Neuron
Article

Six3 Represses Nodal Activity to Establish Early Brain Asymmetry in Zebrafish

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DOI 10.1016/j.neuron.2007.06.037

SUMMARY

The vertebrate brain is anatomically and functionally asymmetric; however, the molecular mechanisms that establish left-right brain patterning are largely unknown. In zebrafish, asymmetric left-sided Nodal signaling within the developing dorsal diencephalon is required for determining the direction of epithalamic asymmetries. Here, we show that Six3, a transcription factor essential for forebrain formation and associated with holoprosencephaly in humans, regulates diencephalic Nodal activity during initial establishment of brain asymmetry. Reduction of Six3 function causes brain-specific deregulation of Nodal pathway activity, resulting in epithalamic laterality defects. Based on misexpression and genetic epistasis experiments, we propose that Six3 acts in the neuroectoderm to establish a prepattern of bilateral repression of Nodal activity. Subsequently, Nodal signaling from the left lateral plate mesoderm alleviates this repression ipsilaterally. Our data reveal a Six3-dependent mechanism for establishment of correct brain laterality and provide an entry point to understanding the genetic regulation of Nodal signaling in the brain.

INTRODUCTION

Brain asymmetry is evident at anatomical and molecular levels and has implications for behavior and cognitive functions. To date, our only insights into the molecular mechanisms underlying the development of brain laterality come from studies of asymmetry in the epithalamus of zebrafish (reviewed in Halpern et al., 2003). The epithalamus contains the centrally positioned pineal organ (a component of the photoneuroendocrine system) and, in many vertebrates, an accessory organ (named parapineal in fish). Additionally, the epithalamus contains the bilateral habenular nuclei, which are part of a conserved conduction system connecting the forebrain and ventral midbrain. Epithalamic asymmetries are conserved throughout many vertebrate species (reviewed in Concha and Wilson, 2001; Harris et al., 1996). In larval zebrafish, they include localization of the parapineal to the left side (Concha et al., 2000; Gamse et al., 2002) as well as differences in size, anatomy, gene expression, and pattern of target innervation between the left and right habenular nuclei (Aizawa et al., 2005; Concha et al., 2000, 2003; Gamse et al., 2003, 2005).

Transient left-sided activation of the Nodal signaling pathway in the developing dorsal diencephalon during mid-late segmentation stages is required to determine the direction of epithalamic lateralities (Concha et al., 2000, 2003; Liang et al., 2000). It has been proposed that diencephalic expression of Nodal pathway genes is first repressed altogether by early Nodal signaling, likely emanating from midline tissues during gastrulation (Concha et al., 2000; Liang et al., 2000). Subsequently, diencephalic Nodal pathway gene expression is activated on the left side during late segmentation, presumably by Nodal signaling from the left lateral plate mesoderm (LPM) (Long et al., 2003). However, the factors that function within the developing brain itself to regulate diencephalic Nodal pathway activity remain unknown.

Six3 is a homeodomain transcription factor that is essential for forebrain and eye development (Carl et al., 2002; Lagutin et al., 2003). Mutations in human *SIX3* can cause holoprosencephaly, the most common congenital malformation of the forebrain (reviewed in Cohen, 2006). We have generated a zebrafish model of Six3 loss of function, which, contrary to previously described Six3-deficient murine and medaka embryos lacking forebrain tissue, enables the study of the roles of Six3 in forebrain patterning. We find that Six3 is also essential for correct left-right epithalamic patterning in zebrafish. The combined inactivation of Six3b and Six7, two zebrafish homologs of Six3 (Kobayashi et al., 1998; Seo et al., 1998a, 1998b), causes brain-specific spatial and temporal deregulation of Nodal pathway activity, as evidenced by excessive, symmetric, and precocious Nodal pathway gene expression during late segmentation, and subsequently results in randomization of epithalamic asymmetries. Rescue experiments, in which Six3 function is restored in a tissue- or time-specific manner, and epistasis experiments support a model whereby Six3 functions in the neuroectoderm no later than early segmentation to bilaterally repress diencephalic Nodal activity. Subsequently, at late segmentation, Nodal signaling from the left LPM is

required to alleviate this repression ipsilaterally. Our results uncover a Six3-dependent regulatory mechanism of Nodal pathway activity in the developing brain that is essential for the establishment of correct brain laterality, without requiring asymmetric expression of *six3*. Identification of Six3 targets in this process should help delineate the molecular genetic hierarchy that governs left-right brain patterning.

RESULTS AND DISCUSSION

six3b/six7-Deficient Embryos

Vertebrate Six3 is expressed in the anterior neuroectoderm during early embryogenesis and afterward in specific forebrain territories. Its loss of function in mice or medaka fish leads to severe forebrain deficiencies by early segmentation (Carl et al., 2002; Lagutin et al., 2003), thereby leaving open the question of the functional significance of Six3 in later forebrain development. In zebrafish, three *six3*-related genes, *six3a*, *six3b*, and *six7*, are similarly expressed in the prechordal plate throughout gastrulation and in the anterior neuroectoderm during late gastrulation and early segmentation (Kobayashi et al., 1998; Seo et al., 1998a, 1998b). We reasoned that loss of function of one or two of these genes may lead to specific forebrain malformations without causing severe forebrain deficiencies. Using Targeting Induced Local Lesions IN Genomes (TILLING) (Draper et al., 2004; Wienholds and Plasterk, 2004), we identified a nonsense mutation in *six3b* (E109→stop), which is expected to result in a truncated protein lacking part of the Six domain and the entire homeodomain (Figure 1A). Whereas misexpression assays suggest that $six3b^{vu87}$ is a null allele (see Figure S1 in the Supplemental Data available with this article online), $six3b^{vu87/vu87}$ embryos appear normal and develop to adulthood, most likely due to redundancy between *six3*-related genes. We therefore used antisense morpholino oligonucleotide translation interference to inhibit specifically Six7 function (MO^{six7}) (see Experimental Procedures). Injection of MO^{six7} into wild-type or $six3b^{vu87/+}$ embryos did not produce any specific defects. However, $six3b^{vu87/vu87}$ embryos injected with MO^{six7} exhibited strongly reduced or no eyes (Figure 1C), a phenotype that could be rescued by coinjection of synthetic RNA encoding Six3b or Six7 (insensitive to MO^{six7}) (Figure S1; data not shown). Morphological and molecular marker analyses revealed that gross regionalization of the brain in these embryos was relatively normal, and the forebrain was not reduced significantly (Figure 1E).

