Sp8 controls the anteroposterior patterning at the midbrain-hindbrain border

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The specification of neuronal cell types in the developing neural tube is orchestrated by signaling centers. However, how patterned territories of the central nervous system (CNS) are organized into structures with appropriate size and shape is still unclear. We report that in the absence of the mouse transcription factor mBtd/Sp8, a posterior shift of the isthmic organizer (IsO) occurs, suggesting a crucial role for Sp8 in this process. In addition, large patches of cells ectopically expressing Fgf8, Otx2 and/or Wnt1 in the rostral hindbrain are detected in Sp8 mutant embryos. In this context, midbrain dopaminergic neurons are found posterior to the IsO. Furthermore, we provide evidence that cell proliferation in the mid- and hindbrain is tightly controlled by Sp8 activity. Our observations are consistent with a role for Sp8 in restricting Fgf8 expression at the IsO.

KEY WORDS: Sp8 (mBtd), Isthmic organizer, Midbrain, AP patterning, Mouse

INTRODUCTION

The isthmic organizer (IsO) represents a signaling center located at the midbrain hindbrain boundary (MHB) and expressing several transcription and secreted factors, including Pax2, Pax5, En1, En2, Fgf8 and Wnt1. All these factors are required for the patterning of the midbrain and cerebellum, and the maintenance of their expression in the MHB is interdependent, as evidenced by loss of gene activity over time once one of them is inactivated (Simeone et al., 2002; Wurst and Bally-Cuif, 2001; Raible and Brand, 2004). However, each factor is induced independently of the others. Fgf8 is the only factor known so far that is able to mimic IsO activity, i.e. exhibiting midbrain and cerebellum inducing properties (Crossley et al., 1996). Gain- and loss-of-function experiments revealed a complex combinatorial genetic network that acts to maintain a functional IsO (Simeone et al., 2002; Wurst and Bally-Cuif, 2001; Raible and Brand, 2004; Wurst et al., 1994; McMahon and Bradley, 1990; Wassarman et al., 1997; Urbanek et al., 1997; Reifers et al., 1998; Schwarz et al., 1999; Hirata et al., 2001). Thus, in the anteroposterior (AP) axis, Otx2 and Gbx2 control, through reciprocal repression, the proper positioning of the IsO and the establishment of a sharp Fgf8 expression domain at the MHB (Acampora et al., 1997; Wassarman et al., 1997; Rhinn et al., 1999; Millet et al., 1999; Broccoli et al., 1999; Li and Joyner, 2001; Martinez-Barbera et al., 2001). However, only in the absence of Pax2, is Fgf8 expression not detected in the IsO of C3H/He mouse embryos (Ye et al., 2001). Interestingly, an Otx2-dependent repressive mechanism is also required for the proper positioning of sonic hedgehog (Shh) expression in the ventral midbrain; hence, specifying neuronal progenitors along the dorsoventral (DV) axis (Puelles et al., 2003).

The murine ortholog of the Drosophila buttonhead gene, Sp8 (previously known as mBtd), is a zinc-finger transcription factor belonging to the Sp family and shares with Fgf8 several domains of expression that have organizer function, including limb and tail bud, anterior neural ridge and MHB (Wimmer et al., 1993; Treichel et al., 2003; Bell et al., 2003). In addition, Sp8 mRNA is also detected in the forebrain, midbrain and spinal cord (Fig. 1A-C; data not shown). In the DV axis, Sp8 is expressed in the basal plate of the spinal cord and in the ventral midbrain (Fig. 1D; data not shown). However, Sp8 is not found in the floor plate. At the MHB, Sp8 is found along the whole neural tube. (Fig. 1A-C; data not shown). As reported previously, Sp8-deficient mice suffer from multiple defects (Treichel et al., 2003; Bell et al., 2003). In this study, we have focused on the role of Sp8 in the establishment of the IsO and the development of the midbrain. Strikingly, the lack of Sp8 activity resulted in a posterior shift of the IsO and ectopic patches of Fgf8-, Otx2- and/or Wnt1-expressing cells were found in the ventral part of the rostral hindbrain. As a consequence, midbrain dopaminergic neurons were detected posterior to the IsO of mouse Sp8-embryos. Moreover, Sp8 was also required to control cell proliferation in the developing mid- and hindbrain. Our findings suggest that Sp8 is a new transcription factor required for normal development of the MHB boundary region.

