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Six3 cooperates with Hedgehog signaling to specify ventral telencephalon by promoting early expression of Foxg1a and repressing Wnt signaling

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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 that results, at least in part, from abnormal telencephalon development. However, the roles of Six3 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 out 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 smaller and dorsal domains are larger. 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 the Wnt/β-catenin pathway.

KEY WORDS: Six3, Telencephalon, Zebrafish

INTRODUCTION

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, whereas 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 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 crucial 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 demonstrate that 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;

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Lacbawan et al., 2009). However, it remains unknown 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 the 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 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 et al. (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 *Nhe*I and *Xho*I 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 *Kpn*I and *Apa*I 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) have been previously described. Embryos were injected at the 1- to 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 carried out 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 (supplementary material Fig. S1). 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 with wild type) and *six3b;six7*-deficient embryos [*six3b*$^{vu87/vu87}$ embryos

injected with MO1-*six7*, which inhibits 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 (Fig. 1A,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 (Fig. 1C,D). Similarly, expression of *isl1*, which encodes 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 (Fig. 1E,F). The expression domain of a third ventral telencephalic marker, the homeobox gene *dlx2a* (Akimenko et al., 1994), was also reduced in *six3b;six7*-deficient telencephalon (Fig. 1G,H). By 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 (Fig. 1I-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 (Fig. 1C-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.

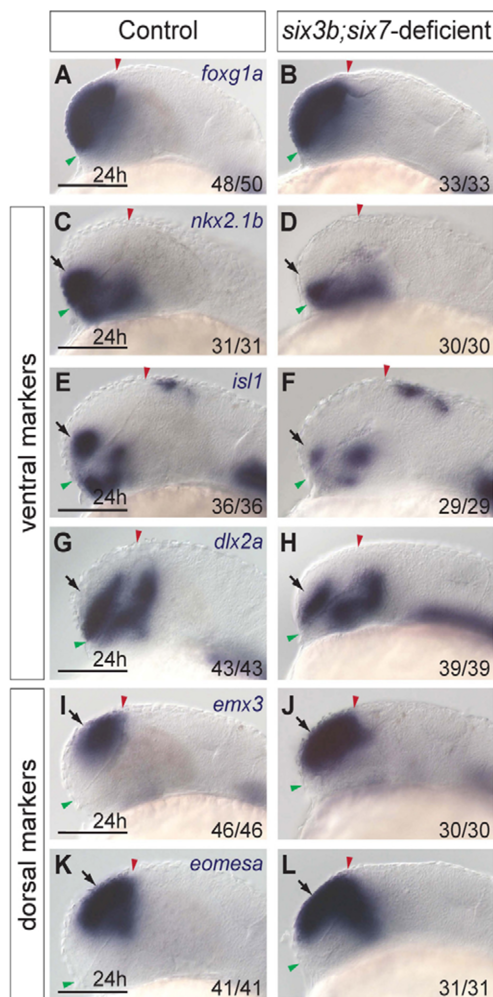

Fig. 1. The telencephalon of *six3b;six7*-deficient embryos is dorsalized. (**A**,**B**) *foxg1a* expression in control (A) and *six3b;six7*-deficient (B) embryos. (**C-H**) Ventral telencephalic expression of *nkx2.1b* (C,D), *isl1* (E,F) and *dlx2a* (G,H) in control (C,E,G) and *six3b;six7*-deficient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

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) onwards. 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 (supplementary material Fig. S1), where ventral telencephalon progenitors are known to reside (Woo and Fraser, 1995). Hydroxyurea/aphidicolin treatment effectively inhibited proliferation within 2 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 (supplementary material Fig. S1A,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 (supplementary material Fig. S1C-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 with vehicle-treated embryos (Fig. 2A,B,G; $P<0.01$). Notably, *emx3* and *nkx2.1b* expression domains appeared relatively normal in the telencephalon in hydroxyurea/aphidicolin-treated embryos (Fig. 2C-F). Quantification of the length of these domains along the telencephalic DV axis showed a slight but statistically insignificant reduction (~5%) compared with vehicle-treated embryos (Fig. 2G; $P>0.27$ for both markers). This discrepancy between the reduction