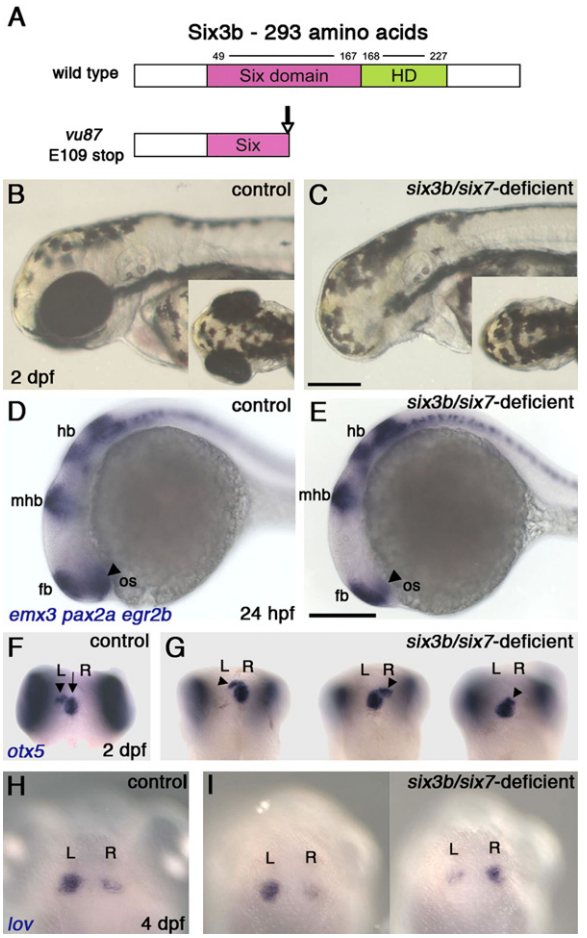

Figure 1. Combined Loss of Six3b/Six7 Function Results in Lack of Eye Tissue and Abnormal Brain Laterality

(A) Schematic presentation of normal Six3b and the predicted truncated Six3b protein, encoded by the $six3b^{vu87}$ allele.

(B and C) Live control embryo (B) and a *six3b/six7*-deficient embryo (B) demonstrating lack of eye tissue.

(D and E) Comparable brain patterning in 24 hpf control (D) and *six3b/six7*-deficient (E) embryos. Forebrain (fb), midbrain-hindbrain boundary (mhb), and hindbrain (hb) are labeled with *emx3*, *pax2a*, and *egr2b*, respectively. The optic stalk (os, arrowheads, *pax2a*) is reduced in the *six3b/six7*-deficient embryo.

(F and G) Pineal (arrow) and parapineal localization (arrowheads) in control (F) and *six3b/six7*-deficient (G) embryos.

(H and I) Habenular nuclei labeled with *lov*, in control (H) and *six3b/six7*-deficient (I) embryos. Control embryos are $six3b^{vu87/+}$ or $six3b^{vu87/vu87}$ and present normal phenotypes.

R, right; L, left. Anterior is to the left in (B)–(E) and up in (F)–(I). (B)–(E) are lateral views, and (F)–(I) and insets in (B) and (C) are dorsal views. Scale bars, 200 μm.

Brain-Specific Randomization of Asymmetry in *six3b/six7*-Deficient Embryos

Detailed analysis of the forebrain of *six3b/six7*-deficient embryos revealed laterality defects. We used molecular markers to examine the developing dorsal diencephalon (epithalamus). At 1 day postfertilization (dpf), expression of *floating head* (*flh*) (Talbot et al., 1995), marking the pineal, was comparable between control and *six3b/six7*-deficient embryos (Figures 2G–2J). At 2 dpf, *otx5* staining, which labels both the pineal and parapineal (Gamse et al., 2002), revealed a normal-looking pineal organ, whereas the parapineal was often found more anteriorly than normal and was sometimes positioned to the right of the pineal (Figure 1G). By 4 dpf, the parapineal was localized more posteriorly, on the left or the right in similar numbers

