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The organizer factors Chordin and Noggin are required for mouse forebrain development

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In mice, there is evidence suggesting that the development of head and trunk structures is organized by distinctly separated cell populations[1,2]. The head organizer is located in the anterior visceral endoderm (AVE) and the trunk organizer in the node and anterior primitive streak. In amphibians, Spemann's organi-zer, which is homologous to the node, partially overlaps with anterior endoderm cells expressing homologues of the AVE markers *cerberus*, *Hex* and *Hesx1* (refs 3–6). For mice, this raises the question of whether the AVE and node are independent of each other, as suggested by their anatomical separation, or functionally interdependent as is the case in amphibians[3–5]. Chordin and Noggin are secreted bone morphogenetic protein (BMP) antagonists[7,8] expressed in the mouse node, but not in the AVE. Here we show that mice double-homozygous mutants that are for *chordin* and *noggin* display severe defects in the develop-ment of the prosencephalon. The results show that BMP antagonists in the node and its derivatives are required for head development.

The signalling activities of BMPs can be antagonized by a diverse group of binding proteins, including Chordin and Noggin[1,2,7,8]. Mouse *chordin* (*Chd*) messenger RNA is first expressed in the anterior primitive streak and then in the node and axial mesendo-derm that derives from it (Fig. 1a), suggesting that *chordin* may play a role in patterning the early embryo. However, targeted inactiva-tion of *chordin* results in stillborn animals that have normal early development and neural induction but display later defects in inner and outer ear development (Fig. 1e and f) and abnormalities in pharyngeal and cardiovascular organization (D.B. *et al.*, manuscript in preparation). At midgastrula, expression of *noggin* (*Nog*)[9] over-laps with *chordin* (Fig. 1b and c). The *noggin* mutants undergo normal gastrulation and anterior central nervous system (CNS) patterning, although at later stages a number of abnormalities are
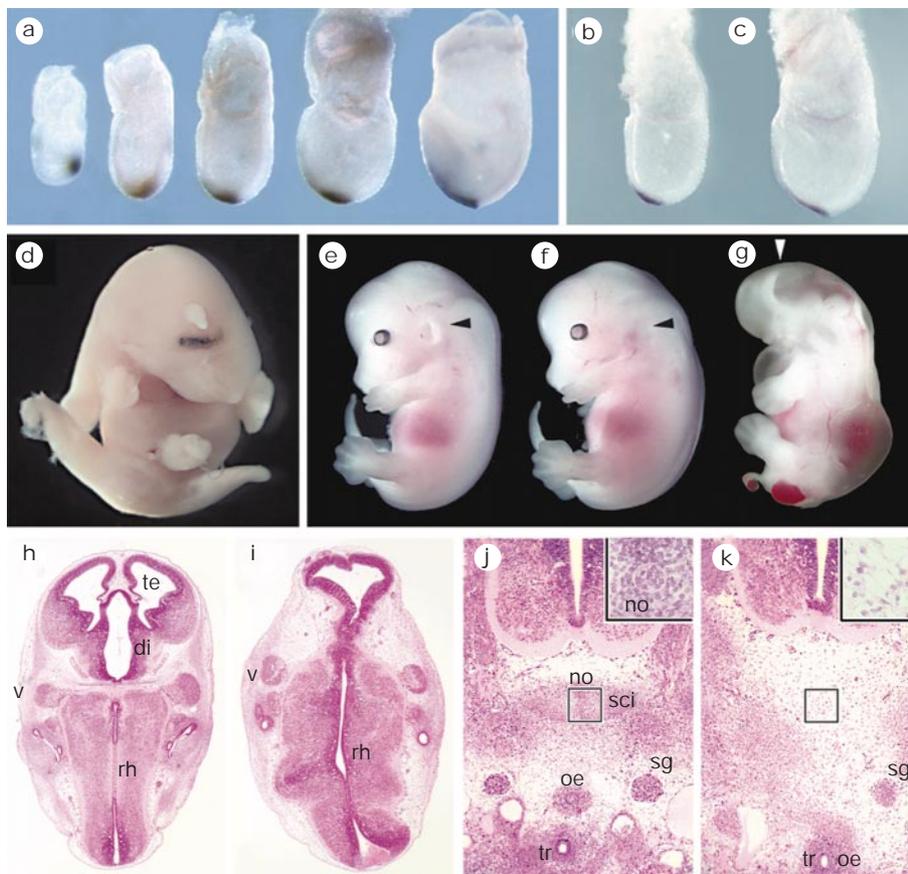
Figure 1 Expression of *chordin* and *noggin* in the node and phenotype of double mutants. **a**, Expression of *chordin* during gastrulation in anterior primitive streak, node and axial mesendoderm. **b** and **c**, Expression of *noggin* at neural plate and head fold stages; expression overlaps with that of *chordin*. We note that *chordin* and *noggin* are not expressed in the AVE. **d**, *Chd*⁻/⁻;*Nog*⁻/⁻ embryo recovered at late gestation, showing single nasal pit (proboscis), cyclopia and agnathia. **e**–**k**, E12.5 embryos. **e**, Wild-type (wt). **f**, *Chd*⁻/⁻;*Nog*⁺/⁺. We note the normal head and defective ear (arrowhead). **g**, *Chd*⁻/⁻ *Nog*⁻/⁻ showing extensive anterior deletions of forebrain, eye, nose and facial structures. White arrowhead indicates the rhombic lip (future cerebellum); all external phenotypes posterior to it are also seen in *noggin* mutants[9]. Of the three double mutants recovered at E12.5 (out of a total of 10 expected, $n = 153$) two had this phenotype, and one retained a small cyclopic eye. **h** and **i**, Coronal sections of the wild-type and double-mutant embryos shown in **e** and **g**; in the mutant the rhombencephalon (rh) is relatively normal, whereas the cerebral hemispheres telencephalon (te) and diencephalon (di) are reduced to a single thin neural vesicle. The fifth (trigeminal) ganglion (v) is indicated. **j** and **k**, Transverse sections of wild-type and double-mutant embryos. We note the absence of notochord (no, see inset) and sclerotome (scl) at this cervical level; the oesophagus (oe) and trachea (tr) form a common duct[20,21] in the mutant. The sympathetic ganglion (sg) is indicated.

