THE REGULATION AND FUNCTION OF SOXB1 GENES AND PROTEINS DURING NEURAL 
INDUCTION AND DEVELOPMENT IN *XENOPUS LAEVIS*

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ABSTRACT

In *Xenopus laevis*, ectodermal cells are induced to form neural tissue when BMP signaling is inhibited. Little is known about the steps following inhibition of BMP that allows for the expression of early neural genes such as *sox2*, *sox3* and *geminin*. This research investigates the regulation and function of the SoxB1 genes and proteins which are activated by BMP inhibition during neural induction in *Xenopus*. We use gain and loss of function assays to determine that BMP signaling inhibits the expression of the *soxB1* gene, *sox3* via its downstream targets, XVent1 and XVent2. Additionally, we demonstrate that FGF signaling is required for the induction of *sox2*, but is only required for the maintenance of *sox3* and *geminin*. Our data indicates that although the same signals and factors are involved in the expression and repression of early neural genes, there is no common neurogenic code regulating neural induction in *Xenopus laevis*. Moreover, since the *soxB1* genes are expressed at the onset of neural induction and are required for normal neural development, we analyze their role in neural induction and formation. We demonstrate that overexpression of Sox2 and Sox3 directly activates expression of the neural progenitor marker, *geminin*, thereby expanding the neural tube and delaying neuronal differentiation. This progenitor expansion occurs at the expense of epidermis and neural crest formation indicating that the SoxB1 proteins are involved in the fate choice between epidermis and neural tissue and they act to promote neurogenesis at the expense of non-neural ectodermal derivatives.
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CHAPTER I: INTRODUCTION: THE REGULATION AND FUNCTION OF SOXB1 GENES AND PROTEINS DURING NEURAL INDUCTION AND DEVELOPMENT
Overview

The purpose of this research is to study the regulation and function of genes and proteins activated during neural induction in *Xenopus laevis*. The neural default model states that ectoderm will become neural tissue in the absence of bone morphogenetic protein (BMP) signaling, in *Xenopus* embryos. When BMP signaling is inhibited by dissociation or antagonists in ectodermal explants, the explants are transformed into neural tissue and if exogenous BMP protein is added to dissociated ectodermal explants, they maintain their epidermal fate (Sato and Sargent 1989; Wilson and Hemmati-Brivanlou 1995). However, there remains controversy about whether BMP inhibition is sufficient to induce neural genes, or if fibroblast growth factor (FGF) signaling and inhibition of Wingless/Int-1 (Wnt) signaling are required as well.

At the onset of gastrulation, early neural genes marking neural progenitors are activated in the dorsal ectoderm in response to the inhibition of BMP signaling by BMP antagonists (Kroll, Salic et al. 1998; Mizuseki, Kishi et al. 1998; Heeg-Truesdell and Labonne 2006). *SoxD, sox2, sox3, geminin* and *zic1* are the earliest neural genes induced in the neural plate and are expressed in neural progenitor cells throughout development (Pevny and Lovell-Badge 1997; Kroll, Salic et al. 1998; Mizuseki, Kishi et al. 1998; Mizuseki, Kishi et al. 1998). Here, we perform gain and loss of function assays to determine the mechanisms that are involved in the activation of three early neural genes, *sox2, sox3* and *geminin*, and to identify the role of Sox2 and Sox3 during neurogenesis.

**Dorsal ventral axis specification: the dorsal organizing centers**
In *Xenopus*, as early as the 32-cell stage, overlapping expression of VegT, Vg1, Nodal-related and β-catenin in the dorsal endoderm form the Nieuwkoop Center (Nieuwkoop 1967; Gerhart, Danilchik et al. 1989; Agius, Oelgeschlager et al. 2000; Vonica and Gumbiner 2007) induces development of dorsal mesoderm in the marginal zone (Figure 1.1A). This dorsal marginal zone, the Spemann organizer in frog (Spemann and Mangold 1924), shield in fish (Oppenheimer 1959; Shih and Fraser 1996), and node in chick and mouse (Waddington 1930; Beddington 1994), is both necessary and sufficient for neural development. Transplantation of the organizer to the ventral side induces a secondary axis (Spemann and Mangold 1924; Storey, Crossley et al. 1992) through the secretion of the BMP antagonists, Noggin (Zimmerman, De Jesus-Escobar et al. 1996), Chordin (Piccolo, Sasai et al. 1996), and Follistatin (Fainsod, Deissler et al. 1997). These proteins bind to and inhibit bone morphogenetic protein (BMP) extracellularly in the ectoderm overlying the organizer thereby inducing the formation of neural tissue (Fig. 1.1B) (Meinhardt 2001; Vonica and Gumbiner 2007). When expression of the secreted organizer molecules is blocked and BMP signaling is maintained on the dorsal side of the embryo, the neural plate and CNS do not form (Wessely, Kim et al. 2004).

**Neural induction and BMP signaling**

Studies using naïve ectodermal explants led to the hypothesis that ectoderm forms neural tissue by default (Sato and Sargent 1989; Hawley, Wunnenberg-Stapleton et al. 1995; Wilson and Hemmati-Brivanlou 1995; Weinstein and Hemmati-Brivanlou
Ectodermal explants dissected from the animal pole of *Xenopus* embryos form atypical epidermis (Fig. 1.2). When dissociated, they become neural tissue indicating that signaling is required for the formation of epidermis (Sato and Sargent 1989; Wilson and Hemmati-Brivanlou 1995). In support of this, inhibition of TGF-β signaling through the use of both dominant negative Activin and BMP (TGFβ) receptors neuralized ectodermal explants (Hemmati-Brivanlou, Wright et al. 1992; Hemmati-Brivanlou and Melton 1994; Hawley, Wunnenberg-Stapleton et al. 1995; Hemmati-Brivanlou and Thomsen 1995). However, the dominant negative Activin receptor interfered with signaling of all of the transforming growth factor–β (TGF-β) family of secreted signaling molecules which include TGF-β, Activin, Nodal and BMP and only overexpression of a dominant negative BMP receptor directly induced anterior neural genes in ectodermal explants, and induced a double axis on the ventral side of whole embryos (Hawley, Wunnenberg-Stapleton et al. 1995).

Mutational analysis and knockdown experiments in zebrafish, frog and mouse embryos also demonstrate a requirement for BMP inhibition to form neural tissue. In zebrafish, double mutants for the BMP antagonists *chordino* (a zebrafish chordin homolog) and *ogon* have a ventralized phenotype (Miller-Bertoglio, Carmany-Rampey et al. 1999). *Xenopus tropicalis* embryos depleted of Chordin, Noggin and Follistatin protein have nearly a complete loss of the CNS. Additionally, mouse mutants lacking the BMP antagonists Chordin (Bachiller, Klingensmith et al. 2000), Noggin (McMahon, Takada et al. 1998), or Cerberus (Belo, Bachiller et al. 2000) express only posterior neural genes, and the double mutants for Noggin and Chordin do not develop anterior
brain structures (Bachiller, Klingensmith et al. 2000). These studies indicate that there is a conserved and necessary role for BMP inhibition in the induction of anterior neural tissue among vertebrates.

There are more than 30 known BMP proteins (Balemans and Van Hul 2002), and it is *bmp4* that is involved in epidermal formation and neural inhibition. Its expression is restricted to the non-neural ectoderm and ventral mesoderm and overexpression ventralizes embryos (Fainsod, Steinbeisser et al. 1994; Hemmati-Brivanlou and Thomsen 1995). BMP signaling is activated when a BMP dimer binds to the BMP type II (BMPRII) receptor, which then recruits the BMP type I (Alk3) receptor creating a tertiary complex. Serine and threonine residues of the type I receptor are then phosphorylated by the type II receptor, thereby activating the BMP signaling cascade (Wrana, Attisano et al. 1994; von Bubnoff and Cho 2001). Once activated, the type I receptor phosphorylates the receptor-regulated Smad proteins (R-Smad) 1, 5, and 8 which can then interact with the common Smad, Smad4, and translocate to the nucleus to activate or repress transcription. Although the Smad proteins can bind to DNA directly, their binding affinities and specificities are low and thus, are proposed to require partner proteins such as OAZ (ten Dijke, Miyazono et al. 2000; von Bubnoff and Cho 2001; Shi and Massague 2003). Very few cofactors that interact with Smad 1, 5 and 8 are known, however the accepted model for BMP signaling suggests that upon activation of the BMP signaling pathway, Smad1, 5, and 8 proteins interact with these cofactors to activate expression of BMP targets genes.

A number of BMP target genes have been identified and these include *msx-1*
(Foerst-Potts and Sadler 1997; Tucker, Al Khamis et al. 1998; Feledy, Beanan et al. 1999; Ishimura, Maeda et al. 2000; Takeda, Saito et al. 2000; Yamamoto, Takagi et al. 2000; Yamamoto, Takagi et al. 2001; Tribulo, Aybar et al. 2003), \textit{msx-2} (Foerst-Potts and Sadler 1997), \textit{Gata-2} (Walmsley, Guille et al. 1994; Friedle and Knochel 2002), \textit{XVent1} (Gawantka, Delius et al. 1995; Friedle, Rastegar et al. 1998; Onichtchouk, Glinka et al. 1998; Rastegar, Friedle et al. 1999) and \textit{XVent2} (Ladher, Mohun et al. 1996; Onichtchouk, Gawantka et al. 1996; Onichtchouk, Glinka et al. 1998; Rastegar, Friedle et al. 1999; Trindade, Tada et al. 1999; Friedle and Knochel 2002). \textit{Msx-2} is not likely to be involved in repressing the expression of early neural genes as it is not expressed until the mid-gastrula stage which is after neural induction. In contrast, \textit{msx-1}, \textit{gata-2}, \textit{Xvent1} and \textit{Xvent2} are expressed in the animal pole of blastula embryos and as gastrulation proceeds, their expressions are restricted to the ventral tissues in a pattern similar to that of \textit{bmp4} (Hemmati-Brivanlou and Thomsen 1995).

Overexpression studies indicate that Msx-1 and Xvent1 and 2 play roles in restricting the expression of neural genes to the dorsal ectoderm. Overexpression of \textit{msx-1} mRNA inhibited neural tissue formation induced by Noggin (Ishimura, Maeda et al. 2000), and overexpression of a dominant activator form of Msx-1 prevented ventralization by BMP overexpression, indicating that it is required for the ventralizing activity of BMP4 (Suzuki, Ueno et al. 1997; Yamamoto, Takagi et al. 2000). However, knockdown of Msx-1 function with morpholino oligonucleotides demonstrated that it is not required for epidermal development or axis formation suggesting it is not required to inhibit neural induction (Khadka, Luo et al. 2006). Similarly, overexpression of Xvent1
(Gawantka, Delius et al. 1995) or Xvent2 (Ladher, Mohun et al. 1996; Onichtchouk, Gawantka et al. 1996; Friedle and Knochel 2002) induced epidermis and inhibited the formation of dorsal mesoderm and the induction of neural tissue. Additionally, both proteins are required for the restriction of *geminin* (Taylor, Wang et al. 2006) and *sox3* reporter constructs (Rogers, Archer et al. 2008) to the dorsal ectoderm.

Several studies showed that even though inhibition of BMP is sufficient to induce neural tissue from unspecified animal ectoderm, it is not sufficient to induce it from ventral ectoderm (Hawley, Wunnenberg-Stapleton et al. 1995; Wilson and Hemmati-Brivanlou 1995; Rogers, Archer et al. 2008). For example, the inhibition of BMP signaling by overexpression of an inhibitory Smad in the non-neural ventral ectoderm represses epidermis formation but fails to induce the ectopic expression of neural markers (Delaune, Lemaire et al. 2005; Chang and Harland 2007). The inability to convert ventral ectoderm to neural tissue indicated that additional factors are required for neural induction in ectodermal cells. This was supported by studies in chick which showed that FGF signaling and inhibition of Wingless/int-1 (Wnt) signaling are also required for neural induction (McMahon and Moon 1989; Christian, Gavin et al. 1991; Rodriguez-Gallardo, Climent et al. 1997; Alvarez, Araujo et al. 1998; Wilson, Rydstrom et al. 2001). Although these studies suggest that the inhibition of BMP protein is not sufficient to induce neural tissue in all ectodermal tissue and that additional instructive signals provided by FGF may be required, recent studies indicate that inhibition of Smad1 and Smad2 signaling is sufficient to induce neural tissue (Chang and Harland 2007).
**FGF signaling: induction or maintenance?**

In frog, there are four known FGF receptors (FGFR1-4a) that may all play an important role in neural induction because they are expressed in the ectoderm prior to neural induction and are subsequently restricted to the neural tissue. However, the expressions of *FGFR2* and *FGFR4α* are up-regulated at the onset of neural induction indicating that they may play a specific role in the process (Hongo, Kengaku et al. 1999). To determine if FGF signaling is required for neural induction, FGF signaling was inhibited by overexpression of dominant negative FGFR1 receptor (XFD) (Amaya, Musci et al. 1991) and/or dominant negative FGFR4a receptor (Δ4a) (Hongo, Kengaku et al. 1999). XFD blocks FGF3 and FGF4 signaling through FGFR1, FGFR2 and FGFR3, whereas Δ4a blocks FGF8 signaling through FGFR4α (Ueno, Gunn et al. 1992; Hongo, Kengaku et al. 1999; Bainter, Boos et al. 2001). Overexpression of XFD or incubation in a chemical inhibitor of FGF signaling, SU5402 (Mohammadi, McMahon et al. 1997), inhibited the induction of neural tissue marked by the pan neural markers, *ncam* and *sox2*, respectively, in neuralized explants (Launay, Fromentoux et al. 1996; Ribisi, Mariani et al. 2000; Delaune, Lemaire et al. 2005). Knocking down FGF8 signaling using Δ4a leads to a loss of anterior neural tissue (marked by *nrp-1*, *bf-1* and *en-2*) in neurula embryos and in tailbud stage explants neuralized by co-culture with cells secreting BMP antagonists (Hongo, Kengaku et al. 1999). Although the neural default model suggests that dissociated ectodermal explants are neuralized by the dispersal of BMP protein and the subsequent reduction in BMP signaling, recent studies
showed that BMP signaling still occurs in dissociated ectodermal explants (Hurtado and De Robertis 2007). Additionally, MAP kinase signaling, which is active in dissociated explants, can function as a neuralizing factor suggesting that ectodermal cells are neuralized by MAP kinase signaling and not the loss of BMP signaling after dissociation (De Robertis and Kuroda 2004; Kuroda, Fuentealba et al. 2005). These authors hypothesized that FGF signaling, and not BMP dispersal, is required for the formation of neural tissue in dissociated ectodermal explants. In contrast, disruption of FGF signaling through the knock down of MAP kinase by inhibition of Ras signaling (by N17Ras), did not affect anterior neural induction but inhibited the posteriorization of neural tissue in explants (Ribisi, Mariani et al. 2000) suggesting that FGF is required for CNS patterning. Moreover, inhibition of FGF signaling via treatment with SU5402 led to a loss of some mesodermal markers including noggin and a subsequent decrease in the induction of the pan neural gene ncam (Delaune, Lemaire et al. 2005). Since expression of critical neural inducers was lost (Dixon and Kintner 1989), the loss of neural tissue caused by SU5402 may be indirect. Taken together, these experiments demonstrate that signaling through FGFR4a may be required for the induction and development of anterior neural tissue and that FGFR1, FGFR2 and MAP kinase signaling are required for posteriorizing anterior neural tissue. FGF can induce neural genes in unspecified ectoderm without inducing mesoderm, but the loss of neural gene expression in FGF knock down experiments may be due to a loss of mesoderm as FGF signaling is required for mesoderm development (Kimelman and Kirschner 1987; Godsave and Slack 1989; Slack, Darlington et al. 1989; Amaya, Musci et al. 1991).
Many studies demonstrated a role for FGF in neural induction, however they do not demonstrate that the function of FGF is independent of its ability to inhibit the transcription of \textit{bmp} or BMP signaling (Baker, Beddington et al. 1999; Hongo, Kengaku et al. 1999; Ishimura, Maeda et al. 2000; Wilson, Graziano et al. 2000; Wilson, Rydstrom et al. 2001; Pera, Ikeda et al. 2003; Sheng, dos Reis et al. 2003; Linker and Stern 2004; Rentzsch, Bakkers et al. 2004; Wittler and Kessel 2004; Delaune, Lemaire et al. 2005). In chick and zebrafish, FGF signaling inhibits \textit{bmp} expression (Furthauer, Thisse et al. 1997; Wilson, Graziano et al. 2000) and in frog and zebrafish FGF signaling inhibits BMP signaling through the phosphorylation and inactivation of Smad1 (Schier 2001; Pera, Ikeda et al. 2003). In this manner, FGF may cooperate or be redundant with neural induction by extracellular BMP antagonists. In total, these data support a role for FGF in patterning of anteroposterior neural tissues but its requirement for neural induction independent of BMP inhibition remains in question (Lamb and Harland 1995; Wilson, Graziano et al. 2000; Takemoto, Uchikawa et al. 2006; Rogers, Archer et al. 2008; Weisinger, Wilkinson et al. 2008).

\textbf{Wnt signaling and neural induction}

Maternal Wnt signaling, and then later in development the inhibition of Wnt are important for the formation of neural tissue. Early Wnt signaling is required to establish the dorsal-ventral axis in \textit{Xenopus} through the accumulation of the Wnt effector \(\beta\)-catenin (Larabell, Torres et al. 1997) on the dorsal side of the embryo. However, studies have investigated additional roles for Wnt signaling in neural induction. To determine if
Wnt signaling or inhibition are involved in *Xenopus* neural development, *Wnt-1* and *Wnt-3a*, which are expressed in neural tissue in *Xenopus* (Wolda, Moody et al. 1993) and *Wnt-8*, which is expressed in ventral tissues (Christian, McMahon et al. 1991) were overexpressed in *Xenopus* embryos. Supporting a requirement for Wnt signaling in axis formation, overexpression of Wnt1 or Wnt3a induced a second axis (Steinbeisser, De Robertis et al. 1993). The *Wnt-8* overexpression phenotype suggested a more specific role in neural induction as *Wnt-8* induced expression of the pan-neural marker *ncam* in ectodermal explants by inhibiting *bmp4* expression at early gastrula stages and sensitizing ectodermal explants to extracellular BMP inhibition (Baker, Beddington et al. 1999). However, Wnt8 overexpression also induced dorsal mesoderm when injected in whole embryos indicating that in vivo, Wnt8 may not induce neural genes in the absence of mesoderm (Otte and Moon 1992).

Studies in both frog and chick support a requirement for the inhibition of Wnt for neural induction and a role for Wnt signaling in anteroposterior patterning of the CNS. In frog, overexpression of a dominant active form of β-catenin inhibited the expression of neural progenitor markers, *sox2* and *sox3*, in neurula embryos and inhibited the induction of *sox3* by Noggin in ectodermal explants at stage 15 (Heeg-Truesdell and Labonne 2006). In chick, overexpression of Wnt3a inhibited the neuralization of ectoderm by BMP inhibition (Wilson, Rydstrom et al. 2001). Wilson and Rydstrom et al. also demonstrated that Wnt signaling must be inhibited for BMP inhibition and FGF signaling to induce neural tissue in chick lateral epiblast (Wilson, Rydstrom et al. 2001).
Is there a neurogenic code in CNS development?

One way to determine which signals are required to induce neural tissue is to determine which transcription factors are required for the induction of early neural genes that are expressed in response to neural inducers, and that are necessary for the formation of a multi-potent neural progenitor population. There are common signals involved in the induction of early neural genes in vertebrates (Fig. 1.3), thus, it is possible that many neural genes are activated and restricted by the same suite of transcription factors such that enhancers of early neural genes are conserved (Markstein, Zinzen et al. 2004).

To identify the signals required for neural induction in vertebrates, experiments focused on the effects of gain or loss of function of BMP, FGF and Wnt signaling on the induction or repression of early neural genes, as well as comparative transgenic analyses on the regulatory regions driving expression of genes such as *sox2* (Mansukhani, Ambrosetti et al. 2005; Takemoto, Uchikawa et al. 2006), *sox3* (Brunelli, Silva Casey et al. 2003; Okuda, Yoda et al. 2006; Rogers, Archer et al. 2008), *zicr-1* (Mizuseki, Kishi et al. 1998; Chang and Harland 2007) and *geminin* (McGarry and Kirschner 1998; Taylor, Wang et al. 2006). Expression of each of these genes is induced by BMP inhibition (Kroll, Salic et al. 1998; Mizuseki, Kishi et al. 1998; Heeg-Truesdell and Labonne 2006). In the *zicr-1* regulatory region, there is a BMP inhibitory response module (BIRM) that is required for expression of a reporter in the absence of BMP signaling (Tropepe, Li et al. 2006). Additionally, transgenic embryos expressing reporter constructs driven by regulatory regions from both *geminin* and *sox3* require XVent1 and XVent2 binding sites for restriction to the dorsal ectoderm (Taylor, Wang et al. 2006;
Rogers, Archer et al. 2008). The regulatory regions of sox2 (Takemoto, Uchikawa et al. 2006), sox3 ((Rogers, Archer et al. 2008), and zicr-1 (Tropepe, Li et al. 2006) also respond to FGF signaling while expression of a geminin transgene requires regulation by the Wnt effector TCF3 for dorsal expression (Taylor, Wang et al. 2006). Although there are similar proteins mechanisms (i.e. BMP inhibition) for the onset of neural induction even in distant organisms, there are still questions about the requirement for FGF and Wnt signaling in neural induction.

