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Hes1 and *Hes3* regulate maintenance of the isthmic organizer and development of the mid/hindbrain

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The isthmic organizer, which is located at the midbrain–hindbrain boundary, plays an essential role in development of the midbrain and anterior hindbrain. It has been shown that homeobox genes regulate establishment of the isthmic organizer, but the mechanism by which the organizer is maintained is not well understood. Here, we found that, in mice doubly mutant for the basic helix–loop–helix genes *Hes1* and *Hes3*, the midbrain and anterior hindbrain structures are missing without any significant cell death. In these mutants, the isthmic organizer cells prematurely differentiate into neurons and terminate expression of secreting molecules such as Fgf8 and Wnt1 and the paired box genes *Pax2/5*, all of which are essential for the isthmic organizer function. These results indicate that *Hes1* and *Hes3* prevent premature differentiation and maintain the organizer activity of the isthmic cells, thereby regulating the development of the midbrain and anterior hindbrain.

Keywords: bHLH/*Hes1*/*Hes3*/isthmic organizer/midbrain–hindbrain boundary

Introduction

Pattern formation of the neural plate is determined initially by vertical signals from the mesoderm and visceral endoderm and then by planar signals from the local organizers within the neuroectoderm (Lumsden and Krumlauf, 1996; Beddington and Robertson, 1998). These signals regulate the anterior–posterior patterning of gene expression: the homeobox gene *Otx2* is expressed in the presumptive forebrain and midbrain, while another homeobox gene, *Gbx2*, is expressed in the presumptive hindbrain and spinal cord. Recent studies revealed that the boundary region between the *Otx2* and *Gbx2* expression domains, which is located at the isthmus (the midbrain–hindbrain boundary), constitutes the organizing center, called the isthmic organizer (Joyner *et al*., 2000; Simeone, 2000; Rhinn and Brand, 2001; Wurst and Bally-Cuif, 2001). Antagonistic regulation between *Otx2* and *Gbx2* regulates the precise position of the isthmic organizer (Broccoll *et al*., 1999; Millet *et al*., 1999; Katahira *et al*., 2000) while other homeobox genes such as *Pax2/5* and *En1/2* are required for isthmic organizer activity (Wurst *et al*., 1994; Schwarz *et al*., 1997, 1999; Urbánek *et al*., 1997; Liu and Joyner, 2001). The isthmic organizer expresses secreting molecules such as Fgf8 and Wnt1 and thereby induces the development of the midbrain and anterior hindbrain (McMahon *et al*., 1992; Bally-Cuif *et al*., 1995; Crossley and Martin, 1995; Crossley *et al*., 1996; Lee *et al*., 1997; Meyers *et al*., 1998; Liu *et al*., 1999; Martinez *et al*., 1999; Xu *et al*., 2000). *Fgf8* expression in the isthmic organizer terminates around embryonic day (E) 13.5, while expression of a related gene, *Fgf17*, continues until E14.5 in mice (Xu *et al*., 2000). Similarly, *Wnt1* expression continues until E13.5 (Wilkinson *et al*., 1987). Thus, the isthmic organizer seems to be maintained at least until E14.5 in mice. Although the genes that establish the isthmic organizer, such as homeobox genes, have been characterized extensively, the mechanism by which the organizer is maintained during embryogenesis is not well understood.

Previous studies showed that the basic helix–loop–helix (bHLH) genes *Hes1* and *Hes3* are expressed in the midbrain–hindbrain region at E8.5 (Lobe, 1997; Allen and Lobe, 1999). By E14.5, *Hes1* expression expands broadly in neural precursor cells of the whole developing nervous system (Sasai *et al*., 1992). *Hes1* is a negative regulator for neuronal differentiation: misexpression of *Hes1* maintains neural precursor cells while inactivation of *Hes1* accelerates neurogenesis and decreases late-born neurons because of depletion of neuronal precursor cells (Ishibashi *et al*., 1994, 1995; Tomita *et al*., 1996; Ström *et al*., 1997; Ohtsuka *et al*., 1999; Cau *et al*., 2000; Nakamura *et al*., 2000). In contrast, *Hes3* expression is down-regulated by E14.5 but reappears in postnatal cerebellar Purkinje cells (Sasai *et al*., 1992; Lobe, 1997; Allen and Lobe, 1999). Recent studies revealed that the embryonal-type and Purkinje-type Hes3 proteins are structurally and functionally different: the embryonal type, which has a complete bHLH domain, can maintain neural precursor cells like Hes1, whereas the Purkinje type, which lacks the N-terminal half of the basic region, cannot (Sasai *et al*., 1992; Sakagami *et al*., 1994; Hirata *et al*., 2000). These two variants are generated by alternative first exons (Hirata *et al*., 2000).

Here, to investigate the functions of *Hes1* and *Hes3* in midbrain–hindbrain development, we performed a targeted gene disruption. We found that, in mice doubly mutant for *Hes1* and *Hes3*, the midbrain and anterior hindbrain structures are missing. In these mutants, cells in the isthmic organizer prematurely terminate the expression

