8. Suda, Y., Matsuo, I. & Aizawa, S. Cooperation between *Otx1* and *Otx2* genes in developmental patterning of rostral brain. *Mech. Dev.* **69,** 125–141 (1997).
9. Wassarmann, K. M. *et al.* Specification of the anterior hindbrain and establishment of a normal mid/hindbrain organizer is dependent on *Gbx2* gene function. *Development* **124,** 2923–2934 (1997).
10. Echelard, Y., Vassileva, G. & McMahon, A. P. Cis-acting regulatory sequences governing *Wnt1* expression in the developing mouse CNS. *Development* **120,** 2213–2224 (1994).
11. Martinez, S., Marin, F., Nieto, M. A. & Puelles, L. Induction of ectopic *engrailed* expression and fat change in avian rhombomeres: intersegmental boundaries as barriers. *Mech. Dev.* **51,** 289–303 (1995).
12. Hogan, B., Beddington, R., Constantini, F. *Manipulating the Mouse Embryo. A Laboratory Manual* (Cold Spring Harbor Laboratory, New York, 1994).
13. Wilkinson, D. *In Situ Hybridization. A Practical Approach* (IRL, Oxford, 1992).
14. Schaeren-Wiemers, N. & Gerfin-Moser, A. A single protocol to detect transcripts of various types and expression levels in neural tissue and cultured cells: in situ hybridization using digoxigenin-labelled cRNA probes. *Histochemistry* **100,** 431–440 (1993).
15. McMahon, A. P., Joyner, A. L., Bradley, A. & McMahon, J. A. The midbrain-hindbrain phenotype of Wnt1-/Wnt1- mice results from stepwise deletion of engrailed-expressing cell by 9.5 days postcoitum. *Cell* **69,** 581–595 (1992).
16. Millen, K. J., Hui, C. C. & Joyner, A. L. A role for *En-2* and other murine homologues of *Drosophila* segment polarity genes in regulating positional information in the developing cerebellum. *Development* **121,** 3935–3945 (1995).
17. Frohman, M. A., Boyle, M. & Martin, G. Isolation of the mouse *Hox-2.9* gene: analysis of embryonic expression suggests that positional information along the anterior posterior axis is specified by mesoderm. *Development* **110,** 589–607 (1990).
18. Toresson, H., Mata de Urquiza, A., Fragerstrom, C., Perlmann, T. & Campbell, K. Retinoids are produced by glia in the lateral ganglionic eminence and regulate striatal neuron differentiation. *Development* **126,** 1317–1326 (1999).
19. Puelles, L. & Rubenstein, J. L. R. Expression of homeobox and other putative regulatory genes in the mouse forebrain suggest a neuromeric organization. *Trends Neurosci.* **16,** 472–479 (1993).

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# The caudal limit of *Otx2* expression positions the isthmic organizer

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**The homeobox gene *Otx2* is expressed in the anterior neural tube with a sharp limit at the midbrain/hindbrain junction (the isthmic organizer)[1]. *Otx2* inactivation experiments have shown that this gene is essential for the development of its expression domain[2]. Here we investigate whether the caudal limit of *Otx2* expression is instrumental in positioning the isthmic organizer and in specifying midbrain versus hindbrain fate, by ectopically expressing *Otx2* in the presumptive anterior hindbrain using a knock-in strategy into the *En1* locus. Transgenic offspring display a cerebellar ataxia. Morphological and histological studies of adult transgenic brains reveal that most of the anterior cerebellar vermis is missing, whereas the inferior colliculus is complementarily enlarged. During early neural pattern formation expression of the midbrain markers *Wnt1* and *Ephrin-A5*, the isthmic organizer markers *Pax2* and *Fgf-8* and the hindbrain marker *Gbx2* are shifted caudally in the presumptive hindbrain territory. These findings show that the caudal limit of *Otx2* expression is sufficient for positioning the isthmic organizer and encoding caudal midbrain fate within the mid/hindbrain domain.**

Early neural-plate pattern formation is determined by exogenous signals from the mesoderm and visceral endoderm, and from planar signals active within the neuroectoderm[3,4]. The combination of these signals influences region-specific gene expression, for instance restricting *Otx2* to the anterior neural plate (presumptive forebrain and midbrain) and *Gx2* to the posterior neural plate (presumptive hindbrain and spinal cord)[4–6]. Subsequently, signalling molecules such as *Fgf-8* and *Wnt1* are expressed on either side of the mid/hindbrain border, controlling patterning and growth of adjacent territories[7,8]. This border acts like an organizing centre (the isthmic organizer). It can induce and reconstruct a mid/hindbrain domain in its vicinity when it is grafted ectopically into the forebrain or hindbrain[9–13]. *Fgf-8* can mimic this effect of the isthmic organizer when released from acrylic beads transplanted into similar positions[8]. It has been postulated that the establishment of such