**Early neural gene expression and regulation**

The onset of neural induction in frog is characterized in part by the expression of sox2 (Uwanogho, Rex et al. 1995; Mizuseki, Kishi et al. 1998; Wood and Episkopou 1999), sox3 (Penzel, Oschwald et al. 1997), geminin (Kroll, Salic et al. 1998), foxd5a (Sullivan, Akers et al. 2001), soxD (Mizuseki, Kishi et al. 1998) and zicr-1 (Mizuseki, Kishi et al. 1998). Although each early neural gene is expressed at the onset of neural induction, their spatiotemporal expression patterns and mechanisms of regulation differ.

**SoxB1**

Sox2 and sox3 are well documented early pan-neural markers (Uwanogho, Rex et al. 1995; Collignon, Sockanathan et al. 1996; Penzel, Oschwald et al. 1997; Rex, Orme et al. 1997; Mizuseki, Kishi et al. 1998; Uchikawa, Kamachi et al. 1999; Graham, Khudyakov et al. 2003; Linker and Stern 2004; Takemoto, Uchikawa et al. 2006; Rogers, Archer et al. 2008) and are part of the SoxB1 (Sox1, 2, & 3) family of transcriptional
activators (Penzel, Oschwald et al. 1997; Uchikawa, Kamachi et al. 1999).

Named for their similarity to the testis determination factor Sry, and a conserved high-mobility group (HMG) (Sry-related HMG-box) domain that confers their DNA binding ability (Gubbay, Collignon et al. 1990; Sinclair, Berta et al. 1990; Wegner 1999). They are transcription factors that bind to the minor groove of DNA, inducing a sharp bend and regulating gene transcription (Guth and Wegner 2008). Of the ten groups of Sox proteins, only SoxD and the SoxB (Sox1, 2, 3) and SoxE (Sox8, 9, 10) groups are involved in neural induction and development (Guth and Wegner 2008). They are both expressed in neural progenitor cells throughout CNS development and required for neural progenitor maintenance (Graham, Khudyakov et al. 2003; Ellis, Fagan et al. 2004; Pevny and Placzek 2005; Rogers, Harafuji et al. 2009). Their expression patterns are similar but there are differences; \textit{sox3} is expressed maternally and is pan-ectodermal until the mid-gastrula stage when expression is restricted to the dorsal ectoderm (Penzel, Oschwald et al. 1997). In contrast, \textit{sox2} expression begins at the onset of neural induction only in the neuroectoderm (Nitta, Takahashi et al. 2006). Furthermore, \textit{sox3} is repressed in the floorplate of the neural tube prior to \textit{sox2}, expressed much more strongly in the otic placodes, and expressed in the developing lens, while \textit{sox2} is expressed in the retina (Penzel, Oschwald et al. 1997; Elkins and Henry 2006; Nitta, Takahashi et al. 2006; Rogers, Archer et al. 2008). These differences in their spatio-temporal expression patterns indicate that they are regulated differently, and promoter studies and computation comparisons indicate that this is true (Uchikawa, Ishida et al. 2003; Rogers, Archer et al. 2008). The \textit{SOX2} regulatory region has a neural induction
module which is conserved across species and in chick this module responds to both FGF and Wnt signaling for activation in the posterior neural plate (Takemoto, Uchikawa et al. 2006). This module is present upstream of *Xtsox2* but is not in the flanking sequence of *sox3*. Thus far, reporter studies in transgenic frog embryos revealed that *Xenopus sox3* expression is induced by the inhibition of BMP signaling and is restricted to the neural plate by the BMP targets, XVent1 and XVent2 (Rogers, Archer et al. 2008).

Rescue experiments indicate that the SoxB1 proteins are redundant in tissues in chick they are co-expressed. Loss of Sox2 in the mouse CNS is compensated for by the up-regulation of *Sox3* expression. Neurogenesis is decreased in these mice, but neural stem cells retain multipotency (Miyagi, Masui et al. 2008). Furthermore, in chick overexpression of Sox1 rescues the loss of Sox2 (Graham, Khudyakov et al. 2003). Although the SoxB1 proteins can compensate for each other, each gene is also expressed in unique tissues during development and loss in that tissue can result in severe phenotypes. Mice mutant for either Sox2 or Sox3 have relatively normal brain formation (Malas, Postlethwaite et al. 2003; Taranova, Magness et al. 2006). However, loss of Sox2 results in diminished neurogenesis in the mouse retina (Ferri, Cavallaro et al. 2004) and anophthalmia in humans (Fantes, Ragge et al. 2003). While Sox3 null mice have craniofacial abnormalities and defective pituitary development (Rizzoti, Brunelli et al. 2004; Rizzoti and Lovell-Badge 2007), Sox1 null mice suffer from epilepsy due to a complete loss of neurons in the ventral striatum, and have lens fiber defects (Nishiguchi, Wood et al. 1998; Malas, Postlethwaite et al. 2003; Ekonomou, Kazanis et al. 2005).
In early neural development vertebrate SoxB1 proteins function as activators (Uchikawa, Kamachi et al. 1999; Bylund, Andersson et al. 2003; Graham, Khudyakov et al. 2003) and it has been proposed that they counteract neuronal differentiation which is induced by SoxB2 repressor proteins (Bylund, Andersson et al. 2003). In this way, SoxB proteins maintain a balance between proliferation and differentiation. In concordance, overexpression of both \textit{sox2} and \textit{sox3} in frog and zebrafish leads to a loss of neurogenesis in the placode (Dee, Hirst et al. 2008; Schlosser, Awtry et al. 2008) and the lens (Kamachi, Uchikawa et al. 1998) and the induction and maintenance of neural progenitors in the frog and chick neural tube at the expense of epidermal development and neuronal differentiation (Kishi, Mizuseki et al. 2000; Graham, Khudyakov et al. 2003; Rogers, Harafuji et al. 2009).

\textit{Geminin}

\textit{Geminin} is a novel coiled coil protein expressed similarly to \textit{sox3}; it is a maternally expressed gene that is ubiquitous in the animal pole until gastrulation when it is first enriched dorsally, and then restricted to the neuroectoderm (Kroll, Salic et al. 1998). After gastrulation, \textit{geminin} expression is pan-neural with an anterior bias and encompasses a region wider than either \textit{sox2} or \textit{sox3} that will become both neural, placodal, and presumptive neural crest tissue (Kroll, Salic et al. 1998; Kroll 2007). Expression is maintained in the proliferative developing central nervous system including the eye, otic placodes and neural tube (Kroll 2007). Like \textit{sox3}, experiments using a human \textit{geminin} regulatory region driving GFP expression indicated that the
BMP targets, XVent1 and XVent2, restrict *geminin* to the dorsal side of the embryo and that *geminin* is inhibited by BMP signaling (Kroll, Salic et al. 1998). Dorsal expression of this *geminin* reporter construct required positive regulation by the Wnt signaling effector, TCF (Taylor, Wang et al. 2006). Additionally, recent experiments demonstrated that *geminin* expression is activated by two other early neural proteins, Sox3 (Rogers, Harafuji et al. 2009) and Foxd5α (Yan, Neilson et al. 2009) in the absence of protein synthesis.

Geminin inhibits re-initiation of DNA replication to maintain chromosomal integrity and prevent cell cycle exit (Seo and Kroll 2006). The latter is controlled by an antagonistic interaction with the catalytic subunits of the SWI/SNF complex, Brg1 and Brahma (McGarry and Kirschner 1998; Seo, Herr et al. 2005). Like Sox2 and Sox3, overexpression of *geminin* mRNA inhibits epidermal development and neuronal differentiation and expands neural and neuronal markers (Kroll, Salic et al. 1998; Papanayotou, Mey et al. 2008).

**Foxd5α**

*FoxD5α* is a maternally expressed gene that is in the fork head gene family (Sullivan, Akers et al. 2001). It is expressed in the oocyte and is localized to the animal pole during cleavage stages. Its zygotic expression begins at the mid-blastula transition when *foxd5α* is localized to the presumptive neuroectoderm. Expression of *foxd5α* is more discrete than other maternal early neural genes as it is not expressed throughout the ectoderm but is expressed in the presumptive neuroectoderm and then restricted to the
neural tube and the paraxial mesoderm (Sullivan, Akers et al. 2001). Additionally, although foxD5α expression is not induced by BMP inhibition via Chordin it is induced by Noggin (Sullivan, Akers et al. 2001). Also, the inhibition of Wnt signaling plays a role in foxd5α expression as it is strongly activated by Siamois, and Cerberus in ectodermal explants (Sullivan, Akers et al. 2001).

Dorsal injections of foxD5α mRNA expanded neural tissue marked by sox3 and otx2 expression repressed the proneural gene ngnr-1, the post-mitotic neuronal marker neuroD, and the neuronal marker n-tubulin (Sullivan, Akers et al. 2001). Microinjections of the foxD5α-VP16 dominant activator reveal that FoxD5α functions as a transcriptional repressor to expand the neural plate. Furthermore, deletion construct experiments revealed that FoxD5α function is dependent on its C-terminal domain. These data indicate that foxD5α is a critical neural gene that maintains proliferation and an undifferentiated neuroectoderm during neural formation. Additional research is necessary to determine the transcriptional machinery and signaling factors which regulate this gene.

**SoxD**

SoxD expression is first detected at late blastula stages. Like maternally expressed early neural genes, its expression begins pan-ectodermally and is later restricted to the presumptive neural plate during gastrulation (Mizuseki, Kishi et al. 1998). Similar to most early neural genes, soxD is induced in response to the inhibition of BMP signaling (Mizuseki, Kishi et al. 1998). Additionally, soxD expression is
reduced upon loss of Foxd5α function via injection of morpholino oligonucleotides (Yan, Neilson et al. 2009) and can be induced in ectodermal explants by zicr-1 (Mizuseki, Kishi et al. 1998). Little is known about the requirement for FGF or Wnt signaling for the expression of soxD.

SoxD acts as a neural inducer in Xenopus embryos and is unique to amphibians (Mizuseki, Kishi et al. 1998; Guth and Wegner 2008). Overexpression of soxD mRNA induces ectopic neural tissue and markers in whole embryos and animal cap assays (Mizuseki, Kishi et al. 1998). Similar to overexpression of Sox2, Sox3 and Geminin, overexpression of SoxD in whole embryos expanded neural progenitors marked by nrp-1, induced the proneural marker ngnr-1, and inhibited epidermal development marked by epi-keratin expression (Kroll, Salic et al. 1998; Mizuseki, Kishi et al. 1998; Rogers, Harafuji et al. 2009). Furthermore, loss-of-function experiments using a dominant-negative soxD construct prevented the induction of neural tissue by BMP inhibition (Mizuseki, Kishi et al. 1998).

**Zicr-1**

Zicr-1 is a member of the zinc finger family of transcription factors. Similar to sox2 and soxD, zicr-1 is a zygotically activated neural transcription factor. Its expression is first detected in late blastula stages and is later restricted to the dorsal ectoderm throughout gastrulation (Mizuseki, Kishi et al. 1998). Zicr-1 is expressed throughout the presumptive neuroectoderm at the onset of neural induction, and is restricted to the anterior neural plate and excluded from the midline during gastrulation.
(Mizuseki, Kishi et al. 1998). During neurulation zicr-1 is expressed in the presumptive neural crest cells but eventually is restricted to the developing brain and neural tube (Mizuseki, Kishi et al. 1998). Additionally, zicr-1 expression is induced in ectodermal explants by the inhibition of BMP signaling via overexpression of Chordin (Mizuseki, Kishi et al. 1998).

Little is known about the role of Zicr-1 during neural induction and development, however, similar to SoxD, overexpression of Zicr-1 results in the activation of the neural progenitor markers, nrp-1 and soxD in ectodermal explants in the absence of mesoderm induction. Also, Zicr-1 it expands the expression of the proneural gene ngnr-1 and the neuronal marker, n-tubulin (Mizuseki, Kishi et al. 1998) earlier than does overexpression of Sox2, Sox3, or Geminin indicating that Zicr-1 is sufficient for neural induction and that it may act upstream of other early neural genes.

Conservation of CNS induction and patterning

Although the mechanisms that regulate the development of the vertebrate CNS vary between organisms, there is a consistent requirement for BMP inhibition and FGF signaling in the process (Harland 1994; Chitnis and Kintner 1995; Hemmati-Brivanlou and Melton 1997; Streit and Stern 1999; Harland 2000; De Robertis and Kuroda 2004; Stern 2005; Stern 2006). Additionally, early neural genes induced by BMP inhibition like the soxB1s are also conserved and have homologs in basal organisms like the arthropod, Drosophila (SoxNeuro) (Cremazy, Berta et al. 2000). In these organisms,
SoxB1 transcription factors are encoded by early neural genes expressed in a subset of ectoderm cells that are directed to become proliferating neural progenitors. Expression of proneural proteins then drive the progenitors to exit the cell cycle, decrease the expression of the SoxB1 genes and differentiate into neurons to form the central nervous system (CNS). Interestingly, although the basic organization of the CNS in vertebrates is conserved, the pathways driving induction and differentiation vary, making it difficult to identify an ancestral molecular mechanism. Study of the regulation and function of the highly conserved SoxB1 transcription factors in organisms with a simple nervous system like the hemichordate, Saccoglossus kowalevskii and the urochordate, Ciona intestinalis, and comparisons to vertebrate neural induction will eventually help to decipher the ancestral mechanism of neural induction and patterning and if the functions of the proteins involved in neural development are conserved.
Figure 1.1. The dorsal ventral axis is specified by the Nieuwkoop center and the Spemann organizer. (A) Prior to gastrulation the dorsal axis is specified by overlapping expression of VegT from the vegetal pole (orange arrows) and β-catenin, which is tethered on the prospective dorsal side of the embryo. The region where both molecules overlap will become the Nieuwkoop center (green circle). (B) The Nieuwkoop center secretes dorsalizing molecules that induce the Spemann organizer (blue oval) in the dorsal mesoderm which expresses Chordin (Chd), Noggin (Nog), Follistatin (Foll), Cerberus (Cer) and Xenopus nodal related-3 (Xnr3). These molecules actively antagonize BMP which is being secreted from the ventral side (red arrows). V is ventral, D is dorsal. Black line above Nieuwkoop center indicates dorsal blastopore lip.
Figure 1.2. The default model of neural induction in *Xenopus laevis*. When ectoderm is dissected from a blastula stage embryo and cultured, the explant becomes epidermis (light blue). If BMP is inhibited in those cells, the explant becomes neural tissue (dark blue). When explants are dispersed, they become neural cells (dark blue), but if exogenous BMP is added to those cells, they become epidermal cells (light blue).
Figure. 1.3. Pathways involved in neural induction and development. Left to right: FGF and Wnt inhibit bmp4 transcription. FGF signaling: After activation of the tyrosine kinase FGF receptor by FGF ligand binding, the Ras-MAPK signaling pathway is activated via interactions with tyrosine-phosphorylated FRS2, Grb2, and SOS (not shown). Ras then interacts with several effector proteins, leading to the activation of the MAPK signaling cascade. (Bottcher and Niehrs 2005). BMP signaling: BMP, BMP2, 4, or 7 dimers bind to the serine-threonine receptor complex, leading to phosphorylation of the type I receptor (RI) by the type II receptor (RII), which in turn phosphorylates an appropriate Receptor regulated (R) -Smad (Smad1, 5, or 8). This phosphorylation enables the R-Smad to form a complex with the Common-Smad, Smad4, and the Smad1,
5, 8/Smad4 complex translocates to the nucleus to activate or repress target genes depending on which nuclear cofactors are present (von Bubnoff and Cho 2001). Wnt signaling: Wnt binds to the Frizzled and LRP receptors and induces phosphorylation of LRP and recruitment of Axin. Dsh is also phosphorylated, and the Axin-APC-GSK-3 complex is inhibited, leading to accumulation of cytosolic β-catenin. Accumulated β-catenin then translocates to the nucleus, takes the place of Groucho with TCF, and activates target genes (Gordon and Nusse 2006).
CHAPTER II: Sox3 expression is restricted to the neural plate by Vent proteins in the Xenopus embryo

Some data shown in this chapter were previously published in: C.D. Rogers, T. C. Archer, D.D. Cunningham, T.C. Grammer and E.S. Casey. Sox3 expression is maintained by FGF signaling and restricted to the neural plate by Vent proteins in the Xenopus embryo. Development. 2008.
Introduction

At the onset of neural induction, genes encoding proteins that are required for the specification of the central nervous system (CNS) are activated in the dorsal ectoderm by the inhibition of bone morphogenetic (BMP) signaling (Harland 1994). Signaling by the secreted BMP actively inhibits the development of neural tissue and drives epidermal formation (Sato and Sargent 1989; Wilson and Hemmati-Brivanlou 1995; Wilson and Hemmati-Brivanlou 1997) and inhibition of BMP by secreted antagonists such as Noggin (Zimmerman, De Jesus-Escobar et al. 1996), Follistatin (Fainsod, Deissler et al. 1997) or Chordin (Piccolo, Sasai et al. 1996), induces neural tissue (Harland 1994; Chitnis and Kintner 1995; Hawley, Wunnenberg-Stapleton et al. 1995; Sasai, Lu et al. 1995; Zimmerman, De Jesus-Escobar et al. 1996). While BMP inhibition is necessary for the formation of the CNS, the mechanisms by which BMP signaling represses neural genes and inhibition of BMP drives neural gene expression are yet to be elucidated.

In frog, the inhibition of BMP signaling is sufficient to induce neural tissue from unspecified ectodermal explants (Hawley, Wunnenberg-Stapleton et al. 1995; Wilson and Hemmati-Brivanlou 1995; Rogers, Archer et al. 2008), but it is not sufficient to induce neural tissue in ventral ectoderm that is specified to become epidermis (Harland 2000; Delaune, Lemaire et al. 2005; Heeg-Truesdell and Labonne 2006; Chang and Harland 2007). Studies in chick have shown that BMP inhibition alone is not sufficient to induce neural tissue from non-neural epiblast cells and that FGF signaling and inhibition of Wnt are required for neural induction (Wilson, Rydstrom et al. 2001). The
focus of this study is to determine the mechanism by which BMP signaling inhibits neural genes. We aim to determine whether BMP signaling inhibits neural gene expression directly or indirectly via BMP targets such as XVent1 or XVent2 (Friedle, Rastegar et al. 1998; Onichtchouk, Glinka et al. 1998).

We studied the regulation of two early neural genes, \textit{sox2} and \textit{sox3}, in response to BMP signaling and inhibition. \textit{Sox2} and \textit{sox3}, two highly conserved members of the SoxB1 (Sox1, 2, and 3) subgroup of HMG-box transcription factors are expressed in neuroectoderm at the onset of neural induction and are maintained there throughout development (Penzel, Oschwald et al. 1997; Brunelli, Silva Casey et al. 2003; Nitta, Takahashi et al. 2006; Rogers, Archer et al. 2008). The SoxB1 proteins act to maintain neural progenitors and are required for normal neural development (Mizuseki, Kishi et al. 1998; Kishi, Mizuseki et al. 2000; Graham, Khudyakov et al. 2003; Wegner and Stolt 2005; Miyagi, Masui et al. 2008; Rogers, Harafuji et al. 2008).

We demonstrate that both \textit{sox2} and \textit{sox3} are expressed in response to BMP inhibition and are inhibited in the neuroectoderm by BMP signaling. Additionally, we show that activation of \textit{sox2} after BMP inhibition requires protein synthesis as does the repression of \textit{sox3} expression. Two BMP targets, XVent1 and XVent2, that share complimentary expression patterns with \textit{sox3} and are inhibited by Noggin, restrict \textit{sox3} expression to the dorsal side of the gastrula embryo.
Materials and Methods:

Embryo culturing and manipulations

*Xenopus laevis* embryos were obtained using standard methods (Sive et al., 2000) and staged according to Nieuwkoop and Faber (1994). Animal ectodermal explants were isolated from stage 8-9 embryos and cultured in 0.75X Normal Amphibian Medium (NAM) with or without 3.1 µg/ml cycloheximide (CHX) (Sigma) and 0.5 µg/ml bone morphogenetic protein (BMP, Sigma) plus 5% BSA (Sigma). CHX stock was dissolved in dimethyl sulfoxide at a 1000X concentration. 0.5% BMP protein and 100mg/ml BSA were dissolved in H2O. Explants were collected between stages 10.5 and 17 based on sibling embryos.