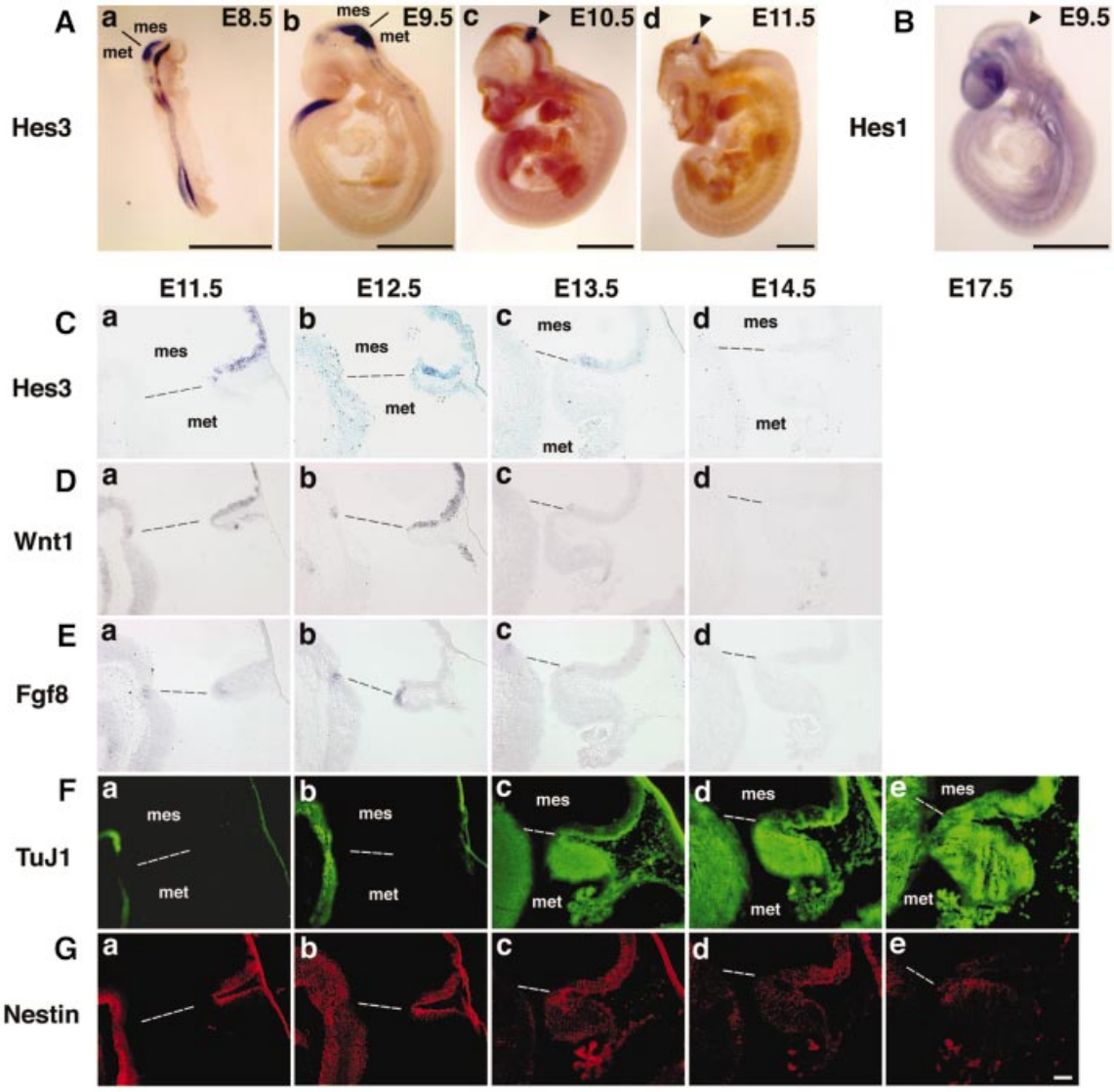


Fig. 1. Spatio-temporal expression of *Hes3*. (**A**) *In situ* hybridization of *Hes3*. (a) At E8.5, *Hes3 is* expressed widely in the midbrain–hindbrain region and spinal cord but not in the forebrain. (b) At E9.5, *Hes3* expression in the spinal cord is down-regulated. (c and d) At E10.5 and E11.5, *Hes3* expression is restricted to the midbrain–hindbrain boundary region. The isthmic organizer is indicated by arrowheads. (**B**) *In situ* hybridization of *Hes1*. At E9.5, *Hes1* is also expressed in the midbrain–hindbrain boundary region (arrowhead). (C–G) *In situ* hybridization (C–E) and immunohistochemistry (F and G) of parasagittal sections. In all sections, ventral is to the left. (**C**) (a–c) *Hes3* is expressed in the ventricular zone of the rostral side of the midbrain–hindbrain boundary at E11.5–13.5. (d) At E14.5, *Hes3* expression almost disappears. (**D**) (a and b) At E11.5–12.5, *Wnt1* is expressed in the ventricular zone of the rostral side of the midbrain–hindbrain boundary. (c and d) *Wnt1* expression is down-regulated at E13.5 and disappears at E14.5. (**E**) (a and b) At E11.5–12.5, *Fgf8* is expressed in the ventricular zone of the caudal side of the midbrain–hindbrain boundary. (c and d) *Fgf8* expression is down-regulated at E13.5 and disappears at E14.5. (**F**) Immunohistochemistry of TuJ1. (a) At E11.5, some TuJ1$^+$ neurons were differentiated in the ventral side of the midbrain and hindbrain but not in the isthmic region. (b) At E12.5, some TuJ1$^+$ neurons were differentiated in the ventral side of the isthmic region. (c and d) At E13.5–14.5, some TuJ1$^+$ neurons were also differentiated in the dorsal side of the isthmic region. (e) At E17.5, almost all cells differentiate into neurons. (**G**) Immunohistochemistry of Nestin. (a–d) At E11.5–13.5, Nestin$^+$ neural precursor cells are present in the ventricular zone of the isthmic region but significantly decreased at E14.5. (e) At E17.5, there are virtually no neural precursor cells left in the isthmic region. mes, mesencephalon; met, metencephalon. Scale bar, 1 mm (A and B); 100 μm (C–G).

of specific genes such as *Fgf8*, *Wnt1* and *Pax2/5*, and differentiate into neurons. These results demonstrate that *Hes1* and *Hes3* prevent premature differentiation and maintain the organizer activity at the isthmus, thereby regulating midbrain and anterior hindbrain development. Moreover, these data also indicate that premature differentiation leads to the patterning defects, in addition to a decrease in late-born neuronal types (Tomita *et al*., 1996), thus pointing to the 2-fold importance of the normal timing of cell differentiation.

Results

Spatio-temporal expression of Hes3

Hes3 expression was first examined by whole-mount *in situ* hybridization. As reported previously (Lobe, 1997; Allen and Lobe, 1999), *Hes3* was expressed at a high level in the midbrain–hindbrain region and the spinal cord at E8.5 (Figure 1Aa). The expression in the spinal cord was soon down-regulated (Figure 1Ab) and, at E10.5 and E11.5, *Hes3* was expressed only around

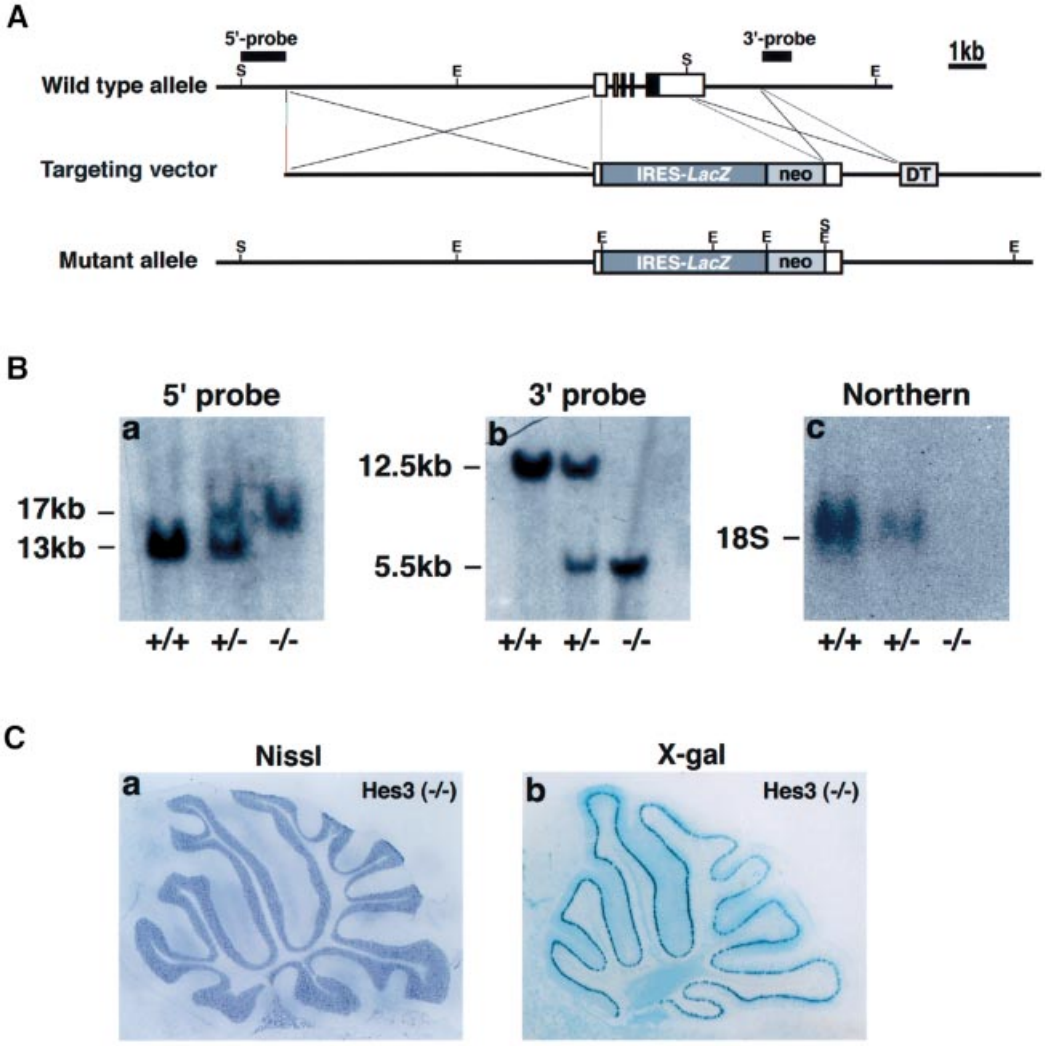


Fig. 2. Targeted deletion of *Hes3*. (**A**) Genomic structure of mouse *Hes3* and targeting strategy. The coding and non-coding regions are indicated by closed and open boxes, respectively. All the coding region is replaced with *lacZ-neo*. *lacZ* expression is under the control of the Purkinje cell-specific promoter (Hirata *et al*., 2000). E, *Eco*RI; S, *Sal*I. (**B**) Southern and northern analyses. (a and b) Genotypes were determined by Southern blot analysis. Genomic DNA was digested with *Sal*I (a) and *Eco*RI (b). (c) Northern analysis shows that *Hes3* expression is decreased in heterozygous and abolished in homozygous embryos. (**C**) Histochemistry of *Hes3*(–/–) mice. (a) Nissl staining. (b) X-gal staining. Purkinje cells (X-gal$^+$) are generated normally in *Hes3*(–/–) cerebellum.

the midbrain–hindbrain boundary region (Figure 1Ac and d, arrowheads). *In situ* hybridization with sections demonstrated that, at E11.5 and E12.5, *Hes3* was expressed in the ventricular zone of the caudal midbrain, which contains neural precursor cells (Figure 1Ca and b). This expression domain overlapped with *Wnt1* expression (Figure 1Da and b) but was rostral to *Fgf8* expression (Figure 1Ea and b). Expression of *Hes3*, *Wnt1* and *Fgf8* was significantly down-regulated at E13.5 (Figure 1Cc, Dc and Ec) and almost disappeared at E14.5 (Figure 1Cd, Dd and Ed), indicating that the time course of expression of the three genes is very similar. This expression pattern suggests that *Hes3*, *Wnt1* and *Fgf8* are expressed by neural precursor cells but not by differentiated neurons. To confirm this possibility, immunohistochemistry was performed with adjacent sections. At E11.5, neurons (TuJ1$^+$) were not generated in the isthmic region, although some neurons were differentiated outside this region (Figure 1Fa). At E12.5, neurons were differentiated in the ventral side of the isthmic region but there still remained many neural precursor cells (Nestin$^+$) (Figure 1Fb and Gb). At E13.5, neurons were also differentiated in the dorsal side of the isthmic region but there were still many neural precursor cells (Figure 1Fc and Gc). However, at E14.5, when *Hes3*, *Wnt1* and *Fgf8* expression almost disappeared, neural precursor cells were significantly decreased in number at the isthmus (Figure 1Gd). At E17.5, most cells differentiated into neurons in the isthmic region, 3 days after the disappearance of *Hes3*, *Wnt1* and *Fgf8* expression (Figure 1Fe and Ge). Thus, *Hes3*, *Wnt1* and *Fgf8* are expressed by neural precursor cells in the isthmic region and the expression is down-regulated when neuronal differentiation proceeds. Since *Hes3* is able to maintain neural precursor cells (Hirata *et al*., 2000), these results raise the possibility that *Hes3* may play a role in maintenance of the

ventricular cells in the isthmic organizer that express *Wnt1* and *Fgf8*.

