THE HOMEODOMAIN TRANSCRIPTION FACTOR Six3 IS REQUIRED FOR

TELENCEPHALON PATTERNING IN ZEBRAFISH

By

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To my mother, Jane Carlin, and my grandparents, Doris and James Carlin and

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LIST OF ABBREVIATIONS

°C	degrees Celsius
γCry	γCrystallin
μm	micrometer
μM	micromolar
A	anterior
An	animal pole
ANB	anterior neural border
Ala	Alanine
AP	anterior-posterior
Bmp	Bone morphogenetic protein
bp	base pair
cDNA	complementary deoxyribonucleic acid
CNS	central nervous system
D	dorsal
Di	diencephalon
Dkk1	Dickkopf1
DNA	deoxyribonucleic acid
dUTP	deoxyuridine triphosphate
DV	dorsoventral
E	eye
EGFP	enhanced green fluorescent protein

eomesa	eomesodermin homolog a
FB	forebrain
Fgf	Fibrblast growth factor
FP	floor plate
GFP	green fluorescent protein
GOF	gain of function
gsc	goosecoid
H	hypothalamus
HB	hindbrain
Hh	Hedgehog
HPE	holoprosencephaly
hpf	hours post-fertilization
hsp70l	heat shock protein 70-like
INT	iodonitrotetrazolium chloride
isl1	islet1
kb	kilobase
L	left
LGE	lateral ganglionic eminence
Mapk	Mitogen-activated protein kinase
MB	midbrain
MGE	medial ganglionic eminence
MHB	midbrain-hindbrain boundary
MIHV	middle interhemispheric variant

mM	millimolar
MO	antisense morpholino oligonucleotide
mRNA	messenger ribonucleic acid
N	notochord
ng	nanograms
NH	non-helical oligopeptide linker
NIH	National Institutes of Health
P	posterior
PCM	prechordal mesoderm
PCR	polymerase chain reaction
pg	picograms
pH3	phospho-Histone H3
PTC1	patched1
ptch2	patched2
R	right
RNA	ribonucleic acid
SC	spinal cord
sFrp	soluble Frizzled-related protein
Shh	Sonic hedgehog
SIX	Sine oculis homeobox
SM	Spemann-Mangold
SMO	Spemann-Mangold organizer
smo	smoothened

T	telencephalon
TB	tailbud stage
Tg	transgenic
Tgf-β	Transforming growth factor β
tp53	tumor protein 53
TUNEL	terminal deoxynucleotidyl transferase dUTP nick end labeling
UAS	upsteam activating sequence
V	ventral
Veg	vegetal pole

CHAPTER I

INTRODUCTION

The vertebrate adult forebrain, comprised of the derivatives of the embryonic telencephalon and diencephalon, which include the cerebrum, basal ganglia, olfactory bulbs, thalamus, hypothalamus and several other adult structures, is complex in both structure and function. This region of the central nervous system (CNS) is responsible for the regulation of many functions including behavioral responses to external stimuli, such as vision and olfaction, maintaining homeostatic mechanisms such as circadian rhythms and hunger, as well as controlling voluntary movement and executive functions. The compartmental organization of the brain helps to facilitate the execution of its many functions. Each compartment contains multiple cell types, and each individual cell can receive many inputs and send signals to potentially many other cells in one or several other regions of the brain. During early embryonic development, a pseudostratified neural epithelium with broad developmental potential generates neural progenitors. As development proceeds, a specific spatiotemporal sequence of transcriptional and signaling programs promotes specification of different cell types based on the location and developmental potential of the progenitors and guides the differentiated cells to their final locations to generate the complex anatomy of the adult forebrain. Insights into the cellular and molecular mechanisms that promote normal brain development may aid in the understanding of diseases with genetic components such as

schizophrenia, autism, holoprosencephaly, and many others, where we currently struggle to provide effective diagnostic tools and therapeutic agents to improve the quality of life for affected individuals. The experiments described in this dissertation provide insight into the mechanisms by which broadly expressed genes encoding transcription factors of the *Six3* family regulate the generation of discrete cell populations within the zebrafish forebrain through their genetic interactions with genes encoding extracellular signaling molecules.

<div align="center">Forebrain Development</div>

<u>Neural induction</u>

Studying early forebrain development enables us to gain insight into how complex neural circuits in adult animals form from a collection of undifferentiated cells in the embryo. The first step towards development of the forebrain is for undifferentiated ectodermal cells to attain neural character during early embryogenesis. Many studies have provided substantial support for the "default model" of neural induction, which states that ectodermal cells will become neurons if they are not exposed to specific extrinsic signals that inhibit this process (Munoz-Sanjuan and Brivanlou, 2002).

Upon fertilization of frog and fish embryos, maternally deposited determinants become apportioned on the future dorsal side of the embryo to promote nuclear localization of β-catenin, a key transcriptional effector of canonical Wnt signaling (Figure 1A), in a relatively small number of cells
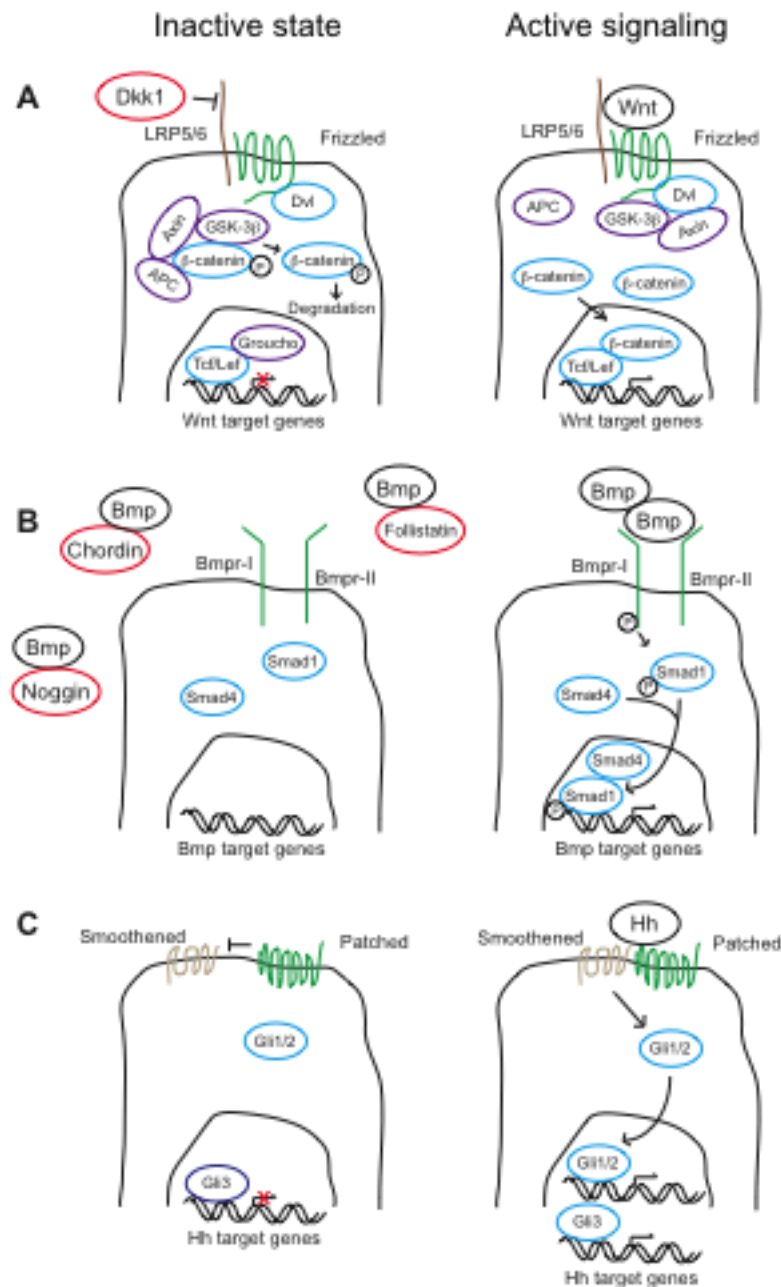
Figure 1. Key developmental signaling pathways in mammals. (**A**) In the absence of Wnt ligand, GSK3β phosphorylates β-catenin (P), marking it for degradation (left). In the presence of Wnt ligand, β-catenin is stabilized, translocates to the nucleus, disrupts Groucho co-repression and binds to Tcf/Lef proteins to activate transcription of Wnt target genes (right). (**B**) When Bmp receptors are not activated, Smads localize to the cytosol (left). When Bmp receptors are activated by ligand, Bmpr-I and Smad1 are phosphorylated. Smad1 binds Smad4 and both proteins translocate to the nucleus and activate transcription of Bmp target genes (right). (**C**) In the absence of Hh ligands, Patched inhibits Smoothened, allowing Gli3 to repress Hh target genes (left). In the presence of Hh ligands, inhibition of Smoothened is released, Gli1 and Gli2 translocate to the nucleus and activate transcription of Hh target genes. Also proteolytic cleavage of Gli3 is inhibited, causing it to function as a transcriptional activator (right).

(Schneider et al., 1996; Larabell et al., 1997; Moon and Kimelman, 1998). At mid-blastula transition which marks the activation of the zygotic genome, nuclear localized β-catenin induces formation of the Nieuwkoop center in blastula cells on the presumptive dorsal side through its transcriptional regulatory activity (Schneider et al., 1996; Wylie et al., 1996; Vonica and Gumbiner, 2007). The Nieuwkoop center then induces and partially differentiates into the Spemann-Mangold (SM) gastrula organizer (the shield in zebrafish), which is a temporally distinct signaling center that shares some molecular components with the Nieuwkoop center (Gimlich and Gerhart, 1984; Gimlich, 1985; Gimlich, 1986; Bauer et al., 1994; Vonica and Gumbiner, 2007). The SM organizer secretes signaling molecules that function to induce the three embryonic germ layers and help establish the anterior-posterior (AP) axis (Figure 2) (De Robertis et al., 2000). When transplanted to the presumptive ventral side of an embryo, the SM organizer is capable of inducing an ectopic neural axis from host tissue (Spemann, 1938), suggesting that signals from the SM organizer are sufficient to specify neural fates.

A number of more recent studies in *Xenopus* and zebrafish have provided support for the "default model" by uncovering the molecular mechanisms behind neural induction. Instead of instructing cells to become neural ectoderm, the SM organizer protects presumptive neural ectoderm from extrinsic signals that induce other nonneural fates. Several Bone morphogenetic protein (Bmp) ligands of the Transforming growth factor β (Tgf-β) superfamily, including Bmp2b, Bmp4 and Bmp7, are expressed on the future ventral and lateral sides of the embryo,

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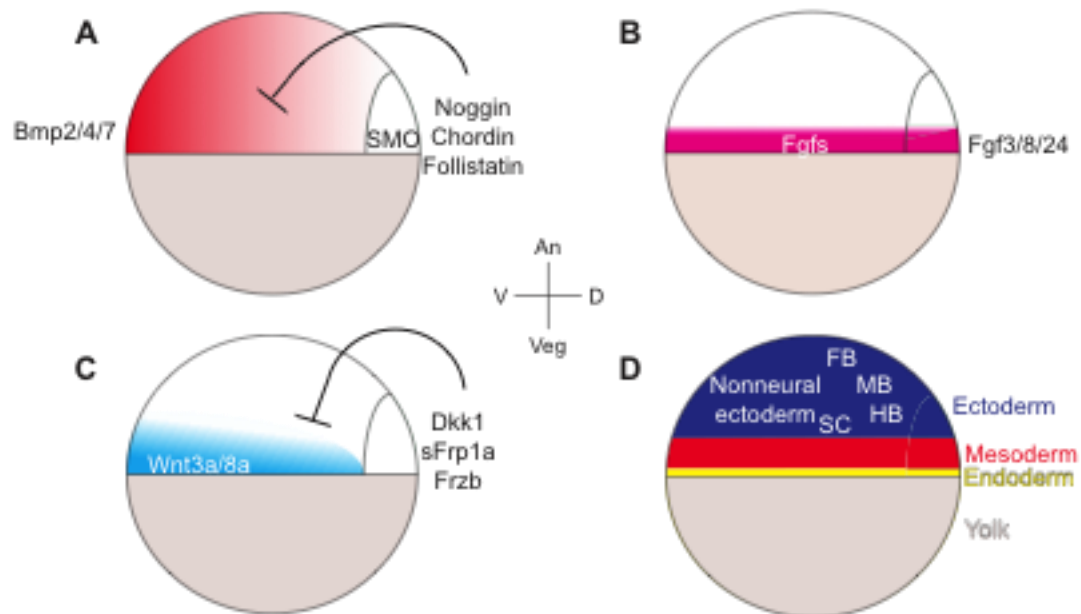
Figure 2. Signaling pathway activity and fate map during neural induction in zebrafish gastrulae. Lateral view, dorsal to the right, animal pole to the top, onset of gastrulation (50% epiboly). (**A**) Bmp ligands are expressed on the ventral side of the embryo and Bmp antagonists are expressed in the SMO establish a ventral to dorsal gradient of Bmp activity (red). (**B**) *fgf3*, *fgf8* and *fgf24* are initially expressed at the dorsal margin during late blastula. Several additional Fgfs are expressed around the entire margin by early gastrulation where Fgf signaling activity is maintained (magenta). (**C**) *wnt3a* and *wnt8a* are expressed at the margin but excluded from SMO where Wnt antagonists are expressed. Wnt activity (blue) extends away from the margin in a gradient towards the animal pole. (**D**) Fate map indicating location of germ layers. Ectodermal fates are arranged along the DV and animal-vegetal axis. Figure panels adapted from Schier and Talbot, 2005. Abbreviations: An, animal pole; D, dorsal; FB, forebrain; HB, hindbrain; MB, midbrain; SC, spinal cord; SMO, Spemann-Mangold organizer; V, ventral; Veg, vegetal pole.

and are sufficient to specify epidermis (Figure 2A,D) (Wilson and Hemmati-Brivanlou, 1995; Nguyen et al., 1998; Schmid et al., 2000). The SM organizer secretes several inhibitors of Bmp signaling including Noggin, Chordin and Follistatin (Figure 1B,2A) (Smith and Harland, 1992; Hemmati-Brivanlou et al., 1994; Sasai et al., 1994). Loss of function of genes encoding Bmp pathway components or misexpression of Bmp antagonists or dominant negative Bmp receptors promote neural fates at the expense of epidermis (Lamb et al., 1993; Hemmati-Brivanlou et al., 1994; Sasai et al., 1994; Xu et al., 1995; Reversade et al., 2005). These studies suggest a role for the SM organizer in protecting presumptive neural ectoderm from the ventralizing Bmp signals through the expression of secreted Bmp antagonists.

Studies using explanted *Xenopus* animal cap tissue advanced the understanding of neural induction by providing a means of analyzing the response of nascent ectoderm to different signals in the absence of potentially confounding signals from other regions of an intact embryo. Explanted *Xenopus* animal cap tissue will differentiate into epidermis if left intact, but will express neural markers if dissociated into individual cells (Sato and Sargent, 1989; Wilson and Hemmati-Brivanlou, 1995). Dilution of Bmp signal in dissociated cells versus intact explant was speculated to be responsible for promoting neural fate in the dissociated explant cells (Hemmati-Brivanlou and Melton, 1997). The Bmp dilution hypothesis was called into question when it was later found that Bmp signaling still occurs in dissociated animal cap cells (Kuroda et al., 2005). Furthermore, inhibition of Bmp signaling is insufficient to promote neural tissue in

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more ventrally located ectodermal blastomeres unless Fibroblast growth factor (Fgf) and possibly other signals are also provided (Linker and Stern, 2004; Delaune et al., 2005). Although these blastomeres would not normally form neuroectoderm, the notion that inhibition of Bmp signaling alone is insufficient to push these cells to adopt a neural fate suggests that additional signaling pathways may also play a role in the dorsal blastomeres that are normally fated to form neuroectoderm.

Additional strong support for the "default model" has been provided by recent experiments in mouse embryonic stem cells. When grown at low density in serum-free, growth-factor free media or saline, embryonic stem cells differentiate into a primitive neural cell type and subsequently neurons or glia (Smukler et al., 2006). This suggests that in the absence of any extrinsic signals, undifferentiated embryonic stem cells will adopt neural fate. However, the environment of an intact vertebrate embryo is quite different from that of dissociated cells in culture. Increasing evidence suggests that additional pathways may be required to induce neural fates in the embryo.

Pathways other than Bmp signaling have been implicated in neural induction, but their roles are much less clear. Fgf signals are also produced by the SM organizer (Figure 2B) (Streit et al., 2000), and may play a permissive role through further inhibition of Bmp signaling. While Bmp binding to its receptor results in activation of the transcriptional effector Smad1 via phosphorylation, the Fgf signaling mediator Mitogen-activated protein kinase (Mapk) inactivates Smad1 by phosphorylating a linker region of the protein, thereby inhibiting its

nuclear import (Kretzschmar et al., 1997; Pera et al., 2003). Inactivation of Smad1 by Fgf signaling reinforces Bmp inhibition on the dorsal side of the embryo. In addition, Fgf signaling has been shown to repress *Bmp* expression (Londin et al., 2005). A Bmp-independent role for Fgf signaling in neural induction remains an open question. Robust induction of neural fates may require inhibition of both Bmp and Wnt signaling along with activation of Fgf signaling. This idea is suggested by the observations that upregulation of canonical Wnt signaling inhibits early neural maker gene expression in *Xenopus* and inhibits neuralization of ectoderm by Bmp inhibition or Fgf signaling in chick (Figure 2) (Wilson et al., 2001; Heeg-Truesdell and LaBonne, 2006). Because of the reiterative nature of this pathway, timing of Wnt signaling manipulations is extremely important and has proven to be a strong technical barrier in dissociating different Wnt functions during early neural development. No studies to date have shown that loss of Wnt signaling results in defects in neural induction independently of its role in SM organizer formation. To this end, Fgf and Wnt signaling also may have indirect roles on neural induction by affecting the amount of dorsal mesoderm tissue available to induce neural fates (Kelly et al., 2000; Wilson et al., 2001; Lee et al., 2011). Other yet undefined pathways may also be involved in neural induction (Linker and Stern, 2004).

Signaling molecules induce expression of specific transcription factors that establish and maintain neural fate. During neural induction, inhibition of Bmp signaling facilitates activation of Geminin and several genes of Sox and Zic families. These transcription factors form a network that specifies neural fate and

maintains neural ectoderm in a proliferative progenitor state in part by inhibiting cell cycle exit (Rogers et al., 2009), thereby establishing the neural plate.

Anteroposterior and mediolateral axial patterning in the neural plate

The complexity of the vertebrate CNS arises from the modification of a relatively uniform neural plate of single cell thickness. The concerted action of a number of signaling molecules functioning in a specific spatiotemporal manner elaborates discrete anatomical and functional domains. The CNS initially establishes an AP and dorsoventral (DV) polarity whereby specific neuronal and glial cell types will arise at different locations along these axes. To establish the AP axis, signals from nonneural ectoderm and mesoderm establish two main signaling centers in the neural ectoderm, the anterior neural border (ANB) and the midbrain-hindbrain boundary (MHB), which are separated by the presumptive forebrain and midbrain (Figure 3). These signaling centers further refine and maintain the initial AP pattern in conjunction with other nonneural signaling centers, resulting in subdivisions of the neural plate into progenitors of the main structural components of the CNS.

The ANB arises at the anterior border of the neural plate between neural and nonneural ectoderm. Because the diffusion of secreted signaling molecules from the SM organizer plays such a prominent role in establishing the neural/nonneural ectodermal boundary, the anterior border of the neural plate is a curve that opens posteriorly. The ANB secretes Bmp and Wnt antagonists as well as Fgfs, and is located in a region between high and low Bmp signaling

activity (Figure 3A). Nonneural ectoderm and anterior mesoderm each express several Bmp ligands, while prechordal mesoderm (PCM) secretes Bmp antagonists, Noggin and Chordin, as it migrates anteriorly during gastrulation (Anderson et al., 2002). Correspondingly, specific levels of Bmp signaling may be required to induce ANB. In zebrafish embryos misexpressing *noggin*, low levels of Bmp signaling activity fail to induce ANB markers (Houart et al., 2002). Conversely, increased Bmp signaling in *Chordin -/-; Noggin +/-* mouse mutant embryos also reduces the ANB (Anderson et al., 2002). Consistent with the notion that moderate Bmp activity induces ANB, Noggin-overexpressing cells transplanted into wild-type embryos can induce ectopic ANB markers in nonneural ectoderm at a short distance from the graft (Houart et al., 2002). Once induced, secreted molecules from the ANB antagonize signals from nonneural ectoderm, mesoderm and MHB to promote the most anterior neural fates.

MHB arises from signals from paraxial and lateral mesoderm and in turn provides a source of Wnt and Fgf signals that pattern the surrounding neural tissue (Figure 3A). During blastula stages, Nodal, Fgf, and Wnt signaling are required for specification of mesoderm (Kimelman and Kirschner, 1987; Slack et al., 1988; Christian et al., 1992; Conlon et al., 1994; Heasman et al., 1994; Jones et al., 1995; Feldman et al., 1998), which becomes a strong source of Fgfs, Wnt8, and retinoic acid. These signals act to specify hindbrain and spinal cord fates by inducing expression of posterior neural transcriptions factors, such as Gbx1 and Hoxb1b, while inhibiting expression of anterior neural transcription factors that specify forebrain and midbrain such as Otx2 and Cyp26
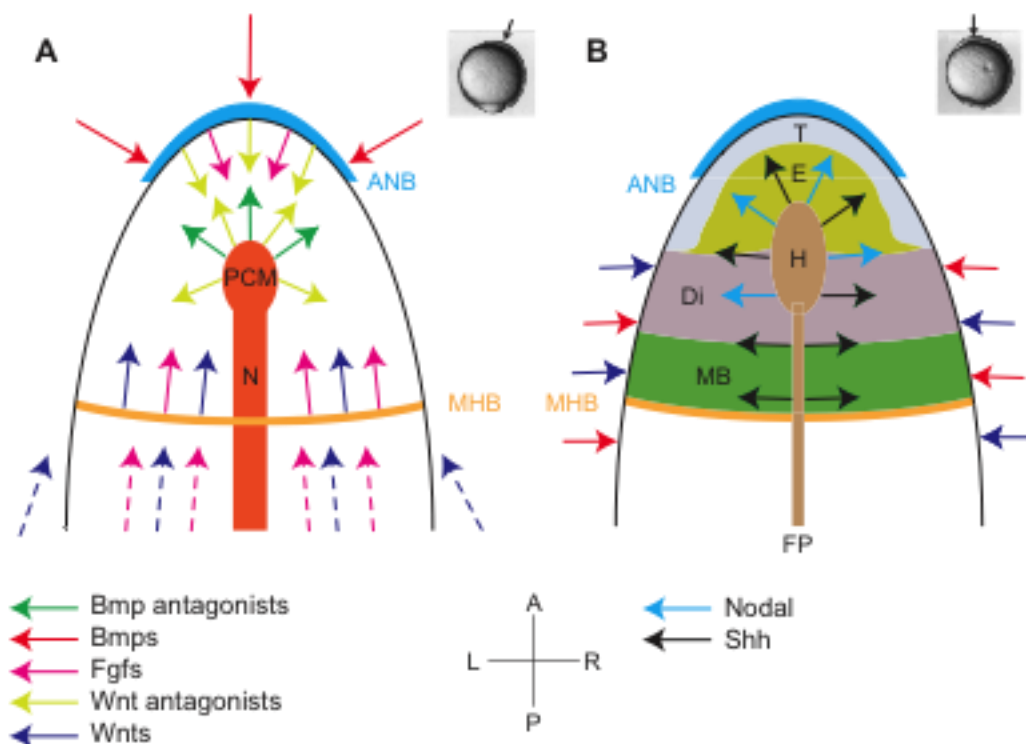
Figure 3. Anteroposterior and mediolateral patterning of neural plate. Embryos are presented as dorsal view with anterior up. Arrow on live embryo denotes view in the diagram. Live images modified from Kimmel et al., 1995. (**A**) Anterior-posterior neural patterning during mid/late gastrulation. ANB (blue) is induced at the anterior neural/nonneural ectodermal boundary between high and low levels of Bmp signaling. ANB expresses Wnt antagonists and Fgfs. PCM expresses Bmp and Wnt antagonists. Fgfs and Wnts expressed in posterior and lateral mesoderm (dashed lines) induce MHB (tan), which also expresses Fgfs and Wnts. Shh and Nodal expression in PCM and N induces H and FP, respectively (not shown). (**B**) Mediolateral neural patterning during early segmentation. Shh and Nodal expression in hypothalamus and floor plate induce medial fates and antagonize Bmp and Wnt expression from nonneural ectoderm and neural crest. Adapted from Wilson and Houart, 2004. Abbreviation: A, anterior; ANB, anterior neural border; Di, diencephalon; E, eye; FP, floor plate; H, hypothalamus; L, left; MB, midbrain; MHB, midbrain-hindbrain boundary; N, notochord; P, posterior; PCM, prechordal mesoderm; R, right; T, telencephalon.

(Woo and Fraser, 1995; Erter et al., 2001; Lekven et al., 2001; Kudoh et al., 2002; Rhinn et al., 2005; Schier and Talbot, 2005; Petersen and Reddien, 2009). The posterior boundary of *Otx2* and anterior boundary of *Gbx1* expression positions the MHB, which itself acts a signaling center within the neural plate (reviewed in Wurst and Bally-Cuif, 2001). Through expression of Fgf and Wnt signals, the MHB further refines AP pattern locally in the neural plate.

By mid-gastrulation, the anterior neural plate is influenced by signals emanating from three main signaling centers: the ANB, PCM, and MHB. The role of the PCM in anterior neural patterning is not clear. PCM secretes Bmp and Wnt antagonists, including the Wnt inhibitor Dickkopf1 (Dkk1), that may have some influence on AP pattern (Figure 3A) (Hashimoto et al., 2000; Mukhopadhyay et al., 2001). However, these molecules likely act on earlier AP patterning events such as positioning the ANB and MHB as described above (Houart et al., 2002; Seiliez et al., 2006). By late gastrulation, PCM predominantly influences mediolateral/dorsoventral neural plate patterning, which will be discussed below.