Plasmid construction

Reporter constructs were generated by E. Casey and internal deletions and binding-site mutations of sox3-GFP were generated by site directed mutagenesis by T.C. Archer.

mRNA Synthesis and Microinjection

Synthetic capped mRNAs were made by *in vitro* transcription using mMessage Machine kits (Ambion) using plasmids from the Casey lab stock. Numbers in parentheses identify specific Casey Lab stock numbers. For explant assays, 25 pg noggin mRNA (#329) (Knecht, Good et al. 1995; Geng, Xiao et al. 2003) was injected into the animal pole of a 1-cell embryo. 1-2.4 ng of vent (#294, #295) or 0.8-2.4 ng of VPvent mRNA (#292, #293) (Onichtchouk, Glinka et al. 1998) with 250 pg of lacZ (#127 or
mRNA was injected into 1 of 2-cells or 0.1 ng of vent1, vent2, and or .05 ng of both was injected into 1 of 32-cells and embryos were cultured until stage 12.5 and analyzed by whole-mount in situ hybridization (WISH) (Hemmati-Brivanlou, Frank et al. 1990; Harland 1991) or reverse transcript polymerase chain reaction (RT-PCR).

**RNA extraction and reverse transcription- polymerase chain reaction (RT-PCR)**

Semi-quantitative RT-PCR was performed as described in (Wilson and Hemmati-Brivanlou 1995) with some modifications. One μl of isolated RNA was used for PCR with primers for ef1a (#86, #87, XMMR) to determine if there was DNA contamination. If there was no contamination, 10 μl of isolated RNA was mixed with 1 μl random hexamers and heated to 65° for five minutes. Primers used: sox2 (#114, #115, F: CTTACATGAACGGCTCGCC, R: CCCAGGGTAGGTACATGC, 58° annealing, 29 cycles), sox3 (#18, F: AGGAGTAAGCCCGGTGGCTAGCA, #19, R: ATGTAGGCATTTGCGAGGGTAGGTCA, 61° annealing and 25 cycles), geminin (#28, #29, XMMR, 58° annealing, 28 cycles), Xvent2 (#153, #154) (Onichtchouk, Gawantka et al. 1996), and Xvent1 (#163, #164) (Rastegar, Friedle et al. 1999) or ODC (#118, #119, XMMR).

**Whole mount in situ hybridization (WISH) and β-galactosidase assay**

Whole mount in situ hybridization (WISH) was performed as described (Hemmati-Brivanlou, Frank et al. 1990; Harland 1991) with the following modifications: embryos were not treated with proteinase K, triethanolamine, or acetic anhydride, and pre-hybridization was shortened to one hour. On day 2, wash in 1X
maleic acid buffer (MAB) with digoxigenin antibody was performed at room temperature for four hours and vials were washed three times at 15 minutes with 1X MAB, and then left overnight at 4° in 1X MAB. Finally, embryos were fixed in 4% formaldehyde, 0.5% acetic acid, and 2X SSC. For lineage tracing, β-galactosidase activity was visualized with Red-gal (Research Organics) or X-gal. We used the following genes as in situ probes: sox2 (#RH1104) (Mizuseki, Kishi et al. 1998), sox3 (#220 and #419) (Pevny and Lovell-Badge 1997), Xvent1 (#294) (Gawantka, Delius et al. 1995), Xvent2 (#295) (Ladher, Mohun et al. 1996; Onichtchouk, Gawantka et al. 1996; Papalopulu and Kintner 1996; Schmidt, von Dassow et al. 1996), geminin (#368) (Kroll, Salic et al. 1998), or GFP (#100) (Kroll and Gerhart 1994).
Results

Comparison of sox3 and sox2 expression in embryos and ectodermal explants

*X. laevis* sox3 and sox2 are both expressed in the presumptive neural ectoderm and throughout the CNS (Penzel, Oschwald et al. 1997; Kishi, Mizuseki et al. 2000; Nitta, Takahashi et al. 2006). To compare their temporal and spatial expression patterns throughout gastrula stages, we analyzed embryos by whole mount in situ hybridization (WISH) and RT-PCR (Fig. 2.1A and 2.1B). Like *SOX3* in chick, *Xenopus* sox3 is expressed initially throughout the animal ectoderm and is later restricted to the dorsal ectoderm (Penzel et al., 1997; Uwanogho et al., 1995). Sox3 expression is stronger on the dorsal side than the ventral side at stage 10 and restricted to the dorsal side by stage 12.5 (Fig. 2.1A). In contrast, sox2 is first detectable by WISH at the onset of gastrulation (stage 10, data not shown) in the dorsal ectoderm. During neurulation, both genes are expressed in the neural tube and the otic placodes (Fig 2.1A, asterisk) (Schlosser and Ahrens, 2004). RT-PCR analysis shows that *Xenopus* sox3 is maternally (Fig. 2.1B and data not shown) and zygotically expressed and levels appear to peak by early gastrula stage (10.5), prior to the peak of sox2 expression at late gastrula (stage 12.5-12, Fig. 2.1B).

To characterize the expression of sox2 and sox3 in response to BMP inhibition, we analyzed their expression by WISH in untreated and *noggin*-injected animal ectodermal explants (caps, Fig. 2.1C, 2.1D). In untreated explants, sox3 and sox2 expression patterns differ; sox3 mRNA is present from stage 8 until stage 11.5, while sox2 expression is not detected. In the presence of Noggin, sox3 message is detectable
through stage 17 and \textit{sox2} is expressed at stage 12.5 and maintained through stage 17 which is in contrast to \textit{sox2} expression in whole embryos which begins at stage 10.5 (Fig. 2.1C). RT-PCR performed on additional stages confirmed that \textit{sox3} is expressed until approximately stage 11 or 12.5 and revealed that \textit{sox2} is expressed at low levels between stages 10 and 12 (Fig. 2.1D, bottom panel). These data demonstrate that both \textit{sox2} and \textit{sox3} are expressed in the neural ectoderm at the onset of neural induction and that both genes are induced in response to BMP inhibition.

**BMP signaling is sufficient to inhibit neural gene expression in neural ectoderm**

Inhibition of BMP signaling by BMP antagonists such as Noggin and Chordin is sufficient to induce neural gene expression in ectodermal explants (Smith and Harland 1992; Sasai, Lu et al. 1995). BMP also induces ventral mesoderm and epidermis at the expense of neural tissue (Hemmati-Brivanlou and Thomsen 1995). However, despite many studies showing that inhibition of BMP can induce neural genes (Harland 2000), we wanted to determine if BMP signaling is sufficient to inhibit expression of early neural genes in the neuroectoderm of whole embryos at the onset of gastrulation and neural formation. We injected constitutively active BMP receptor I (Alk2, CaBMPR) (Macias-Silva, Hoodless et al. 1998) in one of 2-cells and analyzed the embryos for expression of \textit{sox2}, \textit{sox3} and \textit{geminin}. CaBMPR signaling caused a complete loss of \textit{sox2} (Fig. 2.2A, n= 35/56), \textit{sox3} (Fig. 2.2A, n= 62/70) and \textit{geminin} (Fig. 2.2A, n= 96/99) expression in the neural plate as early as stage 10.5 (experiment performed by G.S. Ferzli). This inhibition was maintained in the neural plate through stage 12.5 (Fig.
Therefore, BMP signaling is sufficient to repress neural gene expression in the neural ectoderm of whole embryos and its inhibition is required for neural induction.

Since BMP inhibition induces expression of both sox2 and sox3 in ectodermal explants, we tested whether BMP inhibition by Noggin has the ability to induce premature expression of neural genes in whole embryos. Since sox3 and geminin are maternally expressed and it is difficult to detect increased expression, we assessed the effect of noggin on early sox2 expression. Noggin overexpression did not induce sox2 at stage 9 (Fig. 2.2B) prior to the onset of neural induction, but it did expand sox2 expression by stage 10.5 in whole embryos. This is in contrast to assays performed in ectodermal explants in which noggin-overexpression does not induce sox2 expression until stage 12.5 (Fig. 2.2B and data not shown). This experiment shows that BMP inhibition is not sufficient to induce sox2 prior to the onset of endogenous neural induction.

The inhibition of sox3 by BMP and the induction of sox2 by Noggin require de novo protein synthesis

We have shown that sox2 and sox3 expression is induced by the inhibition of BMP in ectodermal explants (Fig. 2.1C). To understand how inhibition of BMP is translated into a transcriptional response, we analyzed the effect of inhibiting protein synthesis on their expression (Fig. 2.3A, 2.3B). Animal caps from uninjected and noggin-injected embryos were cultured with the protein synthesis inhibitor
cycloheximide (CHX), collected at stages 10.5, 12.5 and 17 and then assayed for \textit{sox3} (Fig 2.3A, B) or \textit{sox2} (Fig. 2.3B) expression by RT-PCR or WISH. To ensure that CHX-treated explants maintained high levels of BMP protein, exogenous BMP protein was added to the media of a subset of explants. In CHX-incubated caps, \textit{sox3} mRNA was detectable from stage 10.5 through stage 17 with or without exogenous BMP and was strongly induced by CHX and \textit{noggin}-overexpression (Fig. 2.3A and 2.3B). These results indicate that a BMP-responsive protein must be synthesized to either degrade maternal \textit{sox3} message or to restrict \textit{sox3} expression to the neuroectoderm. In contrast, \textit{sox2} is not expressed in uninjected or \textit{noggin}-injected caps treated with CHX (Fig. 3C) indicating that expression requires the synthesis of an activator protein.

These data indicate that either the degradation of maternal \textit{sox3} mRNA or the inhibition of zygotic \textit{sox3} mRNA transcription requires protein synthesis. If \textit{sox3} expression is zygotic prior to its restriction, there are two possibilities to explain its dynamic expression pattern. Either zygotic expression of \textit{sox3} is only activated on the dorsal side of the embryo, or the zygotic mRNA is expressed ubiquitously and then restricted. To determine if \textit{sox3} expression in untreated explants is zygotic, we injected a 1.5 kb fragment of the 5’ \textit{sox3} regulatory region driving luciferase (\textit{sox3}-luciferase) into 1-cell embryos, dissected explants at stage 8, performed WISH for \textit{luciferase} expression in whole embryos and explants and compared \textit{sox3}-luciferase expression to that of endogenous \textit{sox3} (Fig. 2.4). \textit{Sox3}-luciferase was expressed in the explants as early as stage 8.5-9 suggesting that \textit{sox3} is expressed zygotically in untreated explants. This experiment supports the hypothesis that in fact, protein synthesis is required to repress
expression of zygotic $sox3$ instead of degrading maternal $sox3$ mRNA.

**The BMP targets are expressed in a complimentary pattern to $sox2$ and $sox3$**

To identify BMP effectors that regulate $sox3$, we analyzed the expression of the BMP targets, $Xvent1$ and $Xvent2$ by WISH and compared their expression to $sox3$ (Fig. 2.5A, B compare to Fig. 2.1A, D). $Xvent1$ and $Xvent2$ are expressed in a complimentary pattern to $sox3$ (Dosch, Gawantka et al. 1997). At stage 10.5, $sox3$ is expressed throughout the ectoderm with a stronger expression in the dorsal neuroectoderm, while $Xvent1$ and $Xvent2$ expression is restricted to the ventral mesoderm and ectoderm (Figure 2.5A). As gastrulation proceeds, $sox3$ expression is restricted to the dorsal neuroectoderm (by stage 12.5), while $Xvent1$ is restricted to the ventral involuting mesoderm and $Xvent2$ is expressed in the ventral ectoderm and paraxial mesoderm (Fig. 2.5A). By neurula stage, $Xvent2$ and $Xvent1$ are restricted to the border of the posterior neural plate, the ventral epidermis and the proctodeum (Fig. 2.5, stage 17).

To characterize the temporal expression of these genes, RT-PCR of untreated whole embryos and ectodermal explants was performed. $Xvent1$ and $Xvent2$ are both zygotically expressed genes (compare Fig. 2.1A and 2.5B). $Xvent2$ directly activates $Xvent1$ expression (Friedle and Knochel) and accordingly it is expressed at stage 8.5 prior to $Xvent1$ which is expressed at stage 10 in whole embryos and ectodermal explants. As expression of the BMP targets increases in untreated explants, $sox3$ expression decreases (compare Fig. 2.1D and 2.5B), supporting a possible regulative relationship in which XVent1 and XVent2 repress $sox3$ expression. Next, we wanted to
determine the effect of Noggin and CHX on Xvent2 expression so we tested Xvent2 expression by RT-PCR in explants from uninjected or noggin-injected embryos and treated a subset of the uninjected explants with CHX. Since XVent2 activates expression of Xvent1 directly, any treatment that inhibits Xvent2 expression should have the same affect on Xvent1 (Friedle and Knochel 2002). Xvent2 is expressed from stage 10.5 through stage 17 (Fig. 2.5C) in uninjected explants and CHX treated explants. Although protein synthesis is not required for Xvent2 mRNA expression, it is not translated into protein in these treatments. Also, Noggin strongly inhibits Xvent2 expression. The induction of sox3 mRNA in CHX and noggin-injected explants (Fig. 2.4A) compared to the loss of Xvent2 in noggin-injected explants, combined with transgenic data using a sox3 reporter construct (data not shown) make is reasonable to assume that XVent2 and XVent1 are the repressors of sox3.

**Xvent1 and Xvent2 repress endogenous sox3 and sox3-GFP expression in gastrula embryos**

Computational analysis of 1.5 kb of regulatory sequence directly upstream of sox3 revealed one XVent1 consensus motif (CTATTCG) (Friedle, Rastegar et al. 1998) and one XVent2 half-site (AATAATAA) (Trindade, Tada et al. 1999). Transgenic analysis completed in our lab by T.C. Archer and E.M. Silva Casey demonstrated that these Vent consensus binding sites are required for restriction of sox3 to the presumptive neural plate (data not shown), thus supporting our interpretation of CHX experiments that sox3 expression is inhibited in ventral ectoderm by a target of BMP. To determine if
XVent1 or XVent2 alters the expression of sox3, we injected 1-cell embryos with sox3-GFP DNA and either Xvent1 (V1), Xvent2 (V2), or the dominant activator VPvent1 (VPV1) or VPvent2 (VPV2) mRNAs. The VPV proteins have the DNA binding domain linked to the strong VP16 activator and therefore should effectively activate the expression of target genes without interaction with another protein. Expression of either VPV protein resulted in an expansion of sox3 in non-neural ectoderm in greater than 90% of late gastrula embryos (Fig. 2.6B). As predicted, overexpression of V1 or V2 decreased expression and VPV1 and VPV2 increased expression of sox3-GFP (Fig. 2.6A). To determine if the two XVent consensus motifs are required for repression of sox3-GFP by XVent proteins, we injected the XVent mRNAs with a sox3-GFP reporter construct that lacked these XVent binding sites (Fig. 2.6A). Overexpression of Xvent1 or Xvent2 mRNA had no effect on the expression of this construct. Therefore, loss of the two XVent consensus motifs prevents XVent1 or XVent2 from repressing sox3-GFP expression.

We next asked whether XVent1 and/or XVent2 are required for repression of endogenous sox3 expression by testing the effect of overexpression of XVent1, XVent2, VPV1 or VPV2 on sox3 expression. Overexpression of XVent1 and XVent2 reduced sox3 levels in 56% and 40% of the embryos, respectively (Fig. 2.6B). These data suggest that while XVent1 and XVent2 may be required for complete restriction of sox3 expression to the neuroectoderm, neither can repress sox3 expression entirely in the neural plate. One possibility is that Vent function requires interaction with another protein such as a co-repressor. XVent2 binds directly to Smad1 to regulate its own
expression (Henningfeld, Friedle et al. 2002) and it interacts with Gata2 to activate Xvent1 expression (Friedle and Knochel 2002). This hypothesis is supported by experiments in which the dominant activator forms, VPV1 and VPV2, were overexpressed (Fig 2.6B).

To determine if overexpression of XVent1 or XVent2 inhibits the induction of sox3 by Noggin, sox3 expression was analyzed in stage 12.5 ectodermal explants from embryos injected with noggin and Xvent1 or Xvent2 mRNA (Fig. 2.6C). Overexpression of XVent2 had no effect, while overexpression of XVent1 decreased the induction of sox3 by Noggin. VPV1 and VPV2 both slightly induced sox3. These responses in explants mimic those in whole embryos with XVent1 decreasing sox3 expression more effectively than XVent2. We also tested the response of geminin to the XVent proteins in explants since a geminin reporter construct was also shown to require XVent binding sites for restriction of expression to the neuroectoderm in transgenic embryos (Taylor, Wang et al. 2006). In this case, both XVent1 and XVent2 reduced geminin expression in response to Noggin but neither VPV1 nor VPV2 induced expression.
**Discussion**

Neural induction occurs in the absence of BMP signaling and conversely, BMP inhibits the expression of neural genes. Are there factors required for *sox3* expression to be activated in response to BMP inhibition, or are neural genes simply de-repressed in the absence of BMP? To identify the mechanisms by which BMP inhibits neural genes we analyzed the endogenous expression of *sox2* and *sox3* and compared this expression to their expression in the absence of protein synthesis. We have also determined the effect of overexpression of two BMP targets, *Xvent1* and *Xvent2* on *sox3* expression. This study has shown that *sox2* and *sox3* are expressed at the onset of neural induction throughout the neuroectoderm, and that they are both induced by BMP inhibition at the onset of neural induction. Moreover, protein synthesis is required for repression of *sox3* expression in explants, and it is required for the induction of *sox2*, but not *sox3*, by Noggin. In support of previous studies, we identified that XVent1 and XVent2 restrict expression of *sox3* to the dorsal ectoderm indicating that there may be a common mechanism for the repression of neural genes in the non-neural ectoderm (Fig. 2.7).

**Comparison of *sox3* and *sox2* expression in embryos and ectodermal explants**

*Sox2* and *sox3* are expressed in similar but distinct patterns in the nervous system (Fig. 2.1A) indicating both that they may be regulated differently and they may have different functions in neural development. While *sox3* mRNA is detectable in ectodermal explants through stage 12.5, *sox2* is detectable by RT-PCR only. The differential expression of these genes may be due to: maternal *sox3* expression that has
yet to be degraded, or the zygotic expression of \textit{sox3} in the absence of BMP inhibition. The first hypothesis indicates that \textit{sox3} expression in the non-neural ectoderm may be maternal while the second hypothesis suggests that zygotic \textit{sox3} may play a transitory role in ectodermal specification, potentially to maintain ectodermal progenitor cells.

**BMP signaling is sufficient to inhibit neural gene expression in neural ectoderm and BMP inhibition cannot induce precocious neural gene expression**

Although inhibition of BMP signaling induces \textit{sox2} and \textit{sox3} expression in ectodermal explants, since expression of \textit{sox2} is delayed in explants, we tested whether BMP signaling was sufficient to inhibit their expression at the onset of neural induction. By overexpressing a constitutively active BMP receptor, we determined that BMP signaling is sufficient to completely inhibit three early neural genes, \textit{sox2}, \textit{sox3} and \textit{geminin} when mis-expressed in the neural plate (Fig. 2.2A). So, \textit{sox2} and \textit{sox3} are not only expressed in the right tissues at the right stages to be involved in neural induction, they also respond to BMP signaling in a similar manner to another early neural gene that is regulated by BMP targets (Kroll, Salic et al. 1998; Taylor, Wang et al. 2006). To determine if BMP inhibition is sufficient to induce \textit{sox2} expression prior to the onset of neural induction, we overexpressed \textit{noggin} and assayed for \textit{sox2} expression by WISH at the mid-blastula transition, stage 9, before the onset of neural induction, and compared that to \textit{noggin}-overexpressing embryos at gastrula stage. Although Noggin induces neural genes in explants, and expands \textit{sox2} expression in gastrula stage embryos, it is not sufficient to induce induction of \textit{sox2} before stage 10.5 (Fig. 2.2B). In toto, these
results indicate that BMP signaling is sufficient to inhibit neural genes in the dorsal ectoderm at the onset of neural induction and that BMP inhibition is not capable of inducing $sox2$ expression until after stage 9.

The inhibition of $sox3$ by BMP and the induction of $sox2$ by Noggin require de novo protein synthesis

To determine if $sox2$ and $sox3$ are inhibited directly by BMP signaling or if they require synthesis of an activator for expression, we blocked protein synthesis in uninjected ectodermal explants and in those injected with noggin mRNA, and performed WISH for $sox2$ and $sox3$ expression. Untreated ectodermal explants express $sox3$ through stage 12.5 and do not express $sox2$ as detected by WISH. Inhibition of protein synthesis extended $sox3$ expression in untreated explants (i.e. in the presence of BMP), and inhibited $sox2$ expression in explants in which BMP signaling was inhibited by Noggin.