MATERIALS AND METHODS

Generation of knockout mice

Embryos were derived from crossings of Sp8 heterozygous animals described previously (Treichel et al., 2003). All knockout animals were of mixed C57/B16 F1 genetic background. Genotyping was performed by PCR.

In situ hybridization and immunohistochemistry

Embryos were prepared at the appropriate time points and subjected to in situ hybridization or immunohistochemistry. Whole-mount in situ hybridization was performed as previously reported (Treichel et al., 2003). In situ hybridization on consecutive sections (18 μm) using digoxigenin-labeled probes was performed according to Moorman et al. (Moorman et al., 2001). Immunohistochemistry was carried out according to the protocol of Kalloniatis et al. (2002).
to manufacturer’s recommendation. Antibodies were anti-serotonin (5-hydroxytryptamine) from Sigma, and TH, TuJ1 and nestin from Chemicon.

BrdU labeling and TUNEL assay
For BrdU labeling, pregnant females of E10.5 and E11.5 were sacrificed 30 and 60 minutes after intraperitoneal injections of BrdU solution (100 µg/g body weight). Apoptosis was detected according to TUNEL method and performed following manufacturer’s recommendation (Chemicon).

RESULTS
Histological analysis of Sp8-deficient embryos
In the absence of Sp8, the brain phenotype is variable and a high incidence of open mid- and hindbrain was observed during embryonic development (Treichel et al., 2003). In the present analysis, we included only embryos with closed mid- and hindbrain. Histological examination of sagittal sections of E10.5 to E12.5 embryos revealed that, in Sp8 mutant embryos, a remarkable overgrowth of the hind- and the midbrain occurred. This is more pronounced in the ventral part of the neuroepithelium (Fig. 1F-H). From 12 analyzed embryos at E17.5 only three were found to develop a cerebellum-like structure. In most embryos at this stage of development, although the neural tube appeared closed, owing to disturbed tissue growth, in histological sections specific structures of the mid- and hindbrain were difficult to identify and were not further analyzed. Therefore, our analysis will be limited to embryos up to E12.5 of gestation.

Sp8 plays a crucial role in positioning of the IsO
To decipher the role of the Sp8 gene in the IsO, we studied the expression of several molecular markers associated with the MHB from E8.5 to E12.5. The homeobox genes Otx2 and Gbx2 are normally expressed in a complementary pattern along the AP axis, thereby controlling the proper positioning of the IsO (Simeone et al., 2002; Wurst and Bally-Cuif, 2001; Raible and Brand, 2004). In the absence of Sp8 activity, this expression boundary was shifted posteriorly, as revealed by whole-mount in situ hybridization at E8.5, and using Krox20 transcripts to label rhombomere 3 and 5 (Wilkinson et al., 1989a) (Fig. 2). At E9.5, this malformation is still evident (Fig. 3; see Fig. S1 in the supplementary material) and the expression of genes transcribed at the MHB, such as Wnt1, Fgf8, En1 and Pax2 (Simeone et al., 2002; Wurst and Bally-Cuif, 2001; Raible and Brand, 2004) is enlarged (Fig. 3, see Fig. S1, S2 in the supplementary material). At E10.5 the expression boundary where Otx2 posterior border and Gbx2 anterior border abut seems normal, as shown on adjacent sagittal sections (Fig. 4E,G and 4F,H). However, ectopic patches of Otx2-, Wnt1- and/or Fgf8-expressing cells are present in the ventral part of the rostral hindbrain at least until E11.5, as shown on adjacent sagittal sections of E10.5 embryos (Fig. 4A-D and 4G,H; data not shown). This patchy expression is barely detectable at E9.5 but clearly evident at E9.7 (data not shown; see Fig. S2 in the supplementary material). Furthermore, it appears that the expression territory of Fgf8 is more dramatically affected and is more widespread. It is expanded in the dorsal part of the rostral hindbrain as well, and some Fgf8-expressing cell patches appear to localize in the most posterior midbrain (Fig. 4, arrowhead). In addition, in the absence of Sp8 the expression of Fgf8 is