observed in posterior spinal cord and somites[9]. These results contrast with overexpression experiments in *Xenopus*, in which the activities of *chordin* and *noggin* have been shown to dorsalize the embryo and induce anterior neural tissue in animal cap explants[7,8]. As both *chordin* and *noggin* have similar biochemical activities and overlapping expression domains during gastrulation, we reasoned that they might have redundant activities during mouse gastrulation, which could be revealed by generating compound mutants.

Intercrosses were set up between compound heterozygous mice and no double-homozygous mutants were recovered among the neonates ($n = 320$, 20 expected). We therefore dissected pregnant mothers at progressively earlier stages of development. Two $Chd^{-/-};Nog^{-/-}$ embryos were found among animals dissected close to term. Both were undergoing resorption, but clearly had holoprosencephaly, with a single nasal pit (proboscis), a cyclopic eye and agnathia (Fig. 1d). These malformations, not observed in either mutant on its own, represent the weakest phenotypes found in $Chd^{-/-};Nog^{-/-}$ mice and resemble embryos lacking *sonic hedgehog* (*Shh*)[10].

At embryonic day 12.5 (E12.5), double-mutant embryos were recovered with more severe phenotypes resembling aprosencephaly. Anterior to the hindbrain (Fig. 1g, white arrowhead), embryos lacked extensive areas of the forebrain, as well as eyes, nasal placodes and facial structures. Histological sections showed that the cerebral hemispheres (telencephalon) and diencephalon were reduced to a small vesicle of thin neuroepithelium, whereas more posterior brain structures were relatively normal (compare Fig. 1h and i).

In double-mutant embryos dissected at E8.5, the forebrain reduction was clearly evident (Fig. 2a–f). Transcripts of *Six-3*, an anteriormost brain marker[11], were almost entirely absent, whereas expression of *Krox-20* in rhombomeres 3 and 5 was present in the hindbrain (Fig. 2a and b). *Engrailed-1* (*En-1*), a marker for midbrain and anterior hindbrain, was expressed in the double mutants, indicating that the neural defects resided in the prosencephalon (Fig. 2c and d). These deficits were also seen in the anterior neural folds at early somite stages (Fig. 2g–l).

To investigate the onset of the forebrain phenotype and the role of the anterior endoderm[1,2], we analysed the expression of a number of markers at earlier stages. In double-mutant embryos dissected at E7.5, the expression of the anterior marker *Hesx1/Rpx* (ref. 12) was undetectable both in the endoderm and in the overlying ectoderm that would become the anterior neural plate (Fig. 3a–c). Interestingly, *Hesx1* was expressed normally in the anterior endoderm at early gastrula stage (Fig. 3h and i). Mutational analysis has shown that *Hesx1* is required for normal forebrain development in mice and humans[13]. The lack of *Hesx1* expression is accompanied by an indentation at the anterior border separating the embryonic and extraembryonic regions (Fig. 3b and c, arrowheads). More severe constrictions of this type are seen in mutants that lack head structures, such as *Lim1*, *Hnf3β* and *Otx2* (refs 14–16) and are caused by defects in the endoderm[16,17]. The expression domains of *noggin* (Fig. 1b) and *chordin* (Fig. 1a, middle embryo) do not overlap with that of *Hesx1* (Fig. 3a), suggesting that the mutations have effects on the anterior endoderm at a distance. To test whether AVE formation is initiated normally but fails to be maintained, we examined expression of the AVE markers *Hesx1*, *Lim1*, *Cer-like* and *Hnf3β* (refs 2, 12 and 18) at E6.5. At these early streak stages, all AVE markers tested were expressed normally in double *noggin* and *chordin* homozygous mutants (Fig. 3d, e, h–k and data not shown). This is in agreement with the recent finding that $Wnt3^{-/-}$ embryos, in which the entire primitive streak is lacking, still form an AVE (ref. 19). As is the case for *Hesx1*, the expression of *Cer-like* fails to be maintained in the anterior visceral endoderm, as well as in definitive endoderm emerging from the node[18], by the late gastrula stage (Fig. 3f and g). We conclude from these data that *chordin* and *noggin* are not necessary for establishing the AVE but are required for subsequent elaboration of anterior pattern.