Signals from the ANB and MHB subdivide the anterior neural plate into telencephalon, eye field, diencephalon and midbrain along an anterior to posterior gradient of Wnt activity (Figure 3B). Data from numerous studies suggests telencephalon is specified at the lowest levels, eye field at low/intermediate levels, and posterior diencephalon and midbrain are specified at relatively high levels of Wnt signaling. Signals from the MHB induce regional expression in dorsal midbrain and diencephalon of several Wnt ligands that are known to activate canonical Wnt signaling, including Wnt1, Wnt3a, Wnt7b, Wnt8b

and others (Lee et al., 2000; Carl et al., 2007). ANB expresses the secreted Wnt inhibitor Tlc, a member of the soluble Frizzled-related protein (sFrp) family, and this molecule is necessary and sufficient to promote the most anterior fates such as telencephalon (Houart et al., 2002). Other Wnt antagonists such as sFrp1 are also secreted in the region of the ANB. Misexpression of *sfrp1* in zebrafish embryos is sufficient to suppress the reduced head and eyes phenotypes resulting from *wnt8b* misexpression (Kim et al., 2007). Additionally, several zebrafish and mouse mutants have been characterized with increased Wnt signaling during gastrulation, and these embryos show various degrees of telencephalon and eye deficiencies, often with expanded posterior diencephalon or midbrain (Fekany-Lee et al., 2000; Kim et al., 2000; Heisenberg et al., 2001; Kiecker and Niehrs, 2001; Kim et al., 2002; Lagutin et al., 2003; Wilson and Houart, 2004; Lavado et al., 2008). Although Fgfs are also secreted from ANB, it appears that these molecules pattern anterior fates rather than induce them (Shimamura and Rubenstein, 1997; Shanmugalingam et al., 2000; Wilson and Houart, 2004). Therefore Wnt ligands from mesoderm during gastrulation and later from MHB and diencephalon limit the most anterior neural patterning programs unless the tissue is protected by Wnt inhibitory molecules such as Dkk1 produced by the SM organizer/PCM and Tlc in the ANB (Figure 2C,3A).

The neural plate mediolateral axis, which subsequently becomes the DV axis of the neural tube, is also patterned by Hedgehog (Hh), Nodal, Bmp, and Wnt ligands from surrounding nonneural tissue. Sonic hedgehog (Shh) is initially expressed during gastrulation in the axial mesoderm, including PCM, and

induces medial neural tissue to become hypothalamus anteriorly and floor plate posteriorly (Krauss et al., 1993; Chiang et al., 1996; Rohr et al., 2001). These medial neural tissues then become sources of Shh, which acts as a morphogen to further specify additional adjacent cell fates (Krauss et al., 1993; Ruiz i Altaba et al., 1995; Poh et al., 2002). Nodal promotes expression of and cooperates with Hh signals to specify and maintain some ventral structures, while functioning independently to specify others (Rohr et al., 2001). These signals function in opposition to Bmp and Wnt morphogen signals from non-neural ectoderm, neural crest and dorsal neural tube (Figure 3B). During early segmentation, the neural plate folds upon itself to form a closed neural tube. This morphogenetic event maintains AP pattern, however medial and lateral neural plate fates become ventral and dorsal fates in the neural tube, respectively. AP and mediolateral patterning establishes discrete competence domains, which comprise cells that respond differently to further developmental signals, thus laying the groundwork for the increasing cellular diversity and complexity to be achieved in the CNS.

Telencephalon development

The mammalian telencephalon will eventually give rise to several structures in the adult brain including the cerebral cortex, basal ganglia, and olfactory bulbs. In anamniotes, the telencephalon will give rise to the pallium and subpallium, which are functionally similar to their mammalian homologs. These regions provide the animal with higher order cognitive and motor abilities. As discussed earlier, in all vertebrates, the telencephalon is specified during

gastrulation at the anterior aspect of the neural plate in a U-shaped domain that opens posteriorly to more posterior neural tissues (Figure 3B). This domain crosses the embryonic midline and is far enough from the Shh-secreting hypothalamus that it is considered to be part of the alar plate, the more lateral region of the neural plate that will generate dorsal structures in the neural tube. During neurulation, which occurs during early segmentation stages, the telencephalon domain "zips" closed into a neural tube, which bends near the midbrain to generate the cephalic flexure. As a result of these morphogenetic movements, the most anterior tip of the neural plate, the curved part of the telencephalic "U", becomes ventral telencephalon and the two posterior segments of the telencephalic anlage come together to form dorsal telencephalon. During and following these morphogenetic movements, the telencephalon becomes patterned into specific domains along its nascent DV axis.

DV patterning in the telencephalon, as in other regions of the embryo, relies on the strict spatiotemporal action of multiple signaling molecules, starting during late gastrulation and continuing through early segmentation stages, to establish the regions of transcription factor expression that specify discrete cell types. Telencephalon DV patterning is achieved through the cooperative action of three major signaling centers: Fgf signaling from ANB and anterior midline, Hh signaling from hypothalamus and Bmp/Wnt signaling from dorsal telencephalon and diencephalon. Whereas Bmp and Wnt signaling both function to promote dorsal and repress ventral telencephalon, Hh and Fgf signaling predominantly

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function to specify ventral and repress dorsal telencephalic fates (reviewed in Lupo et al., 2006; Hebert and Fishell, 2008). These signaling centers serve, in part, to regulate the activity of two transcription factors, Foxg1 and Gli3, which profoundly influence DV patterning (Figure 4A).

The murine Gli3 is a transcriptional repressor that is expressed throughout most of the telencephalon with highest expression dorsally, and is required to promote dorsal fates at the expense of ventral ones (Grove et al., 1998; Theil et al., 1999; Tole et al., 2000; Kuschel et al., 2003). Spinal cord expression of *Gli3* requires Wnt signaling, and levels may be highest near a Wnt source (Alvarez-Medina et al., 2008). If this is also the case in telencephalon, it would set up a feedback loop between Gli3 activity and Bmp/Wnt signaling. In nonexencephalic *extra-toes* mouse mutants, which carry a deletion of the *Gli3* gene, dorsal telencephalic expression domains of *Bmp4* and several Wnt ligands are strongly reduced while anterior midline expression of *Fgf8* is expanded. Together these expression changes result in ventralization of the telencephalon (Grove et al., 1998; Theil et al., 1999; Tole et al., 2000; Kuschel et al., 2003). Activity of Gli3 repressor is also regulated by Hh signaling. Proteolytic cleavage of full length Gli3 protein is required to produce its active transcriptional repressor form, and this cleavage event is inhibited by Hh signaling (Wang et al., 2000). Therefore *Gli3* gene expression seems to be activated by dorsal signals, while the transcriptional repressor activity of its protein product is inhibited ventrally by Hh signaling. In *Shh* mutant mice, ventral telencephalon is absent whereas dorsal fates are expanded (Chiang et al., 1996; Rallu et al., 2002). In the absence of
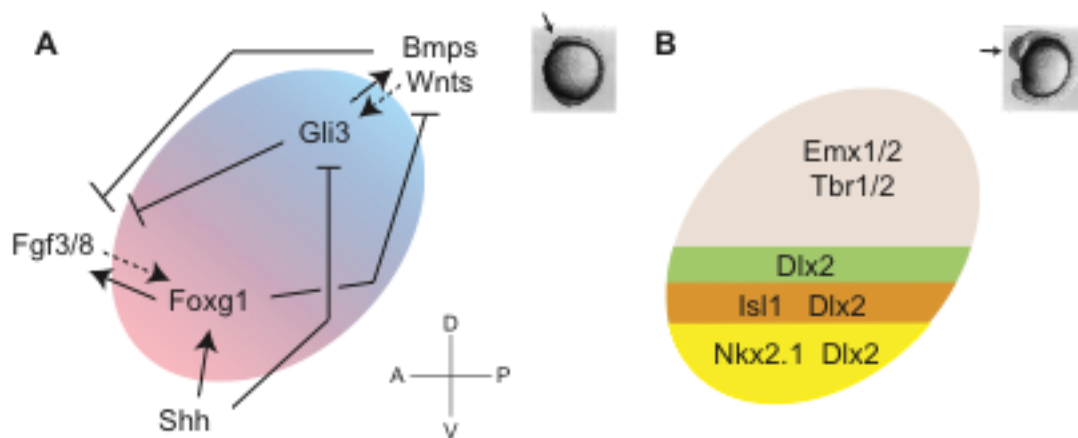
Figure 4. Telencephalon dorsoventral patterning. Model of a sagittal section of telencephalon near the midline. Arrow on live embryo denotes location of of sagittal section. Live images modified from Kimmel et al., 1995. (**A**) Genetic diagram of major signaling pathways and transcription factors affecting telencephalon dorsoventral patterning during early segmentation. *Foxg1* (red) expression is highest in ventral telencephalon. Gli3 (blue) activity is highest in dorsal telencephalon. Solid lines are confirmed interactions. Dashed lines are potential interactions. (**B**) Expression of dorsoventral markers at mid-segmentation. *Emx* and *Tbr* genes are expressed in dorsal telencephalon (tan). In ventral telencephalon, Dlx2 is expressed in Isl1-negative (green) and Isl1-positive (orange) domains in the lateral ganglionic eminence as well as Nkx2.1-positive medial ganglionic eminence (yellow). Abbreviations: A, anterior; D, dorsal; P, posterior; V, ventral.

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Shh, Gli3 repressor activity is expanded and sufficient to repress ventral fates. In *Shh*; *Gli3* double mutant mouse embryos, ventral telencephalic fates are restored, however dorsal telencephalic tissues are absent (Rallu et al., 2002; Rash and Grove, 2007). It follows that loss of Gli3 activity relieves the repression of ventral telencephalon specification. However, dorsal fate specification requires Gli3 function in a cell-autonomous manner (Quinn et al., 2009). Since ventral telencephalic fates are still specified in the absence of both *Shh* and *Gli3* gene function, there must be an additional ventralizing factor that is sufficient to function in the absence of Hh signaling if *Gli3* is also absent. The most likely candidates are Fgf ligands, which can promote ventral telencephalic fates independently of Hh signaling and are negatively regulated by *Gli3* (Aoto et al., 2002; Gutin et al., 2006). Therefore *Gli3* can affect the size of both dorsal and ventral signaling centers, and also functions to interpret signals to promote dorsal and repress ventral telencephalic fates.

In contrast, *Foxg1* is required to promote ventral telencephalic fates while restricting dorsal ones. During late gastrulation in mouse and zebrafish embryos, expression of *Foxg1* is induced in telencephalon specifically through the function of Tlc and Fgf8 in the ANB and Shh in hypothalamus (Shimamura and Rubenstein, 1997; Houart et al., 2002; Storm et al., 2006; Danesin et al., 2009). *Foxg1* is expressed in a gradient with high levels ventrally (Toresson et al., 1998; Dou et al., 1999), which is opposite of the *Gli3* expression pattern. Loss-of-function experiments indicate that *Foxg1* promotes *Fgf8* expression in the anterior midline to generate ventral telencephalic fates (Martynoga et al., 2005).

However, ventral telencephalic precursors also require *Foxg1* to provide competence to respond to ventralizing signals in a cell-autonomous manner (Manuel et al., 2010). Although explanted *Foxg1* mutant cells are able to respond to Hh and Fgf signaling through expression of downstream transcriptional targets, they are unable to adopt ventral fate possibly due to increased Gli3 processing (Manuel et al., 2010). Additionally, *Foxg1* functions to restrict dorsalizing signals such as Bmp and Wnt ligands (Dou et al., 1999; Martynoga et al., 2005; Hanashima et al., 2007; Danesin et al., 2009), thereby further supporting a prominent role for *Foxg1* in ventral telencephalon specification. Interestingly, telencephalon fails to form in mouse embryos deficient in both *Foxg1* and *Gli3* gene functions, further suggesting that these genes are essential for ventral and dorsal telencephalon specification, respectively (Hanashima et al., 2007; Hebert and Fishell, 2008).

During early segmentation stages, dorsal and ventral telencephalic precursors receive signals that specify different cell types in each domain. By mid-segmentation, specific gene expression patterns are evident along the telencephalic DV axis. Dorsal telencephalic precursors predominantly generate projection neurons that comprise the cerebral cortex or pallium. Early markers of this cell fate include transcription factors of the *empty spiracles* gene family, Emx1 and Emx2, as well as T-box transcription factors, Tbr1 and Tbr2 (Figure 4B). *Emx* and *Tbr* genes are expressed during early steps of the neuronal differentiation pathway, in neural epithelium and basal progenitors, respectively, of cells that will eventually become glutamatergic projection neurons. Ventral

telencephalic progenitors predominantly become cortical interneurons, oligodendrocytes, and striatal/subpallial projection neurons. One of the earliest markers of ventral telencephalic fate is the *distalless* family transcription factor Dlx2, a marker of progenitor cells that are still in a proliferative state. In mammals, Dlx2-positive cells populate the medial (MGE) and lateral ganglionic eminences (LGE), which subdivide ventral telencephalon into two morphologically distinct regions on each side of the midline. The MGE is the most ventromedial region in the telencephalon, whereas the LGE is located more dorsolaterally to it. Several genes such as *Dlx2* are expressed in both regions, however each region also expresses characteristic transcription factors that generate specific cell types (Figure 4B). In addition to Dlx2, Nkx2.1 is expressed in MGE, and produces cells that give rise to cholinergic neurons of the pallidum region of the basal ganglia as well as GABAergic neurons that migrate to the cerebral cortex (Sussel et al., 1999). Several genes encoding additional transcription factors are expressed in the LGE, including *Er81* and *Isl1*, which are required to generate olfactory neurons and striatal projection neurons, respectively (Stenman et al., 2003). Additional transcription factors that can influence cell fate are expressed in the MGE and LGE during development (Flames et al., 2007), however high-resolution fate mapping of many of these regions has yet to be carried out.

Ventral and dorsal telencephalon domains are established by transcription factor expression, and the borders of these expression domains are often defined through repression by transcription factor activity in the neighboring domain. For

example, the pallial-subpallial border is aligned by mutual transcriptional repression between LGE-derived Gsh2 and pallial-derived Pax6. In *Gsh2* or *Pax6* single mutant mouse embryos, the pallial-subpallial border is shifted ventrally or dorsally, respectively (Yun et al., 2001). However, significant improvements are seen in the regions surrounding the pallial-subpallial border in *Gsh2*; *Pax6* double mutant embryos, suggesting these neighboring genes regulate opposing genetic programs (Toresson et al., 2000). Thus, signaling centers induce the expression of specific transcription factors at different locations, and these expression domains may then be refined through mutually repressive transcriptional activity.

As development proceeds, cell proliferation and migration facilitate the expansion of bilaterally symmetric telencephalic vesicles. Notch signaling maintains a balance between developing neurons and remaining progenitors by preserving progenitor cells in a proliferative state. Local reduction of Notch signaling promotes neuronal differentiation by relieving repression of proneural basic helix-loop-helix transcription factors such as Neurogenins in dorsal telencephalon or Mash1 in ventral telencephalon (reviewed in Ross et al., 2003). In mammals, cortical progenitors establish layers of the cerebral cortex, which evaginates radially. Starting at mid-segmentation, neurons are born from progenitors at or near the ventricular zone, which is located at the center of the neural tube. They then migrate individually in radial fashion and complete the differentiation program by extending neurites and forming synapses. The earliest born neurons migrate a short distance and populate the deep layers of the

cortex. As additional neurons are born, they migrate to the most superficial layer such that the cerebral cortex is generated in an inside-out fashion. Subpallial cells that become GABAergic interneurons also migrate tangentially from the MGE and LGE to intersperse with projection neurons in the cortex (reviewed in Marin and Rubenstein, 2003). In contrast, the telencephalon of ray-finned fish species such as zebrafish does not evaginate as in mammals. These fish undergo telencephalic eversion where the dorsomedial-most telencephalic cells migrate laterally en masse until they meet ventral pallial cells. Radial migration of individual pallial cells does not occur, but interneurons do undergo tangential migrations as in mammals. This generates adult brain morphology very different from that observed in mammals, however functional similarities are still present in homologous structures (Wullimann and Mueller, 2004). Despite vast differences in morphogenetic movements and adult forebrain anatomical structures, early development and pattern generation of telencephalon is remarkably similar in mammals and ray-finned fish.

Holoprosencephaly

Abnormal forebrain development occurs in a small percentage of human embryos and can produce phenotypes that range from embryonic lethality to mild cognitive impairment. Holoprosencephaly (HPE), whereby the forebrain fails to fully separate into two hemispheres, is the most common birth defect of the human forebrain, arising in 1:250 conceptuses and 1:8,000 live births (Cohen, 2006; Leoncini et al., 2008). HPE encompasses a wide phenotypic spectrum and

can be divided into two categories, classic HPE and middle interhemispheric variant (MIHV) (reviewed in Cohen, 2006; Dubourg et al., 2007). Classic HPE is described according to the level of severity, and has ventrally biased neuropathologies. Its most severe form, alobar HPE, is characterized by a single lobed brain with no separation of deep nuclei, a single brain ventricle, and absence of the olfactory bulbs and corpus callosum. Patients with the somewhat less severe form, semilobar HPE, have partial separation of cerebral hemispheres, variable separation of deep nuclei, and hypoplasia or absence of olfactory bulbs and corpus callosum. Lobar HPE is the least severe form of classic HPE. Patients with lobar HPE generally have two well-developed cerebral lobes with distinct interhemispheric division. However, separation of the frontal lobes may be incomplete. Olfactory bulbs and corpus callosum may be normal, hypoplastic, or absent. MIHV cases show predominantly dorsal neuropathology with a failure in separation of posterior frontal and parietal lobes and a hypoplastic corpus callosum, but with full separation of hypothalamus and deep gray nuclei. In classic HPE, corresponding midline facial defects are also observed including cyclopia or hypotelorism, proboscis, single maxillary incisor, cleft lip and/or palate. In many cases, "the face predicts the brain," which means the severity of facial defects correlates with the severity of brain defects. Severely affected patients typically do not survive infancy, however less severely affected individuals may have relatively normal life spans.

The etiology of classic HPE is very heterogeneous. Approximately 25% of HPE cases arise in syndromes with multiple malformations and normal

karyotypes. Chromosomal abnormalities, most commonly trisomy 13, account for 25-50% of affected individuals. Monogenic lesions account for approximately 25% of cases, and have been identified in genes that modulate the Nodal or Hh signaling pathways, predominantly *SHH*, *ZIC2*, *SIX3*, and *TGIF*. Environmental factors such as maternal diabetes, consumption of alcohol during pregnancy and prenatal exposure to teratogens may increase the risk of HPE by yet undefined mechanisms. Additionally, drugs that inhibit cholesterol biosynthesis increase the risk of HPE, likely by affecting SHH processing and/or signaling (Schachter and Krauss, 2008). The etiology of MIHV is not well understood, however impaired Bmp signaling and/or roof plate development are able to generate MIHV in mouse models (Cheng et al., 2006; Fernandes et al., 2007; Monuki, 2007; Klingensmith et al., 2010).

A main focus of HPE research has been to elucidate the mechanisms by which monogenic lesions generate the phenotype. Most monogenic lesions have been identified as heterozygous hypomorphic mutations in humans that exhibit incomplete penetrance and variable expressivity (Cohen, 2006; Domene et al., 2008; Geng et al., 2008; Lacbawan et al., 2009; Roessler et al., 2009a; Roessler et al., 2009b; Keaton et al., 2010). A conclusion of these genetic studies in humans and animal model systems is that hypomorphic mutations become haploinsufficient when combined with other genetic mutations or environmental insults, thus generating the wide phenotypic spectrum seen in patients. Of the identified and candidate classic HPE genes in humans, most have now been shown to mediate Nodal or Hh signaling. Nodal signaling is required for

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specification of mesoderm, including PCM and notochord, and of floor plate, which are all potent sources of Shh ligand (Krauss et al., 1993; Conlon et al., 1994; Feldman et al., 1998; Schier, 2009). In zebrafish embryos, *shha* misexpression is epistatic to loss of Nodal signaling in telencephalon DV patterning (Rohr et al., 2001). Mutations in human *SHH*, as well as in genes encoding components of the Hh signaling such as *PTC1*, have been linked to HPE (Ming et al., 2002). Zebrafish *zic2* has previously been shown to modulate Hh signaling during forebrain patterning, and mouse Six3 can directly activate transcription of human *SHH* from a remote enhancer that is active in rostral diencephalon ventral midline during embryonic development (Jeong et al., 2008; Sanek et al., 2009). Interestingly, mouse and zebrafish embryos with disruptions in Fgf signaling can also exhibit HPE and associated midline defects, however mutations in genes encoding Fgf pathway components have not been identified in human cases. Fgf signaling controls many other processes in addition to forebrain development, such that human embryos with a genetic disruption of this pathway may be misclassified as syndromic. As Fgf signaling functions downstream of Hh signaling in telencephalon patterning (Gutin et al., 2006), mild disruptions of the Fgf signaling pathway may be a contributing factor to the penetrance and expressivity other monogenic lesions that promote HPE. In addition to patterning signals, several genes that affect cell movements have also been implicated in HPE in model organisms. For example, genetic mutations or misexpression of synthetic RNAs that disrupt convergence and extension movements during gastrulation may induce cyclopia in zebrafish embryos

(Marlow et al., 1998; Heisenberg et al., 2000; Yeo et al., 2001; Ciani et al., 2003; England et al., 2006). One hypothesis to explain the effect of these molecules on separation of the eye field and forebrain is that the molecules are required by progenitors in this region to undergo proper morphogenesis, possibly by maintaining the relative position of the Shh-producing and receiving cells (Marlow et al., 1998). Another plausible mechanism suggests that axial mesoderm extension is required for the PCM to migrate far enough anteriorly to where it can signal to the forebrain and eyes (Heisenberg et al., 2000). The pleiotropy of the genes that affect convergence and extension movements during gastrulation likely preclude their linkage to HPE in humans, however weak hypomorphic mutations may contribute to the HPE phenotype in the presence of additional mutations. Gaining insight into mechanisms and genetic interactions of known HPE genes in normal forebrain development may identify such novel genetic modifiers that contribute to the phenotypic severity or penetrance of HPE.

The Roles of *Six3* in Early Embryogenesis

Six3 is a homeobox gene

Six3 is a gene involved in many aspects of early forebrain development in vertebrates. Importantly, approximately 1.3% of human HPE cases are associated with heterozygous mutations in human *SIX3* (Cohen, 2006). Six3 belongs to the Sine Oculis Homeobox (SIX) family of transcription factors, each of which contains two main evolutionarily conserved regions, a homeodomain and a SIX domain, which are involved in DNA binding and protein-protein

interactions, respectively. In vertebrates, there are six members of the SIX gene family, which group pair-wise into three classes derived from homology to the *sine oculis*, *Six4*, and *optix* genes in *Drosophila melanogaster*. Each pair of vertebrate SIX family members, Six1 and Six2 (most closely related to Sine oculis), Six4 and Six5 (most closely related to *Drosophila* Six4), and Six3 and Six6 (most closely related to Optix), segregate structurally and functionally from the other pairs. In vertebrates and invertebrates, SIX family members have divergent expression patterns, functions and genetic interactions (Christensen et al., 2008). However, the closely related *Six3* and *Six6* genes are both expressed in the developing vertebrate head and have similar DNA binding capabilities (Oliver et al., 1995; Bovolenta et al., 1998; Seo et al., 1998a; Seo et al., 1998b; Jean et al., 1999; Lopez-Rios et al., 1999; Zhou et al., 2000; Jeong et al., 2008). They may have some redundant functions, however *Six3* is expressed much earlier than *Six6*, and their expression domains are overlapping but not identical. Also, mutations in human *SIX6* have not been associated with HPE.

In addition to the SIX domain and homeodomain, Six3 contains several other intriguing structural features. Six3 and Six6 have two eh1-like motifs that have been shown to bind to Groucho co-repressors (Zhu et al., 2002; Lopez-Rios et al., 2003). Indeed, Six3 has been shown to function as a transcriptional repressor, however there is evidence that it can also act as a transcriptional activator in some contexts (Lagutin et al., 2003; Gestri et al., 2005; Jeong et al., 2008; Beccari et al., 2012). The carboxy-terminal domain may play a role in determining protein binding partners, and this region is somewhat divergent

between Six family members, providing a means for diversification of gene function (Hu et al., 2008; Kumar, 2009; Weasner and Kumar, 2009). Also, two possible translation initiation sites have been noted for *Six3* transcripts in several vertebrates, however functional studies have not been performed to assess the relative activity or function of each potential product (Oliver et al., 1995; Seo et al., 1998a).

Six3 regulates multiple stages of vertebrate forebrain development

Six3 homologs have been identified in many metazoans. Studies in invertebrate animals suggest a role for *Six3* homologs in anterior neural patterning. In the sea urchin, *Strongylocentrotus purpuratus*, *six3* is expressed in the animal pole domain, which will specify tissue around the developing mouth including neural ectodermal cells. Loss of *six3* function in these embryos results in a reduction of neurons in this region (Wei et al., 2009). The *Six3* homolog in *Drosophila melanogaster*, *optix*, is expressed broadly in the procephalic neuroectoderm where the brain originates, and in other anterior regions that will give rise to eyes and some mesoderm structures such as the pharynx (Seo et al., 1999). Studies characterizing the function of *optix* have focused on its role in eye development, where *optix* misexpression is sufficient to induce ectopic eyes (Seimiya and Gehring, 2000). No studies on *optix* to date have analyzed loss of function in *Drosophila* embryos or focused on potential roles in anterior patterning or brain development. Together, analyses of expression and function

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of *Six3* homologs in invertebrates suggest that *Six3* may play a conserved role in embryonic patterning of anterior neural ectoderm.

The embryonic expression patterns of *Six3* homologs in vertebrates support a similar role in anterior neural patterning. *Six3* expression is remarkably well conserved across vertebrate species. Initial expression at mid-gastrulation is broadly noted in anterior neuroectoderm, including presumptive telencephalon, eye, diencephalon and midbrain. As segmentation proceeds, *Six3* expression progressively becomes restricted to the ventral forebrain and eyes (Oliver et al., 1995; Bovolenta et al., 1998; Kobayashi et al., 1998; Loosli et al., 1998; Seo et al., 1998a; Seo et al., 1998b; Zhou et al., 2000). By the end of segmentation stages in mouse embryos (embryonic day 14.5), *Six3* expression in forebrain is present in ventral telencephalon, hypothalamus, ventral thalamus and posterior pretectum while eye expression is noted in optic chiasm, optic stalk, neural retina and lens (Oliver et al., 1995). Expression has also been noted in the PCM of some, but not all, vertebrate species during gastrulation stages (Bovolenta et al., 1998; Kobayashi et al., 1998; Seo et al., 1998a; Seo et al., 1998b; Geng et al., 2008). The broad early and more restricted later expression suggest that *Six3* may have multiple roles in anterior neuroectoderm development from induction through later differentiation steps.