Fig. 1. Tissue overgrowth in the mid- and hindbrain. Whole-mount in situ hybridization showing the expression of Sp8 in the central nervous system at E9.5 (A) and E10.5 (B,C). (A-C) Sp8 is found in the forebrain, midbrain, MHB and in the spinal cord. In the spinal cord (B) and in the midbrain, Sp8 is localized to the ventral part of the neuroepithelium, as presented in a frontal section of an E10.5 embryo at the level of the posterior midbrain in situ hybridized with 35S-labeled Sp8 riboprobe (D). At the MHB Sp8 is expressed along the dorsal and ventral neural tube (A,B). Tissue overgrowth occurring in the mid- and hindbrain of Sp8-deficient embryos is apparent on sagittal sections of E11.5 and E12.5 embryos stained with Cresyl Violet (E,F) or Giemsa (G,H). It appears that the tissue thickening which is leading to a reduced aqueduct space, is more prominent in the ventral part of the midbrain and rostral hindbrain.

Fig. 2. Posterior shift of the IsO in Sp8+/−embryos. Whole-mount in situ hybridization showing lateral views of E8.5 control (A) and mutant (B) embryos hybridized with Otx2 and dorsal views of Gbx2 expression in control (C) and mutant (D) embryos at a similar stage of gestation. Rhombomeres (r), 3 and 5 are labeled in all embryos with Krox20 transcripts. The distance between the posterior limit of Otx2-expression and the anterior boundary of r3 is indicated by a red line in mutant and control embryos. This line also indicates the distance between the posterior limit of Gbx2-expression in r1 and the anterior boundary of r3.
upregulated. This is also true for Wnt1 that exhibits an increased, patchy and more widespread expression in the ventral midbrain as well (Fig. 4). It also appears that most of the ectopic cell patches in the rostral hindbrain express Fgf8, thus probably overlapping with Wnt1 and/or Otx2. By contrast, Wnt1- and Otx2-expressing cells are less abundant and may not always overlap. Finally, rhombomere (r) patterning is not perturbed in the absence of Sp8, as revealed by the presence of Krox20 (r3, 5) and hoxa2 (r2, 3, 5) (Wilkinson et al., 1989a; Wilkinson et al., 1989b) (Fig. 2 and data not shown).

In order to elucidate whether the observed early perturbation of Otx2 and Gbx2 expression is accompanied by a change in cell fate in the rostral hindbrain, we used ephrin A5 and the homeobox gene Pitx3 as markers controlling the territory of specific cell types of the dorsal (inferior colliculus) (Donoghue et al., 1996) and ventral midbrain (dopaminergic neurons) (Smidt et al., 2004), respectively. Interestingly, the expression of Pitx3 is found expanded into the ventral domain of the rostral hindbrain (Fig. 4J,K, arrowhead). Although the expression domain of ephrin A5

Fig. 3. At E9.5, the expression domains of genes transcribed at the MHB is enlarged. Whole-mount in situ hybridization showing the expression of Otx2 (A,B), Gbx2 (C,D), Fgf8 (E,F), En1 (G,H), Pax2 (J,K) and Wnt1 (L,M) and transcribed at the MHB, as indicated. At this stage of development (E9.5), the shift of the IsO, detected at E8.5 and shown in Fig. 2, is still evident (A-D), and the expression domains of the presented markers appear enlarged at the MHB of the mutant embryos (D,F,H,K,M). The embryos shown for Wnt1 are between E9.5 and E10.