Targeted deletion of Hes3

To investigate the function of *Hes3* in the isthmic organizer, *Hes3* mutant mice were generated by homologous recombination in embryonic stem (ES) cells. The whole coding region of *Hes3* was replaced by *lacZ-neo* (Figure 2A, and Ba and b). *Hes3*-null mice, which lost *Hes3* expression (Figure 2Bc), were fertile and had no apparent defects. The knocked-in *lacZ* gene was under the control of the Purkinje cell-specific promoter, and Purkinje cells were clearly stained without any defects by X-gal solution in homozygous mutant mice (Figure 2C), suggesting that Purkinje cells developed normally in the absence of *Hes3*.

Because a related bHLH gene, *Hes1*, is also expressed in the embryonal midbrain–hindbrain region (Figure 1B, arrowhead) (Sasai *et al*., 1992; Lobe, 1997; Allen and Lobe, 1999), the lack of phenotypes in the brain of *Hes3*(–/–) embryos is probably due to compensation by *Hes1*. We therefore generated mice doubly mutant for *Hes1* and *Hes3*. The double mutants exhibited patterning defects of the midbrain and anterior hindbrain, as shown below.

Defects of midbrain and anterior hindbrain of Hes1–Hes3 double-mutant mice

The majority of *Hes1–Hes3* double-mutant embryos survived until E10.5 but most of them died by E15.5. All of the double-mutant embryos displayed neural tube defects (40 out of 40) while only some of the *Hes1*-null embryos did (10 out of 68, 14.7%) (Figure 3A). The neural tube defect of the double-mutant embryos exhibited considerable variability: some embryos displayed exencephaly of the whole brain while others had the defect only at the midbrain–hindbrain region. In addition, the double-mutant embryos showed a tendency to growth retardation starting at E10.5 (Figure 3Ad). However, since this tendency was also observed in *Hes1*(–/–) embryos (Figure 3Ac), the growth retardation may be due to *Hes1* deficiency. Because the neural tube defects became severe after E12.5, we examined the mutant phenotypes before E12.5.

Staining in whole mount with antibody to neurofilament revealed that the cranial nerves connected with the hindbrain (such as V, VII, IX and X) developed normally in the double mutants at E10.5 (Figure 3B and data not shown, see Figure 4Ah and l). In addition, *Krox20* was expressed normally in rhombomeres 3 and 5 (data not shown). These results suggest that most of the hindbrain developed normally in the double mutants. In contrast, cranial nerves III and IV, which are derived from the midbrain and the isthmic region, respectively, were missing in the double mutants (Figure 3Bd, asterisks) whereas they were present in the wild-type, *Hes1*(–/–) and *Hes3*(–/–) embryos (Figure 3Ba–c). Agreeing with this observation, oculomotor (III) and trochlear (IV) motor nuclei, which express the homeobox gene *Phox2b* (Pattyn *et al*., 1997), were missing in the double mutant (Figure 3Ed, left asterisk) whereas they were present in the ventral midbrain–isthmus region of the wild-type, *Hes1*(–/–) and *Hes3*(–/–) embryos (Figure 3Ea–c, open arrowheads). To assess the defects of this region further, we analyzed dopaminergic neurons, which differentiate in the ventral midbrain. *Nurr1*, a member of the nuclear receptor superfamily, is essential for generation of midbrain dopaminergic neurons (Zetterström *et al*., 1997; Castillo *et al*., 1998; Saucedo-Cardenas *et al*., 1998). In the wild-type, *Hes1*(–/–) and *Hes3*(–/–) midbrain, *Nurr1* was expressed and dopaminergic neurons (tyrosine hydroxylase$^+$) were generated at E11.5 (Figure 3Ca–c and Da–c). In contrast, in *Hes1–Hes3* double-mutant midbrain, the *Nurr1* expression domain and dopaminergic neurons were completely missing (Figure 3Cd and Dd, asterisks). These results indicate that midbrain development was severely impaired in *Hes1–Hes3* double-mutant embryos.

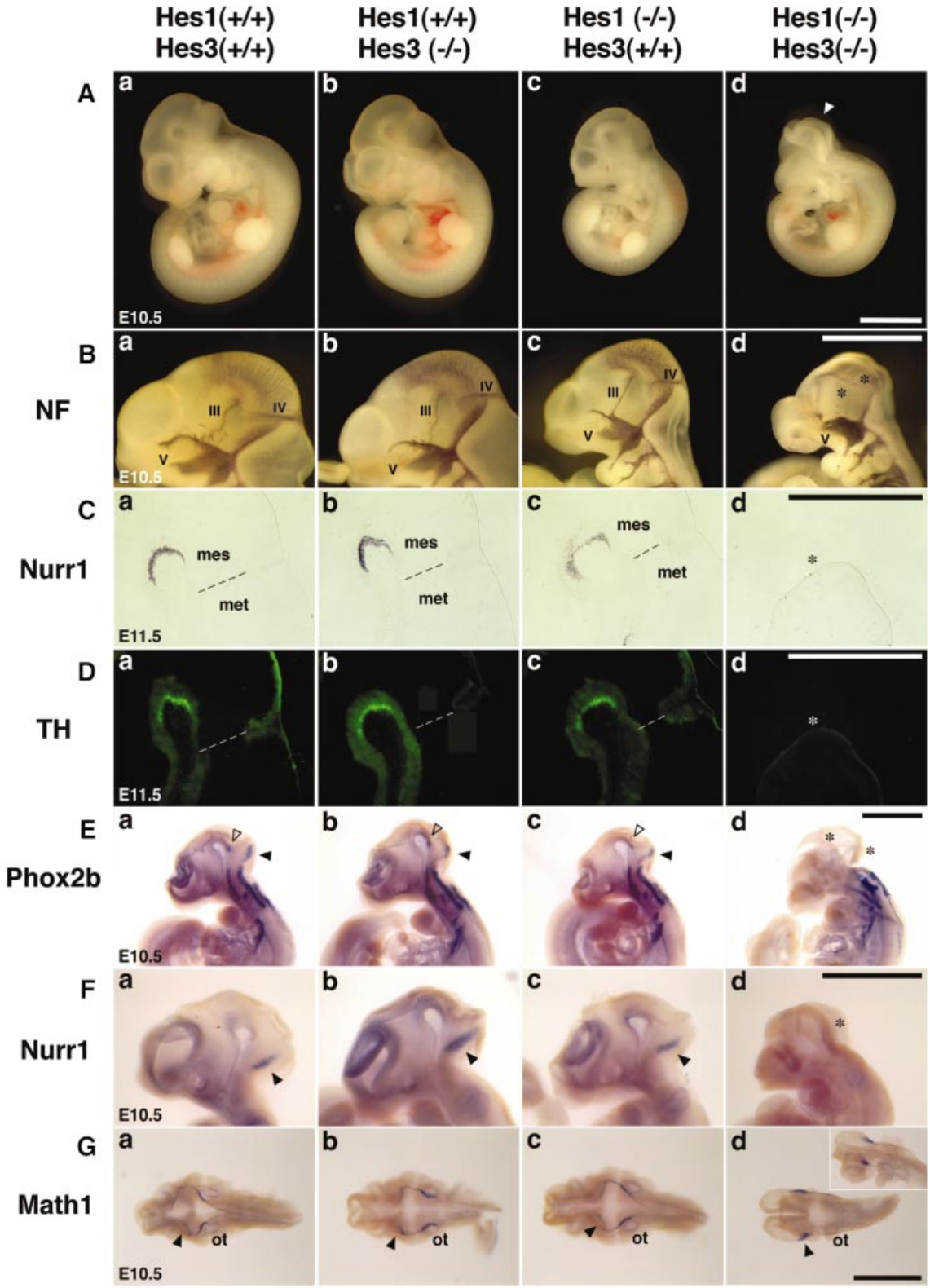
Midbrain defects are often associated with anterior hindbrain defects. We therefore investigated whether the anterior hindbrain is affected in the double mutants. Locus ceruleus, the major noradrenergic center, is located in rhombomere 1 and its development is regulated by the closely related homeodomain genes *Phox2a* and *Phox2b* (Morin *et al*., 1997; Pattyn *et al*., 1997, 1999). In the wild-type, *Hes1*(–/–) and *Hes3*(–/–) hindbrain, locus ceruleus was generated (Figure 3Ea–c, closed arrowheads). In contrast, in *Hes1–Hes3* double-mutant embryos, locus ceruleus was missing (Figure 3Ed, right asterisk). Furthermore, the *Nurr1* expression domain in rhombomere 1 was also absent in the double mutants (Figure 3Fd, asterisk), indicating that anterior hindbrain development was impaired in *Hes1–Hes3* double-mutant embryos. However, the *Phox2b* expression domain in the mid/posterior hindbrain was not affected in the double mutants (Figure 3Ed).