Additionally, a $sox3$ reporter construct containing a 1.5 kb upstream regulatory fragment is sufficient to drive expression of luciferase in whole embryos and explants as early as stages 8.5-9 supporting the hypothesis that $sox3$ is initially zygotically expressed throughout the ectoderm in whole embryos and explants and then later restricted to the dorsal ectoderm. If $sox3$ is expressed in the animal ectoderm zygotically and later restricted to the dorsal ectoderm, it is likely that it may function in a similar manner as maternal Sox3 in axis formation by repressing the expression of $Xnr5$ and 6 and inducing the ectodermal specification genes ectodermin, Xema and Coco (Zhang and
Klymkowsky 2007) in addition to the role of zygotic Sox3 in maintenance of neural progenitors and inhibition of neuronal differentiation (Bylund, Andersson et al. 2003; Rogers, Harafuji et al. 2008). Since zygotic Sox3 functions as an activator, the restriction to the dorsal ectoderm, which requires protein synthesis, may act as a “switch” in function from repression of mesendodermal fates to induction of neural progenitors.

**XVent1 and XVent2 repress the expression of endogenous sox3 and sox3-GFP in gastrula embryos**

XVent1 and XVent2 repress and VPV1 and VPV2 induce sox3 and sox3-GFP expression. XVent1 and its dominant activator form are more effective at altering sox3 expression than XVent2 and VPV2. However, endogenous Xvent1 expression is restricted to the posterior mesoderm while Xvent2 is more widely expressed in the presumptive epidermis making it more likely to repress and restrict sox3 expression to the dorsal ectoderm. Although XVent1 repressed sox3 expression in 56% of embryos, XVent2 only repressed 40%. One possibility for the appearance of a stronger repression of sox3 by XVent1 is that since endogenous Xvent1 expression is so restricted, mis-expression of this protein may be toxic to the cells. It is also possible that XVent1 directly binds to and represses sox3, and that the repression by XVent2 is indirect via activation of Xvent1. However, both VPV2 and VPV1 induce sox3 in explants indicating that they both directly interact with sox3. Studies have shown that XVent2 requires binding partners such as Smad1 and Gata2 to activate expression of itself and Xvent1.
(Friedle and Knochel 2002; Henningfeld, Friedle et al. 2002), whereas XVent1 may not require a binding partner to repress sox3. If Xvent2 is overexpressed in the absence of its binding partners, it may not effectively repress sox3. Studies in which Xvent2 is overexpressed with each potential partner may lead to an equal repression of sox3 expression when compared to Xvent1-overexpression. In addition, Taylor et al. showed that another early maternally expressed neural gene, geminin, is regulated by XVent1 and XVent2 (Taylor, Wang et al. 2006). The role that the Xvent proteins play in the regulation of geminin supports a role for both proteins in the regulation of sox3 as well since both neural genes act to maintain neural progenitors and are expressed at the same stages in similar patterns. These data suggest that there may be redundant programs involved in the restriction of neural genes from the non-neural ectoderm and to the presumptive neural plate. The timing and expression of Xvent1 and Xvent2 are predominantly complementary to sox3, however they appear to overlap with sox3 in the presumptive neural crest. There are two possibilities that may explain this anomaly in expression in light of our data showing that XVent1 and XVent2 repress sox3 expression. It is possible that in the potential overlapping region where Xvent1 and Xvent2 are expressed is the paraxial mesodermal tissues underlying sox3 in the neural plate and therefore in fact, do not overlap. It is also possible that in regions where Xvent2 and sox3 appear to be expressed simultaneously, they may not be in the same cells. Since BMP and its targets are required for the normal development of the neural crest cells (Foerst-Potts and Sadler 1997; LaBonne and Bronner-Fraser 1998), it is likely that Xvent2 is specifically expressed in the presumptive neural crest cells bordering the neural plate.
and its expression is not cell autonomous with the neural plate cells expressing *sox3*.

The BMP targets, *msx-1*, *gata2*, *Vent1*, and *Vent2* are viable candidates for the repressor of *sox3* because they are expressed in the non-neural ectoderm at the same time in development that *sox3* expression is restricted to the dorsal ectoderm (Zon, Mather et al. 1991; Harland 1994; Gawantka, Delius et al. 1995; Hawley, Wunnenberg-Stapleton et al. 1995; Knecht, Good et al. 1995; Schmidt, Suzuki et al. 1995; Onichtchouk, Gawantka et al. 1996; Foerst-Potts and Sadler 1997; Suzuki, Ueno et al. 1997; Friedle and Knochel 2002; Taylor, Wang et al. 2006; Rogers, Archer et al. 2008). However, although Msx-1 is expressed in a complimentary pattern to *sox3* early, it is later restricted to the neural border, required for neural crest development and may not be necessary for epidermal development and neural inhibition (Foerst-Potts and Sadler 1997; Holland and Holland 2001; Sargent 2006). Knock down of Msx-1 in *Xenopus* embryos by injection of morpholino oligonucleotides leads to an expansion of the neural plate border laterally into epidermis, and a loss of neural crest, but does not affect ventral epidermis formation (Khadka, Luo et al. 2006). *Gata-2* is expressed in the ventral and lateral endoderm, mesoderm and ectoderm in *Xenopus* embryos prior to its restriction to the ventral blood islands and is involved in hematopoiesis at tailbud stages (Zon, Mather et al. 1991; Walmsley, Guille et al. 1994). Also, *Gata-2* expression is inhibited by Noggin in explants while overexpression of *Gata-2* in neuralized explants inhibits *ncam* expression at gastrula stage. Having been previously identified as a cofactor in XVent2 function (Friedle and Knochel 2002), Gata-2 may act in conjunction with XVent2 to inhibit early neural genes. Further studies would be needed to determine
if this is the case. Overall, these data support a model in which XVent1 and XVent2 repress expression of \(sox3\) in non-neural ectoderm thereby restricting it to the neural plate and additional yet unknown co-repressors may be required for this action.

**A model for the mechanism of the inhibition of early neural genes by BMP signaling**

The data presented herein indicate that protein synthesis is required for the repression of \(sox3\) in the absence and presence of BMP signaling and both XVent2 and XVent1 repress expression of \(sox3\). Since XVent2 and Gata-2 are activated in response to BMP signaling and together they activate expression of \(Xvent1\), CHX treatments may have blocked the synthesis of either XVent2 or XVent1 to prevent the restriction of \(sox3\). In contrast to \(sox3\), a lack of protein synthesis prevented the induction of \(sox2\) by BMP inhibition indicating that either the synthesis of an activator is required for the expression of some neural genes, or that Smad proteins repress \(sox2\) directly. In the absence of BMP signaling, the \(vents\) are not activated which leads to the expression of \(sox3\). (Fig. 2.6B).

These studies show that \(sox2\) and \(sox3\) are induced by BMP inhibition and that \(sox3\) is repressed by XVent1 and XVent2 proteins. The similarity of \(geminin\) and \(sox3\) regulation by BMP signaling suggests that there may be a conserved mechanism to restrict neural genes to neural ectoderm and repress their expression in non-neural ectoderm. However, this is not necessarily a global mechanism as shown by the different requirements for \(sox2\) expression. This study identifies mechanisms involved in the
expression of two soxB1 genes that are required for neural development at the onset of neural induction.

**Future experiments**

To determine the stage at which maternal sox3 transcripts are degraded in either whole embryos or ectodermal explants, we can treat embryos or explants with a transcription inhibitor such as actinomycin-D at or prior to stage 8 and then perform WISH for sox3 expression at multiple gastrula stages. Although use of microarray technology may be helpful to determine the stage that zygotic sox3 expression is upregulated, recent work in *Drosophila* suggests that degradation of maternal genes often occurs concurrent with zygotic gene transcription (De Renzis, Elemento et al. 2007). If this is the case in *Xenopus*, a reduction in maternal sox3 would be expected to occur at the same stage as an increase in zygotic sox3. Thus, there would be little quantitative change in sox3 expression levels detected by RT-PCR or WISH.

Our experiments show that sox2 activation by BMP inhibition requires protein synthesis and that sox2 may be directly repressed by BMP signaling. In Chapter IV, we show that Sox3 can activate expression of sox2 in the absence of protein synthesis making it a strong candidate for the putative activator. Additionally, to determine if sox2 is directly repressed by BMP signaling via a Smad protein complex, a similar experiment can be performed using a hormone inducible Smad 1,5, or 8 protein and CHX. Although Smad proteins generally interact with p300/CBP to activate transcription of BMP targets (Pearson, Hunter et al. 1999), they may form complexes
with transcriptional co-repressors such as the homeodomain protein interacting factor (TGIF) preventing the activation of $sox2$ expression (Wotton, Knoepfler et al. 2001; Massague, Seoane et al. 2005).
Figure 2.1. A comparison of sox2 and sox3 expression in whole embryos and ectodermal explants. (A) WISH of *Xenopus* embryos using probes for sox3 and sox2 as indicated. Asterisk marks the otic placode. Note: WISH for sox2 on stage 8 was performed by T. Grammer. (B) RT-PCR of whole embryos from stage 8 through 12.5 as indicated on the top. (C) WISH of ectodermal explants from uninjected (UI) embryos or those injected with *noggin* (Nog) mRNA and collected at the stages indicated. (D) WISH for sox3 in ectodermal explants (top panel) with stages indicated in bottom right of each panel. RT-PCR of untreated and uninjected ectodermal explants using primers for sox2, sox3 and *ef1a* as a loading control (bottom panel). Stages are indicated on the top.
Figure 2.2. CaBMPR is sufficient to repress early neural genes in the neural ectoderm and BMP inhibition induces sox2 by stage 10.5. (A) WISH for sox2, sox3 and geminin. Embryos were either uninjected (UI) or injected with CaBMPR and lacZ mRNA and collected at stages 10.5 and 12.5 as indicated on left side of panel. (B) WISH for sox2 expression in uninjected embryos or embryos injected with noggin mRNA. Stages are indicated on left side.
Figure 2.3. Inhibition of *sox3* and induction of *sox2* requires protein synthesis. (A) RT-PCR of ectodermal explants dissected from either uninjected or *noggin*-injected embryos. Some uninjected explants were with CHX. Explants were collected at the stages indicated identified by the whole embryo (WE) sibling controls. WISH for either *sox3* (B) or *sox2* (C) in ectodermal explants from untreated embryos or embryos injected with *noggin* mRNA and/or treated with BMP protein or CHX as labeled on top. The right columns are images of sibling embryos used to stage explants. Explants were collected at stages 10.5, 12.5, and 17.
Figure 2.4. Sox3-luciferase is expressed by stage 9 in ectodermal explants. (A, B) WISH of whole embryos and ectodermal explants using probes for sox3 or luciferase from untreated embryos or embryos injected with sox3-luciferase DNA (50 pg) and collected from stages 6.5 through 23. (A) Whole embryos (top row) and explants (bottom row) expressing sox3. (B) Whole embryos (top row) and explants (bottom row) expressing luciferase. Explants were collected according to sibling embryos stages. Stages are indicated at top and probe indicated on left.
Figure 2.5. *Xvent1* and *Xvent2* genes are expressed in a complimentary pattern to *sox3*. (A) WISH for *sox3*, *Xvent2*, and *Xvent1* (labeled on left) of embryos from stages 10.5 though 17. (B) RT-PCR of untreated embryos and ectodermal explants using primers for *sox3*, *sox2*, *Xvent2*, *Xvent1* and *ef1a* as a loading control. Whole embryos were collected between stages 4 and 12.5 and explants were collected between stages 9 and 12.5. (C) RT-PCR from ectodermal explants for *Xvent2*, *Xvent1* and *ef1a* as a loading control. Explants were dissected from either uninjected embryos (UI) or those injected with
noggin mRNA (Nog). Some uninjected explants were cultured in the presence of CHX. Explants were staged according to untreated sibling whole embryos (WE).
Figure 2.6. XVent1 and XVent2 repress and dominant activator Vents activate sox3 expression. (A) WISH of embryos injected with full length sox3-GFP DNA or a sox3-GFP DNA construct with the Vent binding sites mutated (diagrams of constructs shown below embryo pictures) and mRNA coding for either Xvent1 (V1), Xvent2 (V2), Xvent1-VP16 (VPV1) or Xvent2-VP16 (VPV2). Numbers in bottom right of images indicates the number of embryos out of the total with the pictured phenotype. LacZ was used as a tracer. (B) Embryos are either uninjected (UI) or injected with mRNA coding for Xvent1, Xvent2, VPV1 or VPV2 and WISH was performed for sox3. C. RT-PCR analysis of
ectodermal explants dissected from either uninjected embryos or those injected with noggin, Xvent1, Xvent2, VPV1 or VPV2 mRNA. Primers used were for sox3, geminin or odc.
Figure 2.7. A model for the mechanism of BMP inhibition of early neural genes. (A) BMP signaling activates Smad1, 5, 8 proteins via phosphorylation. The activated Smads then interact with Smad4, translocate to the nucleus, activate transcription of the BMP target genes and may also interact with co-regulators to activate or repress transcription of other genes. The Smad proteins may directly inhibit sox2 and other neural genes. Once XVent1 and XVent2 are translated they return to the nucleus to repress expression of sox3 and geminin. (B) BMP signaling is repressed by organizer proteins such as Noggin. In the absence of an activated BMP pathway, sox3 is expressed.
CHAPTER III: FGF SIGNALING IS REQUIRED FOR THE INDUCTION OF sox2 AND THE MAINTENANCE OF sox3 AND GEMININ EXPRESSION IN Xenopus laevis

Some data shown in this chapter were previously published in: C.D. Rogers, T. C. Archer, D.D. Cunningham, T.C. Grammer and E.S. Casey. Sox3 expression is maintained by FGF signaling and restricted to the neural plate by Vent proteins in the Xenopus embryo. Dev. Bio. Jan 1, 2008. 313, 307-319.
Introduction

The inhibition of BMP signaling is sufficient to induce neural tissue in frog animal ectodermal explants (Hawley, Wunnenberg-Stapleton et al. 1995; Wilson and Hemmati-Brivanlou 1995; Kroll, Salic et al. 1998; Rogers, Archer et al. 2008), however, BMP inhibition is not sufficient to induce neural genes in tissue derived from ventral ectoderm. The induction of neural genes in ventral tissues may require additional signaling by factors such as FGF (Streit and Stern 1999) or the inhibition of TGF-β (Chang and Harland 2007) and Wnt signaling (Heeg-Truesdell and Labonne 2006). There are studies supporting roles for FGF and Wnt in neural induction in frog and other vertebrates. Exposure to a chemical inhibitor of FGF receptor I (SU5402) in frog (Delaune, Lemaire et al. 2005), zebrafish (Dee, Hirst et al. 2008) and chick (Wilson, Rydstrom et al. 2001) embryos lead to a loss of neural induction as marked by sox2 expression. However, FGF signaling is necessary to induce mesoderm development in frog embryos, and dorsal mesoderm is necessary for neural induction (Smith and Slack 1983; Kimelman and Kirschner 1987). Therefore, these studies suggest that inhibition of all FGF signaling prior to gastrulation may affect mesodermal development and subsequently prevent neural induction rather than indicating a requirement for FGF signaling for neural induction independent of its role in mesoderm induction (Kimelman and Kirschner 1987; Kimelman, Abraham et al. 1988; Cornell and Kimelman 1994).

The loss of FGF signaling appeared to inhibit the induction of neural tissue indicating that FGF may be required prior to neural induction. However, studies in chick
determined that FGF signaling alone or in combination with BMP antagonists was not sufficient to induce neural tissue in the prospective epidermis and that inhibition of Wnt signaling is also required for neural induction (Wilson, Rydstrom et al. 2001). The requirement for Wnt inhibition for the induction of neural tissue suggests a more complex model than previous studies suggested. However, the differential requirements for the expression of neural genes between organisms led to a controversy about the requirement for FGF in neural induction. In frog, BMP inhibition is sufficient to induce neural genes in ectodermal explants (Hawley, Wunnenberg-Stapleton et al. 1995), but in chick, FGF is the primary signal involved in the induction of neural tissue with BMP inhibition as a second step (Linker and Stern 2004). The requirement for FGF signaling in neural induction remains a debate. The following question remains: is FGF required to induce neural induction in dorsal ectoderm and if so, what facilitates this induction?

Loss of function studies indicate that FGF signaling is required for neural development, but it has yet to be demonstrated that this requirement for FGF is independent of its ability to inhibit BMP expression and signaling. FGF inhibits the transcription of the \( \text{bmp} \) transcript (Baker, Beddington et al. 1999) and inactivates the BMP effector, Smad1 by phosphorylation (Pera, Ikeda et al. 2003). In support of an instructive signaling requirement in neural induction by FGF, experiments showed that the chicken neural specific \( \text{Sox2} \) enhancer responds to FGF8b signaling in a synergistic reaction with Wnt signaling (Takemoto, Uchikawa et al. 2006). Additionally, experiments in frog using XFD, a dominant negative FGF receptor 1, inhibited ventral but not dorsal mesoderm formation (Amaya, Stein et al. 1993) and repressed the
expression of the anterior markers, \textit{otx-2}, \textit{hf-1} and \textit{rx-1} and the pan neural markers, \textit{nrp-1} and \textit{ncam} (Launay, Fromentoux et al. 1996; Hongo, Kengaku et al. 1999). Therefore, FGF signaling is required for normal neural development, but the aforementioned studies did not focus on a specific requirement for FGF at the onset of neural induction.

Other vertebrate studies investigating the role of FGF signaling in neural induction actually studied its role neuronal differentiation. Instead of testing embryos at the onset of neural induction by analyzing the change in expression of early neural markers (\textit{sox2}, \textit{sox3}, \textit{geminin}), experiments were predominantly designed to assess the effect of loss of FGF in neurula and tailbud embryos by analyzing markers of neuronal differentiation (\textit{n-tubulin} and \textit{ngnr-1}) (Kengaku and Okamoto 1993; Kengaku and Okamoto 1995; Furthauer, Thisse et al. 1997; Alvarez, Araujo et al. 1998; Holowacz and Sokol 1999; Hongo, Kengaku et al. 1999; Hardcastle, Chalmers et al. 2000; Ishimura, Maeda et al. 2000). The experiments described in this chapter were designed to determine if the onset of neural induction by BMP inhibition requires additional signaling through FGF or Wnt. Although BMP inhibition is sufficient to induce neural genes in \textit{Xenopus} animal cap ectoderm, there is a delay in the timing of neural gene induction in explants when compared to the onset of neural induction in whole embryos (Rogers, Archer et al. 2008). One possible explanation for this delay is that the onset of expression requires FGF or Wnt signaling. To test this, we compared the requirements for FGF and Wnt signaling in the induction and expression of three different neural genes that are expressed at the onset of neural induction and mark neural progenitors, \textit{sox2}, \textit{sox3} and \textit{geminin}. Our studies will determine if FGF and Wnt signaling are
required to induce neural genes in the dorsal ectoderm by using multiple inhibitors of FGF and Wnt to inhibit signaling through these pathways.

*Sox2, sox3* and *geminin* are expressed in the developing nervous system at the onset of neural induction (Pevny and Lovell-Badge 1997; Kroll, Salic et al. 1998; Rogers, Archer et al. 2008) and are required for normal neural development (Kroll, Salic et al. 1998; Mizuseki, Kishi et al. 1998; Kishi, Mizuseki et al. 2000; Rogers, Harafuji et al. 2008). While *sox2* expression is zygotic, both *sox3* and *geminin* are maternally and zygotically expressed genes and as such, appear to be regulated differently when comparing induction of expression versus maintenance of expression. Using gain and loss of function studies we show that although BMP signaling is sufficient to inhibit, and the inhibition of BMP is sufficient to induce *sox2, sox3* and *geminin*, both *sox3* and *geminin* are regulated differently than *sox2*. FGF signaling is required for the induction of *sox2* expression but only the maintenance of *sox3* and *geminin*. We also identify the module in the *sox3* regulatory region that may be required to respond to FGF signaling for maintenance.
Materials and Methods

Embryo culturing and manipulations

As in Chapter II.

Plasmid construction

Sox3 regulatory elements were isolated by inverse PCR and subcloned by E. Silva Casey. The PCR fragments were inserted into the plasmid pCR 3.1-TOPO using the TopoTA kit (Invitrogen Corporation) and sequenced. To generate sox3-GFP (#208) and sox3-luciferase (#238), a 1.552 kb fragment upstream of the ATG was fused to EGFP and the SV40 polyA in pCS2+ in which the CMV promoter was removed. A 90 bp minimal cytoskeletal actin promoter (Mohun et al., 1987) was cloned as a blunt-HindIII fragment into the Smal-HindIII sites of pGL3 in which luciferase was replaced with EGFP. Internal deletions and binding-site mutations of sox3-GFP were generated by DPN mutagenesis.

mRNA Synthesis and Microinjection

Capped mRNAs were synthesized by in vitro transcription using mMMessage mMachine kits (Ambion). For explant and gain of function assays, 25 pg noggin mRNA (#329) (Knecht, Good et al. 1995; Geng, Xiao et al. 2003) was injected into the animal pole of a 1 or 2-cell embryo with or without 0.5 ng-1 ng of dominant negative Xfrizzled-8 mRNA (Xfz, #369) (Deardorff, Tan et al. 1998), 0.75-1.5 ng of inhibitory Xtcf-3 mRNA (∆Tcf, #370) (Molenaar, van de Wetering et al. 1996), 0.05 ng - 0.5 ng of dominant negative Xfgfr1 mRNA (XFD, #RH317) (Amaya, Stein et al. 1993) and/or 1-
1.5 ng of dominant negative XFGFR4a mRNA ($\Delta 4a$, #392) (Hongo, Kengaku et al. 1999) with or without 250 pg of lacZ mRNA. Embryos were cultured until stages 10.5-17 (and explants were collected based on stages of sibling embryos) and analyzed by WISH or reverse transcription-polymerase chain reaction (RT-PCR).