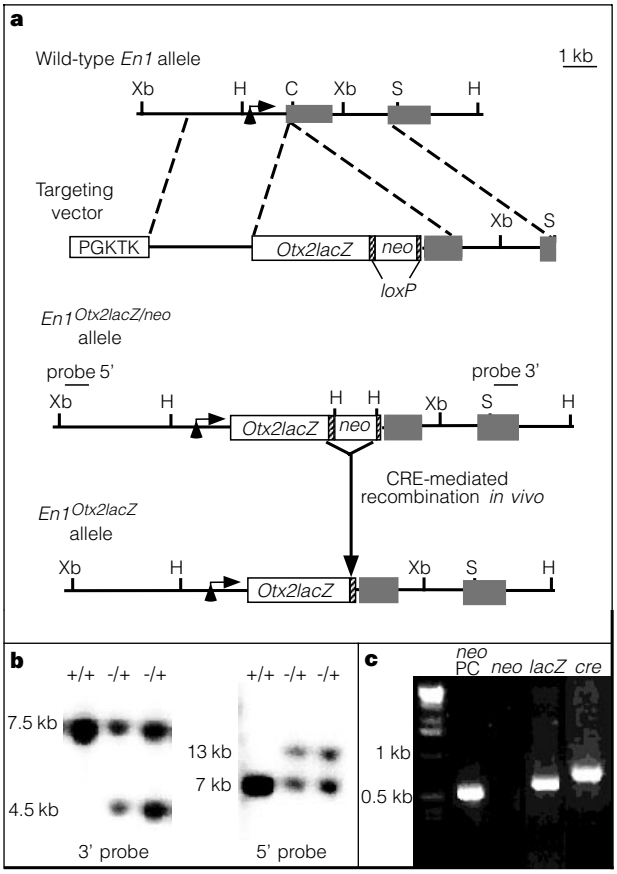


**Figure 1** Knock-in strategy of *Otx2IRESlacZ* (*Otx2lacZ*) into the *En1* locus. **a**, Arrow, *En1* promoter; grey boxes, coding sequences flanking a single intron. The $En1^{Otx2lacZ}$ targeting vector contains *Otx2lacZ* cassette, selectable thymidine kinase (TK) and a neomycin gene flanked by *loxP* sites. Dashed lines show regions of identity between the locus and the targeting vector. After gene targeting, *Otx2lacZ* is inserted into the *En1* locus downstream of the promoter, deleting the first 111 amino acids of *En1* and generating a null mutation. The positions of the 5′ and 3′ probes used to verify homologous recombination are indicated. After *cre* recombinase activation, the *neomycin* selector gene was removed leaving a single *loxP* site (shaded box). C, *Cla*I; H, *Hin*dIII; S, *Sac*I; Xb, *Xba*I restriction enzymes. **b**, Southern blot of genomic DNA isolated from targeted (+/–) ES cells. Digestion with *Hin*dIII and hybridization with a 3′ probe (left panel) revealed an additional 4.5-kb fragment; digestion with *Xba*I and hybridization with a 5′ probe (right panel) revealed an additional 13-kb fragment, indicative of a proper targeting event. **c**, After germline transmission the F1 generation was bred with a *cre* deleter strain to remove the *neo* cassette. Using specific primer sets recognizing *neomycin*, *lacZ* and *cre*, we show that the offspring expressing *cre*, recombinase have lost the *neomycin* gene (right three lanes, *neo* PC represents the *neo* control).

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organizing centres first requires the specification of two different cell populations in adjacent territories[14]. At the presumptive mid/hindbrain border, the juxtaposed expression of *Otx2* and *Gbx2* could perform this function.

To determine whether the sharp caudal limit of *Otx2* expression at the mid/hindbrain border is indeed critical for the formation and localization of the isthmic organizer and specification of midbrain versus hindbrain fate, we ectopically shifted the caudal limit of *Otx2* expression across the mid/hindbrain border into the anterior hindbrain (into the rostral third of rhombomere 1, rh1) by replacing the coding sequence of *En1* with *Otx2* (knock-in approach) using homologous recombination in embryonic stem (ES) cells (Fig. 1a). To allow us to follow easily the ectopic expression of *Otx2*, an internal ribosomal entry site (IRES) containing the *lacZ* gene was included. Thus, *Otx2* and *lacZ* were under the control of the *En1* regulatory elements and were expressed ectopically in the caudal midbrain and the anterior hindbrain domain of *En1* (ref. 15). From 300 recombinant ES cell colonies tested, we obtained 21 colonies carrying the designed modification (Fig. 1b). Germline chimaeras were bred to C57B1/6 or 129/Sv strains to obtain F1 offspring. The F1 animals were crossed with a transgenic line constitutively expressing *cre* recombinase to remove the PGK-promoter neomycin cassette, which might influence *Otx2* expression (Fig. 1c)[16]. Indeed, the expression of *Otx2* was about 2-fold higher after *PGK-neo* removal (data not shown).

Adult F1 heterozygote mutants were readily identified by their altered motor behaviour. They exhibited a gait ataxia and a wide stance. In simple motor behaviour tests such as the stationary rotorod ($n = 6$), horizontal thin rod ($n = 6$) and running rotorod ($n = 6$), the mutants performed significantly less well than wild-type littermates. The performance of the mutants improved slightly on the stationary rod and horizontal thin rod after six consecutive days of testing, but not on the running rotorod (see Supplementary Information). To measure muscle strength, a wire suspension test was applied, on which mutants and wild-type mice performed equally well (data not sown), indicating that the differences observed in the motor behaviour paradigms are due to cerebellar dysfunction.