Fig. 4. At E10.5, the Otx2/Gbx2 expression boundary appears normal but ectopic expression of Fgf8, Otx2 and Wnt1 is found in the ventral part of the rostral hindbrain. The expression of Otx2, Gbx2, Wnt1 and Fgf8 is shown on consecutive sagittal sections of E10.5 control and mutant embryos, as indicated. The embryos in B and D are different from those in F and G. (A-D) Expression of Fgf8 and Wnt1 is presented in consecutive sagittal sections of wild type (A,C) and mutant (B,D) embryos. The expression of Fgf8 appears more affected and therefore more widespread when compared with Wnt1, and ectopic cell patches seem to reach the most posterior midbrain (arrow in B). In the ventral midbrain, the expression of Wnt1 (D) is patchy and more widespread than in the control embryo. (E-H) Expression of Gbx2 and Otx2 on consecutive sagittal sections of wild type (E,G) and mutant embryos (F,H). The ectopic expression of Otx2 and Wnt1 in the rostral hindbrain is found only in the ventral part, while for Fgf8 this is also true for the dorsal part. (J,K) Expression of Pitx3, as a marker for midbrain dopaminergic neurons (Smidt et al., 2004), on sagittal sections of E12.5 embryos. Ectopic Pitx3 signal can be found in the ventral part of the rostral hindbrain of Sp8-deficient embryos, arrowhead in K. (L-O) Expression of ephrin A5 as a marker for the inferior colliculus (Donoghue et al., 1996) was not modified at E11.5 of gestation, as detected on sagittal sections of two mutant embryos (M,O). Ephrin A5 expression is, however, upregulated in the ventricular zone of the ventral midbrain of these mutants (M,O, arrows) when compared with wild type embryos (L,N).
in the dorsal midbrain of E11.5 and E12.5 embryos is normal, it is upregulated in the ventral portion of the midbrain, as shown on sagittal sections at E11.5 (Fig. 4.M.O, arrows). This led us to speculate that Sp8 function may only be required in the ventral part of the neural tube.

To further decipher whether Otx2 and/or Gbx2 control Sp8 expression at the MHB, we analyzed Sp8 expression in embryos lacking Otx2 and/or Gbx2 activity. The results are shown in Fig. 5, where Sp8 transcripts were analyzed by in situ hybridization on sagittal sections of wild-type, Gbx2–/– (B,F,I), Otx1–/–Otx1–/– (C,G) and Otx1–/–Otx1–/–Gbx2–/– (D) embryos. In situ hybridization was performed on sagittal sections and using 35S-labeled Sp8 riboprobe. Although the Otx1–/–Otx1–/–Gbx2–/– double mutant is lethal between E9.2 and E9.5 and therefore cannot be presented at E10.5 and E12.5, the in situ with Otx1–/–Otx1–/– is shown at E10.5 as the E12.5 phenotype is essentially the same as at E10.5. As can be seen, Sp8 is not downregulated in embryos lacking Gbx2, Otx2 or both proteins (D). Sp8 is rather strongly expressed in Gbx2–/–/Otx1–/–Gbx2–/– deficient embryos in the rostral hindbrain committed to be transformed in an expanded posterior midbrain (Wassarman et al., 1997) (B,F,I). In Otx1–/–Otx1–/–, the rostral tip of the central nervous system should correspond to an isthmus-like structure (Martinez-Barbera et al., 2001) and Sp8 expression at the MHB, we analyzed Sp8 expression at the MHB. The expression of Sp8 is shown, where possible, at different stages of development in wild-type (A,E,H), Gbx2–/– (B,F,I), Otx1–/–Otx1–/– (C,G) and Otx1–/–Otx1–/–Gbx2–/– (D) embryos. In situ hybridization was performed on sagittal sections and using 35S-labeled Sp8 riboprobe. Although the Otx1–/–Otx1–/–Gbx2–/– double mutant is lethal between E9.2 and E9.5 and therefore cannot be presented at E10.5 and E12.5, the in situ with Otx1–/–Otx1–/– is shown at E10.5 as the E12.5 phenotype is essentially the same as at E10.5. As can be seen, Sp8 is not downregulated in embryos lacking Gbx2, Otx2 or both proteins (D). Sp8 is rather strongly expressed in Gbx2–/–/Otx1–/–Gbx2–/– deficient embryos in the rostral hindbrain committed to be transformed in an expanded posterior midbrain (Wassarman et al., 1997) (B,F,I). In Otx1–/–Otx1–/–, the rostral tip of the central nervous system should correspond to an isthmus-like structure (Martinez-Barbera et al., 2001) and Sp8 expression is similar to what has been reported previously for other gene functions transcribed at the MHB (Martinez-Barbera et al., 2001; Li and Joyner, 2001).

