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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 CD1X129Sv 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

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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.

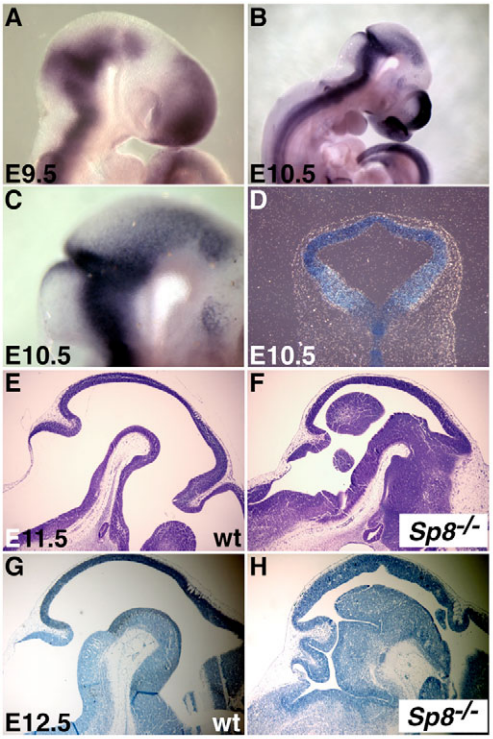

Fig. 1. Tissue overgrowth in the mid- and hindbrain. Whole-mount in situ hybridization showing the expression of *Sp8* in the central nervous system at E9.5 (A) and E10.5 (B,C). (**A-C**) *Sp8* is found in the forebrain, midbrain, MHB and in the spinal cord. In the spinal cord (B) and in the midbrain, *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 ^{35}S-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.

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

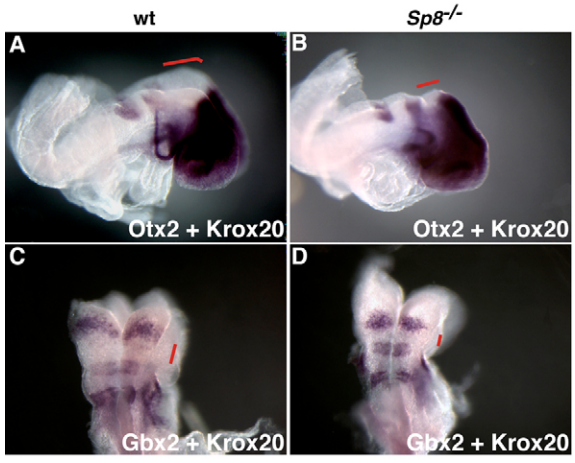

Fig. 2. Posterior shift of the IsO in *Sp8*$^{-/-}$ embryos. Whole-mount in situ hybridization showing lateral views of E8.5 control (**A**) and mutant (**B**) embryos hybridized with Otx2 and dorsal views of Gbx2 expression in control (**C**) and mutant (**D**) embryos at a similar stage of gestation. 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.

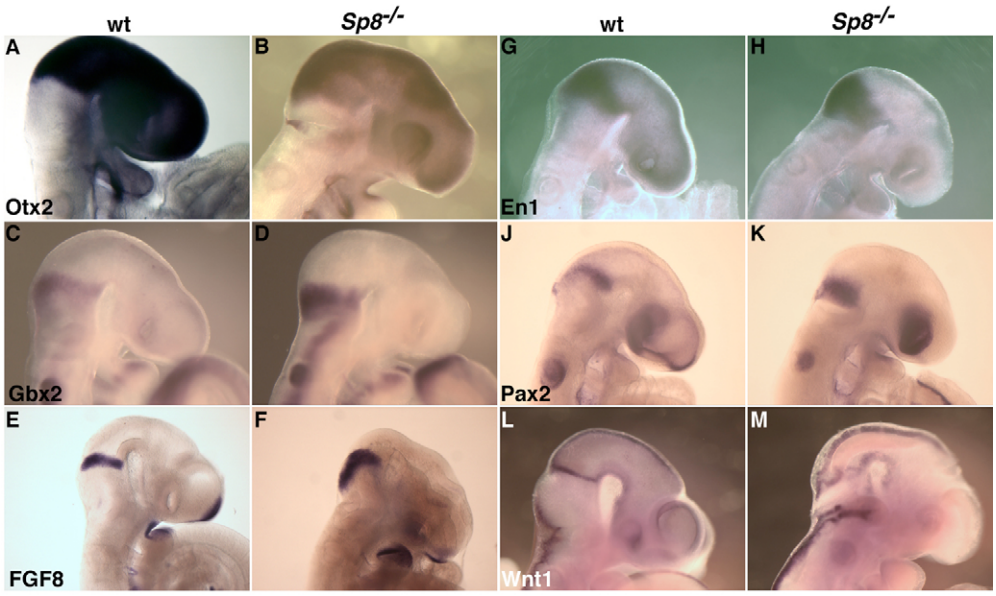

Fig. 3. At E9.5, the expression domains of genes transcribed at the MHB is enlarged. Whole-mount in situ hybridization showing the expression of *Otx2* (**A**,**B**), *Gbx2* (**C**,**D**), *Fgf8* (**E**,**F**), *En1* (**G**,**H**), *Pax2* (**J**,**K**) and *Wnt1* (**L**,**M**) and transcribed at the 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.