We assessed the effect of ectopic expression of *Otx2* on cerebellar development in 12-week-old animals or at E15 by histology, immunohistochemistry and *in situ* hybridization of brain sections or whole mounts. There were clear morphological differences. The superior and inferior colliculi appeared enlarged and were shifted posteriorly in all mutants analysed, and the vermis of the cerebellum was reduced in size and not fused in the midline in $En1^{+/Otx2lacZ}$ transgenic mice (Fig. 2b), unlike in $En1^{+/-}$ and wild-type mice (Fig. 2a). The inferior colliculi of mutants were enlarged and significantly heavier (mean 18.81 mg ± 0.26, $n = 6$) than those of wild-type mice (14.01 mg ± 0.183, $n = 6$, $P < 0.005$, *U*-test). In the cerebellum, most of the anterior vermis was missing in the mutants (Fig. 2d). Unlike the wild-type (Fig. 2c), only the posterior cerebellar lobules VIII, IX and X were present in mutants, but they were not fused in the midline (Fig. 2d). They exhibited longitudinal instead of transverse fissures (Fig. 2c, d). The two longitudinal fissures that normally separate the central vermis from the lateral hemisphere were also missing (Fig. 2d). An additional lobe had formed in the cerebellar hemisphere, between crusII and the paramedial lobe (Fig. 2d). Coronal sections of adult wild-type (Fig. 2e) and mutant (Fig. 2f) cerebellum confirmed the phenotypic alterations observed in the whole-mount cerebellum. In coronal sections, using Zebrin II as a marker for longitudinal compartmentalization of the vermis and hemispheres, we confirmed the loss of anterior vermis tissue and altered foliation patterns in the hemispheres (Fig. 2e, f; and data not shown). The cerebellar commissure, which is normally located within the inner granular cell layer of the anterior cerebellum, was

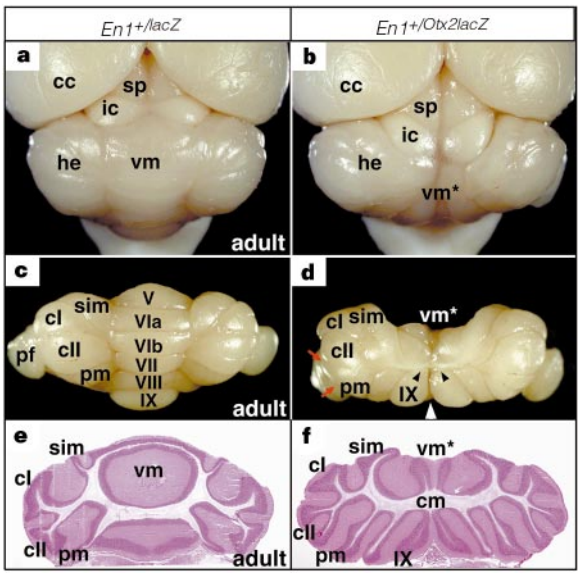


**Figure 2** Ectopic expression of *Otx2* results in phenotypic alterations of midbrain and cerebellum. **a**, **b**, Dorsal view of brains of adult wild-type ($En1^{+/lacZ}$; **a**) and $En1^{+/Otx2lacZ}$ transgenic mice (**b**) show the caudal shift of the superior and inferior colliculi, the loss of most of the anterior vermis and the lack of cerebellar midline fusions in the mutants. **c**, **d**, Dorsal view of the dissected cerebellum shows deletion of most of the anterior vermis in the mutant (**d**) compared with the wild-type (**c**). Lobes VIII, IX and X (hidden) of the posterior vermis are present but not fused in the midline (white arrowhead). The cerebellar commissure appears at the surface (black arrowheads). The foliation patterns of the cerebellar hemispheres are altered in transgenic mice with an additional lobe between crusII and the paramedial lobe (red arrows). **e**, **f**, Coronal sections of wild-type (**e**) and mutant (**f**) cerebellum confirm these interpretations. cc, cerebral cortex; cb, cerebellum; cl, crus I; cII, crus II; he, hemisphere; ic, inferior colliculi; is, isthmus; pf, paraflocculus; pm, paramedial lobe; sim, simplex; sp, superior colliculi; vm, vermis; vermis lobes V–IX are indicated.

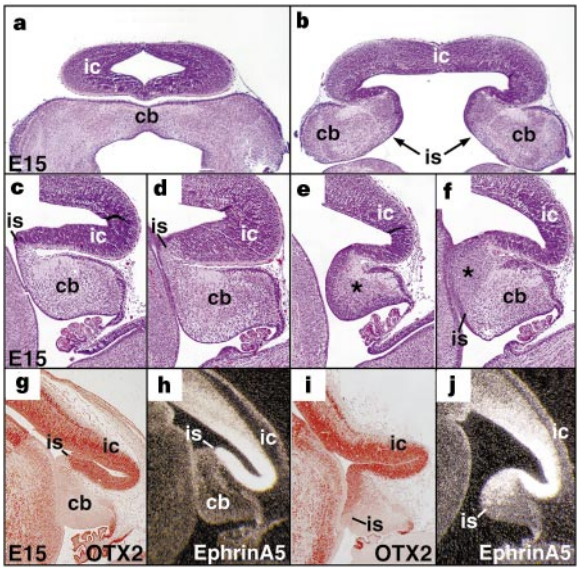


**Figure 3** Ectopic expression of *Otx2* results in phenotypic alterations at E15. Coronal sections of wild-type (**a**) and mutant brain (**b**) at the level of the inferior colliculi at stage E15. In the mutants, the bilateral cerebellar anlagen are not fused in the midline and inferior colliculi and isthmic cells are located in the cerebellar anlage (arrow). **c**–**f**, Sagittal sections of wild-type (**c**, **d**) and mutant (**e**, **f**) brains at E15. The densely packed inferior colliculi cells and the loosely packed isthmic cells (**c**, **d**) are positioned in the anterior cerebellum in the mutants (**e**, **f**; star). **g**, **i**, Anti-α-Otx antibody staining on wild-type (**g**) and mutant (**i**) sagittal brains at E15 indicate extension of *Otx2* expression into the cerebellar anlage. *In situ* hybridization of sagittal sections using *Ephrin-A5* in wild-type (**h**) and mutant (**j**) brains at E15 reveal *Ephrin-A5*-positive cells in the cerebellar anlage in the mutant animals.

displaced ectopically to the surface of the posterior vermis (Fig. 2d). Despite the morphological alterations, the cytoarchitecture of the mutant cerebellum was normal.