**RT-PCR**

Semi quantitative RT-PCR was performed as described in (Wilson and Hemmati-Brivanlou 1995) with some modifications. To make cDNA, 10 μl of isolated RNA was mixed with 1 μl random hexamers and heated to 65° for five minutes. After cooling mixture was combined with 10mM dNTPs, 20mM DTT, Rnase inhibitor, 1X MMLV buffer (Fisher Scientific) and MMLV Rtas e (Fisher Scientific). To determine if there was DNA contamination, 1 μl of isolated RNA was used for PCR with primers for ef1a (#86, #87, XMMR). Primers used were: sox2 (#114, #115, F: CTTACATGAACGGCTCGCC, R: CCCAGGTAGGTACATGC, 58° annealing, 29 cycles), sox3 (#18, F: AGGAGTAAGCGGCTGCTAGCA, #19, R: ATGTAGGCATTGTCGGCGCTAGCA, 61° annealing and 25 cycles), or geminin (#28, #29, XMMR).

**WISH and β-galactosidase assay**

WISH was performed as in Chapter II. We used the following genes as WISH probes: sox2 (#RH1104) (Mizuseki, Kishi et al. 1998), sox3 (#419) (Pevny and Lovell-Badge 1997), and geminin (#368) (Kroll, Salic et al. 1998).
Results

**FGF but not Wnt signaling is required for the maintenance of sox2 and sox3 in ectodermal explants**

Experiments indicate that FGF signaling and the inhibition of Wnt signaling are required for the specification of neural tissue. To further analyze the requirement of either Wnt or FGF signaling for the induction of early neural genes by BMP inhibition, we inhibited signaling through these pathways by overexpression of dominant negative effectors of Wnt signaling. ΔTcf (Molenaar, van de Wetering et al. 1996) is a dominant negative form of a transcription factor downstream of Wnt, TCF3, and ΔXfz (Sokol 1996) is a dominant negative Wnt receptor, Frizzled-8. We also used dominant negative FGF receptor-1, XFD (Amaya, Musci et al. 1991). To demonstrate the functionality of the dominant negative proteins, we overexpressed XFD in *Xenopus* embryos and inhibited neurogenesis marked by *n-tubulin* expression (Hardcastle, Chalmers et al. 2000) and overexpressed ΔTcf3 and ΔXfz which shortened the axis of *Xenopus* tailbud embryos as expected (data not shown) (Wallingford, Vogeli et al. 2001; Roel, Hamilton et al. 2002). However, in ectodermal explants these dominant negative proteins did not affect expression of sox3 or sox2 (Fig. 3.1A and data not shown); as in untreated explants, sox3 was expressed until stage 12.5, while sox2 expression was undetectable by WISH at all stages tested. To determine if inhibition of Wnt or FGF signaling interfered with the ability of Noggin to induce expression of sox2 and sox3, we analyzed expression in animal caps from embryos injected with noggin mRNA and ΔTcf3, ΔXfz or
Neither ΔTcf3 nor ΔXfz altered sox3 or sox2 expression at any of the stages tested (Fig. 3.1B, 3.1C). In contrast, overexpression of XFD mRNA, reduced the expression of sox2 and sox3 in neurula (st. 17) but not gastrula embryos. These data indicate that Wnt and signaling is not required for the induction of sox2 or sox3, but signaling through FGFR-1 and/or 2 is required for the maintenance of sox2 and sox3 in animal caps.

To verify that sox2 and sox3 require FGF signaling for maintenance and not the induction, and to determine if other early neural genes, such as geminin, require FGF signaling, we tested the effect of high levels of XFD, Δ4a (dominant negative FGFR4a), and a combination of both, on the expression of sox2, sox3, and geminin in response to Noggin (compare Fig. 3.1 which used 50 pg of XFD and 3.2 which used 500 pg of XFD). FGFR-4a, the receptor for FGF8, is required for anterior neural induction and its dominant negative form is more effective at knocking down neural development than XFD (Hongo, Kengaku et al. 1999). Ectodermal explants were dissected from embryos overexpressing noggin and XFD, Δ4a, or both. Embryos were collected at stages 11.5, 12.5 and 17 and RT-PCR was performed using primers for sox2, sox3, geminin and either efla or odc as a loading control (Fig. 3.2, and data not shown). Overexpression of noggin induced sox2, sox3 and geminin by stage 12.5 (Fig. 3.2 and data not shown). In contrast to the in situ hybridization data (Fig. 3.1), expression of high levels of XFD marginally reduced the levels of sox2 induced by Noggin at stage 11.5, and Δ4a (or Δ4a +XFD) strongly repressed the levels of sox2 at the early stage. Knocking down signaling
with XFD did not significantly alter the levels of either \textit{sox3} or \textit{geminin} expression at gastrula stages. At stage 17, loss of FGF signaling through FGFR1/2 and/or 4a repressed the expression of \textit{sox2}, \textit{sox3} and \textit{geminin}. These data support a model in which the induction by BMP inhibition of some early neural genes (\textit{sox3} and \textit{geminin}) does not require FGF (Rogers, Archer et al. 2008), but the induction of others (\textit{sox2}) does require FGF signaling. Furthermore, the maintenance of all three genes after induction by BMP inhibition requires FGF signaling.

\textbf{FGF signaling is required for \textit{sox2} expression and the maintenance of \textit{sox3} and \textit{geminin} in neural ectoderm of whole embryos}

Prior studies indicated that FGF signaling is required for neural induction in whole embryos based on the loss of \textit{sox2} induction caused by the addition of SU5402 prior to MBT (Delaune, Lemaire et al. 2005). Early SU5402 treatment prevents mesoderm formation in whole embryos which can lead to a loss of neural tissue. To assess the requirement for FGF signaling for neural induction in whole embryos, we knocked down FGF signaling in 1 of 2 cell embryos by overexpression of XFD and Δ4a in the presence of Noggin. To assess whether signaling is required for the onset of \textit{sox2}, \textit{sox3} and \textit{geminin} expression in the whole embryo, we overexpressed dominant negative Δ4a and assayed at stages 10.5, 11.5 and 12.5 by WISH. We also tested whether FGF signaling is required for the maintenance of expression in whole embryos as it is in explants by stage 17. At stages 10.5 through 12.5, overexpression of \textit{noggin} expanded \textit{sox2} (n= 118/155, 76.1%) and Δ4a inhibited \textit{sox2} (n= 82/109, 75.2%) and this was not
rescued by overexpression of noggin at any stage (Fig. 3.3A, n= 71/93, 76.3%, experiment performed by G.S. Ferzli) indicating that signaling through FGFR-4a is necessary for the induction of endogenous and noggin-induced sox2 expression in whole embryos as well as in explants (Fig. 3.3A).

Explant studies showed that FGF signaling was not required for the induction of sox3 or geminin when BMP was inhibited (Fig. 3.2), therefore we tested if FGF signaling is required for sox3 and geminin expression in whole embryos. As expected there was no change in their expression patterns with the addition of exogenous Noggin (Fig. 3.3B, C, experiment performed by G.S. Ferzli). However, expression of both genes was expanded at stage 12.5 (sox3, n= 10/12, 83.3% geminin, n= 19/22, 86.4%). As in ectodermal explants, overexpression of Δ4a had no effect on the expression of sox3 (n= 133/158, 84%) or geminin (n= 61/64, 95%) at stages 10.5 and 11.5 (Fig. 3.3B, C) but reduced expression of both in late gastrulae (Fig. 3.3B, C; sox3, n= 56/63, 88.9%; geminin 20/27, 74%) which was rescued by noggin overexpression (sox3, n= 17/21, 81% and geminin, n= 61/79, 77%). These experiments demonstrate that FGF signaling is required for the induction of sox2, but is only required for the maintenance of the two other neural genes, sox3 and geminin. Furthermore, BMP inhibition is sufficient to rescue the loss of sox3 and geminin expression in response to ΔFGFR4a indicating that the role of FGF signaling in their expression is to inhibit BMP signaling.

The sox3 regulatory region responds to knock down of FGF signaling

To identify cis-regulatory elements necessary for the response of the sox3
regulatory region to FGF signaling, E. Casey and T.C. Archer created transgenic embryos expressing a 1.5 kb sox3-GFP reporter construct (Rogers, Archer et al. 2008) and analyzed these embryos by WISH for GFP mRNA. The expression of the full length construct was then compared to numerous 5′ end- and internal deletion constructs of sox3-GFP (Fig. 3.5, experiment performed by T.C. Archer). One of these constructs, Δ250sox3-GFP had a 5′ end deletion which deleted a putative Elk site at -1487 bp upstream from the transcription start site (Figure 3.4, red circle). Δ250sox3-GFP expression was compared to wild type sox3-GFP at stages 12.5 and 20. At gastrula stages, Δ250sox3-GFP was expressed in the dorsal ectoderm in a broader domain than sox3-GFP. At neurula stages, the deletion construct was not expressed supporting a role for the putative Elk site in the sox3 regulatory region as the FGF response element responsible for maintenance of sox3 expression.

To determine if the sox3 regulatory region responds to the loss of FGF signaling, we overexpressed sox3-luciferase with or without XFD, Δ4a or a combination of both into one cell embryos, and assayed for Luciferase activity in whole embryos and ectodermal explants at stages 12.5 and 17. In both explants and whole embryos, sox3-luciferase expression is repressed by both dominant negative FGF receptors as early as stage 12.5 and this repression is maintained through stage 17. These data indicate that contrary to endogenous sox3, the short sox3 regulatory region responds to the loss of FGF signaling (Fig. 3.5A) in gastrula stage embryos. However, the sox3-luciferase reporter was not induced by noggin even though its expression pattern in transgenic embryos mimics that of endogenous sox3 (Rogers, Archer et al. 2008) and therefore, we
could not determine if sox3-luciferase was rescued by Noggin. Instead, we tested the activity of a reporter construct which contains a 1.3 kb region upstream of *Xenopus tropicalis* sox3 (*Xtsox3*-luciferase) that is between 50% and 100% conserved with the *Xenopus laevis* sox3 regulatory region (Rogers, Archer et al. 2008). This construct was weakly but consistently upregulated by Noggin at stage 17 but not in gastrulae (Fig. 3.5B and data not shown). We injected *Xtsox3*-luciferase with or without *noggin*, *XFD*, Δ4a and a combination of all three into one-cell embryos, collected at stage 17, and assayed for Luciferase. Overexpression of XFD and Δ4a decreased expression and this was not rescued by Noggin (Fig. 3.3B). This is in contrast to the response of endogenous sox3 to Noggin. These experiments showed the sox3 regulatory region responds to loss of FGF signaling and this fragment will be useful to identify the FGF response element in the sox3 regulatory region (Fig. 3.5B).
Discussion

This study addressed whether FGF signaling is necessary for the induction and/or maintenance of expression of three neural genes, *sox2*, *sox3* and *geminin*, expressed in the neural plate at the onset of neural induction and induced by BMP inhibition. Surprisingly, each of these early neural genes is regulated differently. Whereas the induction of *sox2* requires FGF signaling, even when BMP signaling is inhibited, *sox3* and *geminin* do not require FGF signaling for induction but do require it for maintenance. Even though inhibition of BMP signaling is sufficient to rescue *sox3* and *geminin* expression in embryos depleted of FGF signaling, these experiments support prior studies that indicate that FGF signaling is required for neural induction in *Xenopus* (Launay, Fromentoux et al. 1996; Hongo, Kengaku et al. 1999; Delaune, Lemaire et al. 2005), as Sox2 is required for neural formation (Kishi, Mizuseki et al. 2000). This study is unique in that it analyzes the role of FGF in the induction of three early neural genes, *sox2*, *sox3* and *geminin* that are required for normal neural development and are markers of neural progenitors. In contrast, prior studies have concluded that FGF is required for induction by testing for its effect on CNS patterning (Kengaku and Okamoto 1995; Furthauer, Thisse et al. 1997; Barnett, Old et al. 1998; Holowacz and Sokol 1999; Ribisi, Mariani et al. 2000; Lupo, Harris et al. 2002), the expression of neuronal markers (Pera, Ikeda et al. 2003), or they assayed for neural progenitor markers after the onset of neural induction (e.g. neurula stage) (Pera, Ikeda et al. 2003; Linker and Stern 2004). We show that although *sox2*, *sox3* and *geminin* have similar spatiotemporal expression patterns (Pevny and Lovell-Badge 1997; Kroll, Salic et al. 1998; Rogers, Archer et al. 2008) and
have elicited the same effect when overexpressed (Kroll, Salic et al. 1998; Rogers, Harafuji et al. 2008), they require different signals to be expressed in the neuroectoderm.

**Wnt signaling is not required for the induction of sox2 or sox3 by BMP inhibition**

Knocking down Wnt signaling via overexpression of either dominant negative Frizzled (Sokol 1996) or dominant negative form of Tcf-3 (Molenaar, van de Wetering et al. 1996) did not alter sox2 and sox3 expression in the presence or absence of BMP antagonists (Fig. 3.1B, 3.1C). This is surprising because knocking down Wnt signaling in whole embryos was sufficient to expand expression of sox2 and sox3 in the neural tube, and a constitutively active β-catenin was sufficient to inhibit the induction of sox3 by noggin in explants (Heeg-Truesdell and Labonne 2006) indicating that a loss of Wnt signaling may facilitate induction of neural tissue. The difference may be due to the differences in experimental technique. In the previous experiments, sox3 expression was not assayed neurula stage. Our experiments differed in that we tested the effect of Wnt signaling knock down at an earlier stage (st. 11.5) that corresponds more closely to the stage of neural induction. The ability of Wnt inhibition to expand neural tissue (Heeg-Truesdell and Labonne 2006) supports a role for Wnt in defining neural border cells and specifying prospective neural crest cells (Dorsky, Moon et al. 1998; Abu-Elmagd, Garcia-Morales et al. 2006) but does not specifically show that Wnt inhibition is involved in neural induction. Knocking down Wnt signaling in otherwise untreated explants (Fig. 3.1A), does not induce sox2 and sox3 expression. The previous study used constitutively active β-catenin and showed that Wnt signaling can inhibit neural
induction while our study showed that the inhibition of Wnt signaling is not sufficient to induce neural genes.

**FGF is required for the sox2 expression and the maintenance of sox3 and geminin induced by BMP inhibition**

Our aims were to determine if FGF signaling was required for neural induction independent of inhibiting BMP signaling, and if there is a common neurogenic code involved in the regulation of multiple neural genes. We define a neurogenic code as the same suite of proteins regulating all early neural genes in the same manner at the same stages in development. There is some support for a neurogenic code. In frog, both *geminin* and *sox3* are restricted to the dorsal ectoderm by the BMP targets XVent1 and XVent2 during mid-gastrula stages (Taylor and Labonne 2005; Rogers, Archer et al. 2008). Since these BMP targets play a role in the repression of two early neural genes, we wanted to analyze whether FGF signaling is required for the induction of these same genes. Our studies show that signaling through FGFR-1, 2 and 4a is required for the induction of *sox2* by Noggin and the maintenance of *sox3* and *geminin* after induction by Noggin (Fig. 3.1, 3.2). In this study, we knocked down FGF signaling using XFD, which reduces FGF3 and 4 signaling through both FGFR1 and 2 (Powers, McLeskey et al. 2000; Bainter, Boos et al. 2001), and Δ4a, which reduces FGF8 signaling through FGF4a (Amaya, Musci et al. 1991; Hongo, Kengaku et al. 1999). High concentrations of Δ4a and XFD alone or in combination decreased the induction of *sox2* by Noggin as early as stage 11.5 in ectodermal explants. Also, Δ4a with and without XFD repressed
the expression of *sox2*, *sox3* and *geminin* at stage 17 after they were induced by Noggin indicating a requirement for FGF signaling in maintenance of expression (Fig. 3.2). Although these three early neural genes share similar expression patterns, are induced by BMP inhibition, and even appear to play similar roles in neural development (Kroll, Salic et al. 1998; Rogers, Harafuji et al. 2008), the requirements for their induction appear to differ. These data suggest that even within *Xenopus*, there is no neurogenic code wherein the same proteins are involved in activating and repressing all of the neural genes at the same time during development.

Whole embryos have signaling pathways that are not present in explants and are likely to elicit a different response to inhibition and activation of those pathways. We compared the response of *sox2*, *sox3* and *geminin* expression to the loss of FGF signaling in the absence and presence of Noggin in whole embryos. Since Δ4a more effectively inhibits the induction of anterior neural genes than XFD (Hongo, Kengaku et al. 1999), we overexpressed Δ4a and tested the response of *sox2*, *sox3* and *geminin* in whole embryos. As expected, the expression of each gene was induced by Noggin by stage 12.5. In explants tested at stage 11.5, *sox2* expression was reduced by loss of FGF, and similarly, in whole embryos only *sox2* expression was affected by Δ4a at the onset of gastrulation (compare Fig. 3.2 and 3.3A) while *sox3* and *geminin* remained unaffected. By late gastrula stage, expression of all three genes was inhibited by Δ4a indicating that signaling through FGFR-4a is required for the induction of *sox2* and the maintenance of *sox3* and *geminin*. Also, overexpression of *noggin* was insufficient to rescue the loss of *sox2* but was able to rescue both *sox3* and *geminin* expression at late gastrula indicating
that FGF may only facilitate expression by inhibiting BMP.

Given that sox3 and geminin are both maternally expressed genes, it is possible that zygotic expression of each gene is activated by the maternal protein while induction of sox2 requires a factor downstream of FGF. Sox3, Sox2 and Oct-4 directly activate sox2 expression in frog and stem cells (Tomikawa, Nishimoto et al. 2002; Rogers, Harafuji et al. 2008), while Sox2 and Oct-4 activate FGF4 expression (Yuan, Corbi et al. 1995). It is possible that FGF, SoxB1 proteins and Oct/Pou proteins interact in an auto-regulatory loop in neural stem cell progenitor maintenance. Maternally expressed Sox3 proteins (Penzel, Oschwald et al. 1997) could activate expression of zygotic sox3 with Oct/Pou as a partner in the absence of FGF while FGF signaling is required for the activation of sox2 expression by Sox3. The FGF signaling may be required only to inhibit BMP signaling or to make the tissue competent of sox2 expression. Once Sox2 proteins are present, they then interact with Oct/Pou proteins to activate expression of fgf leading to neural progenitor maintenance. Recent experiments in zebrafish showed that inhibition of FGF signaling by SU5402 led to a loss of sox2 expression in the neuroectoderm, however, overexpression of sox3 mRNA rescued the loss supporting a requirement for FGF in the inhibition of BMP signaling. Exogenous Sox3 may have inhibited BMP sufficiently to allow for the expression of sox2, overriding the requirement for FGF. The studies in this chapter showed that (1) regulation of early neural genes is variable and requires FGF signaling for the expression of sox2 and that (2) all genes studied require FGF signaling for maintenance of expression and (3) sox2, sox3 and geminin are regulated similarly in explants as they are in whole embryos.
The sox3 regulatory region responds to knock down of FGF signaling

To identify cis-regulatory elements necessary for the response of a sox3 reporter construct to FGF signaling, we focused on quantitative and qualitative analysis of the sox3 regulatory region. These experiments suggest that the sox3 regulatory region can respond to FGF signaling and that an effector of FGF signaling binds to the sox3 regulatory region and this interaction is required for expression to be maintained in neurula embryos (Fig. 3.5B). Unlike endogenous sox3, sox3-luciferase expression does not respond to exogenous Noggin. Since there has been an in depth analysis of the sox2 regulatory region in chick showing that there are multiple enhancers responsible for region specific expression of sox2, and that the N-1 region specifically responds to FGF signaling (Uchikawa, Ishida et al. 2003; Takemoto, Uchikawa et al. 2006). These regions are conserved in the frog, so it is not completely surprising that sox2 requires FGF signaling for induction and maintenance (Fig. 3.1C, 3.2, 3.3A).