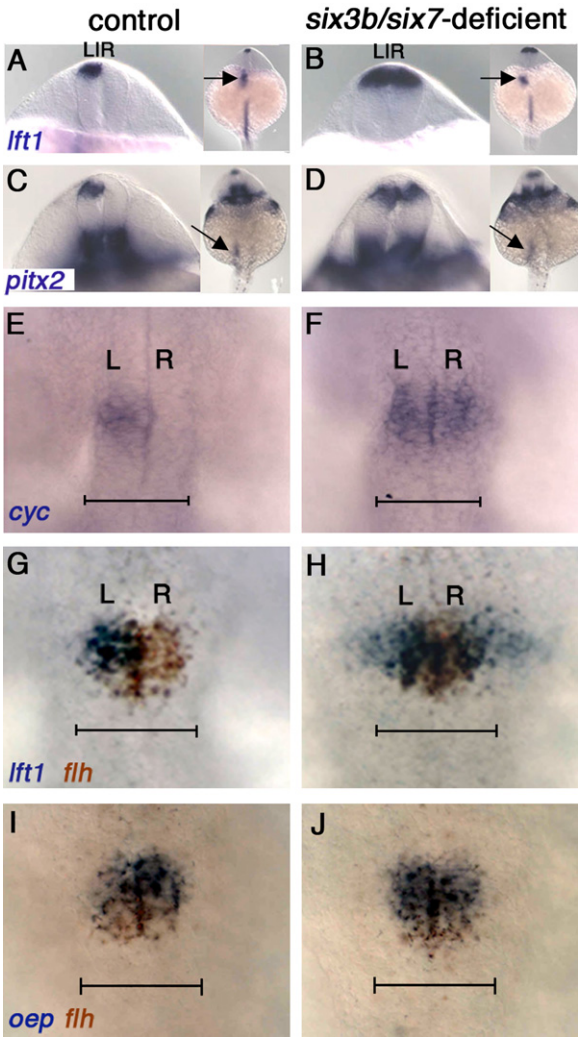

Figure 2. Brain-Specific Loss of Asymmetry and Excessive Diencephalic Nodal Activation in *six3b/six7*-Deficient Embryos

(A–F) Left-sided expression in control (A, C, and E) and bilateral expression in *six3b/six7*-deficient embryos (B, D, and F) of *lft1* (A and B), *pitx2* (C and D), and *cyc* (E and F). In the LPM, expression remains left-sided (arrows in insets [A–D]).

(E–H) The diencephalic domain of Nodal expression and activity is expanded in *six3b/six7*-deficient embryos (compare [F] to [E] and [H] to [G]). *lft1* expression extends outside the *flh*-positive pineal anlage (H).

(I and J) *oep* expression continues to coincide with the pineal anlage in *six3b/six7*-deficient embryos (J). Control embryos in all panels are $six3b^{vu87/+}$ or $six3b^{vu87/vu87}$ and present normal phenotypes.

R, right; L, left. (A–D) Dorsoposterior views; insets show smaller magnification of the same embryo in each panel. (E–J) Dorsal views, anterior is up. All embryos are 21–24s stage. Scale bars, 100 μm.

of embryos (data not shown, n^{left} = 29 [54%], n^{right} = 25 [46%], Figure S2A). Similar defects were observed with *gfi1*, a parapineal-specific marker (Dufourcq et al., 2004) (data not shown, n^{left} = 22 [60%], n^{right} = 15 [40%], Figure S2B). Parapineal laterality is correlated with and affects the asymmetry of the habenular nuclei (Concha et al., 2000, 2003; Gamse et al., 2003). Indeed, consistent with randomization of parapineal localization, habenular nuclei asymmetry was also randomized in *six3b/six7*-deficient embryos, as demonstrated by expression of *leftover* (*lov*), an asymmetric marker of the habenula (Gamse et al., 2003) (Figure 1I, n^{left} = 13 [50%], n^{right} = 13 [50%], Figure S2C). The randomization of parapineal sidedness and habenular asymmetry is indicative of a defect in the establishment of correct brain laterality. By contrast, heart laterality was normal in *six3b/six7*-deficient embryos (data not shown, n^{left} = 21, n^{right} = 0, Figure S2D), suggesting that laterality defects in these embryos were brain specific (also see below).

Loss of Early Brain Asymmetry in *six3b/six7*-Deficient Embryos

Next, we wished to determine when during embryogenesis the brain asymmetry defects arose in *six3b/six7*-deficient embryos. Brain asymmetry can be first detected at the 18 somite stage (18s, 18 hr postfertilization [hpf]), by left-sided expression of *cyclops* (*cyc*), a zebrafish *Nodal* homolog (Rebagliati et al., 1998a; Sampath et al., 1998), followed by left-sided expression of *lefty1* (*lft1*), encoding a Nodal pathway feedback inhibitor (Bisgrove et al., 1999; Thisse and Thisse, 1999), and *pitx2*, encoding a Nodal effector (Campione et al., 1999; Essner et al., 2000), largely within the presumptive pineal (Bisgrove et al., 2000; Concha et al., 2000, 2003; Liang et al., 2000) (Figure 2G). This asymmetric expression is essential for establishment of correct laterality in the epithalamus: both lack of expression of these genes or their bilateral expression lead to randomization of parapineal and habenular laterality (Concha et al., 2000; Gamse et al., 2003). We found that in *six3b/six7*-deficient embryos, *cyc*, *lft1*, and *pitx2* are expressed bilaterally in the dorsal diencephalon (Figures 2B, 2D, and 2F). In accordance with laterality defects being confined to the brain, expression of *lft1*, *pitx2*, and the Nodal homolog *southpaw* (*spaw*) (Long et al., 2003) remained asymmetric in the left lateral plate mesoderm (LPM), as in wild-type (Figures 2B and 2D; data not shown).

In addition to being bilateral, Nodal pathway gene expression is broader and precocious in *six3b/six7*-deficient embryos. Nodal pathway activation, as marked by expression of *lft1*, was also detected well outside the presumptive pineal, marked by *flh* expression (Figure 2H), suggesting that Six3 is required to limit the domain of Nodal pathway activity within the dorsal diencephalon. Interestingly, the expression domain of the *one-eyed-pinhead* (*oep*) gene, encoding an obligatory Nodal coreceptor (Gritsman et al., 1999), was only mildly enlarged and continued to coincide with *flh* (Figure 2J). In addition, loss of Six3b/Six7 function caused precocious diencephalic Nodal activity. Similar to wild-type embryos, at 18–20s Nodal pathway activation was observed in only a small fraction of $six3b^{vu87/+}$ or $six3b^{vu87/vu87}$ embryos, but in *six3b/six7*-deficient sibling embryos, it was significantly more frequent (Figure S3).