At E15, the tectal and lateral cerebellar primordia fused at the midline in the wild-type brains, as shown in coronal sections (Fig. 3a). In contrast, in the mutants the lateral cerebellar anlagen did not fuse in the midline (Fig. 3b), and compact, intensely stained inferior-colliculi-specific and isthmus-specific cells were observed in the anterior cerebellum (compare Fig. 3b, e, f with 3c, d). To confirm that these anterior transformations were due to ectopic expression of *Otx2*, we studied *Otx2* expression at different embryonic stages by mRNA *in situ* hybridization and antibody staining. As shown in Fig. 3i, OTX2 was expressed ectopically in the cerebellum in an *En1*-like manner, confirming the histological observations. Furthermore, using *Ephrin-A5* as a midbrain-specific marker (Fig. 3h; see also Supplementary Information), we detected midbrain-specific cells in the mutant anterior cerebellum (Fig. 3j). These findings agree with fate-map studies performed by chick/quail transplantations, which indicated that the early embryonic, caudal *Otx2* expression domain will give rise to midbrain and the *Otx2*-negative domain in the presumptive isthmic region and anterior rhombomere 1 to the cerebellar vermis in a V-shaped manner[17,18]. To investigate whether these mid/hindbrain transformations are a consequence of repositioning of the isthmic organizer in the rostral hindbrain, we examined E12, E10.5 and E9.5 embryos for isthmic organizer formation and phenotypic alterations using different molecular markers.

Whole-mount *in situ* hybridization at E10.5 in mutant embryos showed that the expression of *Otx2* (Fig. 4b, g), *Pax2* (Fig. 4d, h) and *En1* (Fig. 4j) were shifted caudally into the anterior rh1 domain. At E9.5, *Otx2* (Fig. 5b), *Wnt1* (Fig. 5d), *Fgf-8* (Fig. 5f) and *Ephrin-A5* (not shown) were induced ectopically in the dorsal roof of the hindbrain, whereas *Gbx2* (Fig. 5h) was repressed in the ectopic *Otx2* expression domain (Fig. 5b). These ectopic inductions and repressions were all maintained at E12 (see Supplementary Information). *En2*, which is expressed across the mid/hindbrain junction in a broad domain, seems unaltered (not shown); however, subtle changes within the *En2* expression domain cannot be ruled out, as they would be difficult to visualize. Thus, at early embryonic stages, the expression of all the midbrain- (*Ephrin-A5*, *Wnt1*) and isthmic-specific markers (*Pax2*, *Fgf-8*) was shifted caudally into rh1 as a consequence of the ectopic *Otx2* expression, whereas the hindbrain-specific marker *Gbx2* was repressed. As a result, the isthmic organizer was repositioned in the anterior hindbrain during early development, with the inferior colliculi enlarged and extended caudally at the expense of the anterior vermis in the adult.

Loss-of-function and gain-of-function experiments in mouse,

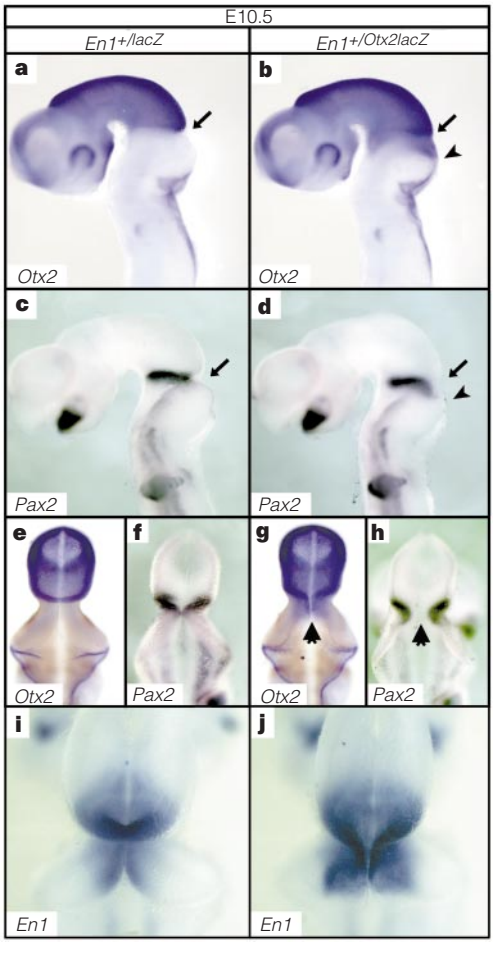


**Figure 4** mRNA *in situ* hybridization of mid/hindbrain-specific markers in wild-type (*En1*$^{+/lacZ}$; **a**, **c**, **e**, **f**, **i**) and mutants (*En1*$^{+/Otx2lacZ}$; **b**, **d**, **g**, **h**, **j**) in a lateral (**a–d**) and dorsal view (**e–j**) at E10.5. Markers used are indicated. **a**, **e**, Wild-type (arrow) and **b**, **g**, ectopic (arrowhead) expression of *Otx2* in the mutants in the midbrain and anterior hindbrain of rh1; ectopic expression of *Pax2* (**d**, **h**) compared with wild-type (**c**, **f**); and expansion of *En1* (**j**) expression in the mutants compared with the wild-type (**i**).