Despite the relative paucity of data on the role of *Six3* homologs in invertebrate development, a number of functional studies in vertebrate species have confirmed a role in anterior neural patterning as well as identified more specific functions in forebrain and eye development. As previously mentioned,

mutations in human *SIX3* have been linked to HPE (Wallis et al., 1999). However, initial loss- and gain-of-function studies in model organisms did not phenocopy human fetuses associated with heterozygosity for *SIX3* mutations. Misexpression of *Six3* led to increased size of the forebrain and eyes in zebrafish embryos (*six3a*) or ectopic retina in *Xenopus* and medaka embryos (*Six3.1*) (Kobayashi et al., 1998; Loosli et al., 1999; Bernier et al., 2000). Increased forebrain and eye size from misexpression of *Six3* may result in part from direct repression of *Bmp4* expression by Six3, thereby expanding the size of the anterior neural plate (Gestri et al., 2005). Conversely, mouse and medaka (*Six3.1*) embryos with a complete or strong loss of *Six3* function lacked forebrain and eyes, suggesting a requirement for *Six3* in early anterior patterning of the neural plate (Carl et al., 2002; Lagutin et al., 2003). However, the size and shape of the neural plate appeared unaffected in *Six3* homozygous mutant mouse embryos, suggesting loss of *Six3* is insufficient to affect neural plate induction (Lagutin et al., 2003). Instead, *Six3* is required to regulate patterning in the anterior neural plate. In *Six3* homozygous knockout mouse embryos, posterior diencephalon and midbrain are expanded at the expense of telencephalon, prethalamus, and eyes. This phenotype was shown to result from a disruption of direct repression of *Wnt1* expression by Six3, whereby *Wnt1* expression was anteriorly expanded in the *Six3* mutant embryos. Interestingly, mice heterozygous for the inactivated *Six3* locus showed a partial expansion of *Wnt1* expression despite appearing morphologically normal (Lagutin et al., 2003). Inactivation of the *Wnt1* locus is sufficient to suppress the *Six3* knockout

phenotype in diencephalon but not telencephalon, suggesting *Six3* has additional roles in establishment or maintenance of telencephalic tissue in addition to repressing *Wnt1* (Lavado et al., 2008). These functional studies outlined a role for *Six3* in early steps of forebrain induction, which may be temporally upstream of its role in HPE.

Several studies have addressed the genetic aspects of *Six3*-mediated HPE. Heterozygous mutations in human *SIX3* have been correlated to HPE, and they have been identified as mainly missense or nonsense mutations (Lacbawan et al., 2009). In medaka embryos injected with MO to disrupt *Six3.1* expression, forebrain and eyes are reduced or absent at high MO doses while at low MO doses embryos develop small eyes or cyclopia, which correlates with increased cell death in the anterior neural tube (Carl et al., 2002). Recent studies have quantitatively tested the activity of human SIX3 mutations correlated with HPE through misexpression of the mutant proteins in zebrafish embryos. The misexpressed mutant proteins had less activity than misexpressed wild-type SIX3 in a variety of assays, suggesting that these mutations result in only partial loss of SIX3 function (Domene et al., 2008; Geng et al., 2008). Mutations exhibiting greater loss of activity in zebrafish misexpression assays correlated with increased severity of classic HPE in human patients (Lacbawan et al., 2009). As previously mentioned, HPE was not observed in initial studies with *Six3* heterozygous knockout mouse embryos (Lagutin et al., 2003). However, additional studies later showed that HPE can be observed in mouse embryos with a heterozygous disruption of *Six3* with incomplete penetrance in a

background-dependent manner. Importantly, heterozygous disruption of *Six3*, achieved by knocking a missense mutation correlated with human HPE into the endogenous *Six3* locus, further validated this as a mouse model of *Six3*-mediated HPE (Geng et al., 2008). The penetrance of *Six3*-mediated HPE in this mouse model was increased to near 100% in heterozygous *Six3* knock-in mouse embryos that were also heterozygous for an inactivating mutation in *Shh*. Moreover, it was shown that *Six3* and *Shh* function in a positive feedback loop to regulate each other's transcription in the rostral diencephalon ventral midline (Geng et al., 2008; Jeong et al., 2008). The activation of *Shh* transcription by Six3 is direct, and the enhancer is evolutionarily conserved from frogs to humans (Jeong et al., 2008). These studies linked Six3 and Hh signaling in the generation of HPE. Together, data characterizing the *Six3* knock-in and knockout mice suggests a model where partial Six3 function is sufficient to specify telencephalon, while full Six3 function is required to ensure its normal midline development.

Recent studies have elucidated additional non-HPE roles for *Six3* in specific tissues in the developing forebrain and eyes. One such role is the maintenance of posterior diencephalic fates. In *Six3* homozygous knockout mouse embryos, thalamus is initially specified but not maintained, and it is replaced by the more posterior pretectum (Lavado et al., 2008). In addition, in mouse embryos with *Rx3-Cre* driving conditional loss of *Six3* function in eye progenitors, specification of neural retina does not occur despite normal optic vesicle development. The loss of neural retina is attributed to an expansion in

Wnt8b expression that Six3 directly represses (Liu et al., 2010). Additional studies are necessary to identify novel functions and further characterize the known roles that *Six3* plays in forebrain and eye development.

<u>*Six3* can regulate cell fate, proliferation and death</u>

The size and number of cell populations are influenced by several cellular mechanisms including cell migration, fate specification, proliferation and apoptosis. In addition to changes in cell fate described above, altered proliferation and apoptosis have been shown to be involved in *Six3* mutant phenotypes. Six3 has been shown to regulate the transition between cell proliferation and differentiation. For example, Six3 physically binds directly to the DNA replication inhibitory protein Geminin, and it is thought that this association sequesters Geminin from its role in inhibiting Cdt1-dependent progression through the cell cycle. The same study supported the physical interaction by analyzing the antagonistic genetic interaction between *Six3* and *Geminin*, whereby loss of *Geminin* function phenocopies the increased forebrain and eye phenotype seen in embryos misexpressing *Six3.1* (Del Bene et al., 2004). This suggests that Six3 may promote proliferation of neural progenitors cell-autonomously and maintain them in an undifferentiated state, while Geminin inhibits proliferation and promotes differentiation. Experiments in mouse cortical progenitors overexpressing Six3 or Geminin support this hypothesis. Cultured cortical progenitors misexpressing *Six3* have an increase in the average number of cells per clone, and these cells are in a less differentiated state.

Overexpression of *Geminin* has the opposite phenotype (Appolloni et al., 2008). Misexpression of *Six3* also promotes increased mRNA levels of positive cell cycle regulators such as *CyclinD1*, *CyclinD2, Cdk4*, and *Cdc37* and may reduce levels of the cell cycle inhibitor *p27* in cultured mouse cortical progenitors and *Xenopus* embryos (Gestri et al., 2005; Appolloni et al., 2008).

In addition to controlling cell proliferation, *Six3* can also inhibit apoptosis. Loss of *Six3.1* in medaka embryos promotes a gross increase in apoptosis in the *Six3.1* expression domain, which correlates with loss of forebrain and eyes (Carl et al., 2002). In addition, excess apoptosis was noted in the diencephalon of *Six3* homozygous knockout mouse embryos undergoing progressive caudalization because of their inability to maintain thalamic tissue (Lavado et al., 2008). However, no direct interaction has been identified between Six3 and any known apoptotic genes. Six3 may also exert influence on cellular mechanisms indirectly through direct regulation of expression of an increasing number of extracellular signaling molecules that have their own influences on cell fate choice, proliferation and apoptosis. Therefore, context plays a prominent role in *Six3* function.

Use of zebrafish to study *Six3* function

Most vertebrates have a single *Six3* homolog, however genome and gene duplication events in teleosts have generated multiple *Six3* homologs in medaka and zebrafish (Loosli et al., 1998; Seo et al., 1998a; Seo et al., 1998b; Beccari et al., 2012). The zebrafish genome contains three homologs of mammalian *Six3*,

which are *six3a*, *six3b*, and *six7*. The homeodomains of the zebrafish *Six3-*related genes each share greater than 95% amino acid identity to mouse *Six3*, and greater than 80% amino acid identity is observed in the SIX domain and homeodomains combined (Seo et al., 1998a; Seo et al., 1998b). The expression patterns of the three zebrafish homologs are strikingly similar to that observed for *Six3* in the course of murine embryogenesis (Oliver et al., 1995; Seo et al., 1998a; Seo et al., 1998b). Additionally, the homeodomains of the zebrafish Six3 homologs are capable of binding the same DNA sequence (Suh et al., 2010). Also, loss of function of either *six3b* or *six7* alone exhibits no embryonic phenotype by morphology or molecular marker analysis (Inbal et al., 2007). Together, this suggests that the zebrafish *Six3* homologs may exhibit strong functional redundancies whereby zebrafish can provide a powerful model system to analyze partial loss of *Six3* function as seen in human HPE.

In medaka, identification and characterization of a second *Six3* homolog has unmasked differences in tissue sensitivity to loss of *Six3* function. Expression of each medaka homolog is largely overlapping but not identical (Loosli et al., 1998; Beccari et al., 2012), similar to the situation in zebrafish. Consistent with their expression patterns in medaka embryos, reduction of eyes is more prominent than defects in forebrain in *Six3.1* morphants, while *Six3.2* morphants present with more prominent defects in telencephalon and hypothalamus compared to eyes (Carl et al., 2002; Beccari et al., 2012). Gene duplication events often lead to changes in the regulatory regions of DNA whereby expression of each homolog is spatially restricted to a part of the whole

domain. For example, the *nkx2.1a* and *nkx2.1b* expression domains in zebrafish together approximate the expression of *Nkx2.1* in mouse embryos (Sussel et al., 1999; Rohr et al., 2001). Alternatively, duplicated genes may have largely overlapping or identical expression patterns and perform redundant functions in the same tissue. Expression levels may be adjusted by evolutionary changes in regulatory regions to maintain overall gene activity within appropriate thresholds. In the latter scenario, loss of one or more such homologs may represent an equivalent of an "allelic series" of a single-copy gene and facilitate characterization of discrete hypomorphic phenotypes as is the case for *Six3* in medaka.

In zebrafish, a third *Six3* homolog gives the potential of additional data points in "a functional allelic series". All three homologs have overlapping expression patterns during gastrulation and early segmentation. However, by the 6-somite stage *six7* expression becomes reduced and is absent by the 12-somite stage, whereas expression of *six3a* and *six3b* continues through adulthood (Seo et al., 1998a; Seo et al., 1998b). This suggests that highest levels of *Six3*-related activity may be required during early development. A previous study using a MO directed against both *six3a* and *six3b* showed reduction of forebrain and eyes similar to, but less severe than, that observed in *Six3* homozygous knockout mouse embryos or medaka *Six3.1* morphant embryos (Carl et al., 2002; Lagutin et al., 2003; Ando et al., 2005). However, in zebrafish embryos with combined loss of *six3b* and *six7*, eyes are reduced or absent but AP patterning in the CNS appears relatively normal. Brain laterality and asymmetric Nodal signaling are

affected in the epithalamus of *six3b;six7*-deficient embryos showing that although AP patterning is normal, specific cell populations are affected by loss of *Six3*-related gene function (Inbal et al., 2007). A thorough analysis of specific cell populations has not been performed in either *six3a;six3b*- or *six3b;six7*-deficient zebrafish embryos despite the relatively mild morphological defects compared to full or partial loss of *Six3* in other model organisms. Further analysis of loss of different combinations of *Six3*-related genes may uncover additional discrete *Six3* functions and provide a zebrafish model of *Six3*-mediated HPE.

Aims of the Dissertation

The broad goal of the studies described in this dissertation is to elucidate the genetic, cellular and embryological mechanisms used by *Six3* in patterning the anterior CNS. The intent was to establish a zebrafish model of *Six3*-mediated HPE. The rapid development and powerful genetics of the zebrafish model offer a means for identifying genetic interactions that could influence *Six3* function in forebrain development and analyzing the cellular mechanisms employed in generating related phenotypes. If function of *Six3* were distributed over several loci in the zebrafish, we hypothesized that we may uncover novel functions of *Six3* by removing one or more homologs. Moreover, we anticipated that one of such genetic scenarios would phenocopy HPE observed in humans. Our group had previously identified a null allele of *six3b*, *six3b^{vu87}*, and characterized a functional MO directed against the 5' untranslated region of *six7* (MO1-*six7*),

which each exhibit no phenotype on their own (Inbal et al., 2007). As described above, initial studies identified a role for these genes in eye development and brain laterality, however further investigation described in the following chapter identified a role for *six3b* and *six7* in generating proper DV patterning in telencephalon, a process disrupted in HPE.

Chapter II of this dissertation continues the characterization of the phenotype of *six3b;six7*-deficient embryos begun by a former post-doctoral researcher, Adi Inbal. My work describes the requirement of *six3b* or *six7* for proper telencephalon patterning. In embryos deficient in both genes, the telencephalon is specified, however markers of subpallial fates are markedly reduced or absent with a concomitant expansion of pallial markers. This phenotype is similar to patterning defects present in HPE, however no midline defects were observed in *six3b;six7*-deficient embryos. Although *Six3* can regulate cellular proliferation and apoptosis, the reduction in ventral telencephalic fates is most likely due to altered fate specification. Unlike in the mouse model of *Six3*-mediated HPE, reduced Hh signaling is not the cause of the disrupted DV patterning. Instead, reduced *foxg1a* expression and expanded *wnt8b* expression may play a role in generating the *six3b;six7*-deficient phenotype. We further sought to address whether all subpallial cell populations respond similarly to misexpression of *six3b*. Misexpression of *six3b* requires both Hh signaling and *foxg1a* function to expand the ventromedial *nkx2.1b* domain. In contrast, *six3b* misexpression can promote the dorsolateral subpallial *isl1*-positive cells in the

absence of Hh signaling or *foxg1a* function. These findings uncover novel roles for *Six3* in telencephalon DV patterning that are independent of Hh signaling.

The differential roles of *Six3* expression in PCM and anterior neuroectoderm have not been determined. *Six3* and *foxg1a* have been shown to directly repress *Wnt8b* expression in mouse and zebrafish embryos, respectively (Danesin et al., 2009; Liu et al., 2010). Therefore, it is likely that anterior neuroectoderm expression of *six3b* and *six7* is responsible for regulation of *wnt8b*. Six3.2 has also been shown to directly activate *Foxg1* expression in medaka embryos (Beccari et al., 2012), however Six3, possibly functioning in PCM, may also regulate expression of both *foxg1a* and *wnt8b* indirectly. It is also possible that signals from PCM may influence DV patterning in telencephalon independently of *foxg1a* and *wnt8b*. Tissue-specific transgenic replacement of *six3b* expression in *six3b;six7*-deficient embryos will help determine whether *six3b* function is required in PCM, anterior neuroectoderm or both regions to promote ventral telencephalon fates. Previous work from our group has characterized a transgenic line that can drive reporter gene expression under the control of the *goosecoid* (*gsc*) promoter specifically in PCM and notochord (Inbal et al., 2006). I generated a stable transgenic zebrafish line that utilizes the regulatory region of the *Hesx1* gene from the chicken, *Gallus gallus*, to misexpress reporter genes in anterior neuroectoderm (Spieler et al., 2004). Chapter III of this dissertation describe novel transgenic lines generated to assess the tissue autonomy of *six3b* and *six7* function in telencephalon DV patterning.

Together my studies establish *Six3*-related gene function as a necessary regulator of DV patterning in telencephalon. Previous work in several model organisms has identified multiple functional interactions of *Six3*. I suggest a novel role for the interaction between *six3* and *wnt8b* in patterning ventral telencephalon independently of Hh signaling, and describe tools to further address the tissue autonomy of *Six3* function. Future research incorporating a combined loss of function including *six3a* may uncover additional interactions to gain a clearer picture of the roles of *Six3* at different stages of forebrain development.

CHAPTER II

Six3 COOPERATES WITH HEDGEHOG SIGNALING TO SPECIFY VENTRAL
TELENCEPHALON BY PROMOTING EARLY EXPRESSION OF Foxg1 AND
REPRESSING WNT SIGNALING

Summary

      Six3 exerts multiple functions in the development of anterior neural tissue of
vertebrate embryos. Whereas complete loss of Six3 function in the mouse results in
failure of forebrain formation, its hypomorphic mutations in human and mouse can
promote holoprosencephaly (HPE), a forebrain malformation resulting, at least in
part, from abnormal telencephalon development. However, Six3's roles in
telencephalon patterning and differentiation are not well understood. To address the
role of Six3 in telencephalon development, we analyzed zebrafish embryos
deficient in two of three *Six3*-related genes, *six3b* and *six7*, representing a partial
loss of Six3 function. We found that telencephalon forms in *six3b;six7*-deficient
embryos, however ventral telencephalic domains are reduced and dorsal domains
are expanded. Decreased cell proliferation or excess apoptosis cannot account for
the ventral deficiency. Instead, *six3b* and *six7* are required during early
segmentation for specification of ventral progenitors, similar to the role of Hedgehog
(Hh) signaling in telencephalon development. Unlike in mice, we observe that Hh
signaling is not disrupted in embryos with reduced Six3 function. Furthermore, *six3b*

overexpression is sufficient to compensate for loss of Hh signaling in *isl1-* but not *nkx2.1b*-positive cells, suggesting a novel Hh-independent role for Six3 in telencephalon patterning. We further find that Six3 promotes ventral telencephalic fates through transient regulation of *foxg1a* expression and repression of Wnt/β-catenin pathway.

<u>Introduction</u>

The telencephalon, located in the anterior-most region of the embryonic neural tube, develops into the cerebral cortex and basal ganglia in mammals and the pallium and subpallium in other vertebrates. During early segmentation stages of vertebrate embryogenesis, the telencephalon becomes patterned along its dorsoventral (DV) axis, as evidenced by restricted expression domains of numerous genes. Dorsally located progenitors generate cortical projection neurons, while ventral progenitors generate striatal projection neurons, as well as interneurons and oligodendrocytes (Wilson and Rubenstein, 2000). Cooperation between many molecules, including ligands secreted from local signaling centers and regionally expressed transcription factors, determines the size and fate of telencephalic progenitor domains (Wilson and Rubenstein, 2000; Hebert and Fishell, 2008). However, the exact functions of genes involved in DV patterning of the telencephalon and the interactions between these genes are still not well understood.

The homeodomain transcription factor Six3 has been shown to regulate a number of events that are involved in telencephalon development in several

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vertebrates. Beginning at late gastrulation, *Six3* is expressed broadly in the anterior neuroectoderm (Oliver et al., 1995; Bovolenta et al., 1998; Kobayashi et al., 1998; Loosli et al., 1998; Seo et al., 1998a; Seo et al., 1998b; Zhou et al., 2000), where it may function in controlling the expression of extracellular ligands that influence telencephalic development, as well as providing competence to respond to such signals (Kobayashi et al., 2002; Lagutin et al., 2003; Gestri et al., 2005; Jeong et al., 2008). Telencephalon induction is severely impaired in *Six3*-null mouse embryos, as well as medaka fish embryos in which Six3 activity is blocked by antisense morpholino oligonucleotides (MOs) (Carl et al., 2002; Lagutin et al., 2003; Lavado et al., 2008). By contrast, dominant mutations in human *SIX3* have been linked to holoprosencephaly (HPE) (Wallis et al., 1999; Lacbawan et al., 2009), a congenital malformation in which the telencephalon forms, but forebrain midline structures are disrupted, resulting in ventrally biased neuropathologies and failure of telencephalic hemispheres to separate (Dubourg et al., 2007; Monuki, 2007). A study with a recently generated mouse model of Six3-mediated HPE suggested that reduced Six3 function disrupts a positive feedback loop between Six3 and Sonic Hedgehog (Shh) (Geng et al., 2008), thereby linking Six3 with Hedgehog (Hh) signaling pathway activity, which is critical for normal telencephalon DV patterning (Chiang et al., 1996). The regulation of the *SHH* gene by SIX3 is conserved in humans, as shown by the ability of mouse Six3 to bind to a conserved enhancer element upstream of the human *SHH* gene and directly activate transcription from this element (Jeong et al., 2008). Together, studies in human, mouse and zebrafish demonstrated that

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most *SIX3* mutations associated with HPE are hypomorphic alleles, that can become haploinsufficient when Shh activity is reduced by other mutations (Domene et al., 2008; Geng et al., 2008; Jeong et al., 2008; Lacbawan et al., 2009). However, it remains an open question whether regulation of Shh expression is the only mechanism by which Six3 influences DV telencephalic development.

In addition to Hh signaling, several Wnt ligands are expressed posterior to the telencephalon anlage, and some have been shown to affect telencephalic DV patterning (Ciani and Salinas, 2005). Six3 has been shown to repress directly expression of both *Wnt1* and *Wnt8b* in mouse embryos, thereby affecting telencephalon induction and patterning of the eye field, respectively (Lagutin et al., 2003; Liu et al., 2010). However, a link between Six3 and Wnt signaling in telencephalon DV patterning has not been established.

Here we use the zebrafish, *Danio rerio*, which has three orthologs of the mammalian *Six3* gene in its genome, *six3a*, *six3b* and *six7* (Seo et al., 1998a; Seo et al., 1998b), to dissect the role of Six3 in telencephalon patterning. Zebrafish embryos that are deficient in both *six3b* and *six7* function exhibit severely reduced eye size and abnormalities in left-right brain asymmetry, yet have largely normal anterior-posterior patterning of the central nervous system (CNS) (Inbal et al., 2007). Our current work shows that *six3b;six7*-deficient embryos have a telencephalon, but with DV patterning defects similar to those found in HPE. Unlike in *Six3* mutant mice, the reduction of ventral cell fates is not mediated by reduced Hh signaling, but may be due to reduced expression during

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early segmentation of *foxg1a*, which is required for telencephalic DV patterning downstream of Hh signaling. Analysis of discrete cell populations in the ventral telencephalon revealed that the telencephalic *nkx2.1b* expression domain requires function of Six3, Foxg1a, and Hh signaling, whereas Six3b overexpression can compensate for loss of Foxg1a or Hh signaling in the more dorsolateral *isl1* domain. We further show that loss of Six3 function leads to expanded Wnt/β-catenin pathway activity in the telencephalon anlage, which could contribute to the DV patterning defects. Our results lend support to the notion that Six3 provides competence for anterior neural tissue to respond to Hh signaling, and uncover new Shh-independent mechanisms through which Six3 mediates telencephalon development.

Materials and Methods

Zebrafish strains, embryo culture, and generation of transgenic fish

Adult zebrafish were maintained according to established methods (Westerfield, 1993). Embryos were obtained from natural matings, grown at 28.5°C and staged according to Kimmel (Kimmel et al., 1995). The following published strains were used and genotyped as previously described: wild-type AB, *tp53^{zdf1}* (Berghmans et al., 2005), *six3b^{vu87}* (Inbal et al., 2007), *smo^{b641}* (Varga et al., 2001), *Tg(hsp70l:Gal4-VP16)vu22* (Shin et al., 2007), *Tg(UAS:six3b)vu156* (Inbal et al., 2007), and *Tg(hsp70l:wnt8a-GFP)w34* (Weidinger et al., 2005).

To generate the *Tg(UAS:shha-NH-EGFP)vu486* transgenic line, the coding sequence for zebrafish *shha* was obtained from zShh-T7TS vector (Ekker et al., 1995). A non-helical oligopeptide linker (NH), APAETKAEPMT (George and Heringa, 2002), was inserted upstream of the EGFP coding sequence isolated from pEGFP-C1 (Clontech). To insert NH-EGFP in frame into the Shha coding sequence, unique NheI and XhoI restriction sites were introduced between Ala192 and Ala193 of Shha and flanking NH-EGFP. GFP was released from pT2-UAS-GFP-γCry-GM2 (Inbal et al., 2006), followed by replacement with the Shha-NH-EGFP construct using KpnI and ApaI restriction sites. Transgenic fish were generated using the *Sleeping Beauty* transposon system (Davidson et al., 2003) by co-injecting 15-20 pg pT2-UAS-Shha-NH-EGFP-γCry-GM2 DNA with synthetic RNA encoding SB10 transposase into one-cell stage embryos. Founder fish were identified as previously described (Inbal et al., 2006). Sequences for PCR primers used for cloning pT2-UAS-Shha-NH-EGFP-γCry-GM2 are available upon request.

Morpholino Oligonucleotides (MOs), cell cycle inhibition, heat shock, and cyclopamine treatment

MOs directed against the translation start site of the *foxg1a* gene (MO2-*foxg1a*) (Danesin et al., 2009) and the 5' untranslated region of the *six7* gene (MO1-*six7*) (Inbal et al., 2007) were previously described. Embryos were injected at the 1-2 cell stage with 2-3 ng MO1-*six7* or 1 ng MO2-*foxg1a* for phenotypic analysis.

To inhibit cellular proliferation, mid-gastrula embryos (80% epiboly) were incubated in 30% Danieau's solution containing 20 mM hydroxyurea (Sigma-Aldrich), 150 µM aphidicolin (Sigma-Aldrich) and 2% dimethyl sulfoxide. Control embryos were incubated with 2% dimethyl sulfoxide alone.

Embryos were heat shocked in prewarmed 30% Danieau's solution at 37°C for 30 minutes, and subsequently developed at 28.5°C.

To inhibit Hh signaling, early gastrula embryos (shield stage) were treated with 30% Danieau's solution containing 10 µM cyclopamine hydrate (Sigma-Aldrich) with 0.1% ethanol. Control embryos were treated with 0.1% ethanol alone.

In situ hybridization, immunohistochemistry and TUNEL

Embryos were fixed in 4% paraformaldehyde in phosphate-buffered saline. Whole mount in situ hybridization was performed according to standard protocols and developed with BMPurple (Roche) and Fast Red (Roche) or INT (iodonitrotetrazolium chloride, Sigma-Aldrich). Digoxigenin- or fluorescein-labeled probes were generated from cDNA templates: *axin2* (Carl et al., 2007), *dlx2a* (Akimenko et al., 1994), *emx3* (Morita et al., 1995), *eomesa* (Mione et al., 2001), *foxg1a* (Toresson et al., 1998), *isl1* (Inoue et al., 1994), *nkx2.1b* (Rohr et al., 2001), *ptch2* (formerly described as *ptc1*) (Concordet et al., 1996), *six3b* (Seo et al., 1998a), *six7* (Seo et al., 1998b), and *wnt8b* (Kelly et al., 1995).

Rabbit polyclonal phospho-Histone H3 antibody (Upstate Biotechnology) and Cy3 conjugated goat anti-rabbit antibody (Jackson ImmunoResearch) were applied at 1:3000 and 1:250, respectively.

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) was performed as described (Verduzco and Amatruda, 2011) using an alkaline phosphatase conjugated anti-digoxigenin antibody (1:5000; Roche) and developed with BMPurple.