To clarify these defects further, the *Phox2b* expression domains were examined in sections and flat-mounted samples. At E10.5, in the wild-type, oculomotor (III) (Figure 4Ba, open arrowhead) and trochlear motor nuclei (IV) (Figure 4Ab and Ba, closed arrowhead) as well as locus ceruleus (Figure 4Ba, arrow) were clearly visible, whereas in the double mutants, they were completely missing (Figure 4Ad and Bc, asterisks), indicating that the midbrain and anterior hindbrain structures were missing in the double mutants. In contrast, three longitudinal columns of *Phox2b* expression domains were generated in the double-mutant mid/posterior hindbrain (compare Figure 4Af and j, with h and l, arrows and arrowheads, and 4Ba with c, open arrows), indicating that the mid/posterior hindbrain structures were generated in the double mutants.

Expression of the bHLH gene *Math1*, which is essential for generation of cerebellar granule cells and pontine neurons (Akazawa *et al*., 1995; Ben-Arie *et al*., 2000), was also examined by whole-mount *in situ* hybridization. At E10.5, *Math1* is expressed in the rhombic lip, the origin of cerebellar granule cells and pontine neurons (Wingate and Hatten, 1999). *Math1* expression was clearly detectable in the rhombic lip of the double mutants, although the position was distorted by the neural tube defect (Figure 3Gd, arrowhead). Taken together, these results demonstrated that, in the double mutants, the midbrain and a part of the anterior hindbrain structures were missing while most other regions of the hindbrain developed normally.

The lack of the midbrain and anterior hindbrain structures of the double mutants is not caused by neural tube defects

The observed abnormality of the double-mutant embryos could be secondary to neural tube defects. To investigate this possibility, we examined *Hes1*(–/–) embryos with severe neural tube defects. Flat- and whole-mount *in situ* hybridization analysis for *Phox2b* expression demonstrated that oculomotor and trochlear motor nuclei and locus ceruleus were generated at E10.5 in *Hes1*(–/–) embryos with severe neural tube defects (Figure 4Bb and Ca, arrows and arrowheads). Furthermore, *Pax5* expression was also observed at the midbrain–hindbrain boundary region of *Hes1*(–/–) embryos with neural tube defects



at E10.5 (Figure 4Cb) like the wild-type (see Figure 7Ia), although it was lost in E10.5 *Hes1–Hes3* double-mutant embryos (see below and Figure 7Id). These results indicate that the lack of the midbrain and anterior hindbrain structures was not the result of neural tube defects but specific to *Hes1–Hes3* double mutations.

Hes1–Hes3 double-mutant embryos display premature neurogenesis but contain many neural precursor cells at E10.5

We found previously that *Hes1* mutant mice display premature neuronal differentiation and contain fewer neural precursor cells (Ishibashi *et al*., 1995; Tomita *et al*., 1996; Nakamura *et al*., 2000). Thus, the lack of the midbrain and anterior hindbrain structures of *Hes1–Hes3* double-mutant embryos could be due to accelerated neuronal differentiation and concomitant depletion of neural precursor cells when specification of the midbrain and anterior hindbrain occurs. To investigate this possibility, we examined whether or not neural precursor cells remain in the double-mutant embryos when midbrain and anterior hindbrain development proceeds.

In the wild-type, *Hes1*(–/–) and *Hes3*(–/–) midbrain, some neurons (TuJ1$^+$) were generated at E10.5 (Figure 5Ba–c). In contrast, in *Hes1–Hes3* double mutants, more neurons were generated prematurely (Figure 5Bd), indicating that *Hes1* and *Hes3* cooperatively prevent premature neuronal differentiation. In spite of this accelerated neurogenesis, there still remained abundant neural precursor cells (Nestin$^+$) in *Hes1–Hes3* double-mutant midbrain at E10.5 (Figure 5Cd). There were also abundant neural precursor cells in the double-mutant hindbrain at E10.5 (data not shown). Thus, the defects of the midbrain and anterior hindbrain specification in *Hes1–Hes3* double-mutant embryos was not due to depletion of neural precursor cells. Another possibility is that the double-mutant cells would die preferentially during embryogenesis. However, TUNEL analysis showed no significant difference between the wild type and mutants at E10.5 (Figure 5Da–d), indicating that cell death is not the primary cause of the patterning defects. These results raised the possibility that isthmic organizer activity may be affected primarily in *Hes1–Hes3* double mutants.

The isthmic organizer is not maintained properly in Hes1–Hes3 double-mutant embryos

To examine whether the isthmic organizer is affected in the double mutants, we investigated the expression of several genes by *in situ* hybridization. The secreting factors Wnt1 and Fgf8 were expressed in the wild-type, *Hes1*(–/–) and *Hes3*(–/–) isthmic organizer at E9.5 (Figure 6Aa–c and Ba–c, arrowheads). They were also expressed at a comparable level in the double-mutant isthmic organizer at E9.5 (Figure 6Ad and Bd, arrowheads). Expression of the homeobox genes *Otx2*, *Gbx2* and *En1* was not significantly changed in the midbrain–hindbrain region of the double mutants (Figure 6C, D and G). These results indicate that the isthmic organizer was established by E9.5 in the absence of *Hes1* and *Hes3*. However, the isthmus-specific paired box gene, *Pax2*, was not expressed in the double mutants at E9.5 (Figure 6Ed, asterisk) although expression of a related gene, *Pax5*, was not significantly changed in the double mutants (Figure 6Fd, arrowhead). These results suggest that the double-mutant isthmic organizer may already be affected at E9.5. This alteration of gene expression is one of theearliest phenotypes of *Hes1–Hes3* double-mutant embryos.

Strikingly, at E10.5, both *Wnt1* and *Fgf8* expression disappeared in the double-mutant isthmic organizer (Figure 7Ad and Bd, asterisks) whereas the two genes were expressed normally in the single mutants (Figure 7Aa–c and Ba–c, arrowheads). In addition, expression of other secreting molecules Fgf17 and Fgf18 was not detectable in the double-mutant isthmic organizer at E10.5 (Figure 7Cd and Dd, asterisks). Furthermore, expression of *Pax2* and *Pax5* was not detectable either and that of *Pax8* was significantly down-regulated in the double-mutant isthmic organizer at E10.5 (Figure 7Hd, Id and Jd, asterisks). Thus, in the absence of *Hes1* and *Hes3*, the isthmic organizer prematurely terminated expression of specific genes such as *Fgf8*, *Wnt1* and *Pax2/5*. In addition, in the double mutants, *Otx2* expression was extended caudally to the anterior hindbrain (Figure 7Fd) while *Gbx2* expression was down-regulated in the anterior hindbrain (Figure 7Gd, asterisk). This deregulation is likely to be the result of disappearance of *Fgf8* expression since *Fgf8* is required for maintenance of *Gbx2* expression and repression of *Otx2* expression (Liu *et al*., 1999; Martinez *et al*., 1999). In contrast, expression of the homeobox gene *En2* was not changed significantly in the double mutants (Figure 7E, arrowheads). All these data demonstrated that the isthmic organizer is not maintained properly in the absence of *Hes1* and *Hes3*.

The premature loss of the isthmic organizer activity could be due to neural tube defects. However, *Pax5* was expressed in the midbrain–hindbrain boundary region of

Fig. 3. Defects of midbrain and anterior hindbrain patterning of *Hes1–Hes3* double-mutant embryos. (**A**) E10.5 embryos. The *Hes1*(–/–)–*Hes3*(–/–) embryo displays the neural tube defect (d, arrowhead). (**B**) Whole-mount immunostaining with anti-neurofilament (NF) antibody of E10.5 embryos. Cranial nerves III and IV are generated in the wild-type, *Hes1*(–/–) and *Hes3*(–/–) embryos whereas they are missing in the *Hes1*(–/–)–*Hes3*(–/–) embryo (d, asterisks), suggesting that midbrain development is affected in the double-mutant embryos. Cranial nerve V is generated normally in the double mutant. (**C**) *Nurr1* expression in the ventral midbrain at E11.5. This expression domain is missing in the *Hes1–Hes3* double-mutant embryo (d, asterisk). (**D**) Immunohistochemistry with anti-tyrosine hydroxylase (TH) antibody. Dopaminergic neurons (TH$^+$) are missing in the ventral midbrain of the *Hes1–Hes3* double-mutant embryo at E11.5 (d, asterisk). (**E**) *Phox2b* is expressed in oculomotor (III) and trochlear (IV) motor nuclei in the ventral midbrain and isthmus, respectively (a–c, open arrowheads), and in locus ceruleus in rhombomere 1 (a–c, closed arrowheads) of the E10.5 wild-type, *Hes1*(–/–) and *Hes3*(–/–) embryos. In contrast, such neurons are missing in the *Hes1–Hes3* double-mutant embryo (d, asterisks). However, *Phox2b* expression domains in the mid/posterior hindbrain are normal in the *Hes1–Hes3* double-mutant embryo. (**F**) The *Nurr1* expression domain observed in the anterior hindbrain of the wild-type, *Hes1*(–/–) and *Hes3*(–/–) embryos (a–c, arrowheads) is missing in the *Hes1–Hes3* double-mutant embryo at E10.5 (d, asterisk). (**G**) *Math1 is* expressed in the rhombic lip (arrowheads) of the wild-type, *Hes1*(–/–), *Hes3*(–/–) and *Hes1*(–/–)–*Hes3*(–/–) double-mutant embryos at E10.5. Note that the rhombic lips are distorted due to the neural tube defect in the double mutant (d). mes, mesencephalon; met, metencephalon; ot, otic vesicle. Scale bar, 1 mm.