Altogether, these data demonstrate that the concerted action of Six3b and Six7 is necessary for spatial and temporal regulation of Nodal activity in the developing diencephalon, so that early brain asymmetry and, afterward, correct brain laterality are achieved. The penetrance of the excessive, symmetric Nodal expression phenotype observed in *six3b/six7*-deficient embryos varied depending on the parents. Incomplete penetrance is also frequently seen in other laterality mutants (Bisgrove et al., 2000), suggesting that the phenotype is influenced by the presence of modifier/s, which are yet to be identified.

Six3 Function Is Required in the Neuroectoderm to Repress Diencephalic Nodal Activity

Diencephalic Nodal pathway gene expression is thought to be controlled by Nodal pathway activity in two phases. Loss of Nodal signaling during both gastrulation and segmentation results in bilateral diencephalic Nodal expression (Concha et al., 2000; Liang et al., 2000). However, loss of Nodal signaling only during segmentation results in the absence of diencephalic Nodal expression (Concha et al., 2000; Liang et al., 2000; Long et al., 2003). Thus, the current model proposes that early Nodal pathway activity is required to repress Nodal pathway gene expression in the brain bilaterally, whereas later Nodal signaling, likely initiated by expression of *spaw* in the left LPM, is required to activate it ipsilaterally (Concha et al., 2000; Liang et al., 2000; Long et al., 2003).

To determine whether and how Six3b/Six7 affect the hierarchy of Nodal signaling in left-right axis formation, we addressed the spatiotemporal requirements for their function in the establishment of early brain asymmetry. *six3b* and *six7* are expressed in the prechordal plate throughout gastrulation and early segmentation and in the anterior neuroectoderm from late gastrulation. By midsegmentation, *six7* expression ceases, whereas *six3b* transcripts become localized mostly to the presumptive telencephalon, eyes, and rostral diencephalon (Kobayashi et al., 1998; Seo et al., 1998a, 1998b). During early segmentation, *six3b* and *six7* expression partially overlaps with the *flh*-positive domain, shown to contribute to the epithalamus and dorsal telencephalon (Masai et al., 1997; Staudt and Houart, 2007) (Figures 3A–3C; data not shown). Importantly, there is no apparent asymmetry in *six3b* or *six7* gene expression. Since deficiencies in axial mesodermal tissues result in bilateral diencephalic Nodal expression (Bisgrove et al., 2000; Concha et al., 2000; Liang et al., 2000; Rebagliati et al., 1998a, 1998b; Sampath et al., 1998), loss of Six3b/Six7 function in the prechordal plate could underlie the loss of early brain asymmetry. Alternatively, asymmetry defects could result from Six3b/Six7 loss of function in the brain/neuroectoderm itself.

Arguing against the first possibility, several lines of evidence suggest that Six3 activity in the prechordal plate does not influence brain asymmetry. We first examined the prechordal plate in *six3b/six7*-deficient embryos using several molecular markers and found no abnormalities in the expression of *gsc*, *cyc*, *lft1*, and *pitx2* (Figure S4), suggesting that structural integrity and Nodal signaling in the prechordal plate are not impaired. Next, we expressed *six3b* from early gastrulation specifically in the anterior axial mesoderm of *six3b/six7*-deficient embryos, harboring *Tg[gsc:Gal4-VP16]*^{vu160} and *Tg[UAS-six3b]*^{vu156} transgenes. The *gsc*:Gal4-VP16 transgene drives expression of UAS-regulated genes in the anterior midline mesoderm, including the prechordal plate (Inbal et al., 2006). However, restoring Six3b function in the prechordal plate failed to suppress the excessive diencephalic Nodal pathway gene expression (Figure 3F).

By contrast, when ubiquitous *six3b* expression was induced by heat shock in *six3b/six7*-deficient embryos harboring *Tg[hsp70l:Gal4-VP16]*^{vu22} and *Tg[UAS-six3b]*^{vu156} transgenes, diencephalic Nodal expression was repressed bilaterally (Figure 4H). Therefore, Six3b/Six7 function is likely required in the neuroectoderm to regulate diencephalic Nodal expression. To test this hypothesis further, we asked whether Six3b misexpression could repress diencephalic Nodal expression in the absence of axial mesodermal tissues. In embryos injected with synthetic *lft1* RNA, early Nodal signaling is inhibited, resulting in deficiencies of axial mesodermal structures including prechordal plate (Bisgrove et al., 1999; Thisse and Thisse, 1999), and later, in bilateral diencephalic Nodal activity (Figure 3G). While ubiquitous *six3b* misexpression did not restore axial mesodermal tissues, it efficiently downregulated diencephalic Nodal expression in these embryos (Figure 3H). This result supports the notion that misexpression of Six3b in the neuroectoderm is sufficient to suppress diencephalic Nodal pathway gene expression. The downregulation of diencephalic Nodal expression by ubiquitous Six3b expression was specific and did not result from loss of presumptive pineal tissue, as judged by *flh* expression (Figure 3J), or from defects in the left LPM *spaw* expression, which is essential for Nodal pathway gene expression in the diencephalon (Long et al., 2003) (data not shown). Taken together, the data are consistent with a model whereby Six3b/Six7 function in the neuroectoderm to repress bilaterally diencephalic Nodal gene expression.

