling are vital for proper maintenance of NPCs and the loss of FGFR3 and HES5-2 might respectively severely compromise each pathway. It is therefore interesting to contemplate whether inhibition of these pathways might represent critical, unappreciated aspects of SOX9 activity. Given that SOX9 is thought to principally act as a transcriptional

activator, neither gene is likely to be a direct target of SOX9. However, the reduction of FGFR3 and HES5-2 are both plausible mechanisms of inhibiting the maintenance of NPCs and thereby facilitating the differentiation of glial precursors. Furthermore, HES5-2 has been found to be highly expressed in OLPs and to oppose both OL differentiation and expression of myelin proteins by repressing targets such as SOX10 (Liu et al., 2006). It is therefore possible that SOX9 inhibition of HES5-2 during its transient expression in early OLPs might facilitate SOX10 expression and OL maturation. The repression of FGFR3 by SOX9 is somewhat surprising given the continued expression of both genes in the cells of the AST lineage (Pringle et al., 2003; Stolt et al., 2003). However, it may be that at endogenous levels, SOX9 merely acts to attenuate FGFR3 expression and thereby negatively fine tune FGF responsiveness of ASPs in a manner similar to the way we have argued PLZF positively adjusts FGF responsiveness.

Given these observations, we propose the following, consolidated model of SOX9 induction of OLP formation (Fig. 5-3). Upon the activation of SOX9 within the ventricular zone of the developing spinal cord, key players in both progenitor maintenance pathways (FGF, NOTCH) and neurogenesis (NEUROG2) are rapidly down-regulated. Subsequently, SOX9 induces the expression of OLIG2, one of the first determinants of OLP identity. Simultaneously with all these developments, SOX9 also induces the expression of SOX6, a feed-forward inhibitor that antagonizes the activation of SOX9 and SOX10 targets and thereby prevents the premature and excessive differentiation of OLPs from the ventricular zone and of OLs from OLPs. However, further stages in OLP development, including cell-cycle exit and terminal differentiation, cannot be efficiently induced by SOX9, whether due to the limited competence of SOX9 at this time, the absence of a vital cofactor, the presence of a potent inhibitor, or some combination of all these explanations.

5-6 Further Developments

Many questions still remain as to how SOX9 induces the neurogenesis/gliogenesis switch. Most of the direct targets by which SOX9 inhibits neuronal differentiation and promotes glial differentiation are unknown. Similarly, the cofactors that influence SOX9 activity and thereby permit it to induce neural crest during one phase of development and gliogenesis at another also need to be identified. Our discovery that SOX9 alone was sufficient to induce precocious gliogenesis led colleagues to investigate whether NFIA might be a target of SOX9 regulation. NFIA, like SOX9, has been shown to be a vital player in promoting the neurogenesis/gliogenesis switch. Misexpression of NFIA induces ASP markers within the ventricular zone whereas knocking-down NFIA results in NPC cell death and excessive neuronal differentiation (Deneen et al., 2006). It was known that NFIA became active in the ventricular zone of the developing spinal cord approximately a day after SOX9. This coupled with our discovery that SOX9 was capable on its own of inducing gliogenesis led Kang et al. (2012) to investigate whether SOX9 might be capable of inducing and acting through NFIA. They discovered that SOX9 directly binds and activates the expression of NFIA and that furthermore, both proteins physically interact and cooperatively activate the expression of several genes that they found to be involved in astrocytes development including APCDD1, MMD2, and ZCCHC24 (Kang et al., 2012).

In addition to establishing the regulatory relationship between two of the most important known factors the neurogenesis/gliogenesis switch, this study also sheds light on aspects of our own study of SOX9. The finding that NFIA and SOX9 cooperatively activate genes necessary for AST differentiation may potentially explain the limited extent of AST formation in our SOX9 misexpression studies. It has been previously reported that ectopic

NFIA is capable of inducing AST formation but that this activity was greatly inhibited by the presence of OLIG2 (Deneen et al., 2006). Given our finding that SOX9 is capable of inducing OLIG2, it is likely that SOX9's limited ability to induce AST formation was due in part to SOX9 activation of OLIG2 interfering with the ability of the SOX9-NFIA complex to initiate astrogliogenesis.

5-7 BIBLIOGRAPHY

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