Mesodermal development is also affected in double-mutant embryos. This is indicated by the lack of *Shh* (Fig. 2b and d) and *Hnf3β* (Fig. 2j and l) expression in the rostral midline. At E12.5 the notochord and surrounding sclerotome are absent in cervical regions (insets, Fig. 1j and k). The anterior notochord may initially form and then degenerate, because we observed some E8.5 embryos in which the notochord was present throughout the length of the animal (Fig. 2e′, f′). In the endoderm, the anterior pharynx is reduced (not shown), and the trachea does not form, with a common tracheo-oesophageal duct connecting the pharynx to the lungs and stomach (Fig. 1j and k), as has been reported for mutations of the *Shh* pathway[20,21]. In addition, the absence of Chordin and Noggin also affects left–right asymmetry. Of seven double-mutant embryos (E8.5) in which heart looping could be
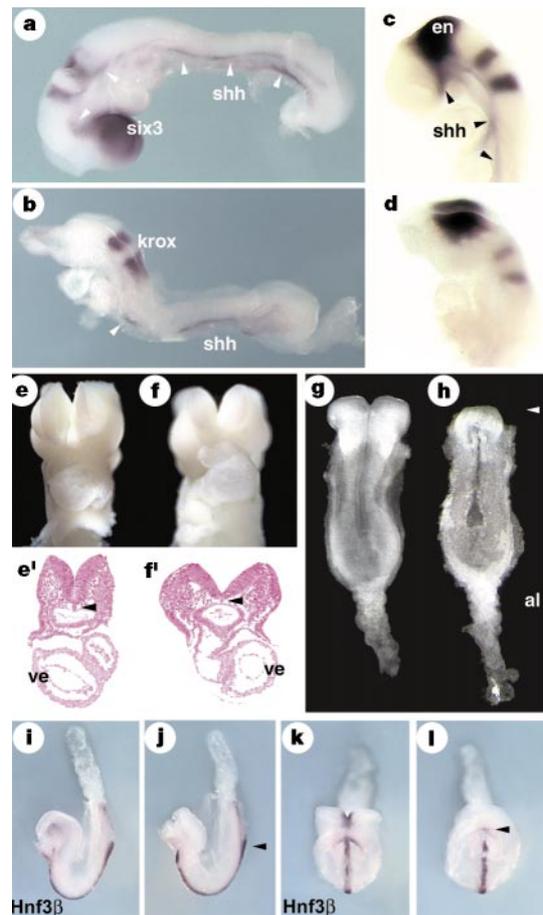

Figure 2 Loss of forebrain in *chordin;noggin* double mutants. **a** and **b**, wild-type and $Chd^{-/-};Nog^{-/-}$ E8.5 embryos hybridized with *Six-3*, *Krox-20* and *Shh*. We note that forebrain tissue expressing *Six-3* is almost completely missing and that *Krox-20* is expressed in the double mutant. The white arrowhead in **b** marks the level of anteriormost expression of *Shh*. **c** and **d**, wild-type and double-mutant embryos showing that *En-1* and *Krox-20* are expressed in the mutant; anterior *Shh* expression (arrowheads) is observed in the wild type but not in the mutant. **e** and **f**, Slightly earlier embryos showing reduction in anterior neural folds and inverted looping of the heart in the double mutant. **e′** and **f′**, Transverse sections of the embryos in **e** and **f**. The prospective heart ventricle (ve) loops to the right in wild-type and to the left in the double-mutant; the arrowhead indicates the notochord. At E8.5 stage 14 double mutants were recovered out of 18 expected ($n = 267$ embryos genotyped). **g** and **h**, Wild-type and double mutant embryos at early somite stage; the anterior neural folds are defective (arrowhead) and the allantois (al) composed of ventral mesoderm is prominent in this double mutant. **i, j, k, l**, Wild-type (**i** and **k**) and double-mutant (**j** and **l**) E8.0 embryos hybridized with *Hnf3β*. The same embryos are shown in lateral and frontal view; *Hnf3β* expression is normal in the node (arrowhead in **j**) and most of the midline, except for the anterior head and foregut pocket region (arrowhead in **l**).