**Sp8-deficient embryos display an expansion of the neural tube in the midbrain and in the rostral hindbrain**

Histological analysis of the Sp8 mutant embryos revealed a remarkable increase in the size of the ventral midbrain and rostral hindbrain. In fact, this leads to a highly reduced aqueduct space (Fig. 6.B,D,F,H,K,M). To explore the molecular mechanism underlying such a defect, we used several markers, including Shh, Nkx6.1, Nkx2.2, Wnt5a, Wnt1, Foxa2, Otx1, Otx2, Grg4, Pax6, Ngn2, Gata2, Phox2a and Pax3, and compared their expression pattern in wild-type and mutant embryos at E10.5 and E11.5 (Fig. 6 and data not shown). In the midbrain of Sp8 mutant embryos, the expression of these markers reflected the overgrowth of the radial neuropil, while it is evident, Sp8 is required for the proper activation but for the restriction of Sp8 expression at the MHB. The expression of Sp8 is shown, where possible, at different stages of development in wild-type (A,E,H), Gbx2–/– (B,F,I), Otx1–/–Otx1–/– (C,G) and Otx1–/–Otx1–/–Gbx2–/– (D) embryos. In situ hybridization was performed on sagittal sections and using 35S-labeled Sp8 riboprobe. Although the Otx1–/–Otx1–/–Gbx2–/– double mutant is lethal between E9.2 and E9.5 and therefore cannot be presented at E10.5 and E12.5, the in situ with Otx1–/–Otx1–/– is shown at E10.5 as the E12.5 phenotype is essentially the same as at E10.5. As can be seen, Sp8 is not downregulated in embryos lacking Gbx2, Otx2 or both proteins (D). Sp8 is rather strongly expressed in Gbx2–/–/Otx1–/–Gbx2–/– deficient embryos in the rostral hindbrain committed to be transformed in an expanded posterior midbrain (Wassarman et al., 1997) (B,F,I). In Otx1–/–Otx1–/–, the rostral tip of the central nervous system should correspond to an isthmus-like structure (Martinez-Barbera et al., 2001) and Sp8 expression is similar to what has been reported previously for other gene functions transcribed at the MHB (Martinez-Barbera et al., 2001; Li and Joyner, 2001).

Taken together, it appears that Sp8 is required for the proper positioning of the MHB and to restrict the expression of gene functions defining the molecular code of this region.

**Ectopic midbrain dopaminergic neurons in the rostral hindbrain of Sp8-deficient embryos**

To investigate whether the observed neural tube defects in the mid- and hindbrain could result in perturbations of neuronal differentiation, we examined the expression of the neuronal markers nestin and βIII-tubulin (TuJ1). The results shown in Fig. 8 indicate that, in contrast to control embryos, in the midbrain of E11.5 Sp8 mutant embryos, nestin-positive cells, present as cell patches outside of the ventricular zone, still express nestin but are devoid of TuJ1 signal (Fig. 8D arrow). This indicates that these cells might...
correspond to neuronal progenitors that are not fully differentiated and could suggest that cell differentiation is at least delayed in the absence of Sp8.

In order to obtain more insight into this process, we analyzed whether the differentiation of dopaminergic and serotonergic neurons was altered. The loss of Sp8 function provoked an alteration of the Otx2/Gbx2 expression boundary and ectopic patches of cells expressing Fgf8, Otx2 and/or Wnt1 are found in the ventral part of the rostral hindbrain. This was further corroborated by the presence of transcripts of the homeobox gene Pitx3, a marker for midbrain dopaminergic neurons (Smidt et al., 2004), in this brain area of mutant embryos (Fig. 4J,K). Accordingly, the tyrosine hydroxylase

Fig. 6. The loss of Sp8 provoked overgrowth of the neuroepithelium. (A-M) Expression analysis of several markers of the ventral midbrain: Shh (A,B), Nkx6.1 (C,D), Nkx2.2 (E,F), Wnt5a (G,H), Wnt1 (J,K) and Foxa2 (L,M) on frontal vibratome sections (45 μm) selected at the level of the posterior midbrain. The expression pattern of all these markers presented here reflects the expansion of the neuroepithelium and does not reveal a patterning defect at this stage of development. The overgrowth of the neuroepithelium results in reduced aqueduct space.