Image acquisition, analysis, and quantitation

Images were acquired using Zeiss Axiophot, Zeiss Imager Z.1 compound microscope, or Zeiss Discovery.V12 stereomicroscope and an Axiocam digital camera. Images from anti-phospho-Histone H3 and TUNEL labeling were taken in a z-series, and a z-projection was generated using the Extended Focus computation in Axiovision software (Zeiss).

Each individual experiment was performed two to four times and the number of affected and observed embryos was compiled from the total over all experiments. All mutant genotypes were confirmed by PCR or morphology.

Quantification of cell counts and measurements was done with Fiji software (NIH). Cells labeled positive by anti-phospho-Histone H3 antibody within an identically sized region of the anterior body axis or anterior neural tube were counted from z-stacks using the cell counter plug-in for Fiji software (Figure 6). One-dimensional measurements along the DV telencephalon axis in vehicle- and hydroxyurea/aphidicolin-treated embryos were also taken with Fiji software. For

each individual experiment, experimental measurements were determined as a fraction of the average control measurement. Statistical significance was determined by a Student's unpaired t-test with a two-tailed distribution.

Results

The telencephalon of *six3b;six7*-deficient embryos is dorsalized

To gain insight into the roles of Six3 in telencephalic development, we compared patterning of the telencephalon at 24 hours post-fertilization (hpf) between control zebrafish embryos (*six3b$^{vu87/+}$* and *six3b$^{vu87/vu87}$*, identified by PCR and comparable to wild type) and *six3b;six7*-deficient embryos (*six3b$^{vu87/vu87}$* embryos injected with MO1-*six7* inhibiting translation of *six7* mRNA (Inbal et al., 2007)). At this developmental stage, subdomains of telencephalon are evident along the DV axis by distinct gene expression (Rohr et al., 2001). We first examined expression of *foxg1a*, a pan-telencephalic marker encoding a forkhead transcription factor (Toresson et al., 1998). *foxg1a* was expressed in *six3b;six7*-deficient embryos, indicating that telencephalic tissue was specified (Figure 5A,B). Next, we examined expression of *nkx2.1b*, encoding a homeodomain transcription factor that is expressed in the most ventromedial domain of the telencephalon and in the hypothalamus (Rohr et al., 2001). In *six3b;six7*-deficient embryos, telencephalic *nkx2.1b* expression was strongly reduced (Figure 5C,D). Similarly, expression of *isl1*, encoding a LIM homeodomain transcription factor expressed in a subpopulation of ventrolateral telencephalic cells (Inoue et al., 1994), was strongly reduced in *six3b;six7*-deficient embryos (Figure 5E,F). The expression domain of a

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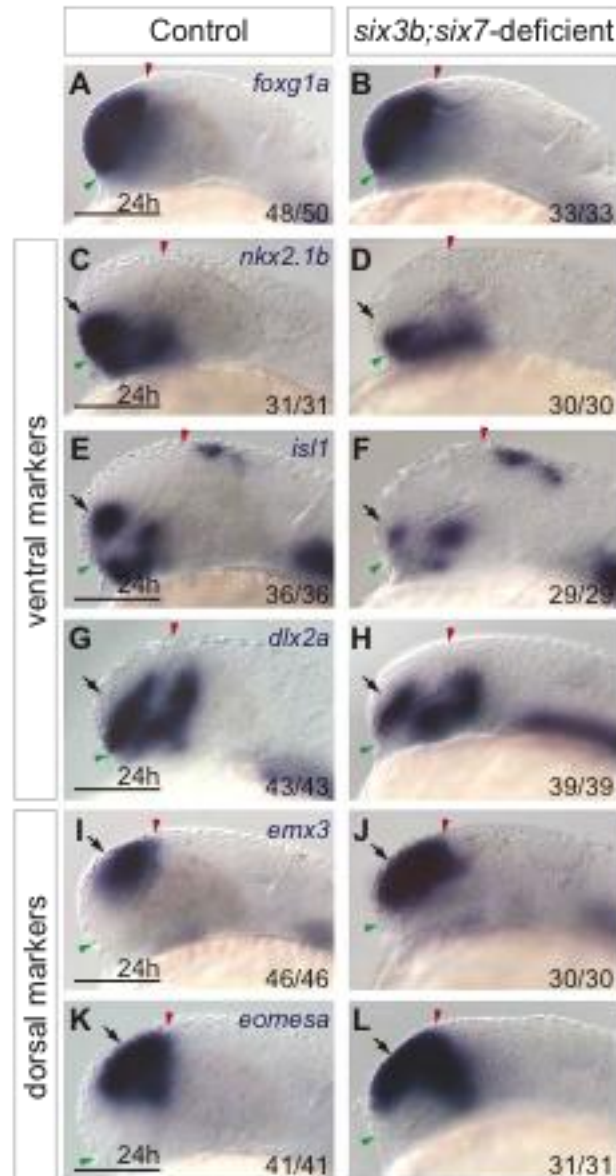
Figure 5. The telencephalon of *six3b;six7*-deficient embryos is dorsalized. (A,B) *foxg1a* expression in control (A) and *six3b;six7*-deficient embryos (B). (**C-H**) Ventral telencephalic expression of *nkx2.1b* (C,D), *isl1* (E,F) and *dlx2a* (G,H) in control (C,E,G) and *six3b;six7*-deficient embryos (D,F,H). (**I-L**) Dorsal telencephalic expression of *emx3* (I,J) and *eomesa* (K,L) in control (I,K) and *six3b;six7*-deficient embryos (J,L). All embryos are 24 hpf. Control embryos denote uninjected *six3b^{vu87/+}* or *six3b^{vu87/vu87}* embryos. Arrows denote telencephalic expression domains. In this and subsequent figures, embryos are shown in lateral view with anterior to the left. Red and green arrowheads point at dorsal and ventral edges of the telencephalon, respectively. Fraction in each panel denotes number of embryos affected over number examined. Scale bars: 100 μm.

third ventral telencephalic marker, the homeobox gene *dlx2a* (Akimenko et al., 1994), was also reduced in *six3b;six7*-deficient telencephalon (Figure 5G,H). In contrast, expression domains of dorsally expressed genes were expanded. We observed that telencephalic domains of both *emx3* and *eomesodermin homolog a* (*eomesa*), which encode homeodomain and T-box transcription factors, respectively, and are normally limited to the dorsal telencephalon (Morita et al., 1995; Mione et al., 2001), were expanded ventrally in *six3b;six7*-deficient embryos (Figure 5I-L). Additionally, the diencephalic domains of *nkx2.1b*, *isl1*, *dlx2a*, and *eomesa* expression were mispatterned in *six3b;six7*-deficient embryos, however in this work we focused our analysis on telencephalon (Figure 5C-H,K,L). Collectively, these data suggest that reduced Six3 function leads to an expansion of dorsal telencephalic fates at the expense of ventral ones, and hence to dorsalization of the telencephalon.

Abnormal proliferation and apoptosis do not significantly contribute to deficiency of ventral telencephalic fates

Decreased proliferation, increased apoptosis, or altered fate specification could contribute to the reduction of ventral telencephalon in *six3b;six7*-deficient embryos. Indeed, Six3 was demonstrated to affect each of these processes (Carl et al., 2002; Lagutin et al., 2003; Del Bene et al., 2004; Gestri et al., 2005; Appolloni et al., 2008). Thus, we first investigated whether decreased proliferation of ventral telencephalic precursors contributed to the reduction in the *nkx2.1b* telencephalic expression domain. Because the exact location of these progenitors at early segmentation stages is not well defined, we could not directly assess their

proliferation. We therefore asked whether global inhibition of cellular proliferation affects telencephalic DV patterning in wild-type embryos. To block proliferation, wild-type embryos were treated with DNA replication inhibitors hydroxyurea (20 mM) and aphidicolin (150 μM) from mid-gastrulation (80% epiboly; 8 hpf) onward. Treatment at this time does not interfere with telencephalon induction and correlates with the onset of *six3b* and *six7* expression in the forebrain anlage (Grinblat et al., 1998; Seo et al., 1998a; Seo et al., 1998b). We counted proliferating cells located within a defined anterior region of the embryos (Figure 6), where ventral telencephalon progenitors are known to reside (Woo and Fraser, 1995). Hydroxyurea/aphidicolin treatment effectively inhibited proliferation within two hours (tailbud stage, 10 hpf), as evidenced by an 81% reduction in the number of cells positively labeled with phospho-Histone H3 antibody, a marker of late G2-M phase (Figure 6A,B,E). Inhibition of proliferation was maintained until the 20-somite stage when we observed a 65% reduction in the number of phospho-Histone H3-positive cells in the anterior neural tube (Figure 6C-E). In 20-somite stage hydroxyurea/aphidicolin-treated embryos, we observed a 9.5% reduction of the DV length of the telencephalon, as defined by *foxg1a* expression, compared to vehicle-treated embryos (Figure 7A,B,G; p<0.01). Notably, *emx3* and *nkx2.1b* expression domains appeared relatively normal in the telencephalon in hydroxyurea/aphidicolin-treated embryos (Figure 7C-F). Quantification of the length of these domains along the telencephalic DV axis showed a slight but statistically insignificant reduction (~5%) compared to vehicle-treated embryos (Figure 7G; p>0.27 for both markers). This discrepancy between the reduction of the

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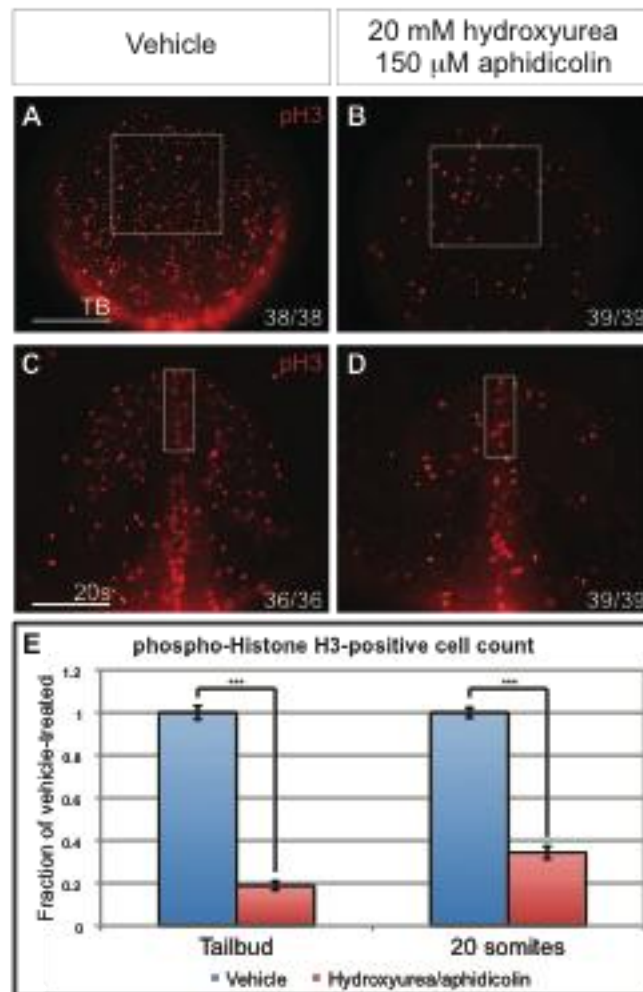
Figure 6. Cellular proliferation is inhibited by hydroxyurea/aphidicolin treatment.
(**A-D**) phospho-Histone H3 labeling in tailbud stage (A,B) and 20-somite stage (C,D) embryos treated with 2% dimethyl sulfoxide alone (A,C) or with 20 mM hydroxyurea and 150 μM aphidicolin from 80% epiboly (B,D). Boxed regions mark anterior neuroectoderm (A,B) and telencephalic region (C,D). Scale bars: 100 μm. (**E**) Graph shows quantification of phospho-Histone H3-positive cells in the boxed regions of panels A-D divided by average cell count in vehicle-treated embryos. n=10 embryos quantified for each sample. Blue and red columns denote vehicle- and hydroxyurea/aphidicolin-treated embryos, respectively. Error bars denote standard error of the mean. *** signifies p < 0.001.
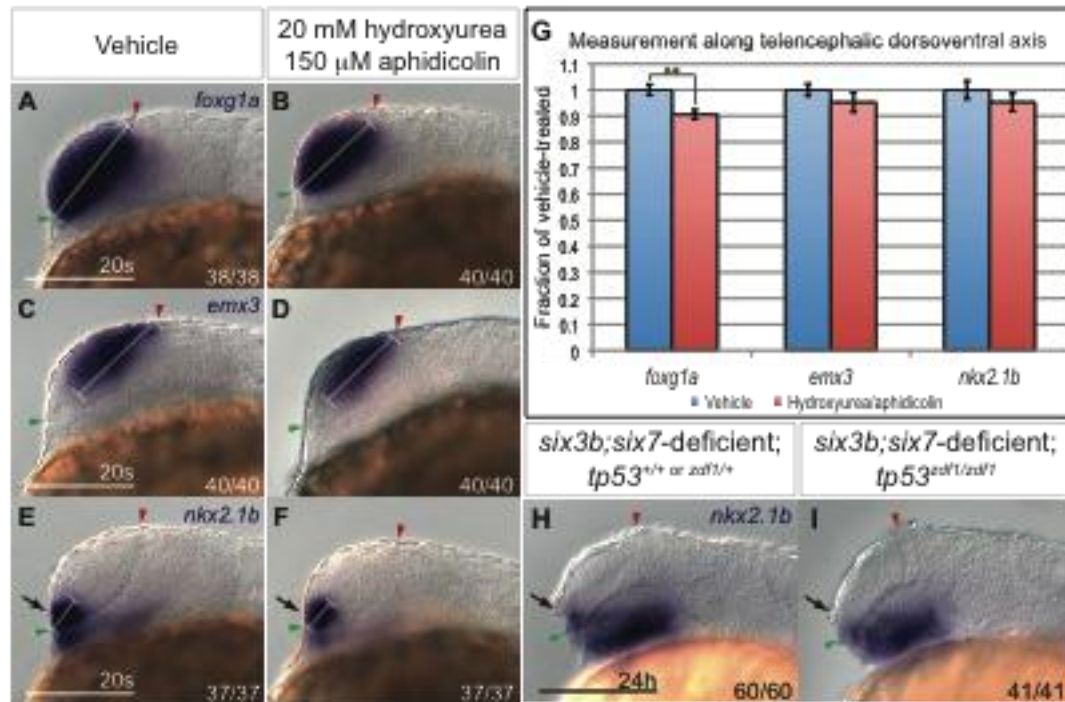
Figure 7. Cellular proliferation and apoptosis do not significantly contribute to reduction of ventral telencephalon. (**A-F**) Expression of *foxg1a* (A,B), *emx3* (C,D), and *nkx2.1b* (E,F) at the 20-somite stage in wild-type embryos treated with 2% dimethyl sulfoxide alone (A,C,E) or 20 mM hydroxyurea and 150 µM aphidicolin at 80% epiboly (B,D,F) . White bracket denotes length of DV domain measured for quantification. (**G**) Graph shows expression domain length along the DV telencephalic axis divided by average DV domain length of vehicle-treated embryos. For each sample, n=11 embryos. Blue and red columns denote vehicle- and hydroxyurea/aphidicolin-treated embryos, respectively. Error bars denote standard error of the mean. ** signifies $p < 0.01$. (**H,I**) Expression of *nkx2.1b* at 24 hpf in *six3b;six7*-deficient embryos (H) that are also *tp53^{zdf1/zdf1}* (I). Arrows in panels E,F,H,I point at ventral telencephalon. Scale bars: 100 µm.

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telencephalon size and the smaller reduction of its ventral and dorsal domains may resolve from analyzing additional domains or additional embryos. As a severe reduction in proliferation did not significantly affect the domain size of ventral telencephalic progenitors in wild-type embryos, we conclude that cellular proliferation does not play a prominent role in generating ventral telencephalon fates during the time when Six3 function is required.

To test whether increased apoptosis is responsible for the reduction of ventral telencephalic progenitors in *six3b;six7*-deficient embryos, we first analyzed apoptosis using terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL). No apparent increase in TUNEL was observed in the anterior neural tube at either the 8-somite or 12-somite stage in *six3b;six7*-deficient embryos (Figure 8A,B,D,E). To more directly address whether apoptosis plays a role in the reduction of ventral telencephalic fates observed in *six3b;six7*-deficient embryos, we introduced the *tp53^{zdf1}* allele, a mutation in the DNA-binding domain of *tp53* (*tumor protein 53*), into *six3b;six7*-deficient embryos to interfere with apoptosis genetically. Similar to *tp53$^{zdf1/zdf1}$* embryos, which are characterized by a dramatic global reduction of apoptosis (Berghmans et al., 2005), *six3b;six7;tp53*-deficient embryos showed a strong reduction or absence of apoptotic cells at the 8-somite stage, as evidenced by TUNEL (Figure 8C). However, global reduction of *tp53*-dependent apoptosis failed to suppress the smaller size of the telencephalic *nkx2.1b* domain in *six3b;six7*-deficient embryos (Figure 7H,I). Collectively, these data support the conclusion that increased apoptosis and reduced proliferation are not major
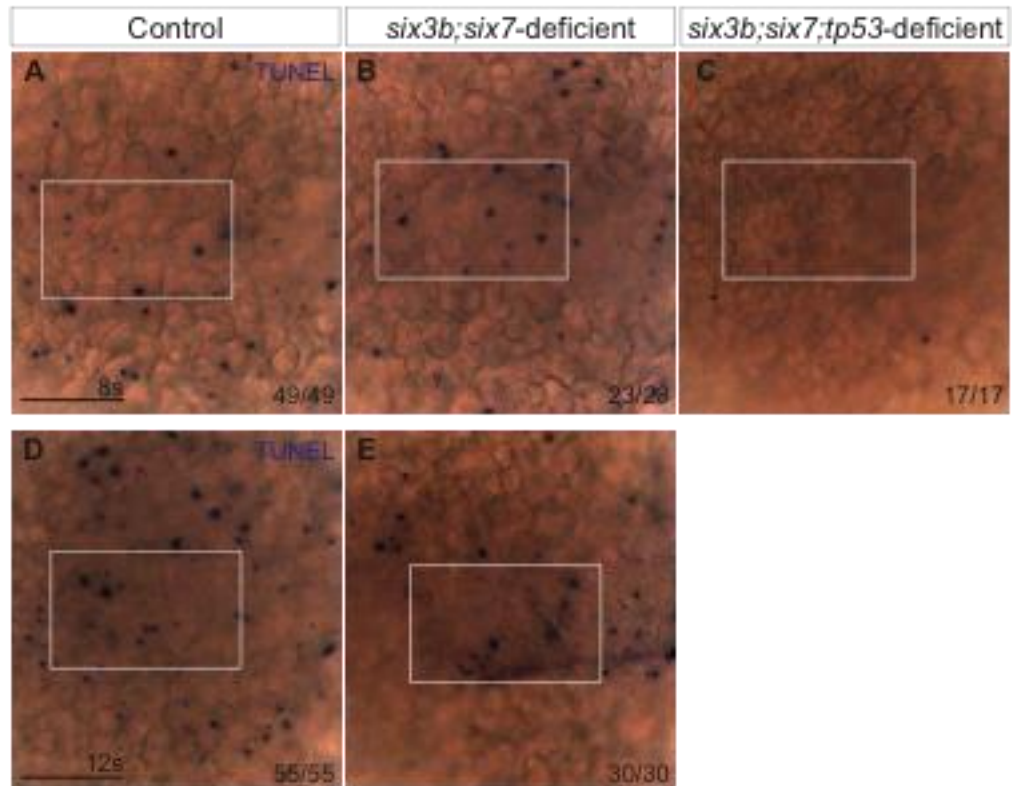
Figure 8. Apoptosis in anterior neuroectoderm is unaffected in *six3b;six7*-deficient embryos. (A-E) Apoptotic cells demonstrated by TUNEL in control (A,D), *six3b;six7*-deficient embryos (B,E) and *six3b;six7;tp53*-deficient embryos (C) at the 8-somite (A-C) and 12-somite (D,E) stages. Boxes outline anterior neural tube. Scale bars: 100 µm.

contributing mechanisms to the reduction of ventral telencephalon cell fates in *six3b;six7*-deficient embryos.

Ventral telencephalon is not properly specified in *six3b;six7*-deficient embryos

A third potential mechanism for Six3 function in DV telencephalon patterning is through specification of ventral telencephalic progenitors. If Six3 specifies ventral fate in the telencephalon, then lack of ventral progenitors in *six3b;six7*-deficient embryos should be evident near the onset of specific marker gene expression. DV polarity in the telencephalon can first be recognized by specific gene expression at the 12-somite stage (Danesin et al., 2009). We examined *six3b;six7*-deficient embryos at the 16-somite stage when expression of dorsal and ventral markers can be unambiguously detected. At this early stage, telencephalic DV patterning was already perturbed, as evidenced by reduced *nkx2.1b* expression domain (Figure 9A,B). Consistent with this result, *emx3* expression, which normally becomes restricted to dorsal progenitors by mid-segmentation, was observed throughout the telencephalon in 12-somite stage *six3b;six7*-deficient embryos (Figure 9C,D). This early patterning defect supports the notion that ventral telencephalic progenitors are not specified properly in *six3b;six7*-deficient embryos.

To test further the idea that Six3 controls fate specification of ventral progenitors, we assessed the ability of *six3b* misexpression to induce ectopic *nkx2.1b* expression. *Six3* overexpression in chick embryos is capable of inducing ectopic *Nkx2.1* expression in more posterior brain regions (Kobayashi et al., 2002). To understand if Six3 regulates expression of *nkx2.1b* similarly in zebrafish, we
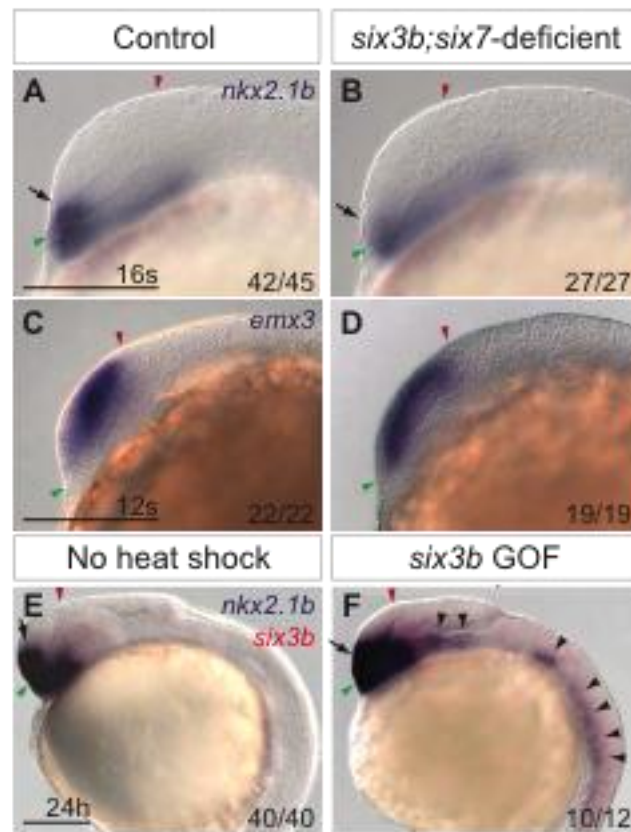
Figure 9. Six3b is required for specification of ventral telencephalon. (**A,B**) *nkx2.1b* expression in telencephalon (arrows) of control (A) and *six3b;six7*-deficient embryos (B) at the 16-somite stage. (**C,D**) *emx3* expression in control (C) and *six3b;six7*-deficient embryos (D) at the 12-somite stage. (**E,F**) *nkx2.1b* (purple) expression in telencephalon (arrows) and ectopic expression (black arrowheads) at 24 hpf in *Tg(hsp70l:Gal4-VP16); Tg(UAS:six3b)* embryos globally misexpressing *six3b* (red) (F) compared to embryos not subjected to heat shock (E). GOF denotes gain of function. Scale bars: 100 μm.

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analyzed *Tg(hsp70l:Gal4-VP16)*; *Tg(UAS:six3b)* embryos that were subjected to heat shock at the tailbud stage to globally misexpress *six3b*. At 24 hpf, these embryos exhibited a dorsally expanded *nkx2.1b* expression domain within the telencephalon, as well as ectopic *nkx2.1b* expression in more posterior regions of the brain and ventral spinal cord (Figure 9E,F). These data provide strong support for Six3's ability to promote specification of *nkx2.1b*-expressing cells, and suggest that the ventral telencephalic deficits observed in *six3b;six7*-deficient embryos are due to impaired cell fate specification.

Six3 function is required during early segmentation for establishing ventral telencephalic cell fates

six3b and *six7* are expressed in the anterior neuroectoderm, including the prospective telencephalon from late-gastrulation (9 hpf) (Kobayashi et al., 1998; Seo et al., 1998a; Seo et al., 1998b), whereas ventral telencephalic progenitors are affected by the 16-somite stage (17 hpf) in *six3b;six7*-deficient embryos. This temporal discrepancy raises the question of when Six3 function is required for specification of ventral telencephalic cell fates. To address this, we induced *six3b* expression at different time points and examined when it was capable of rescuing the reduced *nkx2.1b*- and *isl1*-positive telencephalic cell populations in *six3b;six7*-deficient embryos. To misexpress *six3b* globally in a *six3b;six7*-deficient background, we crossed *Tg(hsp70l:Gal4-VP16)*; *six3b^{vu87/+}* and *Tg(UAS:six3b)*; *six3b^{vu87/+}* lines, injected resulting embryos with MO1-*six7*, and subjected these embryos to heat shock at late gastrulation or early segmentation stages. Telencephalic expression domains of *isl1* and *nkx2.1b* were analyzed at 24 hpf. We

found that inducing *six3b* expression at late gastrulation (10 hpf) restored *nkx2.1b*- and *isl1*-positive ventral telencephalic cell populations in *six3b;six7*-deficient embryos (Figure 10C,G). However, when heat shock was applied at the 4-somite stage, *nkx2.1b* expression was no longer restored (Figure 10D). Similarly, applying heat shock to embryos at the 6-somite stage could not suppress the reduction in *isl1*-positive cells (Figure 10H). Given that strong global *six3b* mRNA induction was not present until 1.5 hours after onset of heat shock (data not shown), these results suggest that Six3 function is required by the 8-somite and 10-somite stage for generation of *nkx2.1b*- and *isl1*-positive telencephalic cell populations, respectively.