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

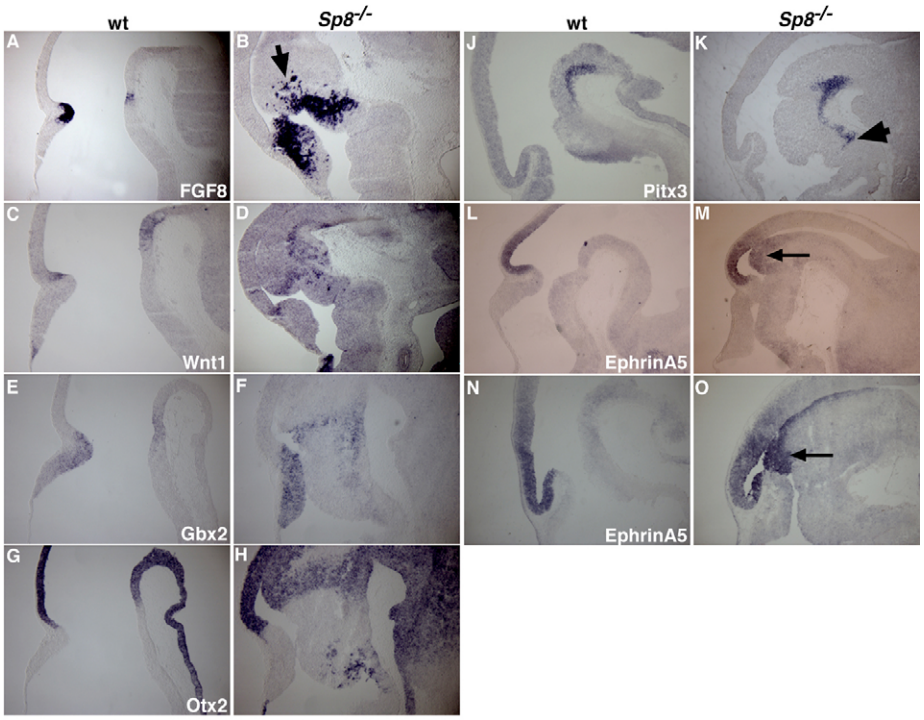

Fig. 4. At E10.5, the *Otx2/Gbx2* expression boundary appears normal but ectopic expression of *Fgf8*, *Otx2* and *Wnt1* is found in the ventral part of the rostral hindbrain. The expression of *Otx2*, *Gbx2*, *Wnt1* and *Fgf8* is shown on consecutive sagittal sections of E10.5 control and mutant embryos, 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).