Repression of Nodal Activity by Six3 Occurs by Early Segmentation

Six3 could repress diencephalic Nodal expression directly, by acting in epithalamic cells during midsegmentation stages just prior to initiation of Nodal expression. Alternatively, Six3 functions at earlier stages, given that the proposed bilateral repression of diencephalic Nodal is induced already during gastrulation (Concha et al., 2000). To determine when Six3 can repress diencephalic Nodal expression, we induced ubiquitous *six3b* expression by heat shock in otherwise wild-type transgenic embryos at different developmental stages. Efficient repression was achieved when heat shock was applied at mid-late gastrulation (8–10 hpf), whereas heat-shocking at early segmentation stages (2s or 4s; 10.6 or 11.3 hpf, respectively) caused only partial repression, and

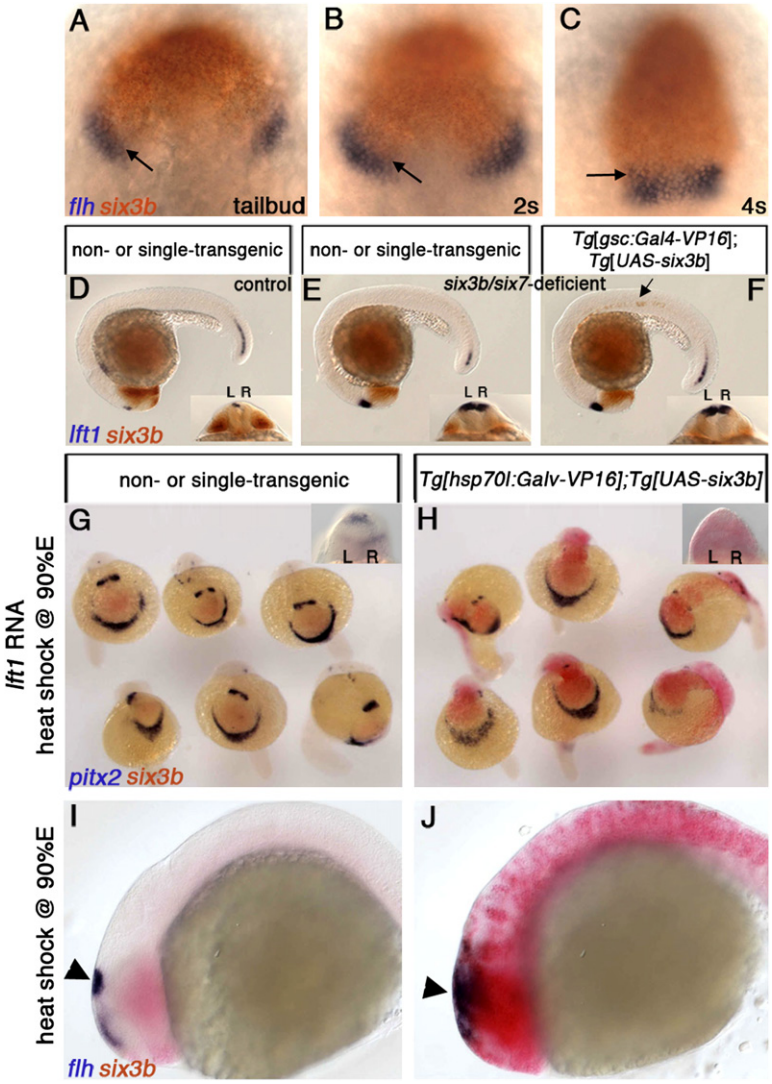

Figure 3. Six3 Function Is Required in the Neuroectoderm to Repress Diencephalic Nodal Activity

(A–C) Wild-type embryos at tailbud (10 hpf) (A), 2s (B), and 4s (C) stages, labeled for *six3b* and *flh* expression, which marks presumptive epithalamic and dorsal telencephalic tissues. Overlap between *six3b* and the *flh*-positive domain is indicated by arrows.

(D–F) Embryos from a cross between *six3b*$^{vu87/+}$;*Tg[gsc:Gal4-VP16]*^{vu160} heterozygous and *six3b*$^{vu87/+}$;*Tg[UAS-six3b]*^{vu156} heterozygous fish. Control embryo (D) not injected with MO^{six7}.

(E and F) *six3b/six7*-deficient embryos. Only the embryo in (F) carries both transgenes, as evidenced by ectopic *six3b* in the notochord (arrow).

(G and H) Embryos from a cross between *Tg[hsp70l:Gal4-VP16]*^{vu22} and *Tg[UAS-six3b]*^{vu156} heterozygous fish that were injected with *lft1* RNA and heat-shocked at late gastrulation (H).

(I and J) Ubiquitous *six3b* does not eliminate presumptive pineal tissue (*flh*-positive domain, arrowheads in [I] and [J]).

R, right; L, left. (A–C) Dorsal views, anterior up. (D–F, I, and J) Lateral views, anterior to the left. Insets in (D)-(H) are dorsoposterior views.

heat-shocking after the 6s stage (12 hpf) no longer repressed diencephalic Nodal expression (Figures 4B, 4D, and 4F; data not shown). Similarly, ubiquitous *six3b* misexpression efficiently suppressed the excessive Nodal expression phenotype of *six3b/six7*-deficient embryos when applied by heat shock at late gastrulation (Figures 4G and 4H), but not when applied at the 4s stage (Figures 4I and 4J). Given that the effector gene is expressed robustly about an hour after heat shock (data not shown), together these results suggest that Six3 functions by the 10s stage to efficiently repress diencephalic Nodal expression, which begins at the 18s stage. Thus, Six3 likely functions as an early bilateral repressor of diencephalic Nodal expression, similar to early Nodal signaling.