Six3 and Hh signaling do not regulate each other during early segmentation stages

The expansion of dorsal telencephalic cell fates at the expense of ventral ones, as observed in *six3b;six7*-deficient embryos, is reminiscent of telencephalic patterning defects observed when Hh signaling is perturbed. For example, zebrafish embryos in which Hh signaling is disrupted due to a mutation in the obligatory Hh pathway mediator *smoothened* (*smo*) or treatment with cyclopamine, a small molecule inhibitor of Smo, exhibit complete loss of telencephalic and diencephalic *nkx2.1b* expression and reduced telencephalic *isl1* domain (Figure 12A,B,J,K,M,N) (Rohr et al., 2001; Danesin et al., 2009). Because Six3 and Shh were reported to positively regulate each other's expression in mice, and simultaneous reduction of function of these two genes results in HPE with reduced ventral and expanded dorsal telencephalic fates (Geng et al., 2008), we asked whether Six3 and Shh also regulate each other in zebrafish. To find if Six3 acts upstream of Hh signaling, we
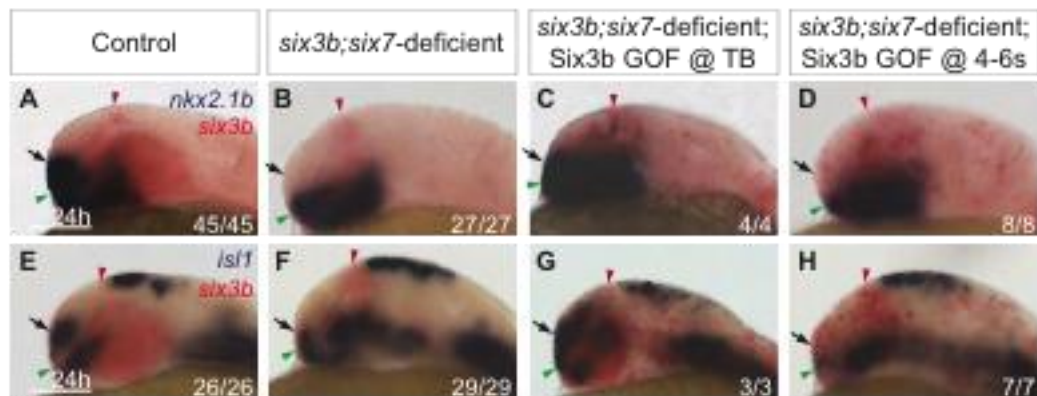
Figure 10. Six3b is required during early segmentation to promote ventral telencephalic fates. (**A-D**) *nkx2.1b* (purple) and *six3b* (red) expression in control embryos (A), *six3b;six7*-deficient embryos (B), *six3b;six7*-deficient *Tg(hsp70l:Gal4-VP16)*; *Tg(UAS:six3b)* embryos induced to misexpress *six3b* at tailbud stage (C), and *six3b;six7*-deficient *Tg(hsp70l:Gal4-VP16)*; *Tg(UAS:six3b)* embryos induced to misexpress *six3b* at the 4-somite stage (D). (**E-H**) *isl1* (purple) and *six3b* (red) expression in control embryos (E), *six3b;six7*-deficient embryos (F), *six3b;six7*-deficient *Tg(hsp70l:Gal4-VP16)*; *Tg(UAS:six3b)* embryos induced to misexpress *six3b* at tailbud stage (G), and *six3b;six7*-deficient *Tg(hsp70l:Gal4-VP16)*; *Tg(UAS:six3b)* embryos induced to misexpress *six3b* at the 6-somite stage (H). Arrows point at the ventral telencephalon in 24 hpf embryos. Scale bars: 100 μm.

examined the expression of *patched2* (*ptch2*, formerly *ptc1*) (Concordet et al., 1996), a downstream transcriptional target of the Hh signaling pathway, in *six3b;six7*-deficient embryos. At early segmentation stages (2- and 8-somite stage), *ptch2* expression appeared normal (Figure 11A,B; data not shown), suggesting that the combined function of *six3b* and *six7* was not required for Hh pathway activity at this time. Similarly, blocking Hh signaling using cyclopamine from early gastrulation (shield stage, 6 hpf) did not affect the expression of *six3b* or *six7* in prechordal mesendoderm at mid-gastrulation (8 hpf) or in anterior neuroectoderm at the 3- and 8-somite stages (Fig11C-F; data not shown). Efficacy of cyclopamine treatment was confirmed by absence of *ptch2* expression in sibling embryos at the same stage (data not shown). Therefore, *six3b* and *six7* expression during gastrulation and early segmentation are independent of Hh signaling. Together, these data suggest that a positive feedback loop between Six3 and Hh signaling, that is dependent on full function of Six3, does not operate during developmental stages when telencephalic DV patterning is established in zebrafish.

Complex interactions between Six3 and Hh signaling promote ventral telencephalic fates

Although Six3 and Hh signaling appear to independently promote ventral telencephalic fates during early segmentation stages in zebrafish, it is possible that they genetically interact in this process. To test this, we generated *six3b^{vu87/vu87}; smo^{b641/b641}* embryos and examined *isl1* telencephalic expression. The *isl1* telencephalic domain appeared similar in wild-type and *six3b^{vu87/vu87}* embryos and was only mildly reduced in *smo^{b641/b641}* embryos (Figure 12A,B). However, in
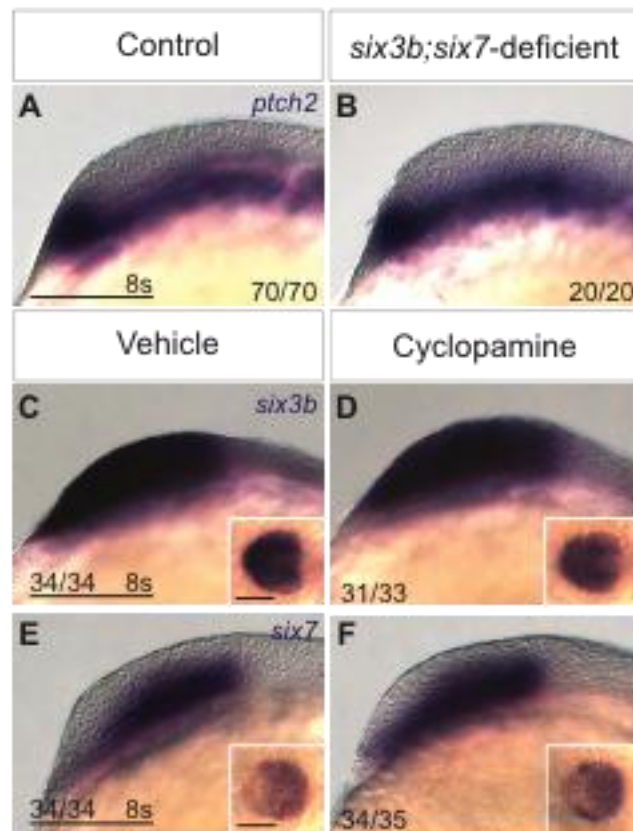
Figure 11. Six3 and Hh signaling do not regulate each other during early segmentation. (**A,B**) Expression of *ptch2* in 8-somite stage control (A) and *six3b;six7*-deficient embryos (B). (**C-F**) Expression of *six3b* (C,D) and *six7* (E,F) in 8-somite stage wild-type embryos treated from 6 hpf with 10 μM cyclopamine (D,F) and embryos treated with 0.1% ethanol alone (C,E). Insets show same embryo as dorsal view with anterior to the left. Scale bars: 100 μm.

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six3b^{vu87/vu87}; smo^{b641/b641} embryos, very few *isl1*-positive telencephalic cells could be detected (Figure 12C). These results demonstrate a synergistic interaction between *six3b* function and Hh pathway activity and suggest that Six3 and Hh signaling cooperate to promote *isl1*-positive telencephalic cells. A similar experiment analyzing the telencephalic domain of *nkx2.1b* is precluded due to the complete loss of *nkx2.1b* expression in Hh signaling-deficient embryos (Figure 12N), as previously described (Rohr et al., 2001).

To better understand the interactions between Six3 and Hh signaling in the generation of ventral telencephalic cells, we tested whether overactivation of the Hh signaling pathway by misexpression of *shha* can compensate for the loss of *six3b* and *six7* function. We crossed *Tg(hsp70l:Gal4-VP16)*; *six3b^{vu87/+}* and *Tg(UAS:shha-NH-EGFP)*; *six3b^{vu87/+}* fish, injected resulting embryos with MO1-*six7*, and induced *shha-NH-EGFP* misexpression by heat shock at late gastrulation (10 hpf). Analysis at 24 hpf showed that *shha-NH-EGFP* misexpression caused strong expansion of *isl1* and *nkx2.1b* expression domains throughout the telencephalon in uninjected embryos as well as wild-type and *six3b^{vu87/+}* embryos injected with MO1-*six7* (Figure 12E,H). In contrast, *isl1* and *nkx2.1b* telencephalic expression domains remained strongly reduced in *six3b;six7*-deficient embryos overexpressing *shha-NH-EGFP* (Figure 12F,I), suggesting that induction of telencephalic *isl1-* and *nkx2.1b*-positive fates by Hh signaling depends on Six3 function.

In a set of reciprocal experiments, we treated *Tg(hsp70l:Gal4-VP16)*; *Tg(UAS:six3b)* embryos with cyclopamine from early gastrulation to block Hh signaling, and induced *six3b* misexpression by heat shock at the end of

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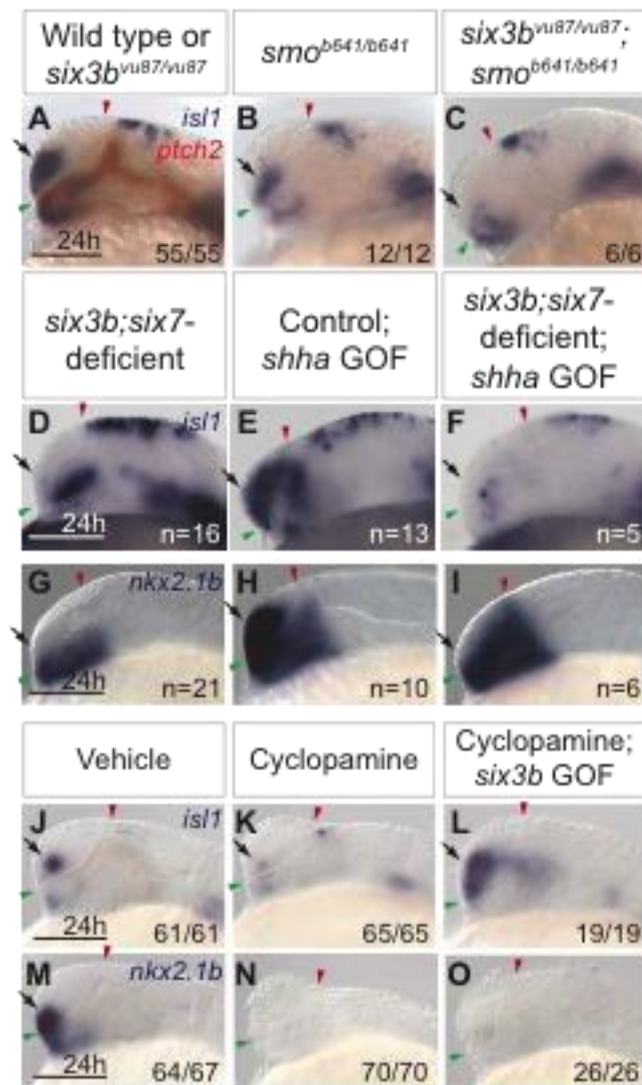
Figure 12. Interactions between Hh signaling and Six3 in ventral telencephalon formation. (A-C) *isl1* (purple) and *ptch2* (red) expression in wild-type and *six3b^{vu87/vu87}* embryos (A), *smo^{b641/b641}* embryos (B) and *six3b^{vu87/vu87}*; *smo^{b641/b641}* embryos (C). (**D-I**) Expression of *isl1* (D-F) and *nkx2.1b* (G-I) in *six3b;six7*-deficient embryos (D,G), control *Tg(hsp70l:Gal4-VP16)*; *Tg(UAS:shha-NH-EGFP)* embryos misexpressing *shha-NH-EGFP* (E,H) and *six3b;six7*-deficient *Tg(hsp70l:Gal4-VP16)*; *Tg(UAS:shha-NH-EGFP)* embryos misexpressing *shha-NH-EGFP* (F,I). (**J-O**) *isl1* (J-L) and *nkx2.1b* (M-O) expression in vehicle-treated embryos (J,M), cyclopamine-treated embryos (K,N) and cyclopamine-treated *Tg(hsp70l:Gal4-VP16)*; *Tg(UAS:six3b)* embryos misexpressing *six3b* (L,O). All embryos are 24 hpf. Arrows point at ventral telencephalon. Scale bars: 100 μm.

gastrulation. All cyclopamine-treated embryos showed identical morphology to *smo* mutant embryos at 24 hpf, confirming a disruption of Hh signaling (data not shown). Control embryos treated with cyclopamine but not subjected to heat shock had a strongly reduced *isl1*-positive telencephalic domain at 24 hpf (Figure 12K). However, in cyclopamine-treated embryos misexpressing *six3b*, expression of telencephalic *isl1* was restored or even expanded (Figure 12L). The same result was obtained when *six3b* was misexpressed in $smo^{b641/b641}$ background (not shown). We also analyzed *nkx2.1b* expression in embryos misexpressing *six3b* in the absence of Hh signaling. In contrast to its ability to promote *isl1*-positive cells, *six3b* misexpression could not restore *nkx2.1b* expression in Hh signaling-deficient embryos (Figure 12M-O), suggesting Six3 promotes telencephalic *nkx2.1b*-positive cell population in an Hh-dependent manner. These results are consistent with the notion that Six3 functions permissively to provide competence for Shh to induce *nkx2.1b* forebrain expression (Kobayashi et al., 2002), yet suggest an instructive role in inducing *isl1*-positive cells in the ventral telencephalon independently of Hh signaling.

foxg1a expression is transiently regulated by Six3 during early segmentation

Foxg1 function is required during early segmentation to promote ventral telencephalic development, and its loss of function results in telencephalic phenotypes highly reminiscent of *six3b;six7-* or Hh signaling-deficient embryos (Xuan et al., 1995; Martynoga et al., 2005; Danesin et al., 2009). We asked if *foxg1a* expression is affected in *six3b;six7*-deficient embryos when telencephalic

DV patterning is established. Indeed, we found that *foxg1a* expression was not established at 1-somite stage and remained strongly reduced in *six3b;six7*-deficient embryos during early segmentation (6-somite stage), compared to control embryos (Figure 13A,B; data not shown). However, by the 12-somite stage, telencephalic *foxg1a* expression had largely recovered (Figure 13C,D). These data demonstrate a biphasic regulation of *foxg1a* expression where its early but not later expression depend on *six3b* and *six7* function.

To test whether *foxg1a* activity also regulates *Six3* expression, we analyzed expression of *six3b* and *six7* during early segmentation in embryos injected with MO2-*foxg1a*. Although *foxg1a* morphant embryos showed a profound reduction of telencephalic *nkx2.1b* at 24 hpf (data not shown) (Danesin et al., 2009), expression of *six3b* and *six7* appeared normal in sibling *foxg1a* morphant embryos at the 4-somite stage (Figure 13E-H), demonstrating that *foxg1a* function is not required for *six3b* and *six7* expression. These results place *foxg1a* function downstream of Six3 in telencephalic DV patterning.

Next we asked whether *foxg1a* function was required for Six3's ability to promote ventral telencephalic fates by injecting MO2-*foxg1a* into *Tg(hsp70l:Gal4-VP16); Tg(UAS:six3b)* embryos and applying heat shock at tailbud stage. Disruption of Foxg1a function did not suppress the expansion of *isl1* in *six3b*-misexpressing embryos (Figure 13I-L). By contrast, telencephalic expression of *nkx2.1b* was strongly reduced in *six3b*-misexpressing embryos injected with MO2-*foxg1a* (Figure 13M-P), while ectopic *nkx2.1b* expression was unaffected, (40/44

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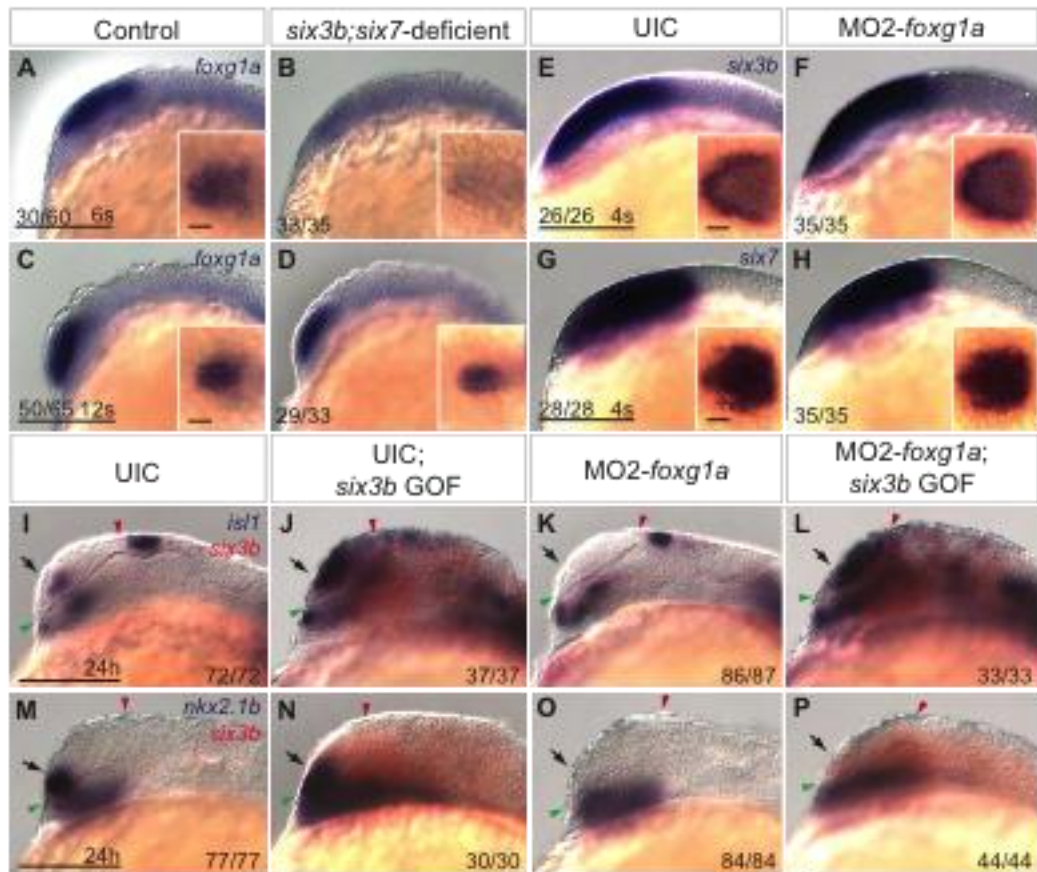
Figure 13. Interaction between Six3 and Foxg1a in ventral telencephalon development. (**A-D**) Expression of *foxg1a* in control (A,C) and *six3b;six7*-deficient embryos (B,D) at the 6-somite stage (A,B) and 12-somite stage (C,D). (**E-H**) Expression of *six3b* (E,F) and *six7* (G,H) in uninjected control (UIC) embryos (E,G) and MO2-*foxg1a* injected embryos (F,H) at the 4-somite stage. Insets in panels E-H are dorsal views of the same embryo with anterior left. Inset scale bars: 50 μm. (**I-P**) *isl1* (purple) (I-L) or *nkx2.1b* (purple) (M-P) and *six3b* (red) expression in UIC embryos (I,M), UIC *Tg(hsp70l:Gal4-VP16); Tg(UAS:six3b)* embryos misexpressing *six3b* (J,N), MO2-*foxg1a* injected embryos (K,O), MO2-*foxg1a* injected *Tg(hsp70l:Gal4-VP16); Tg(UAS:six3b)* embryos misexpressing *six3b* (L,P) at 24 hpf. Scale bars: 100 μm.

embryos; data not shown). These results demonstrate that in telencephalon Six3b can promote *isl1*, but not *nkx2.1b*, expression independently of Foxg1.

Together with previous studies (Kobayashi et al., 2002; Danesin et al., 2009; Beccari et al., 2012), our data place Foxg1a downstream of both Hh signaling and Six3 in promoting *nkx2.1*-positive cells in the telencephalon. Given that Six3 can promote *isl1*-positive cells independent of Foxg1, we asked whether such differential dependence existed also between Hh signaling and Foxg1. Expansion of telencephalic expression of *nkx2.1b* and *dlx2a* due to *shha* misexpression requires *foxg1a* function (Danesin et al., 2009). We tested whether this is also the case for *isl1*. *Tg(hsp70l:Gal4-VP16)*; *Tg(UAS:shha-NH-EGFP)* embryos were injected with MO2-*foxg1a*, heat shocked at tailbud stage and analyzed at 24hpf for *isl1* expression. Unlike Six3, overactivation of Hh signaling could not restore *isl1* expression in *foxg1a* morphants (Figure 14), further supporting the notion that Foxg1a functions downstream of Hh signaling.

wnt8b expression is upregulated in *six3b;six7*-deficient embryos during early segmentation

Wnt ligands are expressed in the dorsal forebrain, and Wnt/β-catenin signaling has been shown to promote dorsal telencephalic fates (van de Water et al., 2001; Carl et al., 2007; Danesin et al., 2009). In mouse, excess Wnt/β-catenin signaling is sufficient to expand dorsal telencephalic fates ventrally and reduce ventral fates (Backman et al., 2005), similar to the phenotype observed in *six3b;six7*-deficient zebrafish embryos. Indeed, we find that overactivation of the Wnt/β-catenin pathway at early segmentation leads to telencephalic phenotypes

UIC	UIC; *shha* GOF	MO2-*foxg1a*; Wild type or *shha* GOF
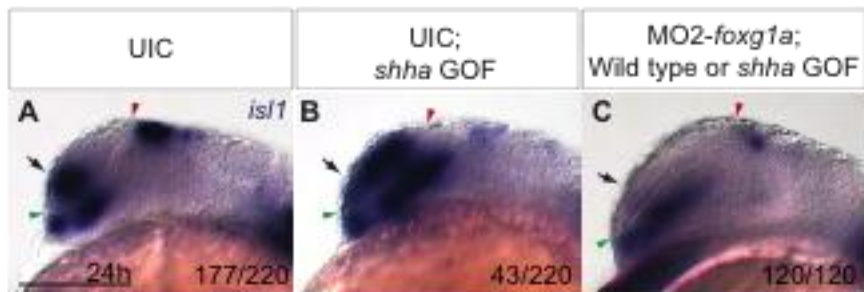
Figure 14. Hh signaling promotes telencephalic *isl* expression in a *foxg1a*-dependent manner. (A-C) Telencephalic *isl1* expression in control embryos (A), *Tg(hsp70l:Gal4-VP16)*; *Tg(UAS:shha-NH-EGFP)* misexpressing *shha-NH-EGFP* (B) and MO2-*foxg1a* injected control and *Tg(hsp70l:Gal4-VP16)*; *Tg(UAS:shha-NH-EGFP)* embryos misexpressing *shha-NH-EGFP* (C). Arrows point at ventral telencephalon. Scale bar: 100 μm.

almost identical to those observed in *six3b;six7*-deficient embryos (Figure 15). We therefore examined expression of the Wnt/β-catenin target gene *axin2* (Kelly et al., 1995; Leung et al., 2002; Carl et al., 2007) in *six3b;six7*-deficient embryos. Whereas at tailbud stage *axin2* expression appeared similar in control and *six3b;six7*-deficient embryos (Figure 16), at the 8-somite stage it was expanded anteriorly into the telencephalon of *six3b;six7*-deficient embryos (Figure 17A,B), suggesting increased Wnt/β-catenin activity in the telencephalon.