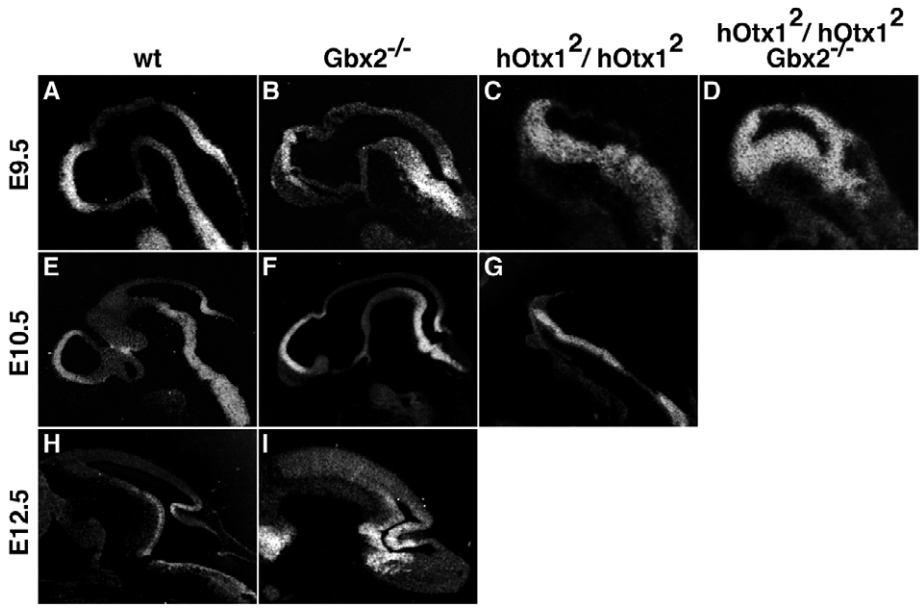

Fig. 5. Otx2 and/or Gbx2 are not required for the activation but for the restriction of Sp8 expression at the MHB. The expression of *Sp8* is shown, where possible, at different stages of development in wild-type (**A**,**E**,**H**), Gbx2$^{-/-}$ (**B**,**F**,**I**), Otx1^{2}/Otx1^{2} (**C**,**G**) and Otx1^{2}/Otx1^{2}/Gbx2$^{-/-}$ (**D**) embryos. In situ hybridization was performed on sagittal sections and using ^{35}S-labeled *Sp8* riboprobe. Although the Otx1^{2}/Otx1^{2}/Gbx2$^{-/-}$ double mutant is lethal between E9.2 and E.9.5 and therefore cannot be presented at E10.5 and E12.5, the in situ with Otx1^{2}/Otx1^{2} 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 (B,F,I), Otx2 (C,G) or both proteins (D). *Sp8* is rather strongly expressed in *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^{2}/Otx1^{2}, the rostral tip of the central nervous system should correspond to an isthmus-like structure (Martinez-Barbera et al., 2001) and *Sp8* is expressed here along all the CNS (C,G). In Otx1^{2}/Otx1^{2}/Gbx2$^{-/-}$ double mutant, *Sp8* is expressed along all the anterior neural plate (D). In this double mutant, this territory fails to activate forebrain- and midbrain-specific markers, while it shows ubiquitous expression of all the genes transcribed at the MHB (Martinez-Barbera et al., 2001) and is therefore considered as an expanded MHB. Thus, *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).

in the dorsal midbrain of E11.5 and E12.5 embryos is normal, it is up regulated in the ventral portion of the midbrain, as shown on sagittal sections at E11.5 (Fig. 4M,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*$^{-/-}$ (Wassarman et al., 1997), *Otx1*^{2}/*Otx1*^{2} (Acampora et al., 1997) and *Gbx2*$^{-/-}$/*Otx1*^{2}/*Otx1*^{2} (Martinez-Barbera et al., 2001) embryos. It is obvious that *Sp8* is not downregulated in embryos lacking Gbx2, Otx2 or both proteins. In fact, *Sp8* is strongly expressed in *Gbx2*$^{-/-}$ embryos in the presumptive rostral hindbrain committed to be transformed in an expanded posterior midbrain (Wassarman et al., 1997) (Fig. 5B,F,I). In *Otx1*^{2}/*Otx1*^{2}, the rostral tip of the CNS should correspond to an isthmus-like structure. In this mutant, *Sp8* is detected along all the CNS (Fig. 5C and G). Finally, in *Gbx2*$^{-/-}$/*Otx1*^{2}/*Otx1*^{2} mutants, *Sp8* mRNA is found along all the anterior neural plate (Fig. 5D). Indeed, in this mutant this territory fails to activate forebrain or midbrain specific markers, while it shows ubiquitous expression of all the genes transcribed at the MHB (Martinez-Barbera et al., 2001; Li and Joyner, 2001). This suggests that in this mutant, which lacks both Gbx2 and Otx2, the rostral neural plate develops as an expanded MHB. Accordingly, *Sp8* is expressed along the entire neural plate. These data indicate that *Sp8* activation is independent of *Otx2* and/or *Gbx2* expression, while these genes have a role in restricting the expression of *Sp8* at the MHB region. Whether, this effect is direct or indirect remains to be elucidated.

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.

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. 6B,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 neuroepithelium (Fig. 6), but did not revealed obvious patterning defects. However, tissue expansion may be a consequence of cell proliferation and/or survival defects. To distinguish between these possibilities, BrdU-labeling and TUNEL assay were performed in mutant and control embryos at E10.5 and E11.5. Although the cell survival does not seem to be affected, BrdU labeling revealed a higher number of proliferating cells in the mid- and hindbrain neuroepithelium of mutant embryos when compared with wild type (Fig. 7A-D). This suggests that in the absence of *Sp8* the control of cell proliferation is disturbed.