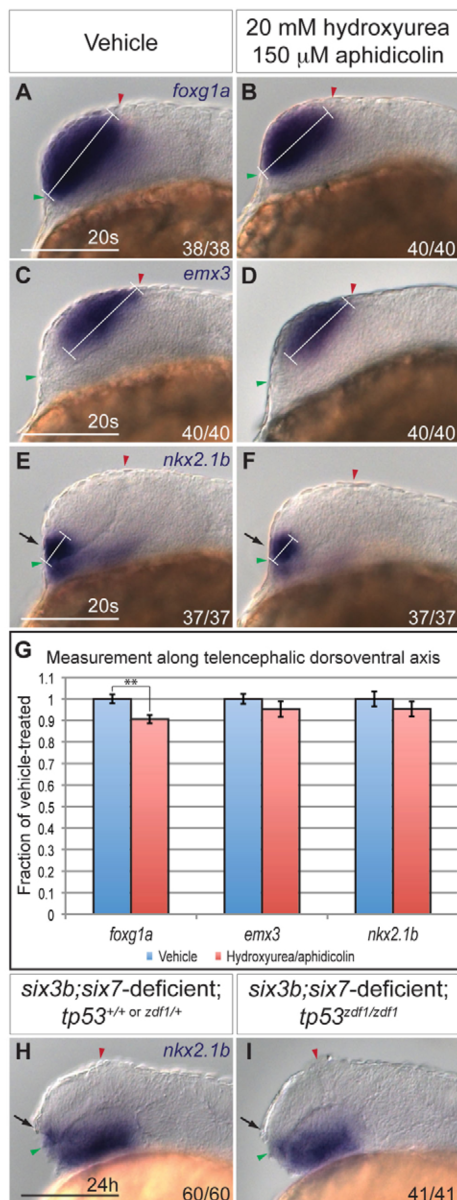

Fig. 2. Cellular proliferation and apoptosis do not significantly contribute to reduction of ventral telencephalon. (**A-F**) Expression of *foxg1a* (A,B), *emx3* (C,D), and *nkx2.1b* (E,F) at the 20-somite stage in wild-type embryos treated with 2% dimethyl sulfoxide alone (A,C,E) or 20 mM hydroxyurea and 150 μM aphidicolin at 80% epiboly (B,D,F). White bracket indicates length of DV domain measured for quantification. Embryos are shown in lateral view with anterior towards the left. Red and green arrowheads indicate dorsal and ventral edges of the telencephalon, respectively. (**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 s.e.m. $^{**}P<0.01$. (**H**,**I**) Expression of *nkx2.1b* at 24 hpf in *six3b;six7*-deficient embryos (H) that are also $tp53^{zdf1/zdf1}$ (I). Arrows in E,F,H,I indicate ventral telencephalon. Scale bars: 100 μm.

of the telencephalon size and the smaller reduction of its ventral and dorsal domains may resolve from analyzing 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 eight-somite or 12-somite stage in *six3b;six7*-deficient embryos (supplementary material Fig. S2A,B,D,E). To address more directly 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 (supplementary material Fig. S2C). However, global reduction of *tp53*-dependent apoptosis failed to suppress the smaller size of the telencephalic *nkx2.1b* domain in *six3b;six7*-deficient embryos (Fig. 2H,I). Collectively, these data support the conclusion that increased apoptosis and reduced proliferation are not major 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 (Fig. 3A,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 (Fig. 3C,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 whether Six3 regulates expression of *nkx2.1b* similarly in zebrafish, we 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 (Fig. 3E,F). These data provide strong support for the ability of Six3 to promote specification of *nkx2.1b*-

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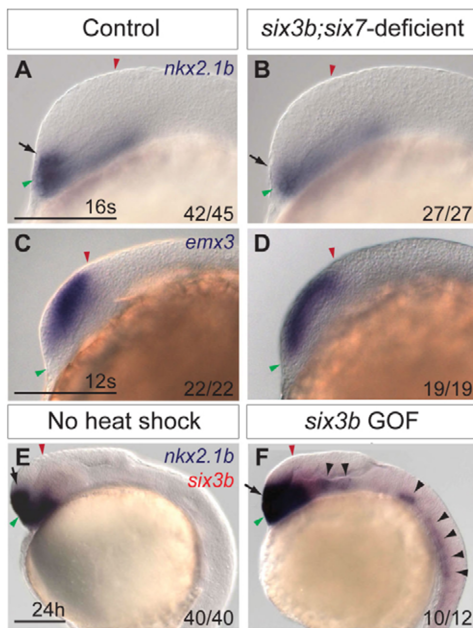