Reporter constructs containing a 1.5 kb Xenopus laevis (sox3-Luciferase or sox3-GFP) or a 1.35 kb Xenopus tropicalis sox3 (Xtsox3-Luciferase) 5’ regulatory region fragment mimics the expression pattern of endogenous sox3 in transgenic embryos, with some differences (Rogers, Archer et al. 2008). For example, our Xenopus laevis sox3-luciferase construct did not consistently respond to noggin-overexpression in whole embryos or explants (data not shown). We tested the response of a Xenopus tropicalis 1.3 kb sox3-luciferase in whole embryos to BMP inhibition and found that Noggin did not rescue the loss of Luciferase expression caused by overexpression of XFD or Δ4a.
Additionally, *Sox3*-luciferase expression is reduced in response to a decrease in FGF signaling in early stage gastrulae in contrast to endogenous *sox3* indicating that although the 1.5 kb regulatory fragment mimics *sox3* expression (Rogers, Archer et al. 2008), there are still missing elements that confer regulatory information. Our reporter construct is driven by a relatively short segment of DNA (1.5 kb), thus there may be other regulatory elements that account for the differential response to FGF signaling when comparing the reporter and endogenous *sox3*.

Identifying these elements will allow us to determine how *sox2* and *sox3* are regulated differently in neural induction and development since the chicken *sox2* neural enhancer has been studied at length (Uchikawa, Ishida et al. 2003; Takemoto, Uchikawa et al. 2006). Because Sox3 is involved in the maintenance of neural progenitors and FGF signaling is implicated in the maintenance of the stem cell fate and CNS patterning, identifying the FGF response element in the *sox3* regulatory region may begin to illuminate how neural stem cell populations are maintained in the developing and adult CNS where *sox2* and *sox3* are expressed.

To identify the actual FGF response element in the *sox3* regulatory region, GFP in transgenic embryos containing *sox3*-GFP was compared to transgenic embryos expressing a construct with 250 bp deleted from the 5’ end (Δ250*sox3*-GFP). There is a putative Elk site in the deleted upstream region that is 85% similar to a published Elk-1 site (Transfac, Fig. 3.4)(Shore, Bisset et al. 1995). The Δ250*sox3*-GFP is expressed at gastrula stages but this expression is lost at neurula and tailbud stages indicating that this is the region that responds to FGF signaling and is required for the maintenance of *sox3*.
expression in neural tissue.

In summary, this study has shown that FGF signaling through FGFR-1 and 4a is required for the induction of *sox2* and signaling through FGFR1 and 4a is required for the maintenance of *sox3* and *geminin* in the absence of BMP inhibition. Additionally, we identified a region of the *sox3*-regulatory region that responds to loss of FGF and is required for the maintenance of expression. These data indicate that early neural genes do not have a neurogenic code; however the same signaling pathways are involved in the regulation of many of these genes at different times in development.

**Future experiments**

It is possible that Wnt signaling was not knocked down completely in our experiments (Fig. 3.1A-C). Use of additional Wnt inhibitors like Dkk1 (Lin and Slack 2008), Gsk3-β (Lin and Slack 2008), or a combination of multiple inhibitors may benefit this experiment. To confirm that Wnt signaling is knocked down by our treatments, we can use a TOP-FLASH assay (Molenaar, van de Wetering et al. 1996) which would allow us to determine if canonical Wnt signaling is occurring by measuring luciferase driven by the TCF regulatory region.

Although our experiments indicate that overexpression of high concentrations of XFD (Fig. 3.2) inhibit the induction of *sox2* by Noggin, and that low concentrations have no effect on this induction, the assays used to analyze loss of FGF signaling were different. Two different assays were utilized to test for *sox2* expression, one being more sensitive than the other (RT-PCR versus WISH). Although there is little question about
the requirement of FGF for the maintenance of sox2, sox3 and geminin after induction by BMP inhibition in explants, quantitative PCR may be necessary to test for the induction of these genes at early stages.

Finally, although our transgenic analysis suggests that there is an FGF response element in the 5’ end of the sox3 regulatory region required for maintenance of expression, we will utilize site directed mutagenesis to identify and confirm the exact site of the FGF response element in the sox3 regulatory region.
Figure 3.1. FGF signaling is not required for induction of *sox2* or *sox3* by *noggin* but is required for the maintenance of *sox3*. WISH for either *sox3* (A, B) or *sox2* (C) in ectodermal explants dissected from uninjected (UI) embryos or embryos injected with *noggin* and/or Δ*TCF*, Δ*Xfz* or *XFD* mRNA as labeled. Explants were collected when sibling embryos were stages 11, 12.5, and 17.
Figure 3.2. FGF signaling is required to maintain expression of *sox2*, *sox3* and *geminin* after induction by BMP inhibition. RT-PCR of ectodermal explants from embryos injected with *noggin* (*nog*) with or without *XFD, Δ4a* or *xfd+Δ4a* mRNA. RT-PCR was used to assay for *sox2*, *sox3*, *geminin* and *ef1a* in stage 11.5 and 17 explants. UI is uninjected, WE is whole embryo and bottom row is RT minus using *ef1a* primers.
Figure 3.3. FGF signaling through FGFR-4a is required for sox2 induction and maintenance of sox3 and geminin expression. WISH for (A) sox2, (B) sox3 and (C) geminin. Embryos were injected in 1 of 2-cells with noggin (nog), with or without Δ4a, or nog + Δ4a and lacZ mRNA and collected at stages 10.5, 11.5 and 12.5. UI is uninjected. All embryos injected on left side as indicated by light blue staining.
Figure 3.4. A 250 base pair fragment of the *Xlsox3* regulatory region is required for maintenance of expression. WISH of transgenic embryos expressing the 1.5 kb *sox3*-GFP or the 1.25 kb Δ250 *sox3*-GFP reporter constructs. Embryos in left row are stage 12 (posterior view) others are stage 20 with center a dorsal view and right a lateral view. Bottom diagram indicates the full length and Δ250 *sox3*-GFP constructs. The red circle indicates putative FGF response element (Elk-1 site) located at -1487 from the transcription start site and the green hexagon indicates region required for early expression.
Figure 3.5. Sox3-luciferase levels are reduced by dominant negative forms of FGFR1/2 and 4a. (A) Luciferase assays of ectodermal explants or whole embryos injected with sox3-luciferase with or without xfd, Δ4a or xfd + Δ4a. Embryos and explants were collected at stages 12.5 and 17. (B) Luciferase assays of ectodermal explants or embryos injected with Xtosox3-luciferase with noggin, xfd, Δ4a or xfd + Δ4a. Embryos were collected at stage 17. All luciferase assays were performed in triplicate and a minimum of 30 embryos or explants was used for each treatment. Error bars are standard deviations.
CHAPTER IV: SOX3 INDUCES GEMININ AND SOX2 EXPRESSION DIRECTLY AND
REPRESSES EPIDERMAL FORMATION VIA XVENT2 INHIBITION

Some data shown in this chapter were previously published in: C.D. Rogers, N. Harafuji, T. Archer, D.D. Cunningham, and E.S. Casey. *Xenopus* Sox3 activates sox2 and geminin and indirectly represses Xvent2 expression to induce neural progenitor formation at the expense of non-neural ectodermal derivatives. Mech Dev. 2008.
Introduction

The development of the central nervous system (CNS) commences when ectodermal cells are specified to become neural precursors (Harland 1994; Chitnis and Kintner 1995; Hemmati-Brivanlou and Melton 1997). These proliferating neural precursor cells will differentiate into all of the different cell types in the nervous system (Edlund and Jessell 1999). Since a small pool of progenitors will make up the entirety of the CNS, progenitor maintenance is necessary for the normal development of the nervous system (Holmberg, Hansson et al. 2008). Evidence indicates that the SoxB1 proteins are responsible for the maintenance of this progenitor cell population in the neural tube (Wegner 1999; Pevny and Placzek 2005; Wegner and Stolt 2005). Thus far, the functions of the SoxB1 proteins have only been partially characterized and none of their direct targets in neurogenesis has been identified.

Sox proteins are members of the Sry-related high mobility group (HMG) box family (Wegner 1999; Wegner and Stolt 2005; Guth and Wegner 2008). Although the 10 Sox protein families play various roles in development, SoxD and the SoxB, C and E families are involved in the specification and development of the central and peripheral nervous systems (Wegner and Stolt 2005). This study focuses on the SoxB1 proteins, Sox2 and 3, which are expressed throughout the nervous system in proliferating neural progenitors (Collignon, Sockanathan et al. 1996; Uchikawa, Kamachi et al. 1999; Wood and Episkopou 1999; Pevny and Placzek 2005). As development proceeds and the neural progenitor cells begin to differentiate, expression of the soxB1 genes is down regulated to allow for neuronal differentiation (Aubert, Stavridis et al. 2003; Bylund, Andersson et
al. 2003; Graham, Khudyakov et al. 2003; Ying, Stavridis et al. 2003).

The SoxB1 proteins are thought to be redundant due to: their overlapping expression in the developing nervous system, the lack of prominent CNS abnormalities in Sox2 or 3 mutant mice (Malas, Postlethwaite et al. 2003; Taranova, Magness et al. 2006), the ability of Sox1, 2 and 3 to bind to the same DNA sequence (Kamachi, Cheah et al. 1999; Graham, Khudyakov et al. 2003), and the compensation by Sox3 in embryos expressing a dominant repressor form of Sox2 (Miyagi, Masui et al. 2008). Also, overexpression of Sox1 can rescue the loss of progenitor cells caused by inhibition of Sox2 (Graham, Khudyakov et al. 2003). However, at the beginning of neural induction the three Xenopus soxB1 genes have different expression patterns indicating that they also have unique roles at this time. Sox3 is maternally expressed while sox2 and sox1 are not expressed until the beginning and end of gastrulation, respectively (Pevny and Lovell-Badge 1997; Nitta, Takahashi et al. 2006; Rogers, Archer et al. 2008). Studies in chick demonstrate that Sox2 and 3 have the same role in maintaining neural progenitors and preventing neuronal differentiation (Bylund, Andersson et al. 2003; Graham, Khudyakov et al. 2003).

Studies also show that Sox2 is involved in patterning the brain (Lupo, Harris et al. 2002; Ferri, Cavallaro et al. 2004; Miyagi, Masui et al. 2008), the development of the lens and retina (Kamachi, Uchikawa et al. 1998; Kamachi, Uchikawa et al. 2001; Taranova, Magness et al. 2006; Donner, Episkopou et al. 2007), and the development of the inner ear (Uchikawa, Kamachi et al. 1999; Kiernan, Pelling et al. 2005). Sox3 regulates development of the cranial placodes (Kamachi, Uchikawa et al. 1998;
Schlosser and Ahrens 2004; Schlosser, Awtry et al. 2008), pituitary gland (Rizzoti, Brunelli et al. 2004), pharyngeal pouches, and craniofacial cartilage (Rizzoti and Lovell-Badge 2007; Rogers, Harafuji et al. 2008). In early development, Sox2 is required for neural specification in *Xenopus* (Kishi, Mizuseki et al. 2000), the individual function of Sox3 in early neurogenesis is yet unknown.

In this study we use gain and loss of function studies to analyze the roles of Sox2 and Sox3 in early neural development and find that Sox2 and Sox3 expand the neural tube and delay neuronal differentiation at the expense of non-neural ectodermal derivatives. We identify the first direct targets of Sox3 by demonstrating that Sox3 can directly activate expression of *sox2* and *geminin*, two markers of neural progenitors. Although Sox2 can also activate *geminin* expression, it cannot activate *sox3* expression indicating that it may act downstream of Sox3 in neural development. Additionally, we show that Sox3 represses the development of non-neural ectodermal derivatives by repressing expression of the BMP target, *Xvent2*, and delaying the specification of neural crest cells.
Materials and Methods

Embryo culturing and manipulations

As in Chapter II.

Plasmid construction

Sox3ΔC-Engrailed was constructed in the same manner as described (Zhang, Basta et al. 2003). Amino acids 1-290 of the Sox3 protein was amplified via PCR adding XhoI and ClaI sites to the 5’ and 3’ ends of the gene, respectively, and deleting the last 20 amino acids. This fragment was then fused in frame to a fragment encoding amino acids 2–298 of the Drosophila engrailed protein (Conlon, Sedgwick et al. 1996) and the resulting Sox3ΔC-Engrailed was subcloned into the pCS2+ expression vector.

Preparation of RNAs and microinjection

Synthetic capped mRNAs were made by in vitro transcription using mMessage mMachine (Ambion). Sox3 (#419), sox3-VP16 (#421) (Zhang, Basta et al. 2003), sox3-GR (#382) (Kishi, Mizuseki et al. 2000), sox2 (#RH1104) (Mizuseki, Kishi et al. 1998), or sox2-GR (#381) (Kishi, Mizuseki et al. 2000) mRNA was injected as described in the figure legends.

WISH and β-galactosidase assay

Whole mount in situ hybridization (WISH) was performed as in Chapter II. We used the following genes as in situ probes: sox2 (#RH1104) (Mizuseki, Kishi et al. 1998), geminin (#368) (Kroll, Salic et al. 1998), zic1 (RH1106) (Mizuseki, Kishi et al. 1998), soxD (#263) (Mizuseki, Kishi et al. 1998), ngnr-1 (#326) (Ma, Kintner et al. 1996), n-

**Luciferase assay**

Luciferase assays were performed as described (Casey, Tada et al. 1999) using the Dual Luciferase Reporter Assay System (Promega). *Xenopus* embryos were injected with 50 pg BRE (1243/-191)x4-luc (BRE-*luciferase*, #174) (Hata, Seoane et al. 2000). Inhibition by Sox3 or Sox2 was tested by injecting 400 pg *sox* mRNA with pRL-CMV and BRE-*luciferase*. For a control, either 400 pg *sox11* (#222) (Hiraoka, Komatsu et al. 1997) or 50 pg of constitutively active BMP-2/4 type I receptor (CABR, #334) (Candia, Watabe et al. 1997) was co-injected. Pools of ten stage 11 embryos were collected in triplicate for each injection mixture. Embryos were mixed with 1 ml of 1x Passive Lysis Buffer and 10µl or 20µl of this supernatant was assayed. Experiments were repeated at least twice and showed similar trends.

**Clearing and cartilage staining**

Cartilage staining was performed as described (Bellmeyer, Krase et al. 2003) with the following modifications: tadpoles were fixed in 1X MEMFA salts and 3.7%
formaldehyde, stained for 3-days with 0.4% alcian blue staining solution and washed and cleared in 2% KOH.

**Cycloheximide experiments**

Embryos were injected with 400 pg of *sox3-GR* (#382) or *sox2-GR* (#381) mRNA. Those analyzed for *sox2* and *Xvent2* expression were incubated in 10 μg/ml cycloheximide at stage 9 and induced with 1μM Dexamethasone (Dex) 30 minutes later. Embryos analyzed for *geminin* and *sox3* were incubated in cycloheximide at stage 11 and induced with Dex 30 minutes later. All embryos were collected at stages 12 and 12.5 and analyzed for β-galactosidase activity and gene expression by WISH. Due to the ubiquitous expression of *sox3* and *geminin* until mid-gastrula stage, (Fig. 4.2 C, D), embryos were treated with CHX at stage 11 to allow for the restriction of their expression to the dorsal side.

**RT-PCR**

Semi-quantitative RT-PCR was performed as described (Wilson and Hemmati-Brivanlou, 1995). Total RNA was extracted from 10-20 whole embryos and cDNA was generated using random hexamer primers. Primers are: *ef1α* (#86, #87, XMMR), *luciferase* (#275, F: CTGTTTCTGAGGAGCCTTCAGG, #276, R: GTAATCAGAATAGCTGATGTAGTTCT, 61° annealing, 28 cycles), *odc* (#118, #119, XMMR) and *geminin* (#28, #29, XMMR).
Results

Sox2 and Sox3 induce neural progenitors and delay neuronal differentiation

Recent experiments showed that maternal Sox3 plays a role in axis determination; sox3 mRNA injected into the equatorial region of dorsal cells, directly inhibited expression of Xenopus nodal (Xnr5) thereby ventralizing the embryo (Zhang et al., 2003). To define the function of Sox2 and Sox3 during early neural development, N. Harafuji injected sox3 mRNA into the animal pole of 1 of 2-cells and analyzed the expression of the early neural genes sox2, geminin, soxD and zic1 which, like sox3, are known to be induced in response to BMP antagonists. Both Sox3 and Sox2 expanded the neural plate as indicated by expanded expression of sox2 (n=42/71, 59%), geminin (n=37/44, 84%), soxD (n = 28/28, 100%) and zic1 (n=126/155, 81.3%) (Fig.4.1A). To verify that the expansion of neural genes by Sox3 is not due to the disruption of the dorsal-ventral axis prior to zygotic transcription, I injected a hormone inducible sox3-GR and induced at stage 9. In the absence of Dex, Sox3-GR had no effect on sox2, geminin or soxD expression in neurula embryos (data not shown). However, in the presence of Dex, sox2 (n= 22/30, 73%), geminin (n= 31/35, 89%), and soxD (n= 12/17, 71%) expression was expanded (Fig. 4.1B). Work done by N. Harafuji demonstrated that Sox3 increased proliferation in neurula embryos as shown by an increase in the level of phosphorylated histone H3 (Rogers, Harafuji et al. 2008). Together, these results indicate that Sox3 expands the neural plate by increasing expression of progenitor markers and by increasing proliferation.
To determine if overexpression of sox3 expanded neural progenitors at the expense of neuronal differentiation, embryos were assayed for the expression of neuronal (n-tubulin) and proneural (ngnr-1) markers at stage 14, the onset of differentiation (Bellefroid, Bourguignon et al. 1996), and at stage 23 during patterning of the CNS. Overexpression of sox3 and sox2 inhibited neurogenesis as marked by a decrease in n-tubulin (n= 88/92) and ngnr-1 (n= 30/32) (Fig. 4.1C, sox3-overexpression experiment performed by T.C. Archer). By stage 23, the embryos had ectopic neurons indicating that the expanded neural progenitor cells eventually differentiated (Fig. 4.1C, n-tubulin: n= 109/125, ngnr-1: n= 95/126). At this stage, only Sox3 embryos had ectopic expression of ngnr-1, and both Sox2 and Sox3 inhibited the neurogenesis of placodes at both stages (Fig. 4.1C, Sox3, n-tubulin: n= 109/125, ngnr-1: n= 95/126; Sox2, n-tubulin: n= 21/53, ngnr-1: n=31/53). In conclusion, overexpression of both sox2 and sox3 expands the neural plate by expanding neural progenitors at the expense of neuronal differentiation.

**Sox3 directly activates geminin and sox2 expression**

Sox1 and Sox2 proteins function as transcriptional activators to drive expression of the chicken δ-crystallin DC5 enhancer (Kamachi, Cheah et al. 1999) and Sox2 and Sox3 function as activators to repress neuronal differentiation in the chick neural tube (Bylund, Andersson et al. 2003). However, the dominant repressor form Sox3-EnR, phenocopies the repression of Xnr5 by overexpression of wildtype Sox3 demonstrating
that Sox3 can function as a repressor to direct axis formation (Zhang, Basta et al. 2003). To determine the transcriptional activity of Sox3 in early neural development, we overexpressed mRNA coding for Sox3, Sox3-VP16, a dominant active protein, or Sox3-EnR, into the animal pole of 1-cell embryos and assayed for the expression of geminin and sox2 in gastrula embryos. Sox3 and Sox3-VP16 expanded expression of sox2 (n=62/67, n= 33/36) and geminin (n=93/108, n= 40/44) (arrows, Fig. 4.2A). In contrast, sox2 expression was repressed in gastrula embryos (n= 33/37, Fig. 4.2A’) and geminin was induced by Sox3-EnR (n= 38/40, Fig. 4.2A). Therefore, these results suggest that Sox3 can function as an activator to induce early neural genes but may also indirectly activate select neural genes such as geminin by functioning as a repressor.

There are no direct targets identified for Sox2 or Sox3 in early neural development in Xenopus. To determine if the induction of geminin by Sox2 and Sox3 was direct or indirect both Sox2-GR and Sox3-GR were overexpressed at the 1-cell stage and induced with Dex at the mid-blastula transition (MBT) in the presence or absence of the protein synthesis inhibitor cycloheximide (CHX; Fig. 4.2C, D). When incubated in Dex, Sox3-GR induced expression of sox2 and geminin in the absence of protein synthesis indicating that Sox3 directly activated the expression of these genes (Fig. 4.2C). In contrast, Sox2-GR did not induce expression of sox3 in the presence or absence of protein synthesis (Fig. 4.2D) but activated expression of geminin (Fig. 4.2D). These experiments show that Sox3 directly activates both geminin and sox2, and Sox2 can also directly activate geminin expression but not that of sox3.
**Sox3 inhibits epidermis development via repression of the BMP signaling cascade**

We demonstrated that overexpression of Sox3 delays neurogenesis (Fig. 4.1C) and increases cell proliferation (data not shown) leading to an expanded neural plate. To determine if that expansion is also due in part to a fate conversion of non-neural ectodermal cells to a neural fate, we analyzed the expression pattern of genes expressed in the developing epidermis, *bmp4*, *Xvent2*, and *epi-keratin* (Fig. 4.3A) in embryos injected with *sox3*, *sox3*-VP16 or *sox3*-EnR mRNA. The expression of all three genes was reduced by overexpression of *sox3* (*bmp4*: n= 64/69, *Xvent2*: n= 52/52, *epi-keratin*: n= 26/28) or *sox3*-VP16 (*bmp4*: n= 35/45; *Xvent2*: n= 50/50, *epi-keratin*: n=13/13) in gastrula (st. 12, *bmp, Xvent2*) and neurula (st. 17, *epi-keratin*) embryos and was unaffected by overexpression of *sox3*-EnR demonstrating that Sox3 functions as an activator to inhibit expression of *bmp4* and *Xvent2* and epidermis formation (Fig. 4.3A).