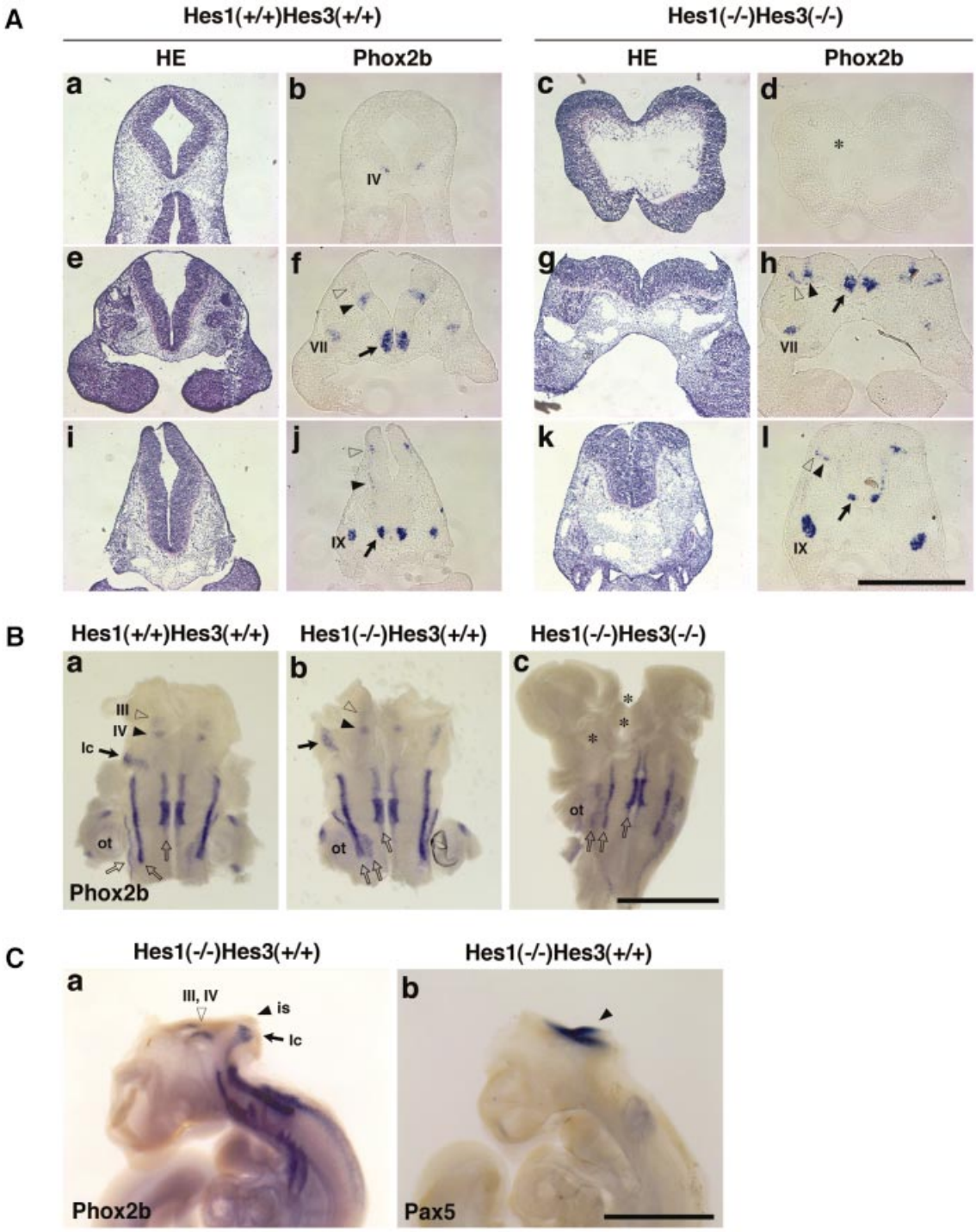


Fig. 4. Patterning defects of the midbrain and anterior hindbrain are specific to *Hes1–Hes3* double-mutant embryos. (**A**) HE staining and *in situ* hybridization for *Phox2b* expression with frontal sections of E10.5 embryos. Trochlear motor nuclei (IV) are missing in the double-mutant isthmus region (d, asterisk). In contrast, three columns of the *Phox2b* expression domains are generated in the double-mutant hindbrain (h and l, arrows and arrowheads). Cranial nerves VII and IX are also present in the double-mutant embryos (h and l). In all sections, dorsal is up. (**B**) Flat-mount *in situ* hybridization of E10.5 wild-type (a), *Hes1*(–/–) (b) and *Hes1*(–/–)–*Hes3*(–/–) (c) embryos. The dorsal side is excised. In *Hes1*(–/–) embryo (b), which has neural tube defects, oculomotor (open arrowhead) and trochlear (closed arrowhead) motor nuclei and locus ceruleus (arrow) are generated. In contrast, in the double mutant (c), those structures are missing (asterisks). However, the three columns of *Phox2b* expression domains are present in the double-mutant hindbrain (open arrows). ot, otic vesicle. (**C**) Whole-mount *in situ* hybridization of E10.5 *Hes1*(–/–) embryos with neural tube defects. (a) The *Phox2b* expression domains (oculomotor and trochlear motor nuclei and locus ceruleus) are present, indicating that neural tube defects do not cause the lack of the midbrain and anterior hindbrain structures at this stage. III, oculomotor; IV, trochlear; is, isthmus; lc, locus ceruleus. (b) *Pax5* is expressed in the midbrain–hindbrain boundary region, indicating that neural tube defects do not cause the lack of the isthmic organizer at this stage. The isthmus is indicated by an arrowhead. Scale bar, 1 mm.

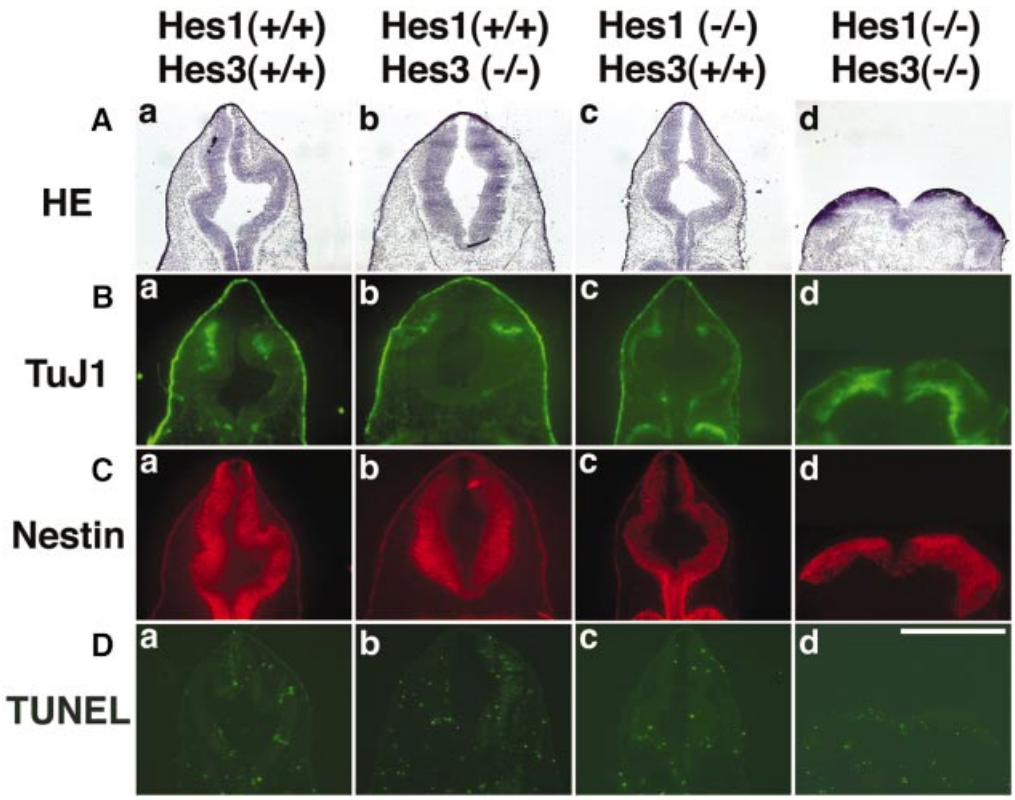


Fig. 5. Premature neuronal differentiation in the *Hes1–Hes3* double-mutant midbrain. (**A**) HE staining of frontal sections of midbrain at E10.5. (**B** and **C**) Frontal sections of midbrain at E10.5 were subjected to immunohistochemistry with TuJ1 (B) and anti-Nestin antibodies (C). In the wild-type, *Hes1*(–/–) and *Hes3*(–/–) midbrain, some neurons (TuJ1$^+$) are generated (Ba–c). In *Hes1*(–/–)–*Hes3*(–/–) midbrain, more neurons are generated prematurely (Bd), suggesting that *Hes1* and *Hes3* cooperatively prevent premature neurogenesis. In spite of this accelerated neurogenesis, there are still abundant neural precursor cells (Nestin$^+$) in the double-mutant midbrain (Cd). (**D**) TUNEL analysis of frontal sections of midbrain at E10.5. No significant difference is observed in the double mutant, indicating that cell death is not induced by *Hes1–Hes3* double mutation. Note that the double-mutant midbrain displays an exencephaly. In all sections, dorsal is up. Scale bar, 1 mm.