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 β-III-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

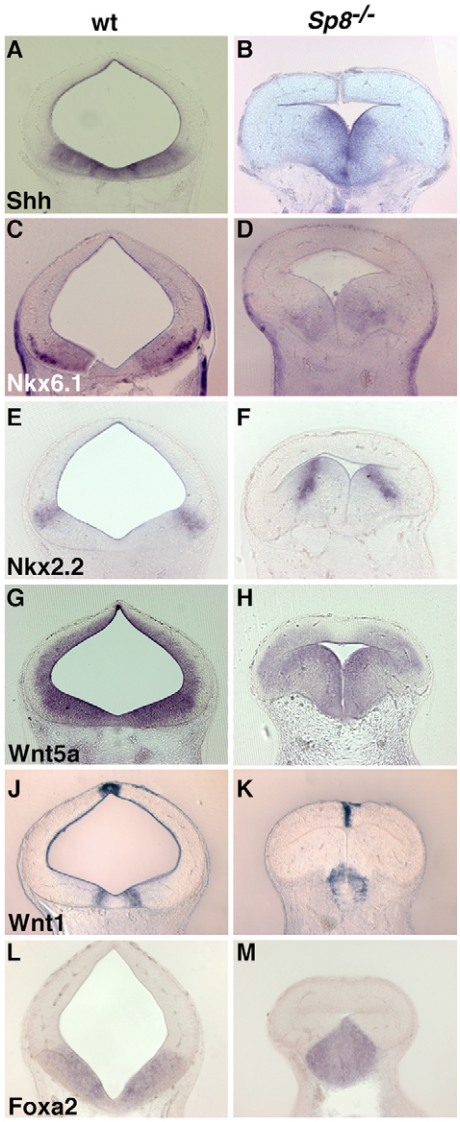

Fig. 6. The loss of *Sp8* provoked overgrowth of the neuroepithelium. (A-M) Expression analysis of several markers of the ventral midbrain: *Shh* (**A**,**B**), *Nkx6.1* (**C**,**D**), *Nkx2.2* (**E**,**F**), *Wnt5a* (**G**,**H**), *Wnt1* (**J**,**K**) and *Foxa2* (**L**,**M**) on frontal vibratome sections (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.

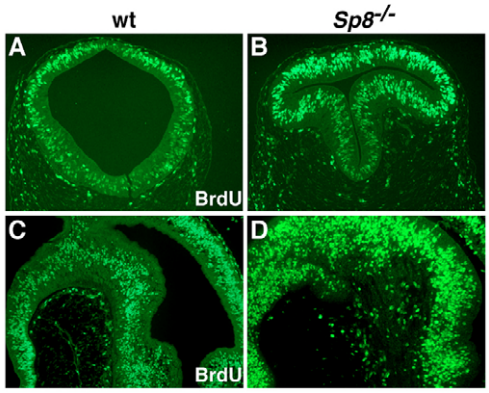

Fig. 7. Enhanced cell proliferation in the mid- and rostral hindbrain of *Sp8*-deficient embryos. (**A-D**) BrdU-labeled cells with a pulse of 30 minutes are shown in frontal sections, at the level of the posterior midbrain (A,B) and in sagittal hindbrain sections (C,D) of 10.5 wild-type and mutant embryos. 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.

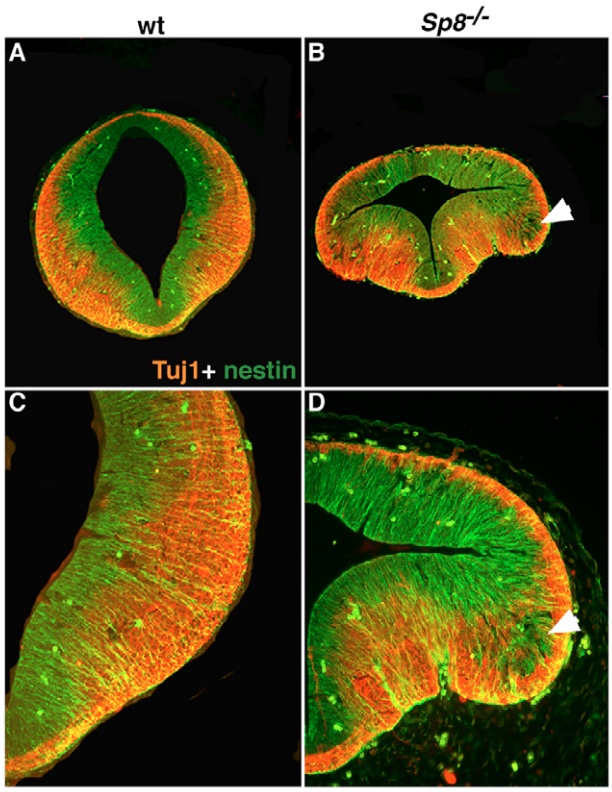

Fig. 8. Cell differentiation may be delayed at E11.5 in the absence of *Sp8*. (**A-D**) Double-immunohistochemical analysis presenting the expression of nestin (green) and β-III-tubulin (Tuj1, red) as markers labeling undifferentiated neuronal progenitors and differentiating neurons, respectively. In *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.