Fig. 7. Enhanced cell proliferation in the mid- and rostral hindbrain of Sp8-deficient embryos. (A-D) BrdU-labeled cells with a pulse of 30 minutes are shown in frontal sections, at the level of the posterior midbrain (A,B) and in sagittal hindbrain sections (C,D) of 10.5 wild-type and mutant embryos. In mutant embryos, a remarkable increase in the number of proliferating cells was evident, thus suggesting that Sp8 negatively regulates cell proliferation. All images are at the same magnification. A and B are 5 μm sections; C and D are 10 μm sections.

Fig. 8. Cell differentiation may be delayed at E11.5 in the absence of Sp8. (A-D) Double-immunohistochemical analysis presenting the expression of nestin (green) and β III-tubulin (Tuj1, red) as markers labeling undifferentiated neuronal progenitors and differentiating neurons, respectively. In Sp8 mutant embryos (E11.5), as indicated by the arrowheads in B and D, there are some cells outside of the ventricular zone that still express nestin but are devoid of Tuj1 signal. This suggests that these cells might correspond to neuronal progenitors that are not fully differentiated. C,D are at higher magnification than A,B.
protein (TH), detected by immunohistochemistry on sagittal sections at E11.5, E12.5 and E17.5, was also found at ectopic sites in the rostral hindbrain of Sp8−/− embryos (Fig. 9C, arrowheads; Fig. 9H, arrowhead; Fig. 9L, arrowhead; Fig. 9P, arrowhead). We then asked whether serotonergic neurons, normally positioned directly posterior to the MHB (Hynes and Rosenthal, 1999; Ye et al., 1998), were affected by the lack of Sp8. As shown in Fig. 9 on the same embryo sections together with dopaminergic neurons, serotonergic neurons were not detected at E11.5 (A, B) 5-HT-expressing cells are found just posterior to the MHB (A), there are no such cells in the mutant (C, at higher magnification in D). At this stage of development, dopaminergic neurons (DA) were detected at ectopic positions in the rostral hindbrain of mutant embryos (C, arrowheads). In contrast to E11.5, at E12.5 in the mutant embryo, serotonergic neurons are detectable (H, arrows). However, in the most rostral domain occupied by ectopic dopaminergic neurons, 5-HT-positive cells are not present (arrowhead in H). At E17.5 serotonergic neurons, although slightly reduced in close vicinity to DA (arrows in K and L), appear similar to controls, while the population of DA is increased. There are also some ectopic DA neurons located posteriorly, where in the control only serotonergic neurons can be detected (arrowheads in L and P). These findings indicate that beside the expansion of midbrain DA in the rostral hindbrain, it seems that the onset of serotonergic neuron differentiation is at least delayed in mutant embryos.

DISCUSSION
In the present study, we have analyzed the consequences of the loss of Sp8 function on the development of the IsO and the midbrain. The lack of Sp8 induced overgrowth of the mid- and rostral hindbrain. Molecular marker analysis revealed that the positioning of the IsO is shifted posteriorly in Sp8 mutant embryos. The observed defects were associated with ectopic Fgf8-, Otx2 and/or Wnt1-expressing cells in the ventral part of the rostral hindbrain, and possibly affecting the differentiation of dopaminergic neurons in this area. Finally, we demonstrate that cell proliferation in the mid- and hindbrain is tightly controlled by Sp8 activity.

Sp8 plays a crucial role in positioning of the IsO
Embryological as well as gain- and loss-of-function studies have established that there is a signaling center (IsO) at the MHB that is required for the patterning of the midbrain and cerebellum (Simeone et al., 2002; Wurst and Bally-Cuif, 2001; Raible and Brand, 2004). The reciprocal repression of the homeobox genes Otx2 and Gbx2 is responsible for the positioning of the IsO along the AP axis (Wassarman et al., 1997; Rhinn et al., 1999; Millet et al., 1999; Broccoli et al., 1999; Li and Joyner, 2001; Martinez-Barbera et al., 2001). The boundary of Otx2/Gbx2 expression plays also a crucial role in defining restricted and juxtaposed expression areas of two secreted factors, Fgf8 and Wnt1, at the MHB (Simeone et al., 2002;
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Wurst and Bally-Cuif, 2001; Raible and Brand, 2004; Wassarman et al., 1997; Li and Joyner, 2001; Martinez-Barbera et al., 2001; Li et al., 2002; Li et al., 2005). In the absence of Gbx2 or both Otx2 and Gbx2, the expression domains of these secreted factors exhibit an overlapping disorganized pattern. Sp8 loss of function provokes an altered positioning of the ISo, which can already be detected at E8.5. This malformation becomes less pronounced after E10.5. Thus, an Sp8-independent mechanism might be responsible for this process at later stages of gestation. Interestingly, the analysis of Gbx2 conditional knockout mutant in r1 uncovered a Gbx2-independent pathway to repress Otx2 after E9 (Li et al., 2002).