The Wnt/β-catenin ligand *wnt8b* is expressed in the dorsal forebrain at early segmentation, and its expression is directly repressed by both Six3 and Foxg1a (Carl et al., 2007; Danesin et al., 2009; Liu et al., 2010). We therefore analyzed *wnt8b* expression in *six3b;six7*-deficient embryos at the 8-somite stage, and found that expression was also expanded anteriorly, and this anterior expansion was noted as early as the 5-somite stage (Figure 17C,D; data not shown). As Six3 has been shown to directly repress *Wnt8b* expression in mouse embryos (Liu et al., 2010), we asked whether Six3b can repress *wnt8b* expression in zebrafish and whether such repression was dependent on Foxg1a function. To test this, MO2-*foxg1a* was injected into *Tg(hsp70l:Gal4-VP16); Tg(UAS:six3b)* embryos and heat shock was applied at tailbud stage. As previously shown, expression of *wnt8b* is anteriorly expanded due to disruption of Foxg1a function (Danesin et al., 2009), however misexpression of *six3b* repressed *wnt8b* in both uninjected and *foxg1a* morphant embryos (Figure 17E-H). Together, these results support the notion that Six3b can repress *wnt8b* expression in a Foxg1a-independent manner, and suggest
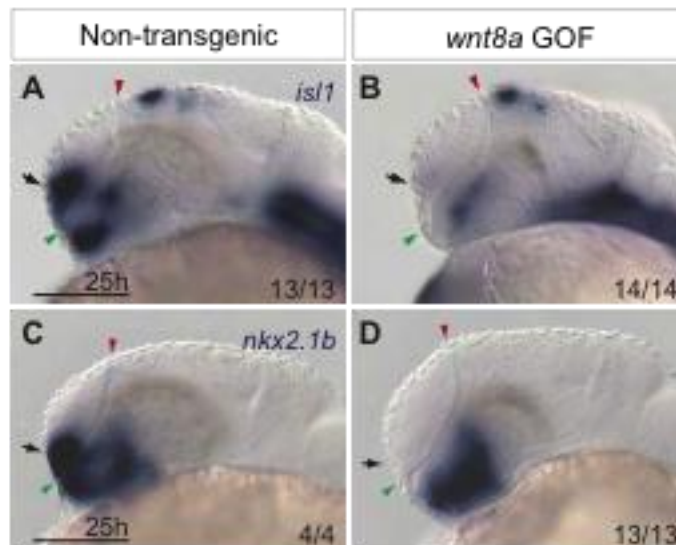
Figure 15. Excess Wnt/β-catenin signaling is sufficient to repress ventral telencepalon. (**A-D**) *isl1* (A,B) and *nkx2.1b* (C,D) expression in non-transgenic control embryos (A,C) and *Tg(wnt8a-GFP)* embryos misexpressing *wnt8a-GFP* after heat shock at tailbud stage (B,D). Arrows point at ventral telencephalon. Scale bars: 100 μm.
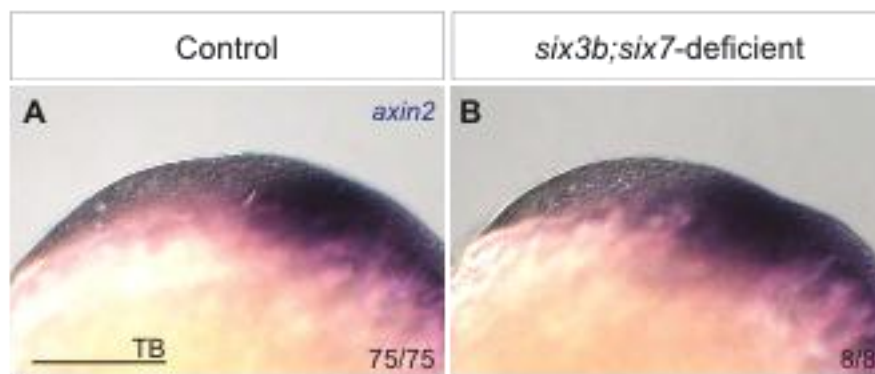
Figure 16. Wnt/β-catenin target *axin2* is unaffected in *six3b;six7*-deficient embryos at tailbud stage. (**A,B**) *axin2* expression in control (A) and *six3b;six7*-deficient embryos (B). 78/78 MO1-*six7* injected embryos from a cross of *six3b^{vu87/+}* with *six3b^{vu87/vu87}* fish showed no observable changes in *axin2* expression compared to control embryos. Scale bar: 100 μm.
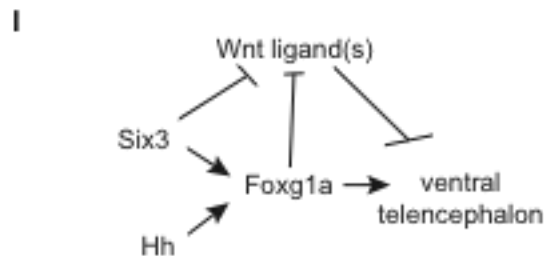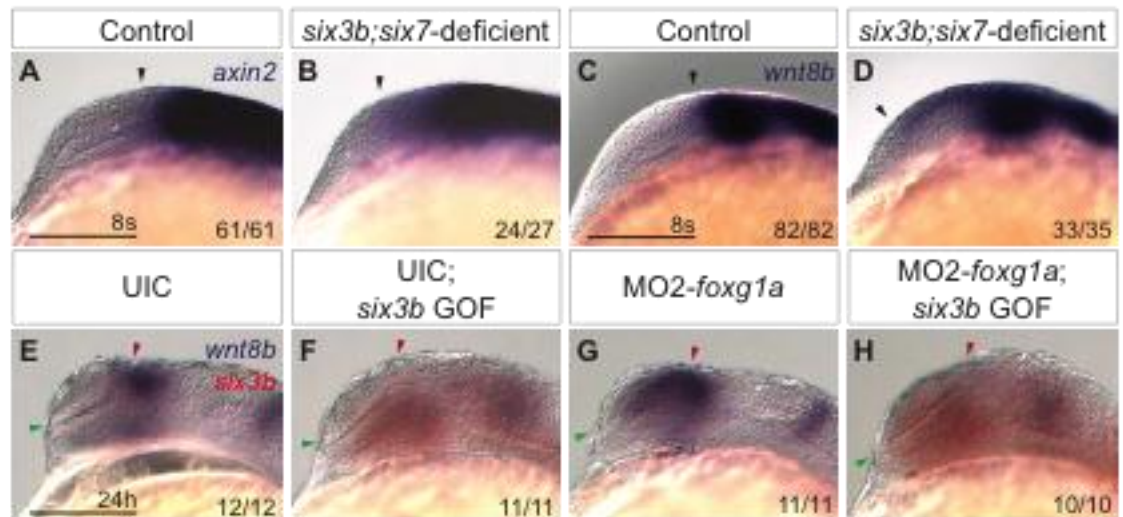
Figure 17. Six3 represses *wnt8b* expression in a *foxg1a*-independent manner.
(**A-D**) Expression of *axin2* (A,B) and *wnt8b* (C,D) in control (A,C) and *six3b;six7*-deficient embryos (B,D) at the 8-somite stage. Black arrowhead denotes anterior limit of expression. (**E-H**) *wnt8b* (purple) and *six3b* (red) expression in UIC embryos (E), UIC *Tg(hsp70l:Gal4-VP16)*; *Tg(UAS:six3b)* embryos misexpressing *six3b* (F), MO2-*foxg1a* injected embryos (G), MO2-*foxg1a* injected *Tg(hsp70l:Gal4-VP16)*; *Tg(UAS:six3b)* embryos misexpressing *six3b* (H) at 24 hpf. Scale bars: 100 μm. (**I**) Genetic model of Six3 function in zebrafish telencephalon DV patterning. Six3 and Hh signaling function in parallel to promote *foxg1a* expression, which in turn promotes ventral telencephalon. Six3 and Foxg1a each can repress expression of Wnt/β-catenin ligands such as *wnt8b*, which can repress ventral telencephalon.

that expanded activity of the Wnt/β-catenin pathway could contribute to the reduced ventral telencephalic fates in *six3b;six7*-deficient embryos.

<u>Discussion</u>

Zebrafish *Six3*-related genes as a tool for dissecting the function of *Six3* in forebrain development

We have taken advantage of the functional redundancies between three *Six3*-related genes in the zebrafish genome to dissect the roles of Six3 in telencephalic development (Seo et al., 1998a; Seo et al., 1998b). The homeodomains of zebrafish *Six3*-related genes can bind the same DNA sequence (Suh et al., 2010), and misexpression of *six3a*, *six3b*, *six7*, or human *SIX3* in zebrafish embryos leads to the same early phenotypes (i.e. dorsalization, increased head and eye size) (our unpublished observations) (Domene et al., 2008; Geng et al., 2008). These data strongly suggest that zebrafish *Six3*-related genes could act redundantly during development and in conserved fashion with mammalian orthologs. Indeed, we have previously shown that whereas the loss of *six3b* or *six7* function alone did not result in observable phenotypes, their combined loss of function resulted in microphthalmia or anophthalmia and brain laterality defects (Inbal et al., 2007). In the current study, we identified abnormal telencephalic DV patterning as a consequence of combined loss of *six3b* and *six7* function. Both eye malformations and telencephalic patterning defects are consistent with phenotypes observed when Six3 function is perturbed in other vertebrates and in cases of HPE (Carl et al., 2002; Lagutin et al., 2003; Ando et al., 2005; Geng et al., 2008).

Since Six3 regulates many processes during early development, three redundant genes in zebrafish afford generation of discrete hypomorphic phenotypes through combinatorial loss of function. For example, loss of Six3 function in mouse results in lack of both forebrain and eyes (Lagutin et al., 2003), whereas the loss of eyes in *six3b;six7*-deficient embryos is uncoupled from lack of forebrain (Inbal et al., 2007). Similarly in medaka fish, differential tissue sensitivities are observed in embryos deficient in *Six3.1* or *Six3.2* (Carl et al., 2002; Beccari et al., 2012). However, certain phenotypes related to loss of Six3 function have not yet been described in zebrafish, such as midline deficiencies seen in HPE, which are not observed in *six3b;six7*-deficient embryos (our unpublished observations). To obtain a more comprehensive understanding of the roles of Six3, it will be important to also analyze loss of *six3a* function alone and in combination with *six3b* and/or *six7*. Indeed, such functional redundancies of three *Nodal*-related genes facilitated dissection of their roles in mesendoderm induction and patterning, and left-right axis specification (Schier, 2009). Overall, zebrafish provide a powerful system in which to study the specific roles of Six3 in early CNS development.

Parallel functions of Six3 and Hh signaling converge on Foxg1a

Several observations suggest Six3 and Hh signaling cooperate in promoting ventral telencephalic fates. First, reduction of Six3 function or Hh signaling, each result in reduction of ventral and expansion of dorsal telencephalic fates (Figure 4; Figure 12K,N) (Chiang et al., 1996; Rallu et al., 2002; Danesin et al., 2009). Conversely, gain of Six3 function or excess Hh signaling each result in an

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expansion of ventral telencephalic fates at the expense of dorsal ones (Figure 10C,G; Figure 12I,H) (Kohtz et al., 1998; Rohr et al., 2001; Rallu et al., 2002). Second, both Six3 and Shh function during early segmentation stages to promote ventral telencephalic fates (Figure 10) (Kohtz et al., 1998; Danesin et al., 2009). Interestingly, our results show *nkx2.1b*-positive cells require Six3 function slightly earlier or longer than *isl1*-positive cells, which suggests that the most ventromedial telencephalic fates may be specified earlier than more dorsally located ventral fates. This is consistent with *nkx2.1b* being expressed earlier than *isl1* in the ventral telencephalon (Rohr et al., 2001), and also with data in rat showing Shh first induces Nkx2.1-positive and later Islet-1-positive ventral telencephalic progenitors (Kohtz et al., 1998). Third, our data show that global misexpression of *six3b* activates ectopic *nkx2.1b* expression only near a source of Shh, similar to what has been previously described in chick embryos (Kobayashi et al., 2002). We interpret this result to mean that Six3 provides competence for cells to respond to Shh by expressing *nkx2.1b*. Fourth, exacerbated deficiency of telencephalic *isl1* expression in *six3b^{vu87/vu87}*; *smo^{b641/b641}* compound mutants demonstrates a strong genetic interaction between Six3 and Hh signaling in formation of these ventral telencephalic progenitors.

In this study, we did not find evidence for Six3 regulating Hh signaling or vice versa. A previous report in zebrafish showed that loss of Hh signaling affects *six3b* expression by mid-segmentation (Sanek et al., 2009), however we observed no significant changes in *six3b* or *six7* expression due to loss of Hh signaling during early segmentation when Six3 regulates DV telencephalon patterning.

As our results suggest that Six3 and Hh signaling function largely in parallel to specify ventral telencephalic fates, we examined the possibility that Foxg1, which is also required to promote ventral telencephalic cell fates, may link Six3 and Hh signaling. Loss of *Foxg1* gene function results in a dorsalized telencephalon almost identical to that observed in *six3b;six7*-deficient or Hh signaling-deficient embryos, and *Foxg1* functions at similar developmental stages (Xuan et al., 1995; Martynoga et al., 2005; Danesin et al., 2009). Our findings show that induction and early maintenance of *foxg1a* expression is affected in *six3b;six7*-deficient zebrafish embryos during early segmentation when ventral telencephalon is specified. These data, together with our observation that *six3b* and *six7* expression is not affected by loss of *foxg1a* function, place Foxg1 downstream of Six3 in telencephalon DV patterning. Consistent with this conclusion, medaka Six3.2 has been shown to bind highly conserved non-coding elements in the *Foxg1* regulatory region *in vitro* (Beccari et al., 2012), and *Six3* misexpression in chick embryos could activate ectopic *Foxg1* expression near the mid-hindbrain boundary (Kobayashi et al., 2002). Expression of *foxg1a* during early segmentation stages is also transiently dependent on Hh signaling, and misexpression of *foxg1a* could restore expression of some ventral telencephalic markers in embryos lacking Hh signaling (Danesin et al., 2009). Consistent with the notion of Foxg1a acting downstream of Hh signaling, misexpression of *shha* is insufficient to promote ventral telencephalon cell fates in *foxg1a*-deficient embryos (Figure 14) (Danesin et al., 2009). Therefore, we propose that *foxg1a* is a common downstream effector of Six3 and Hh signaling in the process of telencephalon patterning during early segmentation (Figure 17I).

Hh signaling- and Foxg1a-independent function of Six3

Our data support the notion that Six3 and Hh signaling cooperate to establish expression of *foxg1a* during early segmentation, which is required to promote expression of *nkx2.1b* in the ventral telencephalon. This is a strict cooperation between Six3 and Hh signaling such that increased activation of one pathway cannot compensate for loss of the other, nor can they compensate for the loss of *foxg1a* function. Surprisingly, Six3 can promote *isl1*-positive cells independently of Hh signaling and *foxg1a*. Although this could be interpreted that Six3 functions downstream of Hh and Foxg1a, given that *six3b* and *six7* expression is not affected by lack of Hh signaling or Foxg1a function during the developmental time window when DV patterning of the telencephalon is established, we favor the interpretation that Six3 acts in parallel to Hh and Foxg1a to specify this cell type.

Both Six3 and Foxg1 have been shown to directly repress expression of *Wnt8b* (Danesin et al., 2009; Liu et al., 2010), and Six3 has also been shown to directly repress *Wnt1* (Lagutin et al., 2003). Wnt/β-catenin activity can promote dorsal and repress ventral telencephalic fates (Figure 15) (van de Water et al., 2001; Backman et al., 2005). We demonstrate here that the Wnt/β-catenin pathway, and specifically *wnt8b* expression, is upregulated in telencephalon of *six3b;six7*-deficient embryos. Given that Six3 and Foxg1a can each repress *wnt8b* expression, regulation of the Wnt/β-catenin pathway by Six3 may be responsible for the Foxg1a- and Hh signaling-independent function of Six3 in promoting telencephalic *isl1* (Figure 17I). As *foxg1a* misexpression is also sufficient to rescue *isl1* expression in embryos lacking Hh signaling (Danesin et al., 2009), it will be

interesting to test if this can also be attributed to Foxg1a repression of Wnt ligands. Since several Wnt ligands are present near the developing telencephalon (Ciani and Salinas, 2005; Carl et al., 2007), reduction of *wnt8b* function alone may be insufficient to suppress the *six3b;six7*-deficient phenotype in ventral telencephalon, as is the case for *foxg1a* morphant embryos (Danesin et al., 2009). The role of Six3 and Foxg1a in regulation of other regionally expressed Wnt ligands remains to be tested, and may provide additional insight into the mechanisms of DV patterning in telencephalon.

CHAPTER III

DETERMINING WHERE Six3b EXPRESSION IS REQUIRED TO EXERT ITS
VARIOUS DEVELOPMENTAL FUNCTIONS

Summary

Six3 has numerous roles in development of the forebrain and eye during gastrulation and segmentation stages. During these stages, Six3 is expressed both broadly in anterior neuroectoderm and in prechordal mesoderm (PCM). Expression of Six3 in either or both tissues may be required to perform its function in different developmental processes. Zebrafish has three genes, six3a, six3b, and six7, with highly conserved sequence and expression pattern homology to mammalian Six3. Analyses of six3b;six7-deficient embryos revealed a requirement for Six3 function in telencephalon DV patterning and left-right brain asymmetry. Previous work has characterized a transgenic zebrafish line Tg(gsc:Gal4-VP16) that can drive gene expression specifically in PCM and its derivatives. Here I describe a transgenic line Tg(hesx1:Gal4-VP16) that drives gene expression in anterior neuroectoderm during late gastrulation and segmentation stages. Also, a novel Tg(UAS:six3b) transgenic line was generated to facilitate genotyping of embryos carrying multiple transgenes. These transgenic lines may prove useful to test the ability of wild-type six3b misexpression in either anterior neuroectoderm, PCM or both tissues to suppress the brain laterality and telencephalon dorsoventral (DV) patterning defects in six3b;six7-deficient embryos.

Introduction

The vertebrate homeobox gene *Six3* has numerous roles in development of the anterior CNS during embryogenesis. Loss of *Six3* function affects various developmental processes such as telencephalon induction and patterning, brain laterality, retina and hypothalamus specification, and forebrain midline formation (Wallis et al., 1999; Carl et al., 2002; Lagutin et al., 2003; Inbal et al., 2007; Geng et al., 2008; Lavado et al., 2008; Liu et al., 2010; Beccari et al., 2012; Carlin et al., 2012). Defects derived from loss of *Six3* function may arise autonomously from deficiencies in affected neural tissue or non-autonomously from deficiencies in surrounding neural or mesodermal tissue.

The diverse functions ascribed to *Six3* are consistent with its complex expression pattern. In all vertebrates studied, *Six3* is expressed during late gastrulation in a broad region of anterior neuroectoderm that includes presumptive telencephalon, eye, diencephalon and anterior midbrain (Oliver et al., 1995; Bovolenta et al., 1998; Kobayashi et al., 1998; Loosli et al., 1998; Seo et al., 1998a; Seo et al., 1998b; Zhou et al., 2000). As somitogenesis proceeds, expression of *Six3* becomes restricted to specific domains within these regions. In embryonic day 14.5 mouse embryos, for example, *Six3* expression in forebrain is detected in ventral telencephalon, hypothalamus, ventral thalamus and posterior pretectum whereas eye expression is noted in optic chiasm, optic stalk, neural retina and lens (Oliver et al., 1995). Expression patterns of *Six3* in other vertebrates are comparable at similar stages, but are less detailed than what has

been reported in mouse embryos. In addition neural tissue, *Six3* has been reported to be expressed in prechordal mesoderm (PCM) of several vertebrate species starting at early gastrulation (Bovolenta et al., 1998; Loosli et al., 1998; Seo et al., 1998a; Seo et al., 1998b; Geng et al., 2008). It is likely that expression ceases in this domain by mid-segmentation as *Six3* expression has not been reported in derivatives of the PCM. To date, it is unknown what function *Six3* expression in PCM serves, however it is tempting to speculate that loss of this expression domain may contribute to several of the neural phenotypes ascribed to *Six3* function.

Zebrafish have three *Six3*-related genes in their genome, *six3a*, *six3b* and *six7* (Seo et al., 1998a; Seo et al., 1998b), and loss-of-function analysis of different combinations of these genes uncovers discrete hypomorphic phenotypes dependent on *Six3* function. Injection of anti-sense morpholino oligonucleotides (MO) to inhibit translation of both Six3a and Six3b generates embryos with reduced head and eye size (Ando et al., 2005). Also, embryos that are both homozygous for a null mutation in *six3b* and injected with MO1-*six7* to inhibit translation of Six7 have defects in brain laterality in the dorsal diencephalon (Inbal et al., 2007) and telencephalon dorsoventral (DV) patterning (Carlin et al., 2012). It is likely that additional phenotypes will be characterized from different loss-of-function combinations.

Given that *Six3*-related genes are expressed in both neuroectoderm and PCM raises the question when and where does it function in these different processes. Ease of generating zebrafish transgenic insertions facilitates

generation of tools to determine when Six3 is required and which tissue requires

Six3 expression to suppress a particular loss-of-function phenotype. Indeed, heat

shock-induced global misexpression of wild-type *six3b* during early segmentation

stages can suppress diencephalic Nodal activity that leads to brain laterality

defects and loss of ventral telencephalon fates in *six3b;six7*-deficient embryos

(Inbal et al., 2007; Carlin et al., 2012). To test if *Six3* function is required in PCM

to suppress these phenotypes, a transgenic line was generated that can drive

reporter gene expression specifically in PCM under the control of the *goosecoid*

(*gsc*) promoter (Inbal et al., 2006). Expression of *gsc* and several other PCM

markers are unaffected at gastrulation stages in *six3b;six7*-deficient embryos,

suggesting that transgene expression would also be unaffected in these mutant

embryos. However, misexpression of wild-type *six3b* specifically in PCM of

six3b;six7-deficient embryos is not sufficient to suppress enhanced diencephalic

Nodal activity (Inbal et al., 2007). This indicates that *six3b* expression is required

in tissue other than PCM to affect left/right brain asymmetry. It is therefore

important to ask whether Six3 is required in anterior neuroectoderm, where it is

also normally expressed, to regulate this process.

To further address the tissue autonomy of *Six3* function in brain laterality

and telencephalon DV patterning in zebrafish embryos, I generated a transgenic

line to drive reporter gene expression in anterior neuroectoderm from late

gastrulation through mid-segmentation stages, when *Six3* is required for these

functions (Inbal et al., 2007; Carlin et al., 2012). A promoter region of the *Hesx1*

gene from the chicken *Gallus gallus* was found to drive reporter expression in

anterior neuroectoderm of zebrafish embryos by transient transgenesis (Spieler et al., 2004). I identified a stable transgenic line that integrated this construct into the genome, and it drives reporter expression in anterior neuroectoderm similarly to transiently transgenic embryos. This may be a useful line to assess whether s*ix3b* in anterior neuroectoderm is sufficient to suppress various Six3-dependent defects.

Materials and Methods

Zebrafish strains, embryo culture, and generation of transgenic fish

Adult zebrafish were maintained according to established methods (Westerfield, 1993). Embryos were obtained from natural matings, grown at 28.5°C and staged according to Kimmel (Kimmel et al., 1995). The following strains were used and genotyped as previously described: wild-type AB, *Tg(UAS:GFP)vu157* (Inbal et al., 2006) and *six3b^{vu87}* (Inbal et al., 2007).

To generate *Tg(hesx1:Gal4-VP16)vu417*, the *Hesx1* promoter from *Gallus gallus* was obtained from the 1kb-lacZ plasmid (Spieler et al., 2004). The 800 bp promoter sequence was amplified by PCR using GANFFOR 5'-ATGATCGTAAGCTTAGGAAAGAAGGAACACCTAAC-3' and GANFREV 5'-ATGACTGGATCCGGTTTCACCTTCAGCCAGC-3' primers. PCR product was cloned into pG1-*gsc:Gal4-VP16* plasmid (Inbal et al., 2006), by HindIII/BamHI digest, to generate pG1-*hesx1:Gal4-VP16*. The *hesx1:Gal4-VP16* cassette was released by HindIII/NotI restriction endonucleases, blunted by Klenow and cloned into an NheI-blunted site of pT2/γ-Cry-GM2 (Davidson et al., 2003). Transgenic

fish were generated using the *Sleeping Beauty* transposon system (Davidson et al., 2003) by co-injecting 15-20 pg pT-*hesx1:Gal4-VP16*-γCry-GM2 DNA with synthetic RNA encoding SB10 transposase into one-cell stage AB embryos. Founder fish were identified as previously described (Inbal et al., 2006).

To generate *Tg(UAS:six3b)s3*, *six3b* coding sequence was amplified from pCS2-*six3b* obtained from Guillermo Oliver's lab using *six3b* TOPOF 5'-CAACATGGTTTTCAGGTCTCCTTTAGAG-3' and *six3b* TOPOR 5'-TCATACATCGAAATCAGAGTCACTG-3' primers. The resulting PCR product was cloned into pENTR/DTOPO vector (Invitrogen). To generate pDestTol2CG2-*UAS:six3b*, pENTR-*six3b* was combined with p5E-UAS, p3E-pA and pDestTol2CG2 (Kwan et al., 2007) using LR Clonase II Plus (Invitrogen). One-cell stage *six3b^{vu87}* embryos were injected with 15 pg pDestTol2CG2-*UAS:six3b* and 10 pg Tol2 transposase RNA (Kwan et al., 2007). Embryos with green fluorescent hearts at 24 hpf were grown to adulthood. Founders were screened for offspring with green fluorescent hearts.

In situ hybridization, image acquisition and analysis

Embryos were fixed in 4% paraformaldehyde in phosphate-buffered saline. Whole mount in situ hybridization was performed according to standard protocols (Thisse and Thisse, 2005)and developed with BMPurple (Roche). Digoxigenin-labeled probes were generated from cDNA templates: *Gal4-VP16* (Inbal et al., 2006), *GFP* (Inbal et al., 2006), and *hesx1* (Kazanskaya et al., 1997).

Images were acquired using differential interference contrast on Zeiss Axiophot or Zeiss Imager Z.1 compound microscope stereomicroscope and captured with an Axiocam digital camera.

<u>Results and Discussion</u>

Characterization of *Tg(hesx1:Gal4-VP16)* zebrafish

To provide a tool to study gene function in anterior neuroectoderm during early segmentation stages, I generated *Tg(hesx1:Gal4-VP16)* transgenic zebrafish that express the transcriptional activator Gal4-VP16 in this region starting at late gastrulation, as the Gal4/UAS system affords an effective means of tissue-specific misexpression in zebrafish (Scheer and Campos-Ortega, 1999). *hesx1* is initially expressed broadly during mid-gastrulation (80% epiboly) in zebrafish anterior neuroectoderm including presumptive telencephalon, eye and diencephalon, and became restricted to telencephalon by early segmentation (1-somite stage) (Figure 18A,B) (Kazanskaya et al., 1997; Thisse and Thisse, 2005). Expression levels of *hesx1* decline during early segmentation and *hesx1* transcripts are no longer detected by mid-segmentation (15-somite stage) (Thisse and Thisse, 2005). A previous study identified a *Hesx1* promoter region from the chicken *Gallus gallus* that is active in transient assays in the anterior neuroectoderm of zebrafish embryos during early segmentation. I cloned an 800 bp region of the chicken *Hesx1* promoter upstream of *Gal4-VP16* coding sequence to generate a *hesx1:Gal4-VP16* cassette that was used to generate *Tg(hesx1:Gal4-VP16)* transgenic fish. Two independent transgenic lines were

maintained and characterized. Similar expression was noted in each line as described below.

To assess transgene expression, I analyzed *Gal4-VP16* mRNA by whole mount in situ hybridization in *Tg(hesx1:Gal4-VP16)* transgenic fish. *Gal4-VP16* transcripts were initially expressed in a similar pattern to *hesx1* at 80% epiboly when endogenous *hesx1* expression was first observed (Figure 18A,C). At the 1-somite stage, when *hesx1* expression becomes restricted to telencephalon, *Gal4-VP16* expression was observed broadly in anterior neuroectoderm (Figure 18B,D). Expression of *Gal4-VP16* in *Tg(hesx1:Gal4-VP16)* transgenic embryos was maintained at the 12-somite stage in eye, diencephalon and possibly telencephalon (Figure 18E). Expression levels of *Gal4-VP16* transcript decreased in CNS during late segmentation stages, however eye expression was maintained at 24 hpf (Figure 18F). The differences between transgene expression and endogenous *hesx1* expression may be due to higher stability of *Gal4-VP16* mRNA versus *hesx1* mRNA, suggesting that *hesx1* has regulatory regions in the untranslated regions of its transcript that affect mRNA stability in more posterior neural tissues. As the endogenous *hesx1* untranslated regions were not used in generating the transgene, these regions are not present in *Gal4-VP16* mRNA. Alternatively, the relatively small promoter region used to make this transgenic line may not contain silencer regions that downregulate endogenous expression of *hesx1* in more posterior neural tissues.