We have shown that Six3 can repress diencephalic Nodal expression bilaterally and can only do so by early segmentation. This temporary ability of Six3 to repress diencephalic Nodal expression suggests that there is transient competence of prospective diencephalic tissue for this function, perhaps due to the requirement for another factor, no longer available after early segmentation. Another question is how Nodal repression is maintained during later segmentation. *six3b* and *six7* expression domains partially overlap with the presumptive pineal domain during early segmentation (Figures 3A–3C). Hence, in one scenario, Six3b/Six7 induce another factor in presumptive dorsal diencephalic cells, which, in turn, represses Nodal expression at later segmentation (Figure 5F). Interestingly, the levels of Six3 activity appear also to be important, since overexpression of Six3 during the limited competence period causes repression that cannot be overcome.

Loss of Six3 Function Is Epistatic to Loss of Spaw Function

Based on our results, we propose that a Six3-dependent prepattern of bilateral Nodal repression in the prospective

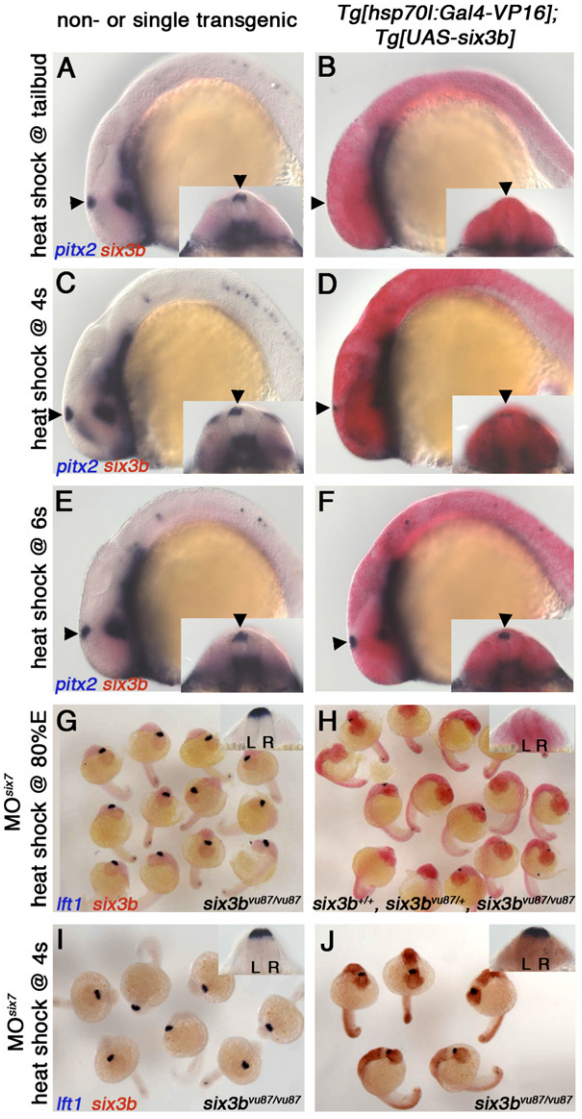

Figure 4. Six3 Misexpression Can Repress Diencephalic Nodal Activity If Induced by the Beginning of Somitogenesis

Tg[hsp70l:Gal4-VP16]^{vu22};*Tg[UAS-six3b]*^{vu156} (B, D, and F) and single or nontransgenic siblings (A, C, and E), heat-shocked at tailbud (10 hpf) (A and B), 4s stage (C and D), or 6s stage (E and F). Arrowheads point at the left-sided dorsal diencephalic domain of Nodal activity represented by *pitx2* expression. (G–J) Embryos from a cross between *six3b*$^{vu87/+}$;*Tg[hsp70l:Gal4-VP16]*^{vu22} heterozygous and *six3b*$^{vu87/+}$;*Tg[UAS-six3b]*^{vu156} heterozygous fish, which were injected with MO^{six7} and heat-shocked at 80% epiboly (80%E) (G and H) or 4s stage (I and J). Insets are dorsoposterior view of the same embryo (A–F) or a representative embryo (G–I). L, left; R, right. Embryos are 22–24s stage. Genotypes in (G), (I), and (J) were inferred from clearly reduced eye size, demonstrating that embryos are *six3b/six7* deficient. Because inducing *six3b* expression by heat shock at 80%E rescues the reduced eye size in *six3b/six7*-deficient embryos, embryos in (H) were PCR genotyped and the presence of all genotypes at expected ratios was confirmed.

dorsal diencephalon is established by early segmentation. The question remains, however, whether later activation of diencephalic Nodal pathway gene expression by Spaw signal from the left LPM is achieved through alleviating the Six3-dependent repression or by directly activating Nodal expression. To distinguish between these two possible models of Spaw action, we inhibited its function using antisense morpholino oligonucleotides in *six3b/six7*-deficient embryos. In this experiment, if Spaw functions by directly activating Nodal expression, then even in the absence of Six3b/Six7 function diencephalic Nodal pathway gene expression would be absent. Alternatively, if Spaw functions by alleviating the Six3-dependent repression, then even without Spaw function diencephalic Nodal expression should be activated. Consistent with a previous report (Long et al., 2003), inhibiting Spaw function abolished diencephalic Nodal activity, as inferred from *lft1* expression, in 99% of progeny from a cross between *six3b*$^{vu87/+}$ and *six3b*$^{vu87/vu87}$ fish (n = 102, Figures 5B and 5E). However, when the function of both Spaw and Six7 was inhibited in sibling embryos, diencephalic *lft1* expression was observed, mostly bilaterally, in 24% of the embryos (n = 101, Figures 5D and 5E). Genotyping confirmed that all embryos showing *lft1* diencephalic expression were *six3b*$^{vu87/vu87}$. Thus, loss of Six3b/Six7 activity can alleviate the need for Spaw-mediated activation of diencephalic Nodal expression. This result supports the notion that Spaw signal from the left LPM promotes ipsilateral diencephalic Nodal expression largely by negatively regulating the bilateral repression set up by Six3. However, because the fraction of *six3b/six7*-deficient embryos exhibiting diencephalic Nodal pathway activation was reduced when Spaw function was inhibited (24% observed compared to almost 50% expected; Figure 5E), loss of Six3b/Six7 function is not completely epistatic to loss of Spaw function. We interpret this incomplete epistasis to mean that Spaw has additional functions, possibly directly activating diencephalic Nodal expression (Figure 5F).