Hes1(–/–) embryos with neural tube defects at E10.5 (see Figure 4Cb). These results suggest that the loss of the isthmic organizer activity in the double-mutant embryos is not secondary to neural tube defects but specific to *Hes1–Hes3* double mutations.

Premature neuronal differentiation in Hes1–Hes3 double-mutant isthmic organizer

The premature termination of *Fgf8/17/18*, *Wnt1* and *Pax2/5* expression in the double-mutant isthmic organizer could be due to apoptosis of the organizer cells. To examine this possibility, a TUNEL assay was performed. This assay demonstrated that there was no significant difference in cell death between the wild type and mutants at E10.5 (data not shown) and E11.5 (Figure 8Ca–d), thus excluding the possibility that cell death is affected in the double mutants. At E11.5, some TuJ1$^+$ neurons were generated in the midbrain and hindbrain but not in the isthmic organizer region of wild-type, *Hes1*(–/–) and *Hes3*(–/–) embryos (Figure 8Aa–c). Furthermore, there were still many Nestin$^+$ neural precursor cells in the organizer region of these embryos (Figure 8Ba–c), which expressed *Fgf8/17/18*, *Wnt1* and *Pax2/5/8* (data not shown, see Figure 1Da and Ea). In contrast, in the double mutants, almost all cells prematurely differentiated into neurons and there were virtually no neural precursor cells left in the isthmic region at E11.5 (Figure 8Ad and Bd). These results indicate that, in the absence of *Hes1* and *Hes3*, the isthmic organizer cells differentiated prematurely into neurons and lost organizer-specific gene expression.

Discussion

Hes1 and Hes3 regulate the maintenance of the isthmic organizer activity

In this study, we found that, in *Hes1–Hes3* double-mutant mice, midbrain and anterior hindbrain structures were missing as early as E10.5. Although neurons were generated prematurely in the double-mutant midbrain and hindbrain, many neural precursor cells still remained at E10.5, thus excluding the possibility that there are no neural precursor cells that could respond to the signals from the isthmic organizer in the double mutants during the midbrain and anterior hindbrain specification. Furthermore, no significant cell death was observed in the double mutants, again excluding the possibility that, once generated, midbrain and hindbrain cells died. In contrast, the double-mutant isthmic organizer cells prematurely terminated expression of secreting molecules such as Fgf8 and Wnt1 and the paired box genes *Pax2/5*, all of which are essential for isthmic organizer function. These results strongly indicate that the defect of the isthmic organizer, rather than the cells that should receive

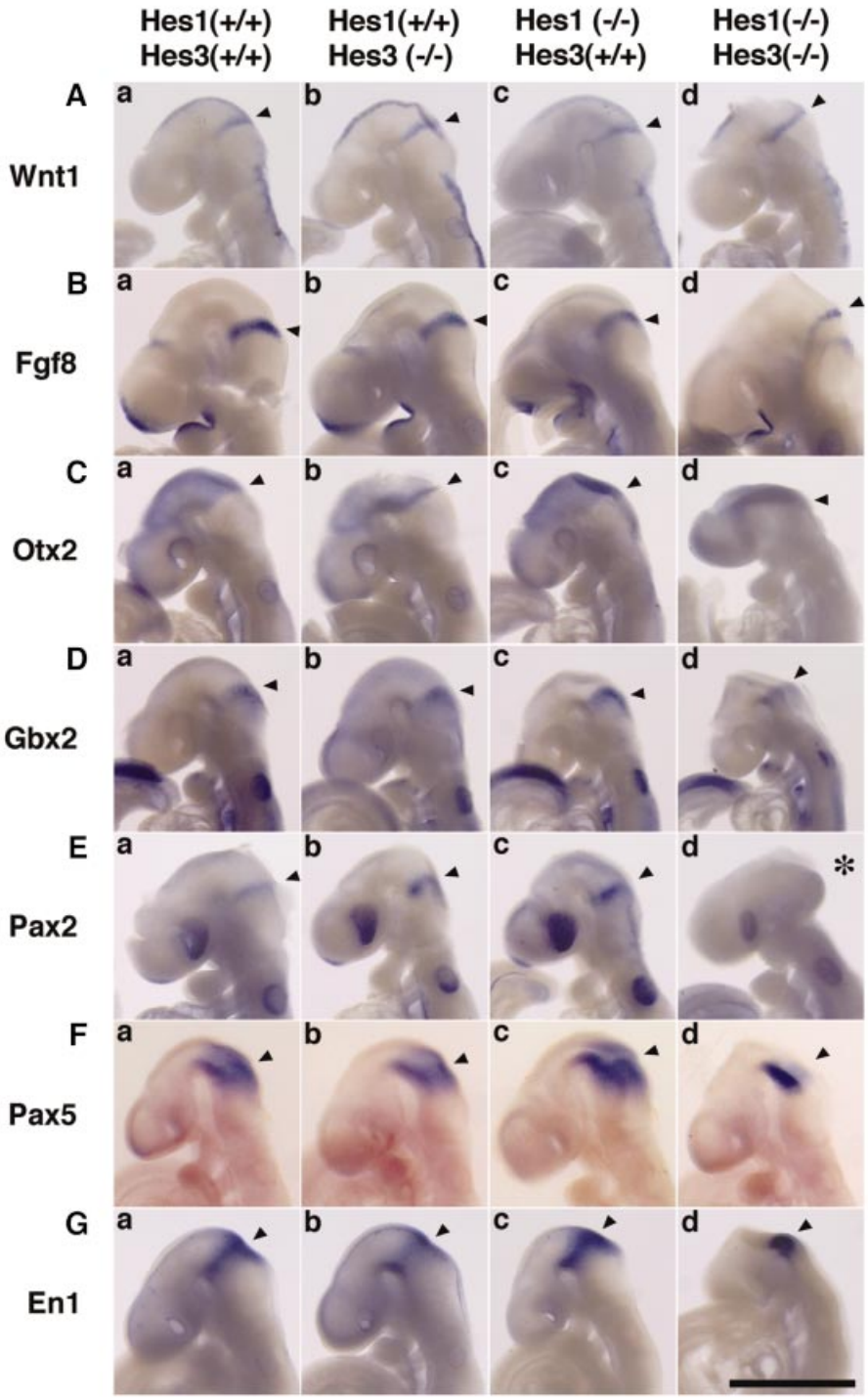


Fig. 6. The isthmic organizer is established in *Hes1–Hes3* double-mutant embryos at E9.5. Whole-mount *in situ* hybridization of E9.5 embryos. (**A–D**) *Wnt1*, *Fgf8*, *Otx2* and *Gbx2* are expressed normally in the double-mutant embryos (arrowheads). (**E**) The paired box gene *Pax2* is expressed in the wild-type, *Hes1*(–/–) and *Hes3*(–/–) isthmic organizer (a–c, arrowheads). In contrast, the expression is missing in the double mutant (d, asterisk). (**F** and **G**) *Pax5* and *En1* expression is not significantly changed in the double mutant although the expression domain is somewhat distorted by the neural tube defect (d, arrowhead). Scale bar, 1 mm.

the organizer signals, is the primary cause for the lack of midbrain and anterior hindbrain structures in *Hes1–Hes3* double-mutant embryos.