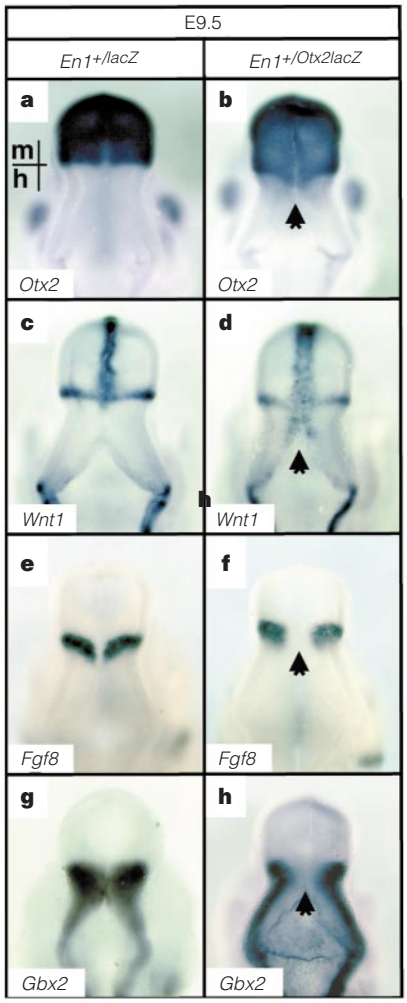


**Figure 5** mRNA *in situ* hybridization of mid/hindbrain-specific markers in control *En1*$^{+/lacZ}$ (**a**, **c**, **e**, **g**) and mutant *En1*$^{+/Otx2lacZ}$ (**b**, **d**, **f**, **h**) embryos at E9.5. Markers used are indicated. **a**, Wild-type and **b**, ectopic expression of *Otx2* in the mutant at the level of the hindbrain of rh1 (arrow). **d**, Ectopic *Wnt1* expression is observed in the dorsal rh1 (arrow) compared with wild-type (**c**). **f**, *Fgf-8* expression is slightly shifted caudally into rh1 abutting the ectopic *Otx2* expression domain but is repressed in the dorsal midline (arrow). **h**, *Gbx2* expression is repressed in the dorsal, anterior hindbrain at the site of ectopic *Otx2* expression. h, hindbrain; m, midbrain.

zebrafish and chicken have shown that *Wnt1* and *Fgf-8* are necessary to maintain the expression of *En1*, *En2* and *Pax2*[7,8,10,19–22]. Moreover, *En1* is necessary to maintain *Pax2* and *Wnt1* (V. Blanquet and W.W., manuscript in preparation) and *Wnt1* and *Pax2* maintain *En1* in a cross-regulatory loop[7,23–25]. The inactivation of one of these genes disrupts the cross-regulatory genetic interactions and, subsequently, causes loss of the mid/hindbrain tissue[10,21,22,24,25]. Studies of $Otx2^{-/-}$ and $Otx1^{-/+}$, $Otx2^{-/+}$ and $Otx1^{-/-}$, $Otx2^{-/+}$ compound mouse mutants indicated that the proper development and patterning of the forebrain, midbrain and anterior hindbrain depends on the *Otx* gene dosage[2,26,27] in the neural plate and/or underlying tissues. Here, we show that the formation and positioning of the isthmus organizer is determined by the caudal expression limit of *Otx2* within the neuroectoderm, which also specifies midbrain versus hindbrain development. Thus, *Otx2* expression and the regulation of its caudal limit could be placed upstream of the genetic events leading to isthmus organizer formation and localization (Fig. 6). □