To test the ability of *Tg(hesx1:Gal4-VP16)* to drive target gene expression in anterior neuroectoderm, I crossed *Tg(hesx1:Gal4-VP16)* and *Tg(UAS:GFP)*
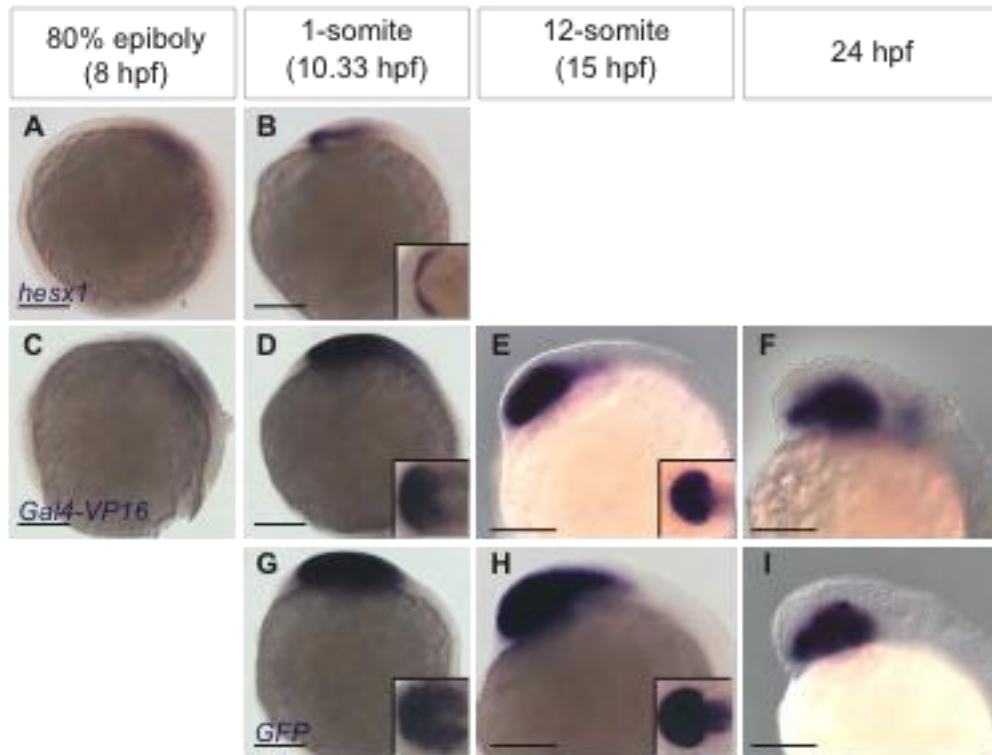
Figure 18. Characterization of *Tg(hesx1:Gal4-VP16)* transgenic embryos. Embryos are *Tg(hesx1:Gal4-VP16)*; *Tg(UAS:GFP)* double heterozygotes. Lateral view with anterior to the left. Insets are dorsal views of same embryo. (**A,B**) Broad, weak anterior neuroectoderm expression of *hesx1* at mid-gastrulation (A) becomes restricted to telencephalon by the 1-somite stage (B). (**C-F**) Broad, weak anterior neuroectoderm expression of *Gal4-VP16* is initiated at mid-gastrulation (C) and gets stronger by the 1-somite stage (D). Expression in eyes and anterior neural tube is maintained at the 12-somite stage (E). By 24 hpf, weaker   expression remains mainly in eyes and ventral forebrain (F). (**G-I**) Expression of *GFP* target gene is established broadly in anterior neuroectoderm at the 1-somite stage (G). Strong expression is maintained in eyes and anterior CNS at 12-somite stage (H), however by 24 hpf *GFP* expression is restricted to eyes and ventral forebrain (I). Scale bars: 100 µm.

transgenic fish and analyzed embryos at gastrulation and segmentation stages for *GFP* expression by whole mount in situ hybridization. *GFP* expression was not observed at 80% epiboly, the onset of *hesx1* and *Gal4-VP16* transgene expression (data not shown). However, robust *GFP* expression in anterior neuroectoderm was observed in a similar expression pattern to *Gal4-VP16* by the 1-somite stage (Figure 18D,G). Expression of *GFP* remained similar to *Gal4-VP16* expression at the 12-somite stage (Figure 18E,H). By 24 hpf, expression of *GFP* remained relatively strong in eyes and ventral diencephalon, however telencephalon expression was rarely observed (Figure 18I). These data present *Tg(hesx1:Gal4-VP16)* transgenic zebrafish as a tool to drive gene expression in anterior neuroectoderm from late gastrulation through 24 hpf.

It is notable that the transgene expression in telencephalon was absent at 24 hpf. Co-localization of *Gal4-VP16* with a marker of telencephalon at early segmentation stages such as *foxg1a* would help to understand if transgene expression in *Tg(hesx1:Gal4-VP16)* embryos includes the telencephalon. Expression *Gal4-VP16* in eye is robust throughout segmentation stages. However, a higher resolution analysis of transgene expression in the telencephalon and diencephalon may be necessary to characterize the expression domain boundaries. It is possible that transgene expression is not completely faithful to endogenous *hesx1* expression. As the promoter used to generate this transgenic line is only 800 bp in length, all regulatory regions instructing the endogenous *hesx1* expression may not be present in the transgenic promoter. Additionally, as the promoter sequence is derived from a

different species, regulatory regions in the promoter may be sufficiently divergent as to reduce activity of zebrafish transcription factors.

Loss of *six3b* and *six7* function in zebrafish generates embryos with brain laterality and telencephalic DV patterning defects. The proposed mechanisms by which *six3b* and *six7* regulate these processes suggest that expression of these genes is required in anterior neuroectoderm during early segmentation (Inbal et al., 2007; Carlin et al., 2012). Global misexpression of wild-type *six3b* in *six3b;six7*-deficient embryos during early segmentation is sufficient to suppress both of these phenotypes, however PCM misexpression of *six3b* is not sufficient to suppress the brain laterality defect (Inbal et al., 2007). To understand if the *Tg(hesx1:Gal4-VP16)* transgenic line could be a useful tool to address whether *six3b* misexpression in anterior neuroectoderm is sufficient to suppress *six3b;six7*-deficient phenotypes, it will be important to analyze *hesx1* expression in *six3b;six7*-deficient embryos. In *Six3* knockout mouse embryos, expression of *Hesx1* is established normally, however it is not maintained as in wild-type embryos (Lagutin et al., 2003). This suggests that initial transgene expression in *Tg(hesx1:Gal4-VP16)* zebrafish embryos may be unaffected by loss of *six3b* and *six7*. Further studies are needed to test transgene expression during early segmentation stages in *Tg(hesx1:Gal4-VP16)* embryos that are *six3b;six7*-deficient.

Generation of a *Tg(UAS:six3b)* transgenic fish

The previously described *Tg(UAS:six3b)vu156* transgenic line was characterized by robust misexpression of *six3b* upon introduction of Gal4-VP16 (Inbal et al., 2007). However, misexpression of *six3b* became weaker in subsequent generations. This is likely due to transgenerational silencing by methylation of CpG-rich regions in the 14 tandem repeats of the UAS sequence (Koster and Fraser, 2001; Akitake et al., 2011). In order to circumvent this decrease in expression, I generated a new *Tg(UAS:six3b)s3* transgenic line. One goal is to test whether misexpression of *six3b* in anterior neuroectoderm is sufficient to suppress *six3b;six7*-deficient phenotypes by crossing with *Tg(hesx1:Gal4-VP16)*; *six3b$^{vu87/+}$* fish with *Tg(UAS:six3b)s3*; *six3b$^{vu87/+}$* fish and injecting MO1-*six7*. As transgene expression of *Tg(hesx1:Gal4-VP16)* embryos is remarkably similar to endogenous *six3b* expression, it was important to generate a *UAS:six3b* cassette that could be distinguished from endogenous expression at 24 hpf. To this end, *Tg(UAS:six3b)s3* embryos also have a *cmlc2:GFP* reporter cassette that drives expression of GFP in heart tissue by 24 hpf facilitating easy identification of embryos carrying the *Tg(UAS:six3b)s3* transgene. Embryos carrying the *Tg(hesx1:Gal4-VP16)* transgene may be identified by in situ hybridization for *Gal4-VP16*. Analysis of phenotypic marker gene expression may also be performed by in situ hybridization in a different color. Future studies may also address the possibility that *Six3*-related gene function is required in both PCM and anterior neuroectoderm by using the transgenic lines described above in conjunction with *Tg(gsc:Gal4-VP16)*.

CHAPTER IV

OVERVIEW AND FUTURE AIMS

Embryonic forebrain development has been heavily studied over the past several decades, and many genes influencing this process have been uncovered. The divisions of the forebrain—telencephalon, eye and diencephalon—are induced during gastrulation, and demarcated by distinct domains of gene expression. During segmentation stages, these regions undergo morphogenetic events that elaborate their morphological distinction, while patterning events generate specific cell populations within each region (Table I). Individual cells then differentiate and migrate to their final destinations throughout embryonic and adult life. It has become clear that genes and signaling pathways are used reiteratively throughout the development of a single structure such as the telencephalon. For example, Wnt signaling has been implicated in telencephalon induction, patterning, and axon guidance (Chapter II) (Kim et al., 2002; Backman et al., 2005; Keeble et al., 2006; Zaghetto et al., 2007). In addition to the reiterative use of any single signaling pathway, multiple signaling pathways are active within a region at any given time. Therefore, cells must interpret both temporal and context-dependent cues to generate the appropriate response, which could be to choose fate, proliferate, differentiate, migrate, etc. Because of the molecular and cellular complexity inherent in forebrain development, gaining insight into the roles of transcription factors and

signaling pathways in later stages of forebrain development has posed a significant hurdle due to the technical effort required to generate tools to address such questions. *Six3* is one such gene that is expressed in forebrain regions, including telencephalon, throughout embryogenesis (Table I) (Oliver et al., 1995; Bovolenta et al., 1998; Kobayashi et al., 1998; Loosli et al., 1998; Seo et al., 1998a; Seo et al., 1998b; Zhou et al., 2000). Studies have shown that Six3 directly represses *Wnt1* expression during late gastrulation to facilitate telencephalon induction. As a result of this interaction, the telencephalon is absent in *Six3* homozygous knockout mouse embryos (Lagutin et al., 2003; Lavado et al., 2008). Because of the severity of this phenotype, study of *Six3* function in later steps of telencephalon development, such as patterning, differentiation, or cell migration, is precluded. Therefore, a complete picture of *Six3* function in telencephalon development remains to be resolved.

After induction, the telencephalon is patterned along its DV axis to generate a number of neuronal and glial cell types. During segmentation stages, the telencephalon is initially divided into dorsal and ventral domains as a result of the activity of Bmp, Wnt, Hh, and Fgf signaling pathways (Table I) (Wilson and Rubenstein, 2000; Wilson and Houart, 2004; Hebert and Fishell, 2008). The dorsal and ventral telencephalon are each subdivided by additional signals to generate numerous gene expression domains that give rise to cells with specific neuronal and glial phenotypes (Flames et al., 2007; Medina and Abellan, 2009; Moreno et al., 2009). In approximately 10% of mouse embryos that are heterozygous for a partial loss of function mutation in *Six3*, telencephalon DV

Table 1. Timing of major events that affect Six3-mediated telencephalon DV patterning in zebrafish embryos. References found in text where appropriate.

Stage	Experimental observations
Early gastrulation (shield)	Germ layer induction, neural induction. *six3a*, *six3b*, and *six7* expression in prechordal mesoderm.
Mid-gastrulation (80% epiboly)	AP neural patterning. Initial expression of broad anterior neural markers such as *hesx1*. Reduction of proliferation can not affect telencephalon DV patterning.
Late gastrulation (90 epiboly to tailbud stage)	Expression of *six3a*, *six3b*, and *six7* in anterior neuroectoderm (~90% epiboly). Subdivision of anterior neural plate evident by molecular marker expression. Expression of *foxg1a* established in telencephalon (~90% epiboly). Excess Wnt signaling can repress ventral telencephalon fates (tailbud). Misexpression of *six3b* can repress *wnt8b* expression at 24 hpf (tailbud).
Early segmentation (1- to 8-somite stage)	Expression of *foxg1a* is reduced in *six3b;six7*-deficient embryos (1-somite). Misexpression of *six3b* is insufficient to suppress reduction of *nkx2.1b* expression in *six3b;six7*-deficient embryos (4-somite). Anterior neural tube closure (~4-somite). Expression of *wnt8b* is anteriorly expanded in telencephalon of *six3b;six7*-deficient embryos (5-somite). Optic vesicle evagination (6-somite). Misexpression of *six3b* is insufficient to suppress reduction of *isl1* expression in *six3b;six7*-deficient embryos (6-somite). Loss of Hh signaling has no effect on ventral telencephalon specification (8-somite).
Mid-segmentation (9- to 18-somite stage)	DV patterning is evident by molecular marker expression (~12-somite). Expression of *six7* is no longer observed. (12-somite) Dorsal telencephalon is expanded in *six3b;six7*-deficient embryos (12-somite). Expression of *foxg1a* recovers in telencephalon of *six3b;six7*-deficient embryos (12-somite). Ventral telencephalon is reduced in *six3b;six7*-deficient embryos (16-somite).
Organogenesis (24 hpf/Prim-5)	Experimental readout of telencephalon DV patterning.

patterning is disrupted whereby ventral fates are reduced while dorsal fates are expanded (Geng et al., 2008). This phenotype has been attributed to a disruption in the direct activation of *Shh* transcription by Six3, and consistent with this notion, the penetrance increases to 100% in *Shh*; *Six3* double heterozygous embryos (Geng et al., 2008; Jeong et al., 2008). These studies did not address potential *Shh*-independent roles of *Six3*. Work in this dissertation investigates additional mechanisms by which *Six3* regulates telencephalon DV patterning.

In zebrafish embryos that are homozygous for a null mutation in *six3b* and injected with MO to inhibit translation of *six7*, thereby constituting a partial loss of *Six3* function, the telencephalon is present although DV patterning is disrupted. Specifically, *six3b;six7*-deficient embryos have an expansion of dorsal telencephalon fates with a reduction of ventral ones. Neither reduced cellular proliferation nor increased apoptosis can account for the reduction of ventral telencephalon fates (Chapter II), despite convincing evidence for the involvement of *Six3* in these cellular processes in other contexts (Carl et al., 2002; Del Bene et al., 2004; Gestri et al., 2005; Appolloni et al., 2008; Geng et al., 2008; Lavado et al., 2008). In *six3b;six7*-deficient embryos, Wnt/b-catenin activity in the dorsal forebrain signaling center is expanded anteriorly as evidenced by expression of *wnt8b* and Wnt/b-catenin transcriptional target *axin2*, however no obvious defects are noted in Hh signaling from the ventral forebrain signaling center. Previously, *foxg1a* was shown to function in the integration of the dorsal and ventral signaling centers. Foxg1a directly represses expression of *wnt8b* dorsally, while Hh signaling ventrally is required for full expression of *foxg1a* transcript

(Danesin et al., 2009). Expression of *foxg1a* is transiently downregulated in *six3b;six7*-deficient embryos. Importantly, this downregulation occurs during the time when telencephalon is being patterned by signals such as Shha and Wnt8b (Table I). These data suggest that *foxg1a* may function downstream of *Six3*-related gene function in zebrafish to promote telencephalon DV patterning. The functional relationships between the aforementioned transcription factors and signaling pathways in specification of the ventral telencephalon cell populations delineated by *isl1* or *nkx2.1b* expression has also begun to be addressed. Although excess Hh signaling is sufficient to expand both ventral cell populations, it requires *foxg1a* and *six3b* or *six7* function to do so. Misexpression of *six3b* or *foxg1a* is sufficient to promote *isl1* but not *nkx2.1b* expression in the absence of Hh signaling (Chapter II) (Danesin et al., 2009). Interestingly, misexpression of *six3b* is also sufficient to promote *isl1* expression in the absence of *foxg1a*, suggesting that the relationship between *six3* and *foxg1a* in promoting *isl1* expression is complex, and may involve the ability of both genes to repress *wnt8b.* Whereas excess Wnt signaling is sufficient to repress ventral telencephalon fates, it remains unclear as to whether reduction of the expanded Wnt signaling in *foxg1a*- or *six3b;six7*-deficient embryos is sufficient to suppress the DV patterning defects in these embryos. The results of Chapter II advance a novel mechanism by which Six3 interacts with Foxg1, Wnt signaling and Hh signaling to regulate telencephalon DV patterning (Figure 19).

Expression of *Six3* has been observed in prechordal plate mesoderm (PCM) of several vertebrate species and anterior neuroectoderm of all vertebrate
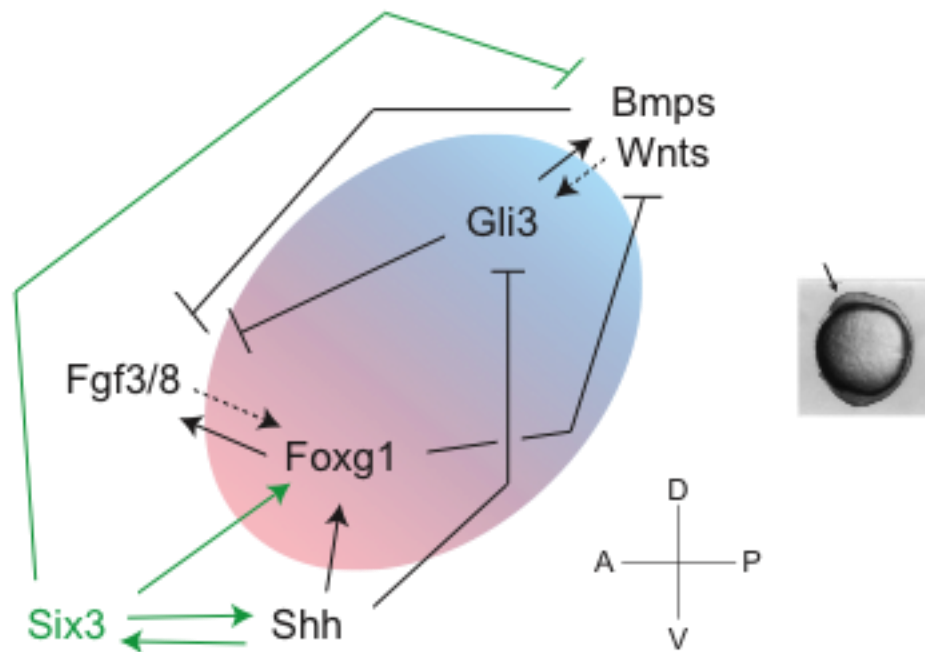
Figure 19. Six3 regulates telencephalon DV patterning during early segmentation. Six3 is required for establishment of *Foxg1* expression and represses expression of Wnt ligands. Studies in mouse embryos have shown that Six3 and Shh form a positive feedback loop, however this has not been demonstrated in zebrafish. Solid lines are supported by experimental data. Dashed lines are potential interactions. Arrow on live embryo denotes location of of sagittal section. Live image modified from Kimmel et al., 1995. Abbreviations: A, anterior; D, dorsal; P, posterior; V, ventral.

species studied (Table I) (Bovolenta et al., 1998; Kobayashi et al., 1998; Loosli et al., 1998; Seo et al., 1998a; Seo et al., 1998b; Zhou et al., 2000; Geng et al., 2008), and it is possible that expression in only one domain is required for DV patterning. As previously mentioned, the role of the PCM in telencephalon DV patterning is not clear. Although it is likely that the telencephalon DV patterning defect in *six3b;six7*-deficient embryos described in Chapter II can be ascribed to neuroectoderm function of *six3b* and *six7*, it is possible that PCM expression may play a role in this phenotype as well. I generated a stable transgenic zebrafish line, *Tg(hesx1:Gal4-VP16)vu416*, which expresses the yeast transcriptional activator Gal4 fused to the activation domain of Herpes simplex virus VP16 under control of the *Hesx1* promoter from the chicken *Gallus gallus* (Spieler et al., 2004). Expression of *Gal4-VP16* is found specifically in the anterior neuroectoderm from mid-gastrulation through mid-segmentation stages when *six3b* is known to be required for telencephalon DV patterning. Use of this transgenic line for tissue specific expression of wild-type *six3b* in *six3b;six7*-deficient embryos may help to address the tissue autonomy of *six3b* function in telencephalon DV patterning as well as other phenotypes that may be identified in additional combinations of *six3* loss of function in zebrafish (i.e. *six3a;six3b*-deficient embryos).

Identifying *Six3* functions in telencephalon

Initial analyses of *Six3* function in vertebrate embryos suggested a prominent role in telencephalon induction. In medaka or mouse embryos with a

strong loss of *Six3* function, telencephalon is strongly reduced in size or absent (Carl et al., 2002; Lagutin et al., 2003). This phenotype is in stark contrast to that identified in humans, where *SIX3* has been correlated with HPE (Wallis et al., 1999). In human HPE patients with heterozygous mutations in *SIX3*, derivatives of the telencephalon such as the cerebral cortex form but fail to separate into two complete hemispheres (Cohen, 2006). HPE-associated *SIX3* mutations have been shown to be hypomorphic or amorphic alleles (Domene et al., 2008; Geng et al., 2008). However, heterozygous knockout mouse embryos exhibited no obvious morphological phenotype in an outbred background (Lagutin et al., 2003). Later work by Geng et al. showed that heterozygous hypomorphic *Six3* mutations can be haploinsufficient in some mouse genetic backgrounds (Geng et al., 2008). The identification of animal models of partial loss of *Six3* function could generate insight into the mechanism by which *Six3* prevents HPE as well as additional roles of *Six3* in telencephalon development.

Given that three *Six3*-related genes are present in the zebrafish genome, loss of one or two of these genes can result in hypomorphic phenotypes. Indeed, early analysis of *six3b;six7*-deficient embryos showed that although eyes are reduced or absent, telencephalon is present, thereby establishing an opportunity to analyze partial loss of *Six3* gene function in that tissue (Inbal et al., 2007). Although midline defects are not observed in *six3b;six7*-deficient embryos, dorsal telencephalon fates are expanded while ventral telencephalon fates are reduced. Similar telencephalon DV patterning defects are consistently seen in human HPE patients and in mouse embryos that are heterozygous for a *Six3* mutation

correlated with human HPE knocked into the endogenous locus (Muenke and Beachy, 2000; Monuki, 2007; Geng et al., 2008). Stronger loss of function mutations in human *SIX3* are correlated with more severe cases of HPE (Lacbawan et al., 2009). Taken together these studies suggest that very low levels of Six3 activity are required for telencephalon induction at late gastrulation, whereas higher levels of activity are required to prevent HPE during segmentation stages. Consistent with this hypothesis, medaka embryos injected with a high dose of *Six3.1* MO manifest reduced telencephalon, while cyclopia is seen in embryos injected with a low dose of the MO (Carl et al., 2002). DV patterning defects may then correlate with a mild HPE phenotype whereby relatively high levels of Six3 activity are required for proper telencephalon DV patterning in zebrafish embryos, and a stronger loss of *Six3* function or loss of a different combination of homologs is required to observe midline defects.

Six3 regulates several cellular mechanisms that may affect the size of specific cell populations. In presumptive telencephalon or telencephalic derivatives, Six3 has been shown to affect fate specification, proliferation and apoptosis (Carl et al., 2002; Lagutin et al., 2003; Del Bene et al., 2004; Gestri et al., 2005; Appolloni et al., 2008; Geng et al., 2008; Lavado et al., 2008; Liu et al., 2010). In homozygous *Six3* knockout mouse embryos, telencephalon is absent despite no obvious changes in cellular proliferation or apoptosis (Lagutin et al., 2003), suggesting loss of *Six3* affects fate specification. Overexpression of Six3 in mouse cortical progenitors in culture promotes proliferation at the expense of differentiation of these cells (Appolloni et al., 2008). In medaka *Six3.1* morphant

embryos with a profound reduction in telencephalic tissue, apoptosis is greatly increased in the presumptive telencephalic region (Carl et al., 2002). Alterations in cellular proliferation and apoptosis were also noted in mouse embryos with a heterozygous HPE knock-in mutation at the *Six3* locus. As previously mentioned, these mouse embryos also have reduced ventral telencephalon and expanded dorsal telencephalon, and it was noted that proliferation is reduced in the anterior telencephalon while apoptosis is increased in the dorsolateral telencephalon (Geng et al., 2008). These cellular changes may contribute to the HPE phenotype in these mutant embryos, but it is unclear how. Telencephalon DV patterning is similarly disrupted in *six3b;six7*-deficient zebrafish embryos, however proliferation and apoptosis do not appear to make significant contributions to this phenotype. Profound reduction in cellular proliferation by treatment with DNA synthesis inhibitors hydroxyurea and aphidicolin in wild-type embryos only produces a mild, statistically insignificant reduction in the size of the *nkx2.1b* expression domain in ventral telencephalon, suggesting that proliferation does not play a significant role in telencephalon DV patterning during segmentation stages (Table 1). In addition, apoptosis is not affected in *six3b;six7*-deficient embryos during segmentation stages as evidenced by TUNEL. Moreover, inhibiting apoptosis does not suppress the reduction of *nkx2.1b* expression in these embryos. Several lines of evidence further support the notion that altered fate specification is the main mechanism whereby telencephalon DV patterning is disrupted in *six3b;six7*-deficient embryos. Additional experiments may be performed to test this hypothesis directly. For

example, a high-resolution fate map of dorsal and ventral telencephalon progenitors can be generated and compared between wild-type and *six3b;six7-*deficient embryos. The lineage of cells can be followed by photo-conversion of light activated molecules such as caged fluorescein or photo-activatible GFP or Kaede.proteins. Single cells could be labeled at late gastrulation or early segmentation stage, and fate would be determined by gene expression in photo-converted cells at 24 hpf. If this mechanistic role for *Six3* is conserved in mammals, it suggests that the DV patterning defect in *Six3*-mediated HPE may also be due to altered fate specification, and the changes in proliferation and apoptosis seen in *Six3* heterozygous mutant mouse embryos may have a more prominent role in affecting telencephalon morphology. Studies suppressing apoptosis or enhancing proliferation in these mutant mouse embryos may shed some light into cellular mechanisms associated with different aspects of HPE.