In the wild-type isthmic organizer region, some neurons were generated at E12.5, but the majority of the cells were still undifferentiated in the ventricular zone. These ventricular cells express secreting molecules such as Fgf8 and Wnt1. Fgf8, Fgf18 and Wnt1 expression continues until E13.5, while Fgf17 expression continues until E14.5 (Figure 1; and Xu *et al.*, 2000), indicating that isthmic organizer activity is maintained at least until E14.5. After termination of expression of such molecules, all these cells differentiate into neurons in the isthmic region by E17.5, whereas, outside the isthmus, most cells

differentiate into neurons earlier, indicating that the ventricular cells of the isthmic region are maintained specifically until the late stages compared with other regions. Strikingly, in the absence of *Hes1* and *Hes3*, all the isthmic cells prematurely lost expression of secreting molecules such as Fgf8 and Wnt1 by E10.5, ~4 days earlier than the wild type, and prematurely differentiated into neurons as early as E11.5, 6 days earlier than the wild type. These results indicate that *Hes1* and *Hes3* are essential for preventing premature differentiation and thereby maintaining the isthmic ventricular cells, which have the organizer activity. Whereas *Hes1* has a more general role in maintenance of various neural precursor cells, *Hes3* seems to be more specific to the isthmic organizer. However, since *Hes1* or *Hes3* single mutation does not affect the organizer activity, the two *Hes* genes are functionally redundant.

The above results also demonstrated that transient expression of *Fgf8* and *Wnt1* at E9.5 is not sufficient for specification of midbrain and anterior hindbrain and that the defects such as the lack of cranial nerves III and IV occur at E10.5, indicating that continued expression of the organizer factors is essential for normal development.

Neural tube defects do not cause the lack of midbrain and anterior hindbrain structures

Since *Hes1–Hes3* double-mutant embryos exhibit neural tube defects, these defects could cause the lack of midbrain and anterior hindbrain structures and of the isthmic organizer. However, in *Hes1* mutant embryos with severe neural tube defects, the *Phox2b* expression domains of oculomotor and trochlear motor nuclei and locus ceruleus as well as the *Pax5* expression domain of the midbrain–hindbrain boundary region were generated at E10.5. In contrast, in *Hes1–Hes3* double-mutant embryos, such structures were completely missing at E10.5. These results strongly indicate that lack of the midbrain and anterior hindbrain structures and premature termination of isthmic organizer activity are not due to neural tube defects but are specific to *Hes1–Hes3* double mutations.

A genetic cascade in the isthmic organizer

Although homeobox and paired box gene expression is deregulated by *Hes1* and *Hes3* double mutation, the relationship between *Hes* and homeobox/paired box genes is not clear. Previous analysis demonstrated that *Hes1* and *Hes3* expression is detectable as early as E7 (Lobe, 1997), and thus the onset of their expression is earlier than that of *En* and *Pax* gene expression, suggesting that *Hes* gene expression during the early developmental stages is likely to be independent of homeobox/paired box genes. However, although *Hes3* is expressed widely in the nervous system during early stages, its expression is soon restricted to the isthmic organizer. This isthmus-

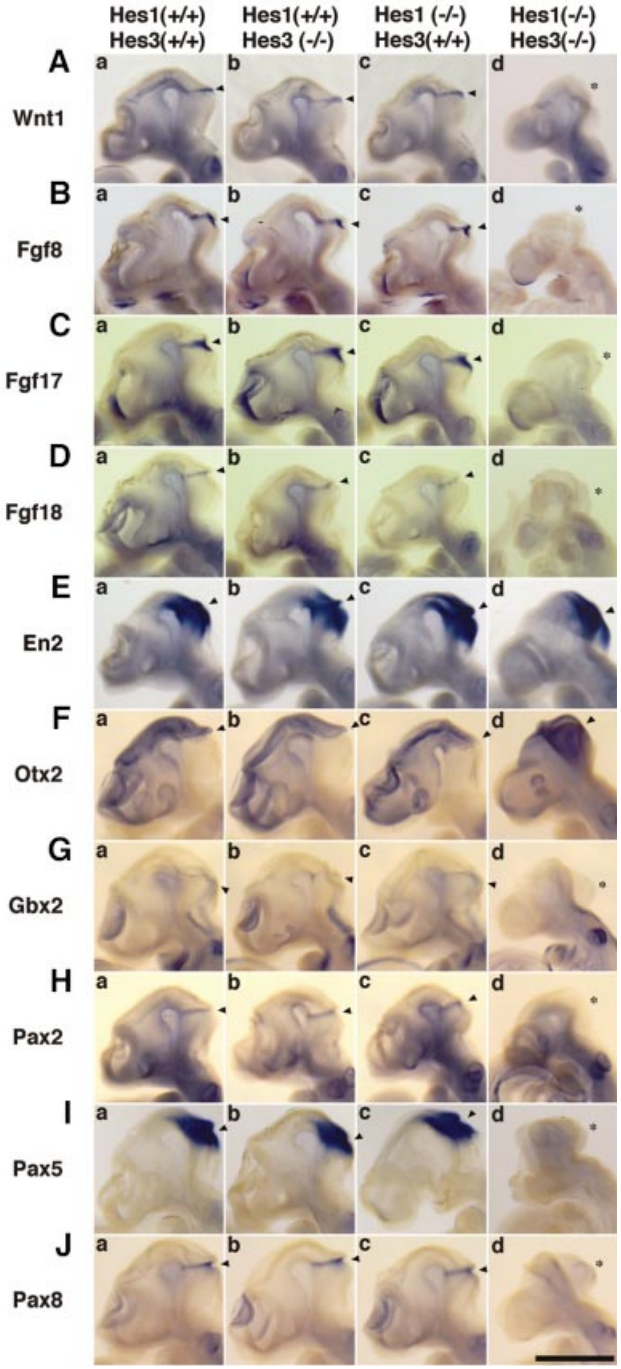


Fig. 7. The isthmic organizer is not maintained properly in *Hes1–Hes3* double-mutant embryos at E10.5. (**A**) *Wnt1 is* expressed in the wild-type, *Hes1*(–/–) and *Hes3*(–/–) isthmic organizer (a–c, arrowheads) whereas the expression disappears prematurely in the double mutant at E10.5 (d, asterisk). (**B–D**) *Fgf8/17/18* are expressed in the wild-type, *Hes1*(–/–) and *Hes3*(–/–) isthmic organizer (a–c, arrowheads) whereas the expression is not detectable in the double mutant at E10.5 (d, asterisks). Note that *Fgf8/17* expression in the forebrain is not affected in the double mutants (Bd and Cd). (**E**) *En2* is expressed normally in the midbrain–hindbrain region of the double mutant (arrowhead). (**F**) *Otx2* expression is restricted to the region rostral to the isthmus of the wild-type, *Hes1*(–/–) and *Hes3*(–/–) embryos (a–c). In contrast, *Otx2* expression is extended caudally in the double mutant (d). The isthmus is indicated by an arrowhead. (**G**) *Gbx2* is expressed in the hindbrain of the wild-type, *Hes1*(–/–) and *Hes3*(–/–) embryos (a–c). In contrast, *Gbx2* expression is not detectable in the anterior hindbrain of the double mutant (d, asterisk). The isthmus is indicated by an arrowhead. (**H–J**) *Pax2/5/8* are expressed in the wild-type, *Hes1*(–/–) and *Hes3*(–/–) isthmic organizer (a–c, arrowheads). In contrast, the expression of *Pax2* and *Pax5* is missing and that of *Pax8* is significantly down-regulated in the double mutant (d, asterisks). Thus, in the absence of *Hes1* and *Hes3*, the isthmic organizer is not maintained properly. The staining of *Pax2* in the floor plate (H) is an artifact. Scale bar, 1 mm.

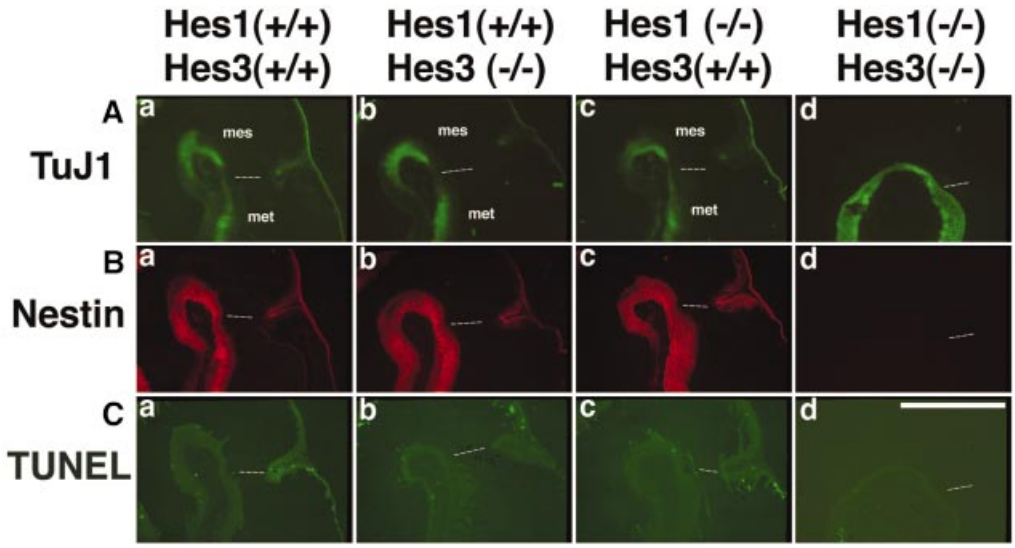


Fig. 8. Premature neuronal differentiation in the *Hes1–Hes3* double-mutant isthmic organizer. Parasagittal sections of E11.5 embryos were examined by immunohistochemistry. (**A**) (a–c) TuJ1$^+$ neurons are not generated in the wild-type, *Hes1*(–/–) and *Hes3*(–/–) isthmic region whereas some neurons are already differentiated outside this region. (d) Almost all cells prematurely differentiate into neurons in the double-mutant isthmic organizer. (**B**) (a–c) There are many Nestin$^+$ neural precursor cells in the wild-type, *Hes1*(–/–), and *Hes3*(–/–) isthmic region. (d) Nestin$^+$ neural precursor cells are virtually absent from the double-mutant isthmus and the surrounding region. (**C**) TUNEL assay. TUNEL$^+$ cells are not increased in the double-mutant isthmic organizer. Scale bar, 1 mm.