Fig. 3. Six3b is required for specification of ventral telencephalon. (**A**,**B**) *nkx2.1b* expression in telencephalon (arrows) of control (A) and *six3b;six7*-deficient (B) embryos at the 16-somite stage. (**C**,**D**) *emx3* expression in control (C) and *six3b;six7*-deficient (D) embryos at the 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 with embryos not subjected to heat shock (E). GOF denotes gain of function. Embryos are shown in lateral view with anterior towards the left. Red and green arrowheads indicate dorsal and ventral edges of the telencephalon, respectively. Scale bars: 100 μm.

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 issue 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 (Fig. 4C,G). However, when heat shock was applied at the 4-somite stage, *nkx2.1b* expression was no longer restored (Fig. 4D). Similarly, applying heat shock to embryos at the 6-somite stage could not suppress the reduction in *isl1*-positive cells (Fig. 4H). 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 (Fig. 6A,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 determine whether Six3 acts upstream of Hh signaling, we 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- to 3- and 8-somite stage), *ptch2* expression appeared normal (Fig. 5A,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 (Fig. 5C-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 promote ventral telencephalic fates independently 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 (Fig. 6A,B). However, in *six3b*$^{vu87/vu87}$; *smo*$^{b641/b641}$ embryos, very few *isl1*-positive telencephalic cells could be detected (Fig. 6C). 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

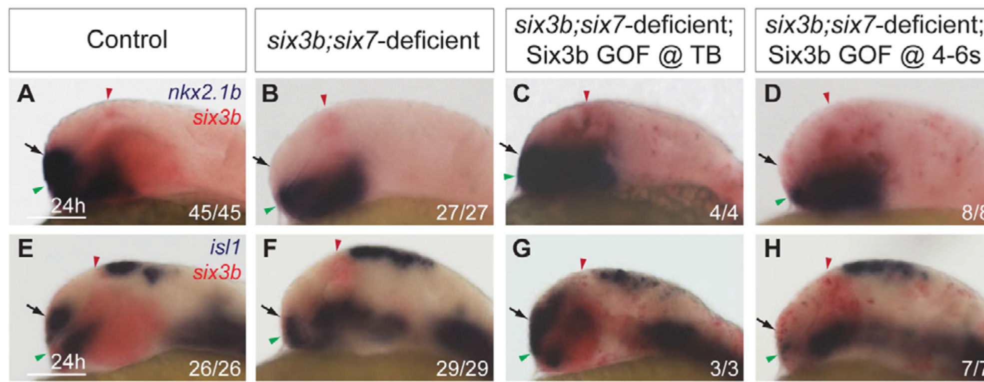

Fig. 4. Six3b is required during early segmentation to promote ventral telencephalic fates. (**A-D**) *nkx2.1b* (purple) and *six3b* (red) expression in control embryos (A), *six3b;six7*-deficient embryos (B), *six3b;six7*-deficient *Tg(hsp70l:Gal4-VP16); Tg(UAS:six3b)* embryos induced to misexpress *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 indicate the ventral telencephalon in 24 hpf embryos. Embryos are shown in lateral view with anterior towards the left. Red and green arrowheads indicate dorsal and ventral edges of the telencephalon, respectively. Scale bars: 100 μm.