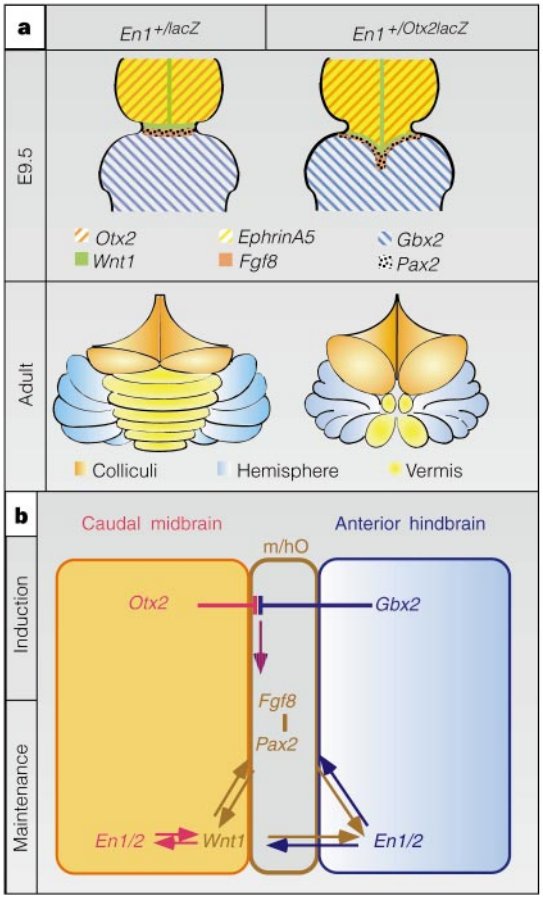


**Figure 6** Genetic interactions leading to isthmic organizer (IsO) formation and mid/hindbrain specification. **a**, At E9.5, *Otx2* is expressed in the midbrain with a sharp limit of expression at the IsO and *Gbx2* is expressed in the hindbrain and IsO with a sharp boundary of expression at the midbrain border abutting *Otx2* expression. *Fgf-8* and *Pax2* are expressed in the IsO overlapping with *Gbx2*. *Wnt1* expression forms a narrow stripe in the caudal midbrain adjacent to the IsO and *Ephrin-A5* is expressed in the midbrain, both overlapping with *Otx2* expression. *En1* and *2* are expressed across the mid/hindbrain region (not shown). Ectopic expression of *Otx2* in the IsO and anterior dorsal hindbrain (right panel) leads to a shift of *Fgf-8*, *Pax2*, *Gbx2*, *Ephrin-A5* and *Wnt1* expression caudally early in embryonic development, resulting in reduction of the vermis and enlargement of the colliculi in the adult brain (bottom right). **b**, Genetic hierarchy determining IsO formation and localization based on gain-of-function and loss-of-function experiments of *En1*, *Pax2*, *Wnt1*, *Fgf-8*, *Gbx2* and *Otx2*. As shown here, ectopic expression of *Otx2* in IsO and rh1 results in a caudal shift of *Gbx2*, *Pax2*, *Fgf-8* and *Wnt1* expression. Thus, *Otx2* can be placed upstream in the hierarchy of genetic interactions specifying the IsO and midbrain versus hindbrain fate. m/hO, mid/hindbrain organizer.

## Methods

### Knock-in of a murine *Otx2* minigene into the *En1* locus.

The *En1* knock-in vector has been described[28]. The *PGK-neo* cassette is flanked by *loxP* sites, allowing the removal of the neomycin gene cassette. A mouse *Otx2* complementary DNA encompassing the entire coding sequences followed by an encephalomyocarditis-virus-derived internal ribosome entry site (IRES) sequence linked to a bacterial *lacZ* reporter gene was inserted into the *Kpn*I site of the *En1* knock-in vector[28], placing the *Otx2* minigene downstream of the *En1* promoter. R1 ES cells were cultured, electroporated and selected as described[24]. Recombinant clones were identified by Southern blotting using *Hin*dIII and *Xba*I restriction enzymes. As a 5′ probe, a 1.2-kb *Sac*I fragment and, as a 3′ probe, a 700-bp *Eco*RI–*Hin*dIII fragment were used to identify a 7-kb or 4.5-kb fragment after a proper homologous recombination event. A total of 300 colonies were analysed, yielding 21 recombinant clones.

Two independent targeted ES cell clones were injected into C57BL/6 blastocysts. Germline chimaeras were bred with either C57BL/6 or 129/Sv mouse strains. Germline transmission was detected by Southern blot analysis after *Hin*dIII digestion of genomic DNA and hybridization with a 3′ probe. F1 offspring were mated with the deleter transgenic mouse line expression cre-recombinase ubiquitously[16]. Cre-mediated *neo* excision was monitored by three PCR reactions detecting the presence of *lacZ* and *cre* and confirming the loss of the *neo* sequence. *LacZ* primers: sense, 5′-GGTGGCGCTGGATGGTAAGC-3′; antisense, 5′-CGCCATTTGACCACTACC-3′. *Neo* primers: sense, 5′-CTGGGCACAACAGACAATCGG-3′; antisense, 5′-CGATAGAAGGC-GATGCGCTGC-3′. *Cre* primers: sense, 5′-GATCGCTGCCAGGATATACG; antisense, 5′-CATCGCCATCTTCCAGCAG.

### Histology, immunohistory and *in situ* hybridization.

Age-matched embryos and tissues were dissected, fixed in 4% paraformaldehyde (PFA), dehydrated and embedded in paraffin for microtome sections. Haematoxylin–eosin was used for staining. For β-galactosidase staining the tissue was fixed in 2% PFA and 0.2% gluteraldehyde for 5–10 min and further processed as described[24]. Immunohistochemistry was carried out on 20-μm sections of fresh tissue or paraffin embedded as described using antisera recognizing *Otx2* (ref. 29), calbindin D-28 (Swant Swiss Antibodies) and calretinin (Swant Swiss Antibodies). *In situ* hybridizations were performed as described[6] with antisense RNA probes transcribed from plasmids containing fragments of *Otx2* (ref. 26), *En1* (ref. 15), *En2* (ref. 15), *Fgf-8* (ref. 8), *Wnt1* (ref. 7), *Ephrin-A5* (ref. 30) and *Gbx2* (ref. 6).