Otx2 and Gbx2 were found to be responsible for the proper positioning of the ISo along the AP axis (Martinez-Barbera et al., 2001; Li and Joyner, 2001; Wurst and Bally-Cuif, 2001). Our findings clearly demonstrate that Sp8 activation is independent of Otx2 and/or Gbx2 expression, although these genes are necessary for restricting the expression of Sp8 at the MHB. Whether this effect is direct or indirect remains to be assessed.

The presence of ectopic patches of cells expressing Fgf8 in the ventral and in the dorsal part, but Otx2 and/or Wnt1 in the ventral part of the rostral hindbrain of Sp8 mutant embryos indicates that its role may be restricted to the ventral neuroepithelium, where it might be required to refine cell identity by repressing the expression of Otx2, Wnt1 and Fgf8. This suggests that Sp8 may contribute to sharpen the molecular code of MHB region on the rostral side of the hindbrain.

By contrast, in Gbx2 conditional mutant, where Gbx2 function in r1 was abolished after E8.5, ectopic patches of cells expressing Fgf8, Otx2 and/or Wnt1 were described to reside in the dorsal part (alar plate) of r1 (Li et al., 2002). Altogether, this may indicate that there may be two separate pathways operating to maintain a normal expression of these genes in the dorsal and ventral part of the neural tube.

Sp8 may restrict the Fgf8 expression domain at the MHB

The perturbed and overlapping expression domains of Fgf8 and Wnt1 in Sp8 knockout embryos are consistent with a role for Sp8 in mediating the segregation of these territories. The establishment of a juxtaposed Wnt1 and Fgf8 expression areas at the mes-met border is required for normal function of the ISo (Wassarman et al., 1997; Li and Joyner, 2001; Martinez-Barbera et al., 2001; Li et al., 2002). Otx2 and Gbx2 were shown to promote stable expression and to define the precise positioning of Fgf8 at the MHB (Wassarman et al., 1997; Li and Joyner, 2001; Martinez-Barbera et al., 2001; Li et al., 2002; Li et al., 2005). In the absence of Otx2 and Gbx2, the expression of Sp8 is similar to that reported for Fgf8 and other genes transcribed at the MHB (Martinez-Barbera et al., 2001; Li and Joyner, 2001). By contrast, in Sp8-deficient embryos no downregulation of the expression of molecular determinant genes of the MHB was observed over time, as reported in embryos lacking one of these factors (Simeone et al., 2002; Wurst and Bally-Cuif, 2001; Raible and Brand, 2004; Chi et al., 2003). Sp8 (Sp8) and Sp9 were reported to positively regulate Fgf8 expression in the limb of mouse and chick as well as pectoral fin of zebrafish (Treichel et al., 2003; Bell et al., 2003; Kawakami et al., 2004). Given the highly GC-rich content of the putative promoter region of Fgf8, which contains Sp1-binding sites, Sp8 was suspected, together with Sp9, to directly interact with Fgf8 (Kawakami et al., 2004). Therefore, one attractive interpretation of the disturbed Fgf8, Wnt1 and Otx2 expression would be the requirement of Sp8 to sustain a normal Fgf8 expression at the MHB. This is in agreement with the more remarkable perturbation and spread of the Fgf8 expression domain observed in the absence of Sp8, when compared to Wnt1 and Otx2. Our findings therefore led us to propose that Sp8 is necessary to restrict the expression of Fgf8 at the MHB. A disturbed Fgf8 expression was reported to be accompanied with Otx2 and Wnt1 perturbed expression at the MHB (Wassarman et al., 1997; Li et al., 2002). In the limb of Sp8–/– embryos, Fgf8 transcripts are properly activated but they gradually disappear during subsequent development, resulting in severe truncations of the appendages (Treichel et al., 2003; Bell et al., 2003; Kawakami et al., 2004). At the MHB, however, Fgf8 expression is upregulated and expanded, suggesting a negative regulation of Fgf8 by Sp8 in this region. Whether Sp8 is directly involved in this process remains to be assessed.