specific expression of *Hes3* during E11.5–13.5 could be regulated by homeobox/paired box genes. In this regard, it is possible that *En* genes are involved in regulation of *Hes* gene expression, since the observed defects of *Hes1–Hes3* double-mutant mice are similar to those of *En1–En2* double-mutant mice, which display a loss of isthmic organizer-specific gene expression (Liu and Joyner, 2001). The onsets of the abnormalities of *En1–En2* double-mutant mice occur earlier (around E8.5; Liu and Joyner, 2001) than those of *Hes1–Hes3* double mutant mice (E9.5). In addition, *En1/2* expression is not altered in *Hes1–Hes3* double-mutant mice, suggesting that *Hes1/3* could function downstream of *En1/2*. However, a sequence search indicated that there is no putative En-binding site in *Hes1* and *Hes3* promoters, and thus further studies are required to determine whether En can up-regulate *Hes* gene expression. In addition to *En* mutation, the phenotypes of *Pax2–Pax5* double mutation (Schwarz *et al*., 1997, 1999; Urbánek *et al*., 1997) are also similar to those of *Hes1–Hes3* double mutation. Because *Pax* gene expression is lost at E10.5 in *Hes1–Hes3* double mutation, it depends on *Hes* genes in the isthmus, indicating that *Hes1* and *Hes3* may, directly or indirectly, maintain *Pax* gene expression in the isthmic organizer. In zebrafish *Pax2* mutants (*no isthmus*), expression of the bHLH gene *her5*, which has structure and expression patterns very similar to *Hes3*, is initiated normally but not maintained properly, indicating that *her5* expression is controlled by *Pax2* (Lun and Brand, 1998). Thus, *Hes*-related bHLH genes and *Pax* genes are likely to regulate each other in the isthmus, suggesting that *Hes* genes may function in concert with the homeobox/paired box gene network to maintain the isthmic organizer.

The *Hes3* expression domain overlaps with that of *Wnt1*. Since *Wnt1* expression is lost at E10.5 in *Hes1–Hes3* double-mutant mice, it is likely that *Hes1* and *Hes3* may up-regulate *Wnt1* expression in the isthmic organizer. Previous analysis demonstrated that Wnt1 can also up-regulate *Hes* gene expression in cultured cells (Issack and Ziff, 1998). Thus, it is possible that Wnt1 and Hes1/3 form a positive regulatory loop in the isthmus. However, misexpression of *Hes3* in the anterior midbrain does not induce *Wnt1* or *Fgf8* expression (our unpublished data), suggesting that this possible regulatory loop may require additional genes such as homeobox genes.

Interestingly, in the double mutants at E10.5, *Otx2* expression is extended caudally while *Gbx2* expression is down-regulated in the anterior hindbrain. This defect is probably the result of loss of *Fgf8* expression since *Fgf8* is required to maintain *Gbx2* expression (Liu *et al*., 1999), which is required to repress *Otx2* expression in the hindbrain (Wassarman *et al*., 1997; Millet *et al*., 1999). Alternatively, it is also possible that deregulation of *Otx2* and *Gbx2* expression leads to disruption of the isthmic organizer and loss of *Fgf8* expression in *Hes1–Hes3* double-mutant mice. However, since this deregulation occurs only after the disappearance of *Fgf8* expression, we prefer the idea that the disappearance of *Fgf8* expression leads to deregulation of *Otx2* and *Gbx2* expression.

The results presented here demonstrate that premature differentiation of the isthmic organizer leads to patterning defects because of premature termination of the organizer activity. It was shown previously that premature differentiation also leads to a decrease in late-born cells because of depletion of precursor cells (Tomita *et al*., 1996). These results together point to the 2-fold importance of the normal timing of cell differentiation. Further characterization of *Hes* genes may be important to understand how the timing of cell differentiation is controlled during development.

Materials and methods

Targeted deletion of Hes3

For the *Hes3* targeting vector, the 9 kb 5′ fragment, IRES-*lacZ*, PGK-neo (in opposite transcriptional orientation), 2.2 kb 3′ fragment and DT were ligated in this order (Figure 2A). *lacZ* expression was under the control of the Purkinje cell-specific promoter (Hirata *et al*., 2000). Mutated ES cells were generated, as described previously (Tomita *et al*., 2000). Briefly, 10^7 TT2 ES cells (Gibco) were electroporated with 30 μg of the linearized targeting vector in 0.8 ml of phosphate-buffered saline (PBS) and selected with 250 μg/ml G418 (Gibco). ES cell lines with a *Hes3* mutation were identified by Southern blot analysis using 5′ and 3′ probes, as shown in Figure 2A, and Ba and b. Chimeric mice were generated by aggregating the *Hes3* mutant ES cells with ICR morula and then by implanting them into the uteri of pseudopregnant foster mothers. The resulting chimeric males were bred with ICR females. Homozygous mice generated from two independent mutated ES cell lines showed identical phenotypes.

Generation of Hes1–Hes3 double mutants

Hes1–Hes3 double-mutant mice were generated by crossing *Hes1*(+/–)–*Hes3*(–/–) or *Hes1*(+/–)–*Hes3*(+/–). Genotypes of *Hes1* and *Hes3* mutant mice were determined by Southern blot analysis or by PCR with the following primers: *Hes1* sense, 5′-ATATATAGAGGCCGCC-AGGGCCTGCGGGATC-3′; *Hes1* wild-type antisense, 5′-CGCAGG-TACTGTCTTACCTTTCTGTGCTCAGAGGCC-3′; *Hes1* mutant anti-sense, 5′-CGCTTCCATTGCTCAGCGGTGCTGTCCATC-3′; *Hes3* sense, 5′-GGCGGGCTGCACGCTTTAATGGGACACATG-3′; *Hes3* wild-type antisense, 5′-CACTATGTCTGCTTGCCAAGTCCTGGCTG-C-3′; and *Hes3* mutant antisense, 5′-ATTACGCCAGCTGGCGAAAGG-GGGATGTGC-3′.

In situ hybridization

In situ hybridization was performed as described previously (Tomita *et al*., 2000). The Wnt1 and Otx2 probes were kindly provided by Dr Shinji Takada and Dr Shinichi Aizawa, respectively, and the other probes used in this study were generated by RT–PCR. The following regions were used: Hes1 (nucleotide residues 71–1460), Hes3 (19–1522), Wnt1 (808–2199), Fgf8 (19–714), Fgf17 (1–683), Fgf18 (1–1080), Gbx2 (117–2010), Pax2 (131–2960), Pax5 (116–1142), Pax8 (91–2393), En1 (6–2311), En2 (91–2060), Nurr1 (647–2456), Phox2b (35–1590), Math1 (9–2200) and Krox20 (446–2246).

Histochemical analysis

Whole-mount immunohistochemistry was performed as described previously (Mark *et al*., 1993). In brief, E10.5 embryos were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) at 4°C for 3 h and were bleached with 0.1% H_2O_2 overnight at 4°C. Then, the embryos were incubated with anti-NF antibody (2H3, Hybridoma Bank) for 3 days at 4°C and next with peroxidase-conjugated secondary antibody (Chemicon) overnight at 4°C. The peroxidase deposits were visualized by 4-chloro-1-naphthol.

Immunohistochemistry with sections was performed as described previously (Tomita *et al*., 2000), with the following antibodies: mouse anti-β-tubulin (TuJ1, BabCO), mouse anti-Nestin (Pharmingen) and rabbit anti-TH (Chemicon). As a secondary antibody, biotinylated goat antibody against rabbit IgG (Vector) was used. The antibody complex was visualized by avidin-labeled fluorescein (Vector), fluorescein-labeled horse anti-mouse IgG (Vector) or Cy3-labeled goat anti-mouse IgG (Amersham).

TUNEL analysis was performed with a detection kit (Boehringer-Mannheim). X-gal staining was performed as follows. Mice were first fixed by perfusion of 4% paraformaldehyde–0.2% glutaraldehyde/PBS for 20 min and then washed by perfusion of PBS for 20 min. Brains were dissected, embedded and frozen in OCT compound. Sections were stained with 1 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside, 35 mM potassium ferricyanide, 35 mM potassium ferrocyanide, 2 mM $MgCl_2$, 0.02% NP-40 and 0.01% deoxycholate in PBS.

Acknowledgements

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