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1. Simeone, A., Acampora, D., Massino, G., Stornainolo, A. & Boncinelli, E. Nested expression domains of four homeobox genes in developing rostral brain. *Nature* **358,** 687–690 (1992).
2. Acampora, D. & Simeone, A. Understanding the roles of Otx1 and Otx2 in the control of brain morphogenesis. *Trends Neurosci.* **22,** 116–122 (1999).
3. Lumsden, A. & Krumlauf, R. Patterning the vertebrate neuraxis. *Science* **274,** 1109–1115 (1996).
4. Beddington, R. S. P. & Robertson, E. J. Anterior patterning in mouse. *Trends Genet.* **14,** 277–284 (1998).
5. Ang, S.-L., Conlon, R. A., Jin, O. & Rossant, J. Positive and negative signals from mesoderm regulate the expression of mouse Otx2 in ectoderm explants. *Development* **120,** 2979–2989 (1994).
6. Wassarman, K. M. *et al.* Specification of the anterior hindbrain and establishment of a normal mid/hindbrain organizer is dependent on *Gbx2* gene function. *Development* **124,** 2923–2934 (1997).
7. McMahon, A. P., Joyner, A. L., Bradley, A. & McMahon, J. A. The midbrain-hindbrain phenotype of Wnt-1$^{-}$/Wnt-1$^{-}$ mice results from stepwise deletion of engrailed-expressing cells by 9.5 days postcoitum. *Cell* **69,** 581–595 (1992).
8. Martinez, S., Crossley, P. H., Cobos, I., Rubenstein, J. L. R. & Martin, G. R. FGF8 induces formation of an ectopic isthmic organizer and isthmocerebellar development via a repressive effect on Otx2 expression. *Development* **126,** 1189–1200 (1999).
9. Bally-Cuif, L. & Wassef, M. Determination events in the nervous system of the vertebrate embryo. *Curr. Opin. Genet. Dev.* **5,** 450–458 (1995).
10. Joyner, A. L. Engrailed, Wnt and Pax genes regulate midbrain-hindbrain development. *Trends Genet.* **12,** 15–20 (1996).
11. Wassef, M. & Joyner, A. L. Early mesencephalon/metencephalon patterning and development of the cerebellum. *Prospect. Dev. Neurobiol.* **5,** 3–16 (1997).
12. Martinez, S., Wassef, M. & Alvarado-Mallart, R. M. Induction of amesencephalic phenotype in the 2-day-old chick prosencephalon is preceded by the early expression of the homeobox gene *en*. *Neuron* **6,** 971–981 (1991).
13. Martinez, S., Marin, F., Nieto, M. A. & Puelles, L. Induction of ectopic engrailed expression and fate change in avian rhombomeres: intersegmental boundaries as barriers. *Mech. Dev.* **51,** 289–303 (1995).
14. Meinhardt, H. Cell determination boundaries as organizing regions for secondary embryonic fields. *Dev. Biol.* **96,** 375–385 (1983).
15. Davis, C. A. & Joyner, A. L. Expression patterns of the homeobox containing genes *En1* and *En2* and the proto-oncogene *int-1* diverge during mouse development. *Gen. Dev.* **2,** 1736–1744 (1988).
16. Schwenk, F., Baron, U. & Rajewsky, K. A cre-transgenic mouse strain for ubiquitous deletion of loxP-flanked gene segments including deletion in germ cells. *Nucl. Acids Res.* **24,** 5080–5081 (1995).
17. Millet, S., Bloch-Gallego, E., Simeone, A. & Alvarado-Mallart, R.-M. The caudal limit of *Otx2* gene expression as a marker of the midbrain/hindbrain boundary: a study using *in situ* hybridisation and chick–quail homotopic grafts. *Development* **122,** 3785–3797 (1996).
18. Marin, F. & Puelles, L. Morphological fate of rhombomeres in quail/chick chimeras: a segmental analysis of hindbrain nuclei. *Eur. J. Neurosci.* **7,** 1714–1738 (1995).
19. Danielian, P. S. & McMahon, A. P. Engrailed-1 as a target of the Wnt-1 signalling pathway in vertebrate midbrain development. *Nature* **383,** 332–334 (1996).
20. Lee, S. M. K., Danielian, P. S., Fritzsch, B. & McMahon, A. P. Evidence that FGF8 signalling from the midbrain-hindbrain junction regulates growth and polarity in the developing midbrain. *Development* **124,** 959–969 (1997).

21. Meyers, E. N., Lewandoski, M. & Martin, G. R. An Fgf8 mutant allelic series generated by Cre- and Flp-mediated recombination. *Nature Genet.* **18,** 136–141 (1998).
22. Reifers, F. *et al.* Fgf8 is mutated in zebrafish acerebellar (ace) mutants and is required for maintenance of midbrain-hindbrain boundary development and somitogenesis. *Development* **125,** 2381–2395 (1998).
23. Lun, K. & Brand, M. A series of no isthmus (noi) alleles of the zebrafish pax2.1 gene reveals multiple signalling events in development of the midbrain-hindbrain boundary. *Development* **125,** 3049–3062 (1998).
24. Wurst, W., Auerbach, A. B. & Joyner, A. Multiple developmental defects in *Engrailed-1* mutant mice: an early mid-hindbrain deletion and patterning defects in forelimbs and sternum. *Development* **120,** 2065–2075 (1994).
25. Schwarz, M., Alvarez-Bolado, G., Urbánek, P., Busslinger, M. & Gruss, P. Conserved biological function between Pax-2 and Pax-5 in midbrain and cerebellum development: Evidence from targeted mutation. *Proc. Natl Acad. Sci. USA* **94,** 14518–14523 (1997).
26. Acampora, D., Avantaggiato, V., Tuorto, F. & Simeone, A. Genetic control of brain morphogenesis through *Otx* gene dosage requirement. *Development* **124,** 3639–3650 (1997).
27. Suda, Y., Matsuo, I. & Aizawa, S. Cooperation between Otx1 and Otx2 genes in developmental patterning of rostral brain. *Mech. Dev.* **69,** 125–141 (1997).
28. Hanks, M., Wurst, W., Anson-Cartwright, L., Auerbach, A. B. & Joyner, A. L. Rescue of the En1 mutant phenotype by replacement of *En1* with *En2*. *Science* **269,** 679–682 (1995).
29. Mallamaci, A., Di Blas, E., Briata, P., Boncinelli, E. & Corte, G. OTX2 homeoprotein in the developing central nervous system and migratory dells of the olfactory area. *Mech. Dev.* **58,** 165–178 (1996).
30. Flenniken, A. M., Gale, N. W., Yancopoulos, G. D. & Wilkinson, D. G. Distinct and overlapping expression patterns of ligands for Eph-related receptor tyrosine kinases during mouse embryogenesis. *Dev. Biol.* **179,** 382–401 (1996).