There is a positive feedback loop in BMP signaling in which BMP activates *Xvent2* expression and XVent2 in turn activates *bmp4* transcription (Ladher, Mohun et al. 1996; Onichtchouk, Gawantka et al. 1996). N. Harafuji performed luciferase experiments testing the effect of *sox3* overexpression on the activity of a BMP response element (BRE)-luciferase construct (Hata, Seoane et al. 2000) which contains four copies of the 152 bp BRE from the *Xvent2* regulatory region. She showed that, Sox3 represses *Xvent2* expression even in the presence of a constitutively active BMP receptor (data not shown). To confirm that Sox3 repressed *Xvent2* as an activator, BRE-luciferase assays were performed in the presence of Sox3, Sox3-VP16 and Sox3-EnR (Fig. 4.3B,
lanes 1-5). Both Sox3 and Sox3-VP16 repressed BRE activity and although the trend for Sox3-EnR was to repress BRE-luciferase, it was not statistically significant.

Since Sox2 (data not shown, N. Harafuji) and Sox3 repressed expression of BRE-luciferase and Sox3-VP16 repressed Xvent2, we tested whether Sox2 and Sox3 directly repressed Xvent2 expression by overexpressing hormone inducible forms of each protein in the presence or absence of Dex and CHX (Fig. 4.3C). As expected, both Sox2-GR (n= 31/31) and Sox3-GR (n= 46/50) repressed Xvent2 in the presence of Dex, but surprisingly, both proteins also repressed Xvent2 in the absence of protein synthesis (Figure 4.3C, Dex+CHX: Sox3-GR, n= 59/63; Sox2-GR, n= 26/26). This result was supported by a second experiment in which we injected BRE-luciferase with or without Sox3-GR and assayed for luciferase expression by RT-PCR. Even though there are no conserved Sox binding sites in the BRE sequence (Fig. 4.3D), Sox3 and Sox3-GR induced by Dex repressed expression of luciferase. Since luciferase expression was repressed when the embryos were treated with CHX it was unclear whether Sox3 can inhibit BRE-luciferase in the absence of protein synthesis (Fig. 4.3D). These data suggest that Sox3 is functioning as an activator to inhibit epidermal formation via inhibition of Xvent2.

**Sox3 inhibits neural crest cell development**

In addition to an expanded neural plate (Fig. 4.1A), sox3-overexpression caused two obvious phenotypes: a concentration of pigment at all stages tested (Fig. 4.4A) and a kinked head in tadpoles (Fig. 4.4D and data not shown, N. Harafuji). To
determine if the excess pigment was caused by the induction of ectopic cement gland cells, we injected Sox3-GR into one blastomere of a 2-cell embryo and performed WISH for Xag-1, a marker of the cement gland. Although the expression of Xag-1 was expanded laterally, the pigment cells in the injected embryos were not Xag-1 positive (Fig. 4.4A). These data suggest that the excess pigment induced by Sox3 is not due to premature differentiation into cement gland cells.

Next, to determine if the pigment was due to the premature differentiation of neural crest cells into melanocytes, we tested the effect of sox3-overexpression on markers of neural crest specification (slug, sox9, and sox10) and progenitor maintenance and melanocyte formation (sox10) (Aoki, Saint-Germain et al. 2003; Kim, Lo et al. 2003). The neural crest markers were not expressed in the pigmented cells, but rather, were inhibited in the injected cells of early neurulae (Fig. 4.4B, and data not shown). Expression of slug, sox9 and sox10 was reduced at the beginning of neural crest migration in early neurula and at the onset of neural crest cell differentiation in early tailbud embryos (Fig. 4.4B, top row); however, by stage 23, slug, sox9, sox10, and hairyA2 were later expanded and disorganized (stage 23; Fig. 4.4B, bottom row, and data not shown). Thus, Sox3-overexpression expanded the neural plate at the expense of the induction of neural crest cells.

To analyze the effect of delayed specification on neural crest migration, embryos were injected with Sox3-GR, induced at stage 9.5 and then assayed for Xtwist, a marker of neural crest migration. Even though anterior migrating neural crest streams were unaffected in the majority of embryos (Fig. 4.4C), the pattern of Xtwist expression was
disorganized and did not extend as far ventrally as controls (Fig. 4.4B, C) specifically in the second, third and fourth neural crest streams that give rise to the branchial cartilage (Schuff et al., 2007). In embryos overexpressing sox3-GR that were uninduced (-Dex) or induced at stage 17, the neural crest cells developed and migrated no differently than the uninjected controls indicating that there is a specific stage by which neural crest cells are specified and can develop normally. Sox3-GR affected neural crest migration only if induced prior to neurula stage (Fig. 4.4C). The delay in neural crest specification and inhibition of migration caused by Sox3 indicates that neural crest cells must be induced at the correct stage in development for normal migration and differentiation.

To investigate the effect that the inhibition of migration had on neural crest differentiation, N. Harafuji analyzed the sox3 and sox3-VP16-overexpression phenotypes in late tailbud and tadpole embryos. By stage 32 the embryos had a kinked head phenotype which was more severe in Sox3-VP16 injected embryos. To confirm that the phenotype was specific to sox3 and sox3-VP16 embryos, I performed the same experiment using Sox3-EnR and the craniofacial defects were absent (Fig. 4.4D, n= 6/8). Alcian blue staining of cartilage revealed that the ceratobranchials, neural crest derivatives (Sadaghiani and Thiebaud, 1987), were severely reduced only in sox3 (n = 10/15) and sox3-VP16 (n = 3/5) injected embryos (Fig. 4.4D). Therefore, overexpression of Sox3 delays neural crest development resulting in reduced migration and increased cell death (data not shown, D. Cunningham) which leads to a loss of craniofacial cartilage.
Discussion

SoxB1 genes are expressed prior to and at the onset of neural induction and have been proposed to be involved in neural progenitor maintenance (Bylund, Andersson et al. 2003; Graham, Khudyakov et al. 2003). Loss of SoxB1 proteins leads to a host of neural developmental abnormalities (Kamachi, Uchikawa et al. 1998; Nishiguchi, Wood et al. 1998; Rizzoti, Brunelli et al. 2004; Taranova, Magness et al. 2006). These are less severe than expected based on their expression throughout the CNS suggesting that they have redundant functions in neural development. To date, no direct targets of Sox2 or Sox3 in early neural development have been identified. This study has shown that both Sox2 and Sox3 expand neural tissue at the expense of non-neural ectoderm and neuronal differentiation, and they appear to have different effects on expression of ngnr-1 at tailbud stage. Sox2 and Sox3 activate expression of geminin directly, yet while Sox3 can activate sox2 expression in the absence of protein synthesis, Sox2 cannot activate sox3 expression. Furthermore, even though maternal Sox3 functions as a repressor in dorsal-ventral axis specification prior to MBT (Zhang, Basta et al. 2003), zygotic Sox3 functions as an activator during neurogenesis. Of note, Sox3 and Sox3-VP16 repress BMP signaling via Xvent2 expression even in the absence of protein synthesis and inhibit neural crest induction (Fig. 4.5). These data suggest that levels and timing of the SoxB1 proteins are important and can override the non-neural ectodermal program.

Sox2 and Sox3 induce neural progenitors and delay neuronal differentiation

This study analyzed the role that the SoxB1 proteins play in early neural
development. There is little known about the roles of Sox2 and Sox3 prior to neuronal differentiation. Previous studies in frog demonstrated that Sox2 is required for neural specification in ectoderm (Kishi, Mizuseki et al. 2000), and in zebrafish Sox3 expands the neural tube at the expense of epidermis (Dee, Hirst et al. 2008). There are more studies which identify the role that Sox2 and Sox3 play in maintenance of neural progenitors, the specification of sensory placodes, and in the patterning of the CNS (Kamachi, Uchikawa et al. 1998; Bylund, Andersson et al. 2003; Donner, Episkopou et al. 2007; Schlosser, Awtry et al. 2008). Overexpression of sox3 and sox2 expanded the neural plate as marked by the expression of early neural markers n-cam, geminin, zic1, and soxD (Fig. 4.1A). This neural tube expansion was due in part to increased proliferation caused by the expanded neural progenitor pool marked by sox2 and geminin expression (demonstrated by N. Harafuji). The expanded neural tube also occurred when Sox3-GR was induced after the mid blastula transition (Fig. 4.1B) indicating that it was independent of the role of Sox3 in dorsal-ventral axis formation (Zhang, Basta et al. 2003). Concordantly, Sox2 and Sox3 inhibited neuronal markers during primary neurogenesis as was previously shown in the chick spinal cord (Bylund, Andersson et al. 2003; Graham, Khudyakov et al. 2003). Eventually, both ngnr-1 and n-tubulin were expressed indicating that when Sox3 levels drop, the progenitors upregulate bHLHs and differentiate into neurons. Sox3 function is similar to Geminin, a maternally expressed neural protein that counteracts Brg-1 to keep progenitor cells in the cell cycle (Seo, Herr et al. 2005). Like Sox3, overexpression of Geminin expanded neural tissue at the expense of epidermal formation, neuralized ectodermal explants as marked by ngnr-
expression (Kroll, Salic et al. 1998). In contrast, Sox2 induced ectopic *n-tubulin* positive neurons but did not induce *ngnr-1*. Therefore, it is possible that the SoxB1 proteins act through unique bHLHs to inhibit neuronal differentiation, and that Sox2 represses expression of *Xath5* or *neuroD* to prevent exit from the cell cycle while Sox3 represses *ngnr-1* (Logan, Steele et al. 2005; Seo, Lim et al. 2007).

**Sox3 directly activates *geminin* and *sox2* expression**

Use of dominant repressor and dominant activator forms of Sox3, demonstrated that it performs as an activator to expand the neural tube. *Sox3-VP16* mimicked Sox3 and expanded or induced *sox2* and *geminin* expression (Fig. 4.2A). Unexpectedly, even though Sox3-EnR repressed the expression of *sox2*, it induced expression of *geminin* ectopically (Fig. 4.2A, A’). This may be explained in two ways. One possibility is that induction of *geminin* by Sox3 does not require Sox3 to bind to DNA and activate or repress transcription. Since *geminin* is repressed by XVent1 and XVent2 (Taylor, Wang et al. 2006), Sox3 may be interfering with Smad activation of BMP targets and lifting the repression of *geminin* indirectly. A second possibility is that Geminin and Sox3 act redundantly in neural development such that when Sox3 activity is reduced, Geminin expression is increased. If this is the case, knocking down the function of Sox3 via dominant repressor may induce *geminin* expression to rescue the loss of neural progenitors caused by the loss of both *sox2* expression (Fig. 4.2A’) and Sox3 function. Maintaining a neural progenitor population is necessary for the proper development of the CNS and the SoxB1 proteins are involved in this process (Bylund, Andersson et al.
Although *geminin* is not a SoxB1 protein, *sox3* and *geminin* are both maternally expressed genes that are ubiquitous and then restricted to the dorsal ectoderm by the XVent proteins (Taylor, Wang et al. 2006; Rogers, Archer et al. 2008), and they have similar overexpression phenotypes (Kroll, Salic et al. 1998). It is possible that they act in the same or a parallel pathway to maintain neural progenitors.

Using a hormone inducible Sox3-GR, we demonstrated that Sox3 directly activates both *geminin* and *sox2* expression in gastrula embryos and Sox2 activated *geminin* directly. These data support a model in which maternal Sox3 activates zygotic *sox3* which then activates expression of *sox2* and *geminin*. Sox2 is also able to activate *geminin* expression. Geminin, which is required for the specification of neuroectoderm, activates expression of *Sox2* in chick (Papanayotou, Mey et al. 2008), and is required for the induction of *sox3* and other early neural genes. The data in this study suggest that Geminin and Sox3 may have redundant functions in neurogenesis.

**Sox3 inhibits epidermis development via repression of the BMP signaling cascade**

Sox3 and Sox3-VP16 repressed epidermal development as marked by loss of *Xvent2*, *bmp*, and *epi-keratin* expression. Although Sox3-EnR repressed BRE-luciferase activity in most experiments, it was not statistically significant (Fig. 4.3B) and there was no noticeable effect on *Xvent2* expression as analyzed by WISH. Unexpectedly, Sox3-GR repressed *Xvent2* expression in the absence of protein synthesis and decreased
luciferase activity driven by a BMP-response element that does not contain a consensus Sox binding element. Furthermore, Sox3 repressed BRE-luciferase gene expression in the absence of protein synthesis (Fig. 4.3D). These experiments showed that Sox3 represses Xvent2 as an activator, indicating that it may not bind DNA to repress expression (Fig. 4.3A). Since CHX treatment repressed expression on its own, the decrease in Xvent2 expression in the presence of Sox3 was weak, but consistent between experiments (data not shown). Since there is no consensus Sox binding site in the BRE, one possibility is that Sox3 binds to and blocks the activity of proteins required for expression of Xvent2, such as Smad 1,5,8 the Smad cofactors p300/CREB binding protein (CBP) (Fujii, Tsuchiya et al. 1998; Pearson, Hunter et al. 1999; von Bubnoff and Cho 2001) or Olf-1/EBF associated zinc finger (OAZ) (Hata, Seoane et al. 2000). Inhibition of p300/CBP neuralizes embryos supporting a role for it in epidermal development and the inhibition of neural development (Kato, Shi et al. 1999). Sox proteins have been shown to interact with the p300 Smad cofactor in previous studies. Sox-2 and Oct-3 interact with p300/CBP to activate FGF4 expression in embryonic stem cells (Nowling, Johnson et al. 2000; Nowling, Bernadt et al. 2003; Bernadt, Nowling et al. 2004). Also, Sox9 interacts with p300/CBP, a histone acetyl transferase, to activate neural crest gene expression. As Sox9 binds to p300/CBP to hyper-acetylate histones and activate transcription (Furumatsu, Tsuda et al. 2005), Sox3 may also interact with p300/CBP to activate neural gene expression. However, although Sox2 and Sox9 interact with p300 to activate gene expression as a transcription complex (Furumatsu, Tsuda et al. 2005; Chen, Xu et al. 2008), a second possibility is that Sox3 interacts with
p300 to sequester the Smad cofactor away from the Smad complex, thereby repressing epidermal development. In support of this hypothesis, when Sox2 is either down regulated or overexpressed in stem cells, FGF4 expression is repressed due to the ability of Sox2 to interact with each of its cofactors individually without necessarily creating a complete transcription complex (Bernadt, Nowling et al. 2004). No Sox proteins have yet been shown to interact directly with Smads or OAZ.

**Sox3 inhibits neural crest cell development**

Sox3-overexpression was able to increase pigmentation in embryos as early as the gastrula stage and that pigment was maintained throughout development (Fig. 4.4A). The pigmented cells did not express a cement gland marker, Xag-1, nor a marker of melanocyte precursors, sox10 (Fig. 3A, B) (Aoki, Saint-Germain et al. 2003). The source of the pigment is yet unknown although sox3-overexpression looks similar to the pigment-inducing overexpression phenotypes of both zic1-4, which induces neural crest derived pigment, and shroom, which is involved in the localization of pigment granules in apical cells (Pevny and Lovell-Badge 1997; Nakata, Nagai et al. 1998; Fairbank, Lee et al. 2006; Rogers, Archer et al. 2008). Additional gain-of-function experiments need to be performed to determine the origin of these cells.

Although Sox3 repressed slug, sox9, and sox10 expression in gastrula, neurula and early tailbud embryos, by mid to late tailbud, all three were expressed in an expanded domain (Fig. 4.4B). Sox3-overexpression also expands expression of zic-1 as early as stage 10.5 (Fig. 4.1A and data not shown, N. Harafuji). Since zic1-
overexpression has been shown to induce pigmentation and *slug* expression (Mizuseki, Kishi et al. 1998; Nakata, Nagai et al. 1998), it is possible that the expansion of the neural crest is in response to increased *zic1* expression. It is also possible that the Sox3 protein and mRNA were degraded accounting for the expression of neural crest genes. The effect of Sox3 on neural crest was dependent on the timing of expression. When Sox3-GR was induced after stage 12.5, it had no effect on neural crest development (Fig. 4.4C). These data indicate that Sox3 inhibits the specification of neural crest, but once neural crest cells are specified, Sox3 has no affect on their migration or differentiation.

We showed that the cells expressing *slug*, *sox9*, and *sox10* at tailbud stage did not develop into neural crest derivatives as indicated by the lack of migration, increased apoptosis (data not shown, D.C. Cunningham) and eventual loss of branchial cartilage in tadpoles (Fig 4.4B- E). One possibility is that the cartilage defect is due only to cell death. In experiments using avian trunk neural crest cells, delayed induction or decreased migration of the neural crest lead to a higher density of premigratory cells which lead to increased cell death and therefore decreased neurogenesis (Vogel and Weston 1988; Vogel, Marusich et al. 1993; Maynard, Wakamatsu et al. 2000). The lack of craniofacial cartilage in *Xenopus* supports this hypothesis since our experiments show similar delay in specification and disorganized non-migrating neural crest cell “patches”. Another possibility is that there is inhibition of the epithelial-mesenchymal transition (EMT) of the *slug*-positive and *sox3*-positive cells which means that the neural crest cells are specified, but they do not delaminate from each other allowing for normal migration and differentiation. Studies in chick showed that misexpression of Sox2
decreases \textit{slug} expression in early embryos that later failed to undergo EMT (Wakamatsu, Endo et al. 2004). Normal neural crest development proceeds with the induction of \textit{slug} in neural crest progenitors and then the subsequent down regulation of cell adhesion molecules leading to EMT (reviewed in (Taneyhill 2008).

In summary, this research demonstrated that Sox3 delays neuronal differentiation, inhibits epidermis, and delays neural crest specification. Sox3 directly activates early neural genes \textit{sox2} and \textit{geminin}, which are required for neural development and inhibits epidermal development via inhibition of the BMP target, \textit{Xvent2}, in the absence of protein synthesis. Finally, Sox3 represses the formation of non-neural ectodermal derivatives including neural crest. Future experiments including identification of co-factors required for the activator and repressor functions of Sox3 will clarify the molecular steps required for neural development and neural stem cell formation.