Conclusions

We have described an essential role for Six3 in left-right brain patterning, which provides a mechanism for the regulation of diencephalic Nodal activity within the neuroectoderm. Despite being expressed symmetrically, Six3 is required for brain asymmetry by ensuring repression of diencephalic Nodal activity on both sides of the brain, thus allowing the setting of asymmetric activation later on. That Six3 functions much earlier than the appearance of brain asymmetry is surprising, yet consistent with a similar role proposed for early Nodal signaling (Concha et al., 2000). In agreement is also the recent finding that excessive Wnt activity in the anterior neuroectoderm during a limited time window at late gastrulation leads to brain-specific bilateral Nodal expression, as seen in Six3 loss of function (Carl et al., 2007 [this issue of *Neuron*]). Because high levels of canonical Wnt signaling in the neuroectoderm have been shown

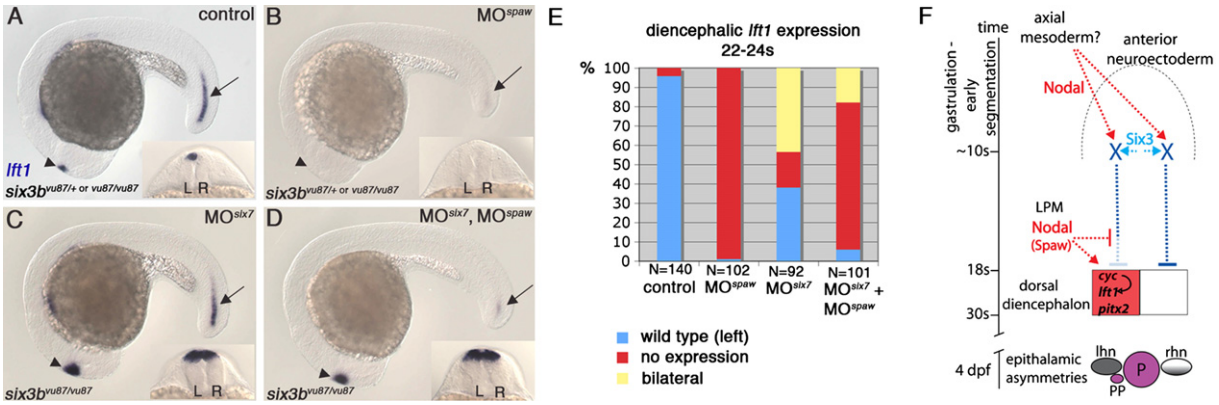

Figure 5. Loss of Six3b/Six7 Is Epistatic to Loss of Spaw Function, and a Model for Six3 Function in Regulation of Diencephalic Nodal Activity

(A) Control, uninjected embryo.

(B) *six3b*$^{vu87/+}$ or *six3b*$^{vu87/vu87}$ injected with Spaw-MO1 (Long et al., 2003) (MO^{spaw}). *lft1* expression is abolished in the diencephalon (arrowhead) and very strongly reduced in the posterior notochord (arrow).

(C) *six3b*/*six7*-deficient embryo.

(D) *six3b*/*six7*-deficient embryo injected with Spaw-MO1. Excessive, bilateral diencephalic *lft1* expression is not abolished, but *lft1* posterior notochord expression is strongly reduced.

(E) In clutches comprising approximately 1:1 ratio of *six3b*$^{vu87/+}$ and *six3b*$^{vu87/vu87}$ embryos, inhibiting Spaw function abolishes *lft1* diencephalic expression in 99% of embryos, inhibiting Six7 function causes bilateral *lft1* expression in more than 40% of embryos (all *six3b*$^{vu87/vu87}$), and inhibiting both Spaw and Six7 results in restoring *lft1* expression in 24% of embryos (all *six3b*$^{vu87/vu87}$).

(F) A model of Six3 function and interaction with Nodal signaling during the establishment of early brain asymmetry. Six3 activity in the anterior neuroectoderm (light blue) and early Nodal signaling, presumably from the axial mesoderm (red), induce by early segmentation repressor/s of Nodal expression (X, dark blue) in the prospective dorsal diencephalon. Later, diencephalic left-sided expression of Nodal is achieved by ipsilateral Spaw, repressing X and activating Nodal. This results in correct epithalamic asymmetries (left-sided parapineal; higher *lov* levels [gray] in left habenular nucleus). P, pineal; pp, parapineal; lhn, left habenular nucleus; rhn, right habenular nucleus.

to repress *six3* expression (Braun et al., 2003; Lagutin et al., 2003; Wilson and Houart, 2004), Six3 may be the main target through which Wnt signaling influences left-right brain asymmetry.

Interestingly, unlike mice or medaka embryos lacking Six3 function, *six3b*/six7-deficient embryos have a relatively intact forebrain. A possible explanation for this difference is the activity of the *six3a* gene, which is highly similar to *six3b* and *six7* in both sequence and expression pattern during early development. In the future, it will be interesting to test whether loss of *six3a* function, alone or in combination with other *six3*-related genes, affects brain asymmetry as well.