telencephalic domain of *nkx2.1b* is precluded owing to the complete loss of *nkx2.1b* expression in Hh signaling-deficient embryos (Fig. 6N), 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* (Fig. 6E,H). By contrast, *isl1* and *nkx2.1b* telencephalic expression domains remained strongly reduced in *six3b;six7*-deficient embryos overexpressing *shha-NH-EGFP* (Fig. 6F,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 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 (Fig. 6K). However, in cyclopamine-treated embryos misexpressing *six3b*, expression of telencephalic *isl1* was restored or even expanded (Fig. 6L). 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 (Fig. 6M-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 whether *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 the 1-somite stage and remained strongly reduced in *six3b;six7*-deficient embryos during early segmentation (6-somite stage), compared with control embryos (Fig. 7A,B; data not shown). However, by the 12-somite stage, telencephalic *foxg1a* expression had largely recovered (Fig. 7C,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 (Fig. 7E-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 the ability of Six3 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 (Fig. 7I-L). By contrast, telencephalic expression of *nkx2.1b* was strongly reduced in *six3b*-misexpressing embryos injected with MO2-*foxg1a* (Fig. 7M-P), whereas ectopic

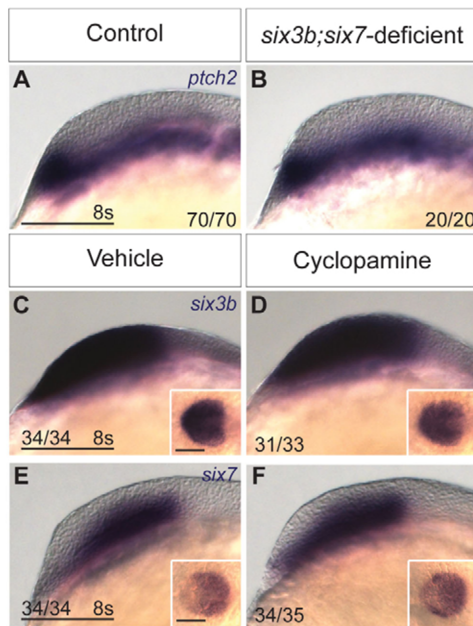

Fig. 5. Six3 and Hh signaling do not regulate each other during early segmentation. (**A**,**B**) Expression of *ptch2* in eight-somite stage control (A) and *six3b;six7*-deficient (B) embryos. (**C-F**) Expression of *six3b* (C,D) and *six7* (E,F) in eight-somite stage wild-type embryos treated from 6 hpf with 10 μM cyclopamine (D,F) and embryos treated with 0.1% ethanol alone (C,E). Embryos are shown in lateral view with anterior towards the left. Insets show same embryo as dorsal view with anterior towards the left. Scale bars: 100 μm.

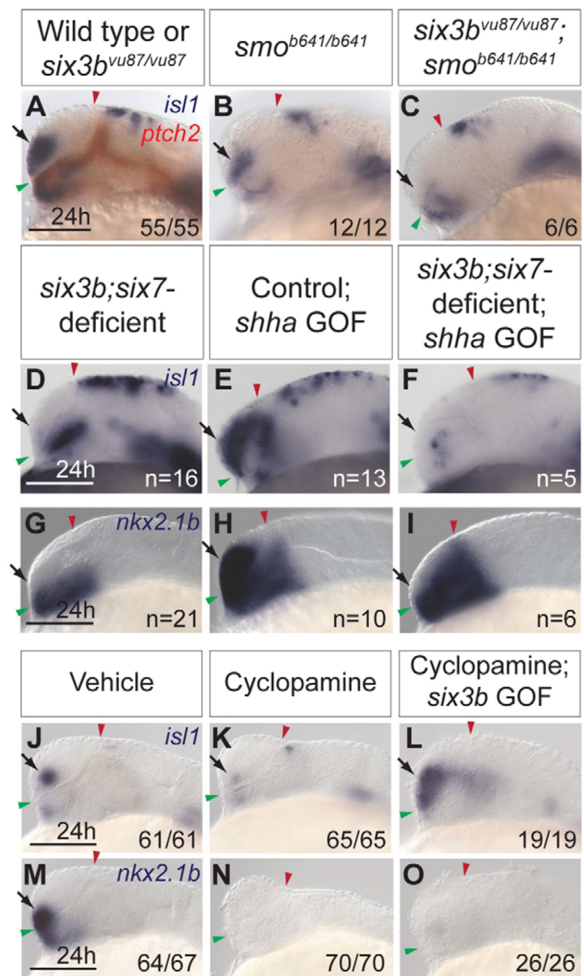

Fig. 6. Interactions between Hh signaling and Six3 in ventral telencephalon formation. (**A-C**) *isl1* (purple) and *ptch2* (red) expression in wild-type and *six3b*$^{vu87/vu87}$ embryos (A), *smo*$^{b641/b641}$ embryos (B) and *six3b*$^{vu87/vu87}$;*smo*$^{b641/b641}$ embryos (C). (**D-I**) Expression 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. Embryos are shown in lateral view with anterior towards the left. Red and green arrowheads indicate dorsal and ventral edges of the telencephalon, respectively. Arrows indicate ventral telencephalon. Scale bars: 100 μm.