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# Acinus is a caspase-3-activated protein required for apoptotic chromatin condensation

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**Apoptosis is defined by several unique morphological nuclear changes, such as chromatin condensation and nuclear fragmentation[1]. These changes are triggered by the activation of a family of cysteine proteases called caspases[2,3], and caspase-activated DNase (CAD/DFF40)[4,5] and lamin protease (caspase-6)[6,7] have been implicated in some of these changes. CAD/DFF40 induces chromatin condensation in purified nuclei, but distinct caspase-activated factor(s) may be responsible for chromatin condensation[8]. Here we use an *in vitro* system to identify a new nuclear factor, designated Acinus, which induces apoptotic chromatin condensation after cleavage by caspase-3 without inducing DNA fragmentation. Immunodepletion experiments showed that Acinus is essential for apoptotic chromatin condensation *in vitro*, and an antisense study revealed that Acinus is also important in the induction of apoptotic chromatin condensation in cells.**

To identify the molecules acting downstream of caspases that are responsible for apoptotic changes of the nucleus, we have developed an *in vitro* system using digitonin-permeabilized cells, that has been widely used to study active nuclear transport[9]. When permeabilized human HeLa cells were incubated with a lysate prepared from apoptotic Jurkat cells, apoptotic morphological changes of the nucleus, including chromatin condensation, were induced (Fig. 1a). The lysate from live Jurkat cells treated with caspase-3 induced chromatin condensation, but the lysate or the caspase-3 alone failed to induce this (Fig. 1a) indicating that the target molecule of caspase-3 that is responsible for chromatin condensa-

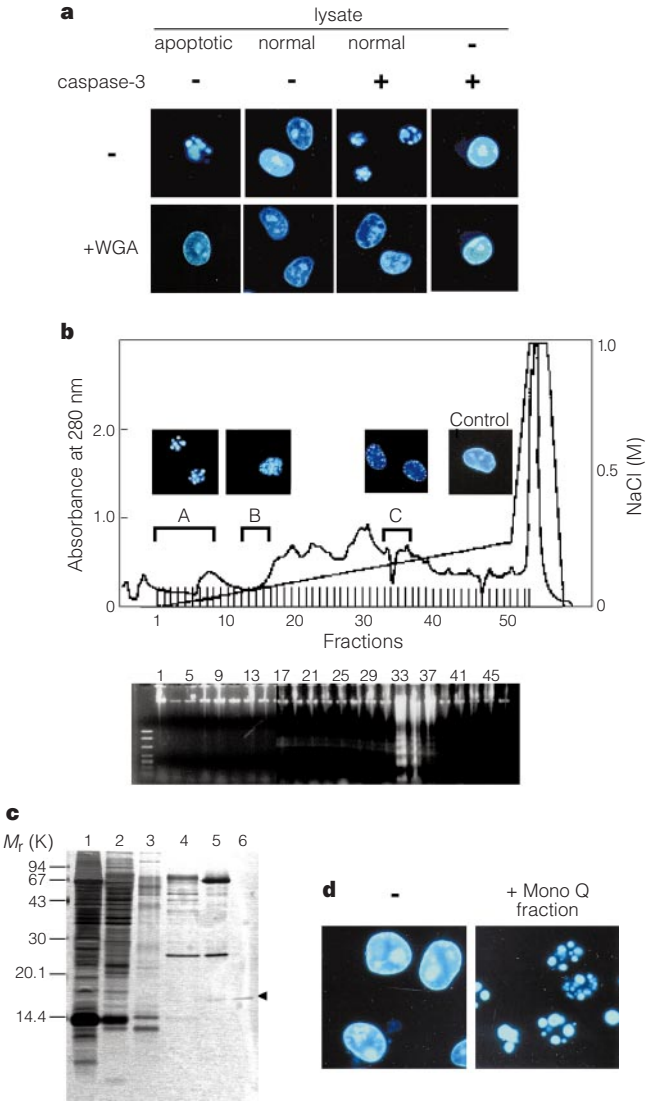


**Figure 1** Purification of an apoptotic chromatin-condensation factor from bovine thymus. **a**, *In vitro* apoptosis system using permeabilized HeLa cells. Permeabilized cells were incubated for 2 h with apoptotic Jurkat lysate, normal Jurkat lysate, normal Jurkat lysate plus caspase-3, or caspase-3 only, with or without 0.1 mg ml$^{-1}$ wheatgerm agglutinin (WGA), an inhibitor of active nuclear transport, and examined under a fluorescence microscope after Hoechst 33342 staining. **b**, Separation of three distinct activities inducing chromatin condensation by HiTrap-Q column chromatography of bovine thymus lysate. Fractions (2.5 μl) were assayed for induction of chromatin condensation using the *in vitro* system. Chromatin condensation caused by three fractions (A, B and C) are shown. The DNA laddering activity of every other fraction was also assayed[25]. Protein concentration was monitored by absorbance at 280 nm. **c**, Portions of pooled fractions with chromatin-condensing activity at each step of purification were subjected to SDS–PAGE and silver staining. Lane 1, 100,000*g* supernatant (3.4 μg); lane 2, HiTrap-Q (1.7 μg); lane 3, Heparin Sepharose after passing the hydroxyapatite column (150 ng); lane 4, Phenyl Sepharose (70 ng); lane 5, Superose 12 (50 ng); lane 6, Mono-Q (2.5 ng). Arrowhead, position of purified Acinus p17 protein. **d**, Nuclear changes caused by the Mono-Q fraction. Permeabilized HeLa cells were incubated with (right) or without (left) the active fraction from Mono-Q column chromatography in the absence of caspase-3.