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

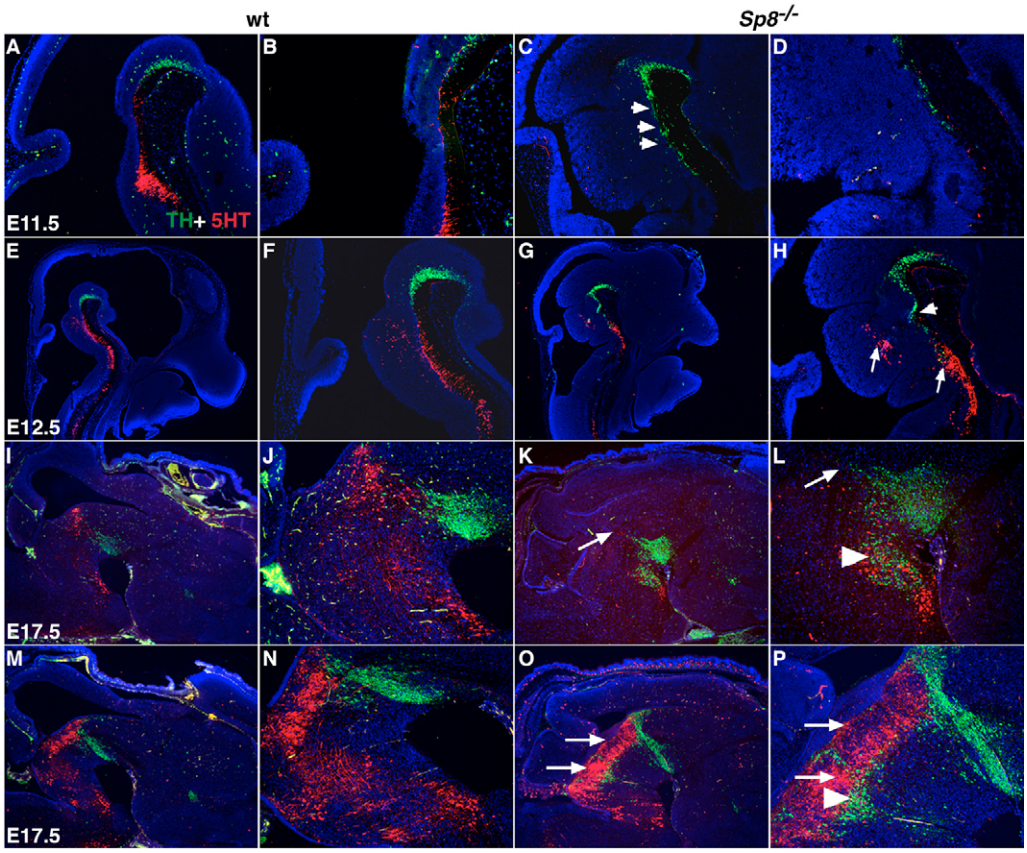

Fig. 9. Midbrain dopaminergic neurons are ectopically expressed in the rostral hindbrain of Sp8$^{-/-}$embryos. Sagittal sections of E11.5 (**A-D**), E12.5 (**E-H**) and E17.5 (**I-P**) control and mutant embryos showing the expression of tyrosine hydroxylase (TH, green) and 5-hydroxytryptamine (5-HT, red) revealed by double immunohistochemistry. The expression is also shown at two different magnifications (low: A,C,E,G,I,K,M,O; high: B,D,F,H,J,L,N,P). At E11.5, although in the control embryo (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.

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 (Fig. 9C,D). However, at E12.5 and E17.5, serotonergic neurons were found (Fig. 9G, see arrows in Fig. 9H,O,P) although the number of 5HT-positive cells is reduced in the area where some ectopic dopaminergic neurons emerge (Fig. 9H, arrowhead; see arrowheads in Fig. 9L,P). This suggests that the development of serotonergic neurons is at least delayed in *Sp8* 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;

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 Capecchi, 1990). The lack of *Wnt1* activity in mice results in the complete loss of mid- and hindbrain territories (McMahon and Bradley, 1990; Thomas and Capecchi, 1990). Interestingly, this factor may also act on cell proliferation, as evidenced in embryos carrying a knock in of *Wnt1* in the *En1* 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 Sp/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 Fgf8, 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 Fgf8, 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

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