nkx2.1b expression was unaffected (40/44 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 24 hpf for *isl1* expression. Unlike Six3, overactivation of Hh signaling could not restore *isl1* expression in *foxg1a* morphants (supplementary material Fig. S3), 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 almost identical to those observed in *six3b;six7*-deficient embryos (supplementary material Fig. S4). 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 (supplementary material Fig. S5), at the 8-somite stage it was expanded anteriorly into the telencephalon of *six3b;six7*-deficient embryos (Fig. 8A,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

DEVELOPMENT

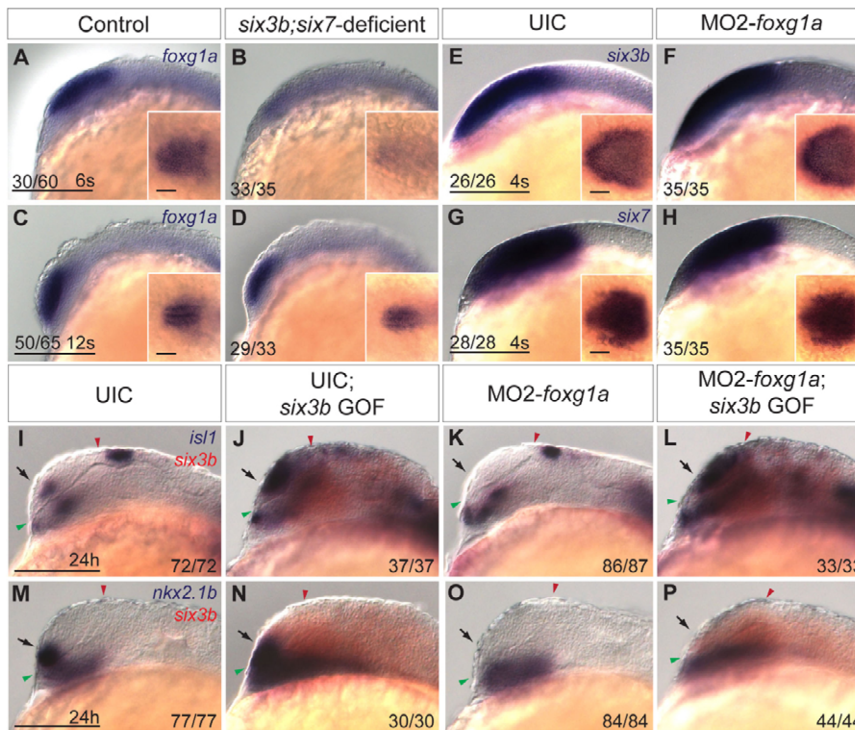

Fig. 7. Interaction between Six3 and Foxg1a in ventral telencephalon development. (**A-D**) Expression of *foxg1a* in control (A,C) and *six3b;six7*-deficient (B,D) embryos at the 6-somite stage (A,B) and 12-somite stage (C,D). (**E-H**) Expression of *six3b* (E,F) and *six7* (G,H) in uninjected control (UIC) embryos (E,G) and MO2-*foxg1a* injected embryos (F,H) at the 4-somite stage. Insets in E-H are dorsal views of the same embryo with anterior leftwards. 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) and MO2-*foxg1a* injected *Tg(hsp70l:Gal4-VP16); Tg(UAS:six3b)* embryos misexpressing *six3b* (L,P) at 24 hpf. Embryos are shown in lateral view with anterior towards the left. Red and green arrowheads indicate dorsal and ventral edges of the telencephalon, respectively. Arrows indicate telencephalic expression domains. Scale bars: 100 μm.