\textbf{Future experiments}

These studies indicate that Sox3 directly inhibits \textit{Xvent2} but that it does so when in a dominant active form. To determine if Sox3 interacts with Smad proteins or their cofactor p300/CBP; first, co-immunoprecipitation assays can be used to determine whether Sox3 can bind to Smad or p300/CBP. If Sox3 binds to and inhibits Smad 1, 5, 8 function, additional Smad targets would be down regulated in Sox3 gain of function assays. Although Sox3-VP16 phenocopies overexpression of Sox3, in that it induces expression of neural genes and inhibits non-neural ectodermal derivatives, Sox3-EnR
induced expression of *geminin*. To reconcile the fact that a form of Sox3 that represses *sox2* expression and has little to no effect on epidermal genes, it would be necessary to determine if overexpression of p300/CBP or Smad1, 5, or 8 is sufficient to rescue the induction of *geminin* in the presence of Sox3-EnR. Future research may suggest that Sox3 can act as a direct transcriptional activator of neural genes with the ability to interact with proteins historically involved in BMP signaling.
Figure 4.1. Sox3 expands the expression of early neural markers and delays the expression of proneural and neuronal markers. (A) WISH of stage 17 neurulae injected with sox2 or sox3 mRNA for early neural markers as indicated in lower right corner. (B) WISH of stage 17 neurulae for early neural markers as indicated in lower right corner. Embryos injected into 1 of 2-cells and induced with Dex at stage 9. (C) WISH of stage 14 neurulae and stage 23 tailbud embryos for the pan-neuronal marker: n-tubulin and pro-neural marker: neurogenin r-1 (ngn). Embryos were injected with sox2 or sox3 (400 or 500 pg) and lacZ (100 pg) mRNA in 1 of 2-cells. Images are a dorsal view with
anterior to the bottom, shown with the injected side on the right marked by a black asterisk in the first panel.
Figure 4.2. Sox3 activates *geminin* and *sox2* expression in the absence of protein synthesis. (A) WISH of stage 12 embryos injected with *sox3*, *sox3*-VP16, or *sox3*-EnR mRNA at the 1-cell stage. Sox3 and Sox3-VP16 expand expression of *sox2* and *gem* (arrows) and Sox3-EnR induces *gem*. Images are ventral view with the anterior to the top. (A’) WISH for *sox2* of stage 11.5 embryos injected with *sox3* or *sox3*-EnR mRNA. Embryos are a dorsal view with anterior to the top. (B, C) WISH of stage 12 embryos injected with either Sox3-GR or Sox2-GR and incubated without or with Dex and CHX. Region in hatched rectangle is shown below whole embryo images. Sox3-GR induces expression of *sox2* (Dex, n = 22/30; Dex + CHX, n = 12/20) and *gem* (Dex, n = 31/35;
Dex + CHX, n = 46/54). Sox2-GR induces *gem* in the absence of protein synthesis (Dex, n = 25/43; Dex + CHX, n = 35/47).
Figure 4.3. Sox3 and Sox3-VP16 inhibit Xvent2 expression and epidermal formation. (A) WISH for bmp4, Xvent2, or epi-keratin in embryos injected with sox3, sox3-EnR or sox3-VP16 mRNA and lacZ mRNA into one of a 2-cells. Bmp4 and Xvent2 (st. 12) embryos are animal pole view with the dorsal side to the top and epi-k embryos (st. 17) are a lateral view with anterior to the left. (B) One-cell embryos were injected in the animal pole with BRE-luciferase and sox3, sox3-VP16, sox3-EnR or sox11 mRNA (lanes 1-5). The graph represents one experiment done in triplicate. Lines represent standard deviations. *p < 0.05 indicates values that differ significantly from lane 1, Student’s t-test. (C) WISH of stage 12 embryos injected with Sox3-GR or Sox2-GR into 1-cell embryos and incubated without or with Dex and CHX (D) RT-PCR with primers to luciferase, odc, (used as a loading control) and efla (for RT-minus samples). Embryos were injected with BRE-luciferase and sox3-GR mRNA and were incubated without or with Dex and CHX.
Figure 4.4. Sox3 interferes with neural crest formation and migration. (A)
Overexpression of sox3-GR induces ectopic pigment (129/181) and expands the cement gland but does not induce ectopic cement gland as indicated by WISH of Xag-1 (n = 49/77) in two right panels. (B) WISH of sox9 (n = 47/68), slug (n = 36/57), and sox10 (n = 46/56) in embryos injected with sox3-GR and lacZ mRNA and was induced at stage 9. Embryos are a dorsal view of stages 13–21 embryos with anterior to the top (top row). Asterisk in first panel marks injected side. After stage 21, expression is expanded (bottom row) sox9 (n = 33/38), slug (n = 50/52), sox10 (n = 50/50), but migration is inhibited (Xtwist: n = 96/130). Uninjected (UI) and injected (I) sides of same embryo shown. (C) WISH of Xtwist in embryos injected with sox3-GR and lacZ mRNA. Sox3-GR was induced with Dex at stage 9, 12.5 or 17. Neural crest cell migration was inhibited when Sox3-GR was induced at stages 9 (n= 23/54) and 12.5 (n= 21/34) but not at stage 17 (n= 27/35). Lateral view of stage 23 embryos with anterior to the left. Uninjected (UI) and injected (I) sides of same embryo shown. (D) Development of branchial cartilage is disrupted by Sox3 and Sox3-VP16. Embryos are injected in 1 of 2-cells with sox3 or sox3-VP16 mRNA, cultured until stage 48 and stained using Alcian blue for cartilage. Embryos are dorsal view with anterior to the top and cartilage is ventral view with anterior to the top.
Figure 4.5. Model of Sox3 function during neural development. When BMP signaling is inhibited in dorsal tissues, *sox3* is expressed (Fig. 2.7). Sox3 protein directly activates the expression of both *sox2* and *geminin* thereby inducing neural progenitors at the expense of proneural gene expression and neuronal differentiation. The induction of progenitors by Sox3 is at the expense of the formation of the non-neural ectodermal derivatives, epidermis and neural crest. Sox3 represses the formation of epidermis by repressing BMP target gene expression.
APPENDIX A: THE EVOLUTION OF SOXB1 FUNCTION AND REGULATION
Introduction

The central nervous system (CNS) is composed of the brain and spinal cord. Although the basic organization of the central nervous system in vertebrates is conserved, the pathways driving neural induction and neuronal differentiation vary between organisms, making it difficult to identify the ancestral molecular mechanism involved in neural development. It is unclear which network of neural inducing proteins was inherited from more primitive organisms and which are derived in vertebrates.

The process regulating neural induction in *Xenopus laevis* has yet to be completely characterized. In frog, inhibition of BMP signaling has been shown to be sufficient to induce neural tissue from ectoderm (Hawley, Wunnenberg-Stapleton et al. 1995; Wilson and Hemmati-Brivanlou 1995; Uchikawa, Kamachi et al. 1999). Although many studies showed that FGF and Wnt signaling are involved in neural induction in vertebrates, their main role may be to decrease BMP signaling (Hawley, Wunnenberg-Stapleton et al. 1995; Wilson and Hemmati-Brivanlou 1995; Baker, Beddington et al. 1999; Wilson, Graziano et al. 2000; Pera, Ikeda et al. 2003; Sheng, dos Reis et al. 2003; Rentzsch, Bakkers et al. 2004; Rogers, Archer et al. 2008). We have analyzed the expression of the early neural genes *Xsox2* and *Xsox3* (*XsoxB1s*) to define the mechanism of neural induction by BMP inhibition. These studies revealed that BMP signaling represses expression of the *XsoxB1* gene, *sox3*, through two of its targets, XVent1 and XVent2 (Rogers, Archer et al. 2008). Thus there are different requirements for neural induction within vertebrates, one question we posed is: what is the basal mechanism of induction of neural tissue? One possibility is that FGF signaling is the
basal neural inductive signal and that vertebrates evolved the requirement for BMP inhibition. However, since there are similar dorsal-ventral BMP-Chordin (a BMP antagonist) gradients in the more basal organisms (Darras and Nishida 2001), another possibility is that the inhibition of BMP signaling is the ancestral mechanism for neural induction.

Neural development begins when ectodermal cells receive signals from neighboring cells that lead to the expression of the early neural soxB1 genes (sox1-3 in vertebrates), and these genes encode proteins that are highly conserved (Fig. A.1), have overlapping expression (Hawley, Wunnenberg-Stapleton et al. 1995; Rogers, Archer et al. 2008) (Fig. A.4), and are required for the development and maintenance of neural progenitors in the CNS (Graham, Khudyakov et al.) (Fig. A.2). When SoxB1 expression is down regulated, neural progenitors exit the cell cycle and differentiate into every type of cell of the central nervous system (Uchikawa, Kamachi et al. 1999; Sandberg, Kallstrom et al. 2005).

To determine how the central nervous system evolved, we have begun to study organisms that may be more similar to the last common deuterostome ancestor due their lack of complexity in comparison to vertebrates. Interestingly, the urochordate, Ciona intestinalis requires FGF to induce early neural genes (Hudson, Darras et al. 2003; Shi, Levine et al. 2005; Hudson, Lotito et al. 2007). In this chordate model, FGF activates its downstream effectors Ets and Gata which then activate the early neural gene CiOtx (Bertrand, Hudson et al. 2003). CiOtx has orthologs in vertebrates and hemichordates, but unlike the CiOtx gene, XOtx and SkOtx are only expressed in the anterior portion of
the brain or nerve net respectively, are involved in patterning instead of induction (Lowe, Wu et al. 2003), and are expressed later than the onset of neural induction (Pannese, Polo et al. 1995; Hudson and Yasuo 2005). Due to the differences in the expression and function of *Otx* genes between organisms, this study used *soxB1* as a model gene to identify a common neural induction mechanism. *SoxB1* genes and their homologs are expressed in early neural precursors in many organisms (Pevny and Lovell-Badge 1997; Cremazy, Berta et al. 2000; Miya and Nishida 2003), and can respond to the inhibition of BMP signaling (Rogers, Archer et al. 2008). If there is a conserved mechanism involved in the induction of the CNS between these organisms it would most likely be involved in the regulation of a more widely expressed gene like SoxB1.

To determine if development of the nervous system occurs via a conserved mechanism, this study focused on determining whether the functions of *Ciona* *SoxB1* and *Saccoglossus* *SoxB1* are conserved with those of *Xenopus* *Sox2* and *XlSox3* and we begin to study the regulation of the *Ciona soxB1* gene in order to compare its regulation to that in *Xenopus* as a means of identifying whether the same proteins are involved in the regulation of neural development in both organisms. To this end, we utilized overexpression studies and found that the *SoxB1*s from *Ciona* and *Saccoglossus* expand neural tissue marked by *geminin* and *soxD* at the expense of epidermal tissue marked by *epi-keratin*. However, only vertebrate *SoxB1*s affected neural crest development and induced ectopic neurons at late stages. We also showed that a 3.5 kb and a 7 kb fragment of the *Ciona soxB1* regulatory regions are sufficient to drive expression of lacZ in the neural tissue in a pattern similar to that of endogenous *soxB1* of *Ciona* embryos.
Materials and Methods

Embryo culturing and manipulations

*Xenopus laevis* eggs were isolated and fertilized using standard methods (Sive, Grainger et al. 2000) and staged according to Nieuwkoop and Faber (Nieuwkoop and Faber 1994). Animal ectodermal explants were isolated from stage 8–9 embryos, cultured in 75% NAM (Slack 1984; Peng 1991) and collected according to sibling embryos. Adult *Ciona intestinalis* animals were ordered from M-Rep in Long Beach, California and kept in light until sperms and eggs were harvested. Fertilization was performed as described in Corbo et. al (1997).

Plasmid construction

*Ciona intestinalis* (c1001) and *Saccoglossus kowalevskii* soxB1 (#434) genes were isolated from genomic DNA via nested PCR and cloned into either the *Ciona* expression vector pCESA or via digestion from these sites in this lab by N. Harafuji and D. C. Cunningham, respectively. The *Ciona* SoxB1 regulatory region was amplified via nested PCR and cloned into the *Ciona* expression vector pCESA and the *Saccoglossus* in pCS2. Plasmids were electroporated as described in Corbo et. al (1997).

Preparation of RNAs and microinjection

Synthetic capped mRNAs were made by in vitro transcription using mMMessage mMachine (Ambion). *CiSoxB1* (#c1001, 0.4-0.8 ng), *SkSoxB1* (#434, 0.4-0.8 ng) and *XlSox2* (#RH1104, 0.4-0.8 ng), and *XlSox3* (#419, 0.4-0.8 ng) mRNAs were injected into one of two cell embryos with lacZ mRNA (#128, 0.15 ng) as a tracer.
**WISH and β-galactosidase assay**

WISH of frog embryos was performed as described in Chapter II. We used the following genes as in situ probes: *geminin* (#368) (Kroll, Salic et al. 1998), *soxD* (#263) (Mizuseki, Kishi et al. 1998), *epi-keratin* (#330) (Jonas, Sargent et al. 1985), *n-cam* (#311) (Kintner and Melton 1987), *sox9* (#385) (Spokony, Aoki et al. 2002), *Xtwist* (#637) (Hopwood, Pluck et al. 1989), *ngnr-1* (#326) (Ma, Kintner et al. 1996), and *n-tubulin* (#431) (Richter, Grunz et al. 1988). *Ciona* embryos were fixed and lacZ staining was performed as in (Corbo, Levine et al. 1997).
Results

To understand how different nervous systems are generated in divergent organisms like *Xenopus*, *Ciona* and *Saccoglossus*, we focused on the role and regulation of SoxB1 proteins and genes. If the SoxB1 proteins have dissimilar sequences with little similarity and have evolved different functions, or the genes are expressed in different tissues, then the nervous systems may develop differently. I utilized ClustalW software to compare the protein sequences of all three *Xenopus* SoxB1 proteins to *Ciona* and *Saccoglossus* SoxB1 (Fig. A.1). The *Xenopus* SoxB1 proteins are highly conserved with at least 95% sequence similarity in the high mobility group domain (HMG) (Kamachi, Uchikawa et al. 2000). The *Xl*SoxB1s HMG domains are 86.1% identical to *Ci*SoxB1 and *Sk*SoxB1 (Fig. A.1). Additionally, the group B homology domain is only 15% conserved between all organisms but is approximately 80% identical between *Xenopus* and *Saccoglossus* SoxB1s indicating that the *Ciona* SoxB1 sequence has diverged.

Since the SoxB1 proteins share a high similarity, we tested their function in *Xenopus laevis* embryos. *Xenopus* embryos were used due to their large sizes and the ease of microinjection. First, we tested the effect of *CisoxB1* and *SksoxB1* overexpression on neural development and compared the phenotype to that of *Xlsox2* and *Xlsox3* overexpression. *Xl*SoxB1s expand neural progenitors and delay neurogenesis which leads to ectopic neurons by neurula stage (Rogers, Harafuji et al. 2008). Overexpression of any of the *soxB1s* induced expression of *geminin* by stage 17 (Fig. A.2A), however only *Xlsox3* induced ectopic expression of *geminin* at gastrula stage. Additionally, although the *soxB1s* caused a minimal expansion of *ncam*, all expanded
expression of the neural inducer soxD, and CiSoxB1 induced ectopic expression of this gene (Fig. A.2B, see inset). Thus, the SoxB1s appear to share a conserved function in expanding neural progenitors.

To determine if CiSoxB1 and SkSoxB1 induce ectopic neurons, we tested the effect of overexpression on the expression of the proneural gene neuroD and the neuronal marker n-tubulin. XlSox2, and XlSox3 and CiSoxB1 caused disorganized and ectopic expression of n-tubulin by stage 17, but only Xenopus SoxB1s induced ectopic neurons in stage 23 embryos. SkSoxB1 did not induce ectopic n-tubulin expression (Fig. A.2). Though all of the SoxB1s are able to expand the neural tube, the expansion does not always lead to ectopic neurons.

To determine if the Ciona and Saccoglossus SoxB1s induce neural tissue at the expense of non-neural ectodermal derivatives similarly to the XlSoxB1s (Rogers, Harafuji et al. 2008), they were overexpressed in 1 of 2 cells. CiSoxB1 and SkSoxB1 inhibited epidermal development as marked by epi-keratin, however they had little effect on the expression of the neural crest specifier, sox9, or on migratory neural crest cells as marked by Xt Twist expression (Fig. A.3). This was unlike the phenotype caused by XlSox2 and Sox3 (Fig. A.3).

Because CiSoxB1 phenocopies the ability of XlSoxB1s to expand the neural plate at the expense of epidermal formation, we determined that they may function similarly in the development of the Ciona and Saccoglossus nervous systems indicating that their dissimilar morphologies may be due to differences in gene regulation. As such, we next compared the differences in the regulation of Ciona and Xenopus soxB1 genes
because \textit{Xlsox3} and \textit{CiSoxB1} are both expressed in neural precursors throughout development (Fig. A.4, \textit{XlsoxB1} WISH performed by E.C. Casey).

To determine if \textit{CisoxB1} is regulated in a manner similar to \textit{Xlsox2} and \textit{Xlsox3}, N. Harafuji isolated a 7 kb 5’ \textit{soxB1} regulatory fragment from \textit{Ciona intestinalis} genomic DNA and created 7 kb and 3.4 kb reporter constructs with the regulatory fragments driving expression of \textit{lacZ}. DNA was electroporated into \textit{Ciona intestinalis} embryos to determine if these regulatory sequences were sufficient to drive expression in a pattern that mimicked that of \textit{CisoxB1} endogenous expression (Fig. A.5). The embryos with both the 3.4 kb and 7 kb constructs lead to anterior specific expression of \textit{lacZ} by the neural plate stage (3.4 kb) or 110-cell stage (7 kb) similar to \textit{CisoxB1} endogenous expression (Fig. A.4). By tailbud stage, expression of the 3.4 kb construct was restricted to the dorsal nerve chord and anterior head mesenchyme. The 7 kb fragment had a similar expression but was also expressed in the epidermis of the embryos.

This study shows that \textit{CiSoxB1} and \textit{SkSoxB1} share many functions with \textit{XlSoxB1} when overexpressed in \textit{Xenopus} embryos. Also, we have identified a regulatory region from \textit{Ciona intestinalis} that drives expression of a reporter gene similar to endogenous \textit{CisoxB1}. Future experiments will utilize this regulatory region to decipher the differences involved in development of the vertebrate and urochordate nervous systems. Since frog and sea squirts both have dorsal central nervous systems, these studies will allow us to determine if the \textit{soxB1} genes are regulated by the same signaling pathways (i.e., both BMP and FGF). Eventually, the conclusions of these studies will be compared to regulatory studies in \textit{Saccoglossus kowalevskii}. Differences
in the regulation of SksoxB1, which has a nerve net instead of a dorsal CNS, can help to determine how the dorsal central nervous system evolved.
Figure A.1. SoxB1 proteins are highly conserved across species. An alignment of *Xenopus laevis* Sox2, Sox3, Sox1 and *Ciona intestinalis* and *Saccoglossus kowalevskii*
Sox1/2/3 proteins using ClustalW. XlSox2, 3 and 1 are most similar to each other. Yellow highlighting indicates HMG domains that are 86.1% conserved (identical or similar) and pink indicates the Group B homology which is 15% similar. CiSoxB1 is the least similar to other proteins. An asterisk, "*" indicates conserved amino acid identity, a colon, “:” indicates a conservative amino acid substitution and a period "." indicates amino acid semi-conservative substitutions between the three sequences.
Figure A.2. *CisoxB1* and *SkSoxB1* expand neural tissue and delay neuronal differentiation like *XlSoxB1*. (A) WISH of embryos injected with *lacZ* with *Xlsox2*, *Xlsox3*, *CisoxB1*, or *SksoxB1* and probed for *geminin* (stage 12.5; Sox2= 22/33, Sox3= 7/7, CiSoxB1= 25/26, SkSoxB1= 19/19, animal pole view and 17; Sox2= 15/15, Sox3= 23/23, CiSoxB1= 14/14, SkSoxB1= 7/7, lateral view), *ncam* (stage 17; Sox2= 59/66, Sox3= 36/62, CiSoxB1= 23/44, SkSoxB1= 15/28, dorsal view) or *soxD* (stage 17, dorsal view; Sox2= 9/10, Sox3= 7/12, CiSoxB1= 16/18, and SkSoxB1= 4/8). (B) WISH of embryos injected with *lacZ* with *Xlsox2*, *Xlsox3*, *CisoxB1*, or *SksoxB1* and probed for *neuroD* (stage 17, dorsal view; Sox2= 7/9, Sox3= , CiSoxB1= 12/20, SkSoxB1= 9/14) or *n-tubulin* (stage 17; Sox2= 15/22, Sox3= 16/24, CiSoxB1= 23/27, SkSoxB1= 11/17; and stage 23; Sox2= 23/36, Sox3= 21/24, CiSoxB1= 31/37, SkSoxB1= 52/52, dorsal view). Lateral views are anterior to the left and dorsal views are anterior to the top.
Figure A.3. CiSoxB1 and SkSoxB1 proteins mimic XlSoxB1s to repress non-neural ectodermal derivatives in *Xenopus* embryos. WISH of embryos injected with lacZ with *Xlsox2, Xlsox3, CiSoxB1,* or *SksoxB1* and probed for *epi-keratin* (17; Sox2= 25/25, Sox3= 25/27, CiSoxB1= 28/30, SkSoxB1= 16/20, lateral view), *sox9* (stage 16, dorsal view; Sox2= 17/30, Sox3= 33/41, CiSoxB1= 21/37, SkSoxB1= 17/30) or *Xtwist* (stage 25; Sox2= 14/30, Sox3= 19/24, CiSoxB1= 34/34, SkSoxB1= 26/28, lateral view, UI side on top row and I side on bottom row). UI is uninjected, I is injected. Lateral views are anterior to the left and dorsal views are anterior to the top.
Figure A.4. SoxB1 genes are expressed in neural precursors. WISH of *Xenopus laevis* sox3 (left column, performed by E.C. Casey) and *Ciona intestinalis* soxB1 (right column). Stages are indicated on left. Stages are: *Xenopus* (early gastrula: stage 10.5, late gastrula: stage 11, neurula: stage 17 and tailbud: stage 35) and *Ciona* (early gastrula: 110-cell stage, late gastrula, neurula: neural plate, and tailbud: mid-late tailbud).
A.5. A CiSoxB1 upstream regulatory sequence drives expression of lacZ in Ciona embryos. Expression of sox3-β-galactosidase (lacZ) as visualized by X-Gal (left and middle panels) or WISH of CisoxB1 expression. Embryo stages are on the left side. Embryos are electroporated with a 3.4 kb CiB1-lacZ DNA construct (left column) or a 7 kb CiB1-lacZ DNA construct (middle column).
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Sox3 expression is maintained by FGF signaling and restricted to the neural plate by Vent proteins in the Xenopus embryo

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