Sp8 may control cell proliferation in the midbrain and rostral hindbrain

In the mutant midbrain, the expression domain of Wnt1 was increased and ectopic patches were detected in the ventral part. Wnt1 has been already demonstrated to play a crucial role in midbrain patterning (McMahon and Bradley, 1990; Thomas and Cepacchi, 1990). The lack of Wnt1 activity in mice results in the complete loss of mid- and hindbrain territories (McMahon and Bradley, 1990; Thomas and Cepacchi, 1990). Interestingly, this factor may also act on cell proliferation, as evidenced in embryos carrying a knock-in of Wnt1 in the Enl locus (Panhuysen et al., 2004). In fact, the overgrowth provoked in the rostral hindbrain by the absence of Sp8 is reminiscent of similar malformations described in these embryos (Panhuysen et al., 2004). Thus, it is conceivable that the perturbed and increased Wnt1 expression could be responsible for the overgrowth observed in the mid- and hindbrain. Therefore, Sp8 may act as a negative regulator of Wnt1. However, in the rostral hindbrain the normally very restricted expression territory of Fgf8 was highly enlarged, in the absence of Sp8. Therefore, the cell proliferation defect detected in this rostral hindbrain may be rather related to Fgf8 that to Wnt1 expression. It was demonstrated that Fgf8 mimics isthmic organizer activity, inducing midbrain and cerebellum, and controlling anterior hindbrain development (Simeone et al., 2002; Wurst and Bally-Cuif, 2001; Raible and Brand, 2004; Crossley et al., 1996; Martinez et al., 1999; Irving and Mason, 2000). Furthermore, at the MHB and in other tissues, Fgf8 has been shown to act on cell survival and/or proliferation (Chi et al., 2003; Lee et al., 1997; Trump et al., 1999; Sun et al., 2002; Boulet et al., 2004). Accordingly, this is consistent with an enhanced or even uncontrolled growth and correlates with the high incidence of exencephaly at the level of the mid- and hindbrain of Sp8-deficient embryos. Whether the upregulation of ephrin A5 in the ventral midbrain is responsible for cell proliferation, tissue segregation or other not yet identified defects is not clear at this stage. Finally, it can be assumed that the deregulated expression of Fgf8, Otx2 and Wnt1 in Sp8-deficient embryos is responsible for the appearance of cells with mesencephalic cell fate in the rostral hindbrain. Indeed it is well accepted that the expression of Wnt1 and Otx2 are indicative for a midbrain territory (Puelles et al., 2004). Fgf8 and Shh were shown to confer dopaminergic neuron identity (Hynes and Rosenthal, 1999; Ye et al., 1998). Moreover, it has been very recently shown that ectopic expression of Wnt1 in the ventral region of the rostral hindbrain is sufficient to activate in the same cells Otx2 and convert serotonergic precursors to a dopaminergic fate (Prakash et al., 2006). Thus, the induction and differentiation of dopaminergic neurons in the hindbrain of Sp8 mutant embryos might probably be related to both over expression of Fgf8 and ectopic activation of Wnt1 and Otx2.
Finally, the zebrafish Sp8/btd homolog Bts1 has been implicated in the regulation of Pax2 expression (Tallafuss et al., 2001). Whether Sp8 is involved in this process remains to be assessed. In summary, our study provides evidence that Sp8 is involved in positioning of the ISO. This factor is required to maintain a normal expression of Fgft8, Otx2 and Wnt1 at the MHB. Our findings are consistent with a role for Sp8 in sharpening the MHB expression code by restricting the expression of Fgft8, Otx2 and Wnt1 in this area. We further demonstrate that Sp8 controls cell proliferation in the midbrain and rostral hindbrain. Our findings sustain the notion that tissue patterning and cell proliferation and/or survival are tightly co-regulated.

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Supplementary material
Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/133/9/1779/DC1.

References

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