To gain insight into additional roles of *Six3* in telencephalon development, future studies may analyze conditional mutants where *Six3* is deleted specifically in telencephalon. A *Foxg1-Cre* mouse strain is available to generate a conditional deletion of the *Six3* locus (Hebert and McConnell, 2000). Using this driver to delete *Six3* is insufficient to generate HPE (Geng et al., 2008), however detailed analysis of telencephalon in these mutant embryos was not performed. It would be interesting to assess DV patterning in the telencephalon of these mutant embryos as telencephalon induction occurs during gastrulation, and is marked by expression of *Foxg1* by late gastrulation stages (Table 1) (Woo and Fraser, 1995; Grinblat et al., 1998; Stigloher et al., 2006). Genetic and pharmacological

manipulations of transcription factor or signaling pathway activity after late gastrulation can disrupt telencephalon DV patterning (Chapter II) (Table 1) (Kobayashi et al., 2002; Backman et al., 2005; Danesin et al., 2009), suggesting that DV patterning can be temporally uncoupled from telencephalon induction. Tissue-specific gain-of-function studies can be performed by generation of a zebrafish transgenic line using an *emx3* enhancer region to drive *six3b* expression specifically in telencephalon from late gastrulation onwards. In addition to identifying gain-of-function phenotypes, this line may allow for tissue-specific rescue experiments by misexpressing wild-type *six3b* in telencephalon of *six3b;six7*-deficient embryos to identify functions of *six3b* that are autonomous to telencephalon. Recent innovations in tissue-specific RNA interference have been demonstrated in zebrafish facilitating the converse experiment. Transgenic lines can be generated expressing small hairpin RNA constructs under the control of a tissue-specific promoter to knockdown gene function in that tissue (Rienzo et al., 2012). Also, photo-activation of caged MOs can knockdown gene function in a defined region or group of cells (Ando et al., 2005). These experiments can uncover the tissue- or cell-autonomy of Six3 function in the specification of different cell types. Cell transplantations could further address whether *six3b* is acting cell-autonomously in regulating specific gene expression and/or cell specification. Homochronic, homotopic transplantation of cells from the animal pole of a mid-blastula stage zebrafish embryos would predominantly integrate into anterior neuroectoderm, and could then be assayed by fluorescent in situ hybridization. Conditional loss or gain of *Six3* may also uncover its novel roles in

maintenance, differentiation, migration or axon pathfinding in specific cell populations.

Six3 regulation of telencephalon signaling centers

A number of previous studies have identified several signaling molecules, including Bmp4, Wnt1, Wnt8b and Shh, whose expression is directly regulated by Six3 during forebrain development (Lagutin et al., 2003; Gestri et al., 2005; Jeong et al., 2008; Liu et al., 2010). As most of these reports do not focus specifically on telencephalon development, an understanding of how *Six3* may function in regulating the signaling centers affecting telencephalon DV patterning has not been fully elucidated. The dorsal (Bmp/Wnt), ventral (Shh), and anteromedial (Fgf) signaling centers function to generate proper DV pattern in the telencephalon, and in doing so, each signaling center may also have a cooperative or antagonistic role in regulating the expression of signals from other signaling centers (Figure 4). For example in mouse embryos, Six3 activates *Shh* expression in the rostral diencephalon ventral midline to ensure proper midline development and DV patterning in telencephalon (Geng et al., 2008; Jeong et al., 2008). In mouse embryos with partial loss of both *Six3* and *Shh* function, *Wnt8b* is expressed normally, but *Bmp4* and *Fgf8* expression are reduced in the dorsal telencephalon and anterior midline, respectively (Geng et al., 2008). It is unclear if the alteration of these signaling centers is a function of the loss of *Six3* or secondary to the reduction of Hh signaling in these embryos (Figure 19). As Hh signaling functions normally in *six3b;six7*-deficient zebrafish embryos, zebrafish

provide a useful system for investigation of *Six3* function in the other telencephalic signaling centers.

Although Bmp expression has not been documented in the telencephalon of zebrafish, genes encoding several Wnt ligands are expressed in the dorsal telencephalic signaling center, including *wnt8b* (Carl et al., 2007). In mouse, direct repression of *Wnt8b* by Six3 is required for specification of the neural retina (Liu et al., 2010), however no effects of this interaction have been characterized in the telencephalon. Excess Wnt signaling during early segmentation is sufficient to disrupt telencephalon DV patterning by expanding dorsal and repressing ventral fates (Table I) (Chapter II) (Backman et al., 2005). The interaction between Wnt ligands in the dorsal telencephalon and diencephalon to control the size of this dorsal signaling center has not been well studied. *Wnt8b* mutant mouse embryos have no obvious phenotype in hippocampus, a derivative of the dorsal telencephalon. The lack of phenotype in these mutant embryos may be due to compensatory changes in the expression levels of other Wnt ligands in dorsal telencephalon (Fotaki et al., 2010). Loss of *Wnt3a* has more deleterious effects on development of the mouse hippocampus, and also causes a slight reduction in the expression of telencephalic *Wnt8b* (Lee et al., 2000). These data suggest that the levels Wnt activity may feed back to regulate Wnt ligand expression. The effect of excess Wnt activity on the dorsal telencephalon signaling center has not been characterized, and it is possible that loss of *Six3* gene function and/or increased Wnt signaling may upregulate expression of additional Wnt ligands. A detailed analysis of all relevant Wnt

ligand expression in the dorsal telencephalon and diencephalon of *six3b;six7*-deficient zebrafish embryos may yield insight into this question. In one scenario, *wnt8b* expression alone may be affected, suggesting that *six3a* function provides sufficient levels of *Six3* activity to regulate other components of the signaling center or that additional Wnt ligand expression is not affected by changes in *wnt8b* or *Six3*-related gene function. Alternatively, expression of additional Wnt ligands may be affected by loss of *six3b* and *six7*, and further studies may address whether this is a result of losing *Six3* function directly, reduction of *foxg1a* expression, or disrupted autoregulation of the dorsal signaling center.

Studies have described the role of Six3 in directly regulating *Shh* expression in the ventral signaling center in mouse embryos, and further suggested that partial loss of *Six3* function can reduce *Shh* expression with incomplete penetrance (Geng et al., 2008; Jeong et al., 2008). However, analysis of *six3b;six7*-deficient zebrafish embryos as a partial loss of *Six3* function has not identified a similar connection with Hh signaling. A previous study reported that *six3b* expression is reduced in the telencephalon of mid-segmentation stage zebrafish embryos that lack Hh signaling (Sanek et al., 2009). However, it is likely that this is a secondary effect of temporally upstream DV fate changes in telencephalon, a possibility that was left unaccounted for in the study. The simplest explanation for Hh signaling being unaffected in *six3b;six7*-deficient embryos is that these or possibly all *Six3*-related genes do not activate s*hh* expression in zebrafish as in other vertebrates. In mouse embryos, Six3 directs *Shh* expression by binding to a conserved enhancer element 460 kilobases

upstream of the transcription start site of *Shh*, and this element is conserved in human, chicken and frog (Jeong et al., 2008). Alignment of DNA sequence from any of the above species against zebrafish, fugu or tetraodon failed to identify a conserved element in these fish species using the National Center for Biotechnology Information's ECR browser (www.dcode.org). This suggests that the two pathways may not directly interact in zebrafish as they do in other vertebrates, but may only function in parallel to promote ventral telencephalon fates. Alternatively, a conserved element may exist, but is located far enough away or with sufficiently divergent sequence from the mammalian enhancer as to escape identification by the software. Chromatin immunoprecipitation with an antibody recognizing one or more zebrafish Six3-related proteins, followed by sequencing of fragments, may uncover such a site. If zebrafish Six3-related proteins do directly regulate *shh* expression, then expression of Six3a alone is sufficient to activate expression during forebrain development. A more complete loss of *Six3* function in zebrafish may provide insight into whether the direct interaction between Six3 and *Shh*, as observed in other vertebrates, is conserved in fish.

Several additional lines of evidence support a Hh signaling-independent role for Six3 in zebrafish embryos. There is a strong implication from analysis of *Six3*; *Shh* double heterozygous mouse embryos that the reduction of *Shh* expression can account for the telencephalon DV patterning defects and HPE, however rescue experiments with *Shh* misexpression were not performed in these mutant embryos (Geng et al., 2008). In *six3b;six7*-deficient embryos,

misexpression of *shha* was insufficient to suppress the reduction of ventral telencephalon fates. In addition, misexpression of *six3b* can expand *isl1* expression in the absence of Hh signaling. Together these data suggest that Six3 has functions in telencephalon DV patterning that are not strictly performed by regulation of *shh* expression or Hh signaling. In zebrafish embryos homozygous for strong mutations in both *six3b* and the obligate Hh mediator *smo*, no exacerbation of the *smo*-dependent partial cyclopia was noted despite an synergistic reduction of telencephalic *isl1* expression. Lack of an observed interaction between *six3b* and *smo* in promoting cyclopia may result from there being no interaction between Six3 and Hh signaling in this process or further reduction of Six3 function is required to enhance the phenotype. Synergistic reduction of telencephalic *isl1* expression is consistent with the notion that Six3 and Hh signaling act in parallel, not directly, to specify these cells. It will be important to test which aspects of the *Six3*; *Shh* double heterozygous mouse phenotype are related to reduction of *Shh* expression and which are Shh-independent.

To date, no direct relationship between Six3 and Fgf signaling has been characterized. Previously, expression of *Fgf8* was shown to be reduced in anterior midline of mouse embryos with partial loss of *Six3* function (Geng et al., 2008), but it is likely that this is a secondary effect of the reduction of Hh signaling in the telencephalon of these embryos. Expression of *Foxg1* is dependent on Fgf signaling (Storm et al., 2006), and is reduced in *six3b;six7*-deficient embryos. In mouse embryos, excess Fgf signaling is sufficient to reduce

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Wnt ligand expression in dorsal telencephalon, and *Wnt8b* expression in the forebrain is expanded in *Fgf8* mutant embryos (Shimogori et al., 2004; Storm et al., 2006). Therefore, a contributing mechanism to the telencephalon DV patterning defects in *six3b;six7*-deficient embryos may be disruption of Fgf signaling from the anterior midline. As Fgf ligand expression is downstream of Hh signaling (Aoto et al., 2002; Ohkubo et al., 2002), which is unaffected in *six3b;six7*-deficient embryos, any changes in Fgf signaling in these embryos may result directly from loss of Six3 function. However, maintenance of *Fgf8* expression also requires Foxg1 (Martynoga et al., 2005), so Foxg1-dependence must be tested in any interaction between Six3 and Fgf signaling. Experiments to explore a link between Six3 and Fgf signaling may uncover a novel interaction and implicate Six3 in the direct regulation of all three major telencephalic signaling centers.

Six3 provides competence to respond to telencephalon DV patterning signals

In addition to regulating expression of extracellular signaling molecules, *Six3* has been shown to provide competence for responding cells to attain anterior neural character. While it is unlikely that *Six3* provides competence for Bmp or Wnt signals due to its known repressive effects on these pathways, *Six3* can function as a competence factor for Fgf and Hh signals. Six3 promotes expression of downstream targets of Fgf and Hh signaling, such as *Foxg1* and *Nkx2.1*, that are normally expressed only in the anterior region of the embryo (Shimamura and Rubenstein, 1997; Kobayashi et al., 2002; Vokes et al., 2007).

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Ectopic expression of *Six3* in more posterior regions of the embryo is sufficient to induce ectopic *Foxg1* and *Nkx2.1* near endogenous sources of Fgf and Hh signaling, respectively (Kobayashi et al., 2002). Interestingly, ectopic expression of *Six3* and constitutively active *Fgfr1* or *Six3* and *Shh* can induce *Foxg1* and *Nkx2.1* expression, respectively, at a distance from an endogenous signaling source (Kobayashi et al., 2002). The mechanism of how *Six3* promotes cells to attain anterior character when responding to Fgf and Hh signaling remains to be resolved.

As expression of *Foxg1* in telencephalon anlage is a common downstream effect of both *Six3* gene function and Fgf signaling (Figure 19) (Chapter II) (Kobayashi et al., 2002; Storm et al., 2006; Beccari et al., 2012), it may serve as a mediator for both of their activities. In medaka, Six3.2 has been shown to directly bind and activate a conserved enhancer element in the *Foxg1* promoter region (Beccari et al., 2012). As the *Six3* expression domain is initially much larger than the *Foxg1* expression domain, it is likely that Fgf signaling sets the limits for size of the *Foxg1* domain. However, it is not known how Fgf signaling mediates *Foxg1* expression. One mechanism may be that Six3 protein binds to the *Foxg1* enhancer and awaits an unidentified trigger from Fgf signaling to initiate transcription, although it remains to be tested whether *Six3*-deficient cells are capable of responding to Fgf signaling. Six3 may be required for receipt or mediation of Fgf signaling by the responding cell. Additional downstream targets of Fgf signaling should be analyzed in s*ix3b;six7*-deficient embryos to address the functional response to Fgf signaling in telencephalic precursors. These

potential roles of Six3 are not mutually exclusive, so Six3 may be required for direct activation of *Foxg1* and intracellular mediation of Fgf signaling.

In the case of *Nkx2.1*, its expression is directly regulated by Gli transcription factors, which are Hh signaling mediators (Vokes et al., 2007). However, it is not known how Six3 promotes *Nkx2.1* expression in response to Hh signaling. Six3 may bind directly to enhancer regions in the *Nkx2.1* promoter or activate another protein to do so indirectly. Additionally, Six3 may affect the activity or DNA binding site preference of Gli proteins. Any or all of these mechanisms may also contribute to ectopic *Nkx2.1* expression in response to *Six3* misexpression.

Foxg1 also regulates the competence of telencephalic precursors to respond to Hh and Fgf signaling. Expression of *Shh* and *Fgf8* is reduced in *Foxg1* mutant mouse embryos (Huh et al., 1999; Martynoga et al., 2005), however, this is not the only mechanism by which *Foxg1* affects DV patterning in telencephalon. *Foxg1*-deficient telencephalic progenitors are able to respond to exogenous Shh and Fgf8 by expressing downstream target genes, however these cells exhibit a cell-autonomous defect in ventral telencephalon fate acquisition (Manuel et al., 2010). Therefore, in the absence of *Foxg1*, telencephalic progenitors cannot execute the proper response to these ventralizing signals. The ability of s*ix3b;six7*-deficient cells to respond to Hh and Fgf signaling has not been exhaustively tested, and neither has the cell-autonomy of *Six3* function in telencephalon fate acquisition. A similar phenotype between *Foxg1*-deficient and *six3b;six7*-deficient telencephalic progenitors would

further support *Foxg1* as a primary mediator of *Six3*-dependent telencephalon DV patterning, or suggest that the two genes function cooperatively in the process.

It is unlikely that *Foxg1* is the sole effector of *Six3*-mediated competence, since ectopic *nkx2.1b* expression is observed in *foxg1a*-deficient embryos misexpressing *six3b* (Chapter II). Also, ectopic *Foxg1* and *Nkx2.1* expression due to *Six3* misexpression in chick embryos are not seen in overlapping regions (Kobayashi et al., 2002). Ectopic *Nkx2.1* has not been reported in *Foxg1* misexpressing embryos. This suggests that in CNS regions outside of the telencephalon, additional unidentified genes may function with or downstream of *Six3* to provide competence to respond to local signals influencing fate specification.

Six3 promotes striatal development independently of *Foxg1* and Hh signaling

Data in this dissertation have uncovered a novel role for *six3b* in promoting striatal development. Telencephalic expression of the LIM-domain containing transcription factor, *Isl1*, is mainly outside of the proliferative ventricular and subventricular zone, and these neurons will populate the striatum (caudate and putamen), central amygdala and septum (Stenman et al., 2003; Moreno et al., 2008). Previous studies have shown that *Isl1*-positive cells give rise to cholinergic neurons in these regions, and loss of *Isl1* in progenitor cells results in a profound loss of cholinergic innervation to the cerebral cortex (Wang and Liu, 2001; Elshatory and Gan, 2008). Future experiments in *six3b;six7-*

deficient zebrafish embryos could assess the structural integrity of the striatum at larval stages, when it is well defined, and the expression of characteristic neurotransmitters of this region using a marker such as Choline acetyltransferase, an enzyme required for acetylcholine synthesis. Conversely, the ability of *six3b* misexpression to drive telencephalon cells to a cholinergic fate could be addressed. Importantly, the genetic mechanism by which *six3b* misexpression promotes *isl1* expression remains to be uncovered.

One potential mechanism by which *six3b* misexpression may promote *isl1* expression independently of Hh signaling is through upregulation of *foxg1a* expression. In zebrafish embryos, *foxg1a* expression is reduced in embryos where Hh signaling has been disrupted, and misexpression of *foxg1a* is sufficient to suppress the reduction of telencephalic *isl1* expression in Hh signaling-deficient embryos (Danesin et al., 2009). It remains to be tested whether *six3b* misexpression is capable of producing increased levels of *foxg1a* expression. If this were the case, direct activation of *foxg1a* by Six3 may also be independent of Hh signaling. Control of *foxg1a* expression levels by Six3 may also provide a plausible mechanism for the expansion of *isl1* expression due to *six3b* misexpression in *foxg1a* morphant embryos. Experiments to reduce *foxg1a* function in this dissertation utilized MO2-*foxg1a*, a MO designed to block translation of Foxg1a protein, however the relative reduction of protein levels at different MO2-*foxg1a* doses has not been reported (Danesin et al., 2009). It is therefore possible that misexpression of *six3b* upregulates *foxg1a* expression beyond the capacity of MO2-*foxg1a* at the dose used in this study. However, this

seems an unlikely scenario as misexpression of *six3b* is unable to suppress the loss of telencephalic expression of *nkx2.1b* in *foxg1a*-deficient embryos.

While *Foxg1*-dependent specification of ventral telencephalon cells occurs in a cell-autonomous manner, a small population of *Foxg1* mutant cells that express *Isl1* was identified (Manuel et al., 2010). This suggests that there may be cell-autonomous and cell non-autonomous effects of *Foxg1* on the specification of *Isl1*-positive cells or that there is a sub-population of *Isl1* cells that are specified *Foxg1*-independently. These findings in mouse embryos have not yet been tested in zebrafish. If confirmed, *six3b* misexpression in zebrafish may selectively expand a *foxg1a*-independent *isl1*-positive population and/or suppress the loss of *foxg1a* in *foxg1a*-dependent *isl1*-positive cells through the mechanisms described below.

Repression of Wnt signaling may account for the Hh signaling- and *foxg1a*-independent role of *six3b* in promoting *isl1* expression in telencephalon. Mouse embryos with excess β-catenin activity in telencephalon during early segmentation stages show a reduction of ventral telencephalic fates, whereas embryos with a conditional loss of β-catenin function using the same Cre driver show an expansion of ventral fates (Backman et al., 2005). The relationship between Wnt/β-catenin activity during early segmentation stages and telencephalon DV patterning in zebrafish is less clear. Wnt/β-catenin activity affects dorsal telencephalon fates similarly in mouse and zebrafish embryos (van de Water et al., 2001; Backman et al., 2005). Excess Wnt signaling during early segmentation is sufficient to repress ventral telencephalon fates, however it is

unknown if reduction of Wnt/β-catenin activity at this stage is sufficient to expand ventral fates in zebrafish embryos. Expression of *wnt8b* is repressed by Six3 in a Foxg1-independent manner (Chapter II) (Liu et al., 2010), however reduction of *wnt8b* is insufficient to expand ventral telencephalic fates (Danesin et al., 2009). Functional redundancy of Wnt ligands may require reduction of Wnt/β-catenin activity during early segmentation stages as opposed to reduction of a single Wnt ligand or transcriptional mediator in order to affect ventral telencephalon. Dickkopf1b (Dkk1b) is a secreted negative modulator of Wnt/β-catenin activity, and functions by binding and internally sequestering the transmembrane proteins LRP5/6, obligate co-receptors for Wnt/β-catenin activity (Figure 1A) (Kawano and Kypta, 2003). A transgenic zebrafish line with a heat shock inducible *dkk1b* that can temporally disrupt Wnt/β-catenin activity in a ligand-independent fashion has been generated (Stoick-Cooper et al., 2007). This line could address whether disruption of Wnt/β-catenin activity is sufficient to expand ventral telencephalon fates such as *isl1*-positive cells. In addition, it could also serve to directly address whether reduction of Wnt/β-catenin activity can suppress the *six3b;six7*-deficient loss of ventral telencephalon fates. Together that could suggest that modulation of Wnt/β-catenin activity would be sufficient to expand *isl1* expression in telencephalon independently of Hh signaling or *foxg1a* function.

It is also possible that *six3b* promotion of *isl1* expression occurs through a cell-autonomous mechanism. In zebrafish, Six3-related proteins bind to closely related DNA sequences with slightly different affinities (Suh et al., 2010), such that there is a range of affinities of Six3 proteins for specific DNA sequences that

may be influenced in a cell-specific cofactors. Loss of Hh signaling and/or *foxg1a* gene function may affect the choice of enhancer regions by Six3b. Excess Six3b due to overexpression may enable binding to weaker affinity DNA binding sites without modulation from Hh signaling or *foxg1a*. Comparison of chromatin immunoprecipitation results with anti-Six3 antibodies between control, *six3b* misexpressing, Hh signaling-deficient and *foxg1a*-deficient embryos would begin to address this.

Changes in DNA binding affinities of Six3b may be mediated by changes in protein binding partners. Members of the Groucho/Tle family of co-repressors are candidate binding partners as Six3, Six6 and Foxg1 have been shown to physically interact with these molecules (Zhu et al., 2002; Lopez-Rios et al., 2003). A role for Groucho family members in telencephalon DV patterning has been identified in *Xenopus* embryos where reduction of *Tle2* function by MO injection reduces the telencephalic expression domain of *Nkx2.1* (Roth et al., 2010). Additional work on the interaction between Six3, Foxg1 and Groucho/Tle proteins may prove fruitful in identifying additional Six3 target genes.

Lastly, it is possible that Six3 and Foxg1a have overlapping functions in telencephalon DV patterning in zebrafish embryos. Previous studies have already shown that Six3 and Foxg1a both directly repress expression of *Wnt8b* (Danesin et al., 2009; Liu et al., 2010). Six3b can repress *wnt8b* expression likely in a *foxg1a*-independent fashion in zebrafish (Chapter II), consistent with the direct repression in mouse embryos. It seems likely that Foxg1a and Six3 function are each required to repress *wnt8b* expression independently of each

other (Figure 19), but overexpression of one may be capable of functionally substituting for the other. This may also be true for *isl1* expression, where it was shown that excess *six3b* can compensate for loss of *foxg1a* function. However, it remains to be tested if overexpression of *foxg1a* can suppress loss of *isl1* expression in *six3b;six7*-deficient telencephalon. If that is the case, it will be interesting to identify a common target of both transcription factors, whether it is *isl1* itself or some upstream activator of *isl1* expression.

Not all ventral telencephalon cell types are specified by Six3 in Hh signaling- and *foxg1a*-independent fashion. Expression of the ventromedial telencephalon marker *nkx2.1b* requires Hh signaling, *foxg1a*, and either *six3b* or *six7*. *Nkx2.1* is a direct target of Gli proteins (Vokes et al., 2007), but additional studies are required to understand how Six3 or Foxg1 promote *Nkx2.1* expression. It will be interesting to see the mechanistic differences in how Six3, Foxg1 and Hh signaling generate the adjacent *Nkx2.1*- and *Isl1*-positive cell populations.

Zebrafish as a model for *Six3* function in development and disease

Zebrafish have many advantages as a model system to study embryonic brain development. However, the adult zebrafish brain differs morphologically from the adult mouse or human brain as would be expected from the different behavioral requirements for each to interact and flourish within their respective environments. Nevertheless, early development is largely conserved on the molecular level between fish and humans. However, there are some notable

differences (Wullimann and Mueller, 2004). For example, the zebrafish pallium lacks expression of *pax6a*, however expression is observed in the homologous mammalian cortex (Wullimann and Rink, 2001; Wullimann and Mueller, 2004). As discussed previously, the phenotype and mechanism derived from the partial loss of *Six3* function in *six3b;six7*-deficient embryos are not wholly consistent with similar studies in mouse embryos, and it is possible that *Six3* does not play a role in HPE in zebrafish. Mutations in Hh pathway components such as Smo or environmental insults such as ethanol exposure do lead to HPE in zebrafish (Blader and Strahle, 1998; Barresi et al., 2000), suggesting that zebrafish may be a useful model to study some mechanisms by which HPE is generated. However, additional discrepancies between the forebrain development of zebrafish and mammals may arise and must be addressed to further the goal of understanding human forebrain development.

The central hypothesis in this dissertation is the functional redundancy of the three *Six3*-related genes in zebrafish, and a substantial amount of evidence exists to support this claim (discussed in Chapter I). Despite the numerous structural and functional similarities between the *Six3*-related genes in zebrafish and their mouse homolog, contrasts also exist. In the amino-terminal domain of mouse Six3, there is a glycine-rich region that is also present in the *Drosophila* homolog *sine oculis* but is not present in other mouse Six genes or in any of the zebrafish Six3 homologs (Oliver et al., 1995; Seo et al., 1998a; Seo et al., 1998b). The functional significance of this domain is unclear as no protein or DNA binding targets have been mapped to this region. The carboxy-terminal

domain of Six family members may play a role in determining protein binding partners and DNA binding specificity (Hu et al., 2008; Weasner and Kumar, 2009). This region of zebrafish Six7 is particularly divergent from Six3a, Six3b, and mouse Six3 (Seo et al., 1998b). The level of conservation is much higher between the carboxy-terminal domain of mouse Six3 and zebrafish Six3a and Six3b (Seo et al., 1998a), suggesting that these homologs may be more functionally conserved with mammals. The evolutionary divergence of Six7 may indicate that it has developed functions specific to zebrafish development. A comparative analysis of the DNA binding specificity and protein-protein interactions between full length mouse Six3 and zebrafish Six3a, Six3b and Six7 may uncover gene- and/or species-specific gene expression cassettes regulated by each of these proteins. In a pioneering study on the physical interactions of Six3, medaka Six3.1 was used as bait in a yeast two-hybrid screen to identify candidates for physical interaction, and interactions with several basic helix-loop-helix transcription factors were verified (Tessmar et al., 2002). This study provides a proof of concept and some positive controls to test mouse and zebrafish Six3-related proteins in similar fashion. Because of the differences in the number of Six3 homologs in mammals and zebrafish as well as the divergent protein sequence of the zebrafish homologs, it will be important to verify any functional findings from zebrafish in mouse or other mammals.

As *six3b* homozygous mutant embryos exhibit no observable phenotype, they may prove a fruitful tool for identifying novel genetic interactions through their use in a forward genetic screen for phenotype enhancers. A standard three-

generation breeding scheme can generate embryos that are homozygous for both *six3b* and an induced mutation. These embryos may be analyzed morphologically for forebrain or eye defects and cyclopia through several days of development. Motivated by this logic, approximately 50 F2 families were screened in this fashion, and no enhancing mutations were identified. In future screens, a fluorescent transgene could provide higher resolution for such processes as telencephalon DV patterning and midline defects in telencephalon. Such transgenes have been characterized for *dlx* genes, *isl1*, *emx3*, and *eomes*. Analyzing embryos by morphology and transgene expression may facilitate the identification of novel genes that interact with *six3b* in promoting proper forebrain development. These interactions could then be verified functionally in zebrafish and mammalian embryos.

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