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 (Fig. 8C,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 owing to disruption of Foxg1a function (Danesin et al., 2009); however, misexpression of *six3b* repressed *wnt8b* in both uninjected and *foxg1a* morphant embryos (Fig. 8E-H). Together, these results support the notion that Six3b can repress *wnt8b* expression in a Foxg1a-independent manner, and suggest that expanded activity of the Wnt/β-catenin pathway could contribute to the reduced ventral telencephalic fates in *six3b;six7*-deficient embryos.

DISCUSSION

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) (D.C., L.S.-K. and A.I., unpublished) (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).

As 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 (D.C., L.S.-K. and A.I., unpublished). 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 with 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

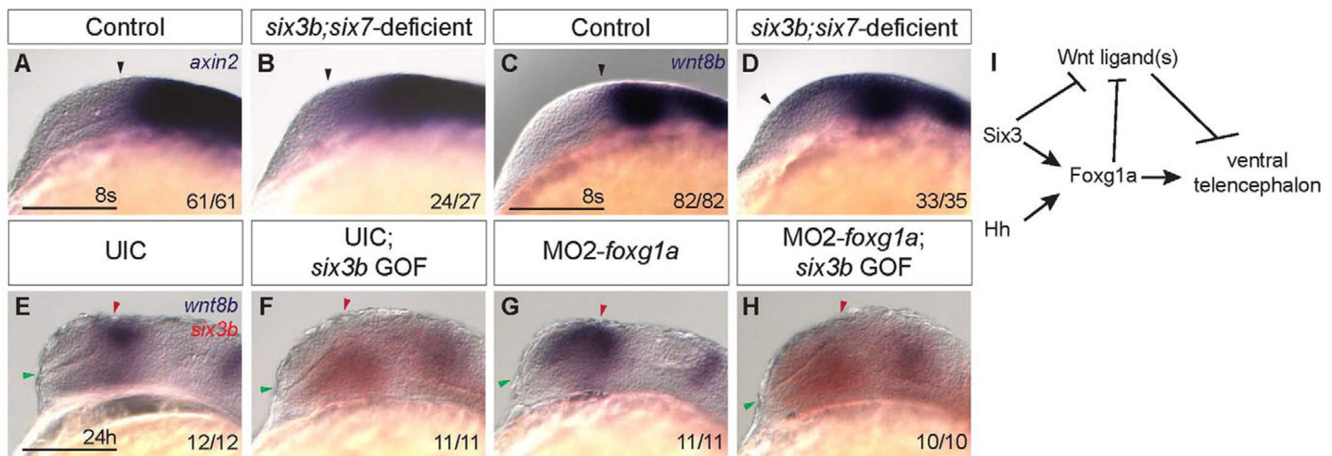

Fig. 8. Six3 represses *wnt8b* expression in a Foxg1a-independent manner. (**A-D**) Expression of *axin2* (A,B) and *wnt8b* (C,D) in control (A,C) and *six3b;six7*-deficient embryos (B,D) at the 8-somite stage. Black arrowhead indicates anterior limit of expression. (**E-H**) *wnt8b* (purple) and *six3b* (red) expression in UIC embryos (E), UIC *Tg(hsp70l:Gal4-VP16); Tg(UAS:six3b)* embryos misexpressing *six3b* (F), MO2-*foxg1a*-injected embryos (G) and MO2-*foxg1a*-injected *Tg(hsp70l:Gal4-VP16); Tg(UAS:six3b)* embryos misexpressing *six3b* (H) at 24 hpf. Embryos are shown in lateral view with anterior towards the left. Red and green arrowheads indicate dorsal and ventral edges of the telencephalon, respectively. 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.

expansion of dorsal telencephalic fates (Fig. 1; Fig. 6K,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 expansion of ventral telencephalic fates at the expense of dorsal ones (Fig. 4C,G; Fig. 6E,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 (Fig. 4) (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 the 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 midsegmentation (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 that lack 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 (supplementary material Fig. S3) (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 (Fig. 8I).

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 (supplementary material Fig. S4) (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* (Fig. 8I). 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 whether this can also be attributed to Foxg1a repression of Wnt ligands. As 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.

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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

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