The finding that Six3, a transcription factor, is a neuroectodermal repressor of diencephalic Nodal activity should help to delineate the molecular genetic hierarchy that establishes left-right brain asymmetry by identification of Six3 target genes and by testing its functional interactions with other signaling pathways in this process. It is also intriguing that both left-right asymmetry defects and holoprosencephaly are associated with anomalies in Nodal, Hedgehog, and Six3 function (Roessler and Muenke, 2001). Hence, future studies of functional interactions between these genes might be of particular importance to our understanding of normal forebrain development and its pathologies.

EXPERIMENTAL PROCEDURES

Fish Lines and Genotyping

six3b^{vu87} mutation was identified using TILLING as previously described (Draper et al., 2004; Wienholds and Plasterk, 2004) and maintained in AB background. The mutation introduces a g325 > t transversion resulting in gaa (glutamic acid, E109) to taa (stop) change. *vu87* genotypes were identified using the following protocol: a 590 bp fragment from the *six3b* locus was amplified from genomic DNA by PCR using the forward primer 5′ ACGACGTCGGGTTTTCCGTCTTT 3′ and the reverse primer 5′ GTTCGTTCCTTGAAACAGTGC 3′. Because the mutation introduces an MseI restriction site, the PCR product of the *six3b*^{vu87} allele, but not the wild-type allele, is cut to 378 bp and 212 bp fragments.

Tg[UAS-six3b]^{vu156} fish: *six3b* coding sequence was cloned into pT2-UAS-pA-γCry-GM2 to generate pT2-UAS-*six3b*-pA-γCry-GM2 (further details of constructs will be provided upon request). Transgenic fish were generated using the *Sleeping Beauty* transposon system by injecting construct DNA (15–20 pg) and synthetic RNA encoding SB10 transposase (200 pg) into one-cell stage embryos, as previously described (Inbal et al., 2006). Founder fish were identified as previously described (Inbal et al., 2006). Other fish lines used in this work were *Tg[gsc:Gal4-VP16]*^{vu160} (Inbal et al., 2006) and *Tg[hsp70l:Gal4-VP16]*^{vu22} (provided by J.S. and B. Appel).

Heat Shock Treatment

Embryos were placed in prewarmed 30% Danieau's solution in a water bath at 37°C for 30 min. Subsequently, the embryos were allowed to develop at 28.5°C or room temperature until reaching the desired developmental stage, when they were fixed in 4% paraformaldehyde.

Morpholino Oligonucleotide Information, RNA Synthesis, and Injection

MO^{six7} targets the *six7* gene 5′ untranslated region (UTR), whereas $MO^{six7\text{-}ATG}$ was designed to overlap the *six7* gene translation start site. Injection of either MO into *six3b*$^{vu87/vu87}$ embryos resulted in reduced eye phenotype; however, $MO^{six7\text{-}ATG}$ was less effective and yielded nonspecific defects more often. Therefore, we used MO^{six7} in this work, at 1 ng per embryo. MO sequences: MO^{six7}, 5′ CCAACGG CATTCCAGTGTGAGTAAC 3′; $MO^{six7\text{-}ATG}$, 5′ TGAACATCGGCAGAG GAAACATGGC 3′. Spaw-MO1 has been described (Long et al., 2003) and was used at 10 ng per embryo.

MO efficacy and specificity: injection of 30 pg synthetic *six7* RNA caused severe dorsalization and embryo death during segmentation. Coinjection of MO^{six7} and 300 pg synthetic *six7* RNA containing the MO binding site did not have any effect on embryos, whereas coinjection of MO^{six7} and synthetic *six7* RNA lacking the MO^{six7} binding site resulted in the same effects as injection of *six7* RNA alone.

Synthetic capped RNA was prepared using the mMESSAGE mMACHINE Kit (Ambion) from *six3b* (Kobayashi et al., 1998) and *lft1* (*antivin*) (Thisse and Thisse, 1999) expression constructs. pCS2-*six7*: total mRNA was extracted from gastrulation stage embryos using Trizol, and *six7* coding sequence with partial 5′ UTR was amplified by RT-PCR (SuperScript, Invitrogen). The amplified fragment was cloned into pCS2+ between BamHI and XbaI sites. To generate pCS2-*six7-ATG*, which lacks the MO^{six7} binding site, only the coding sequence of *six7* was amplified by PCR from pCS2-*six7* and was cloned into pCS2+ between BamHI and XbaI sites. Sequence was confirmed for both constructs, and synthetic RNA was prepared by NotI digestion and transcription with SP6 RNA polymerase. MOs and synthetic RNA were injected into one- to four-cell-stage embryos.

In Situ Hybridization and Imaging

Single and double whole-mount in situ hybridization using riboprobes was performed according to standard protocols. BMPurple (Roche) was used as blue substrate and either Fast Red (Roche) or INT (iodonitrotetrazolium chloride, Sigma) and BCIP (Roche) mixture were used as red substrates. Images were acquired using a Zeiss Axiophot compound or Zeiss dissecting microscope and Axiocam digital camera.

Statistical Analysis

In all cases, we used the nonparametric χ^2 analysis to test for goodness of fit. P values were determined according to the χ^2 value and degrees of freedom.

Supplemental Data

The Supplemental Data for this article can be found online at http://www.neuron.org/cgi/content/full/55/3/407/DC1/.

ACKNOWLEDGMENTS

We thank J. Gamse and S. Wilson for suggesting experiments; M. Carl and S. Wilson for discussions and sharing unpublished data; and S. Wilson, C. Wright, J. Gamse, B. Appel, G. Levkowitz, X. Geng, and LSK group members for critically reading the manuscript. We thank A. Bradshaw for technical assistance and our entire fish facility staff for excellent fish care. This work is supported by NIH and the Zebrafish Initiative—Vanderbilt University Academic Capital Venture Fund.

Received: April 10, 2007
Revised: May 30, 2007
Accepted: June 26, 2007
Published: August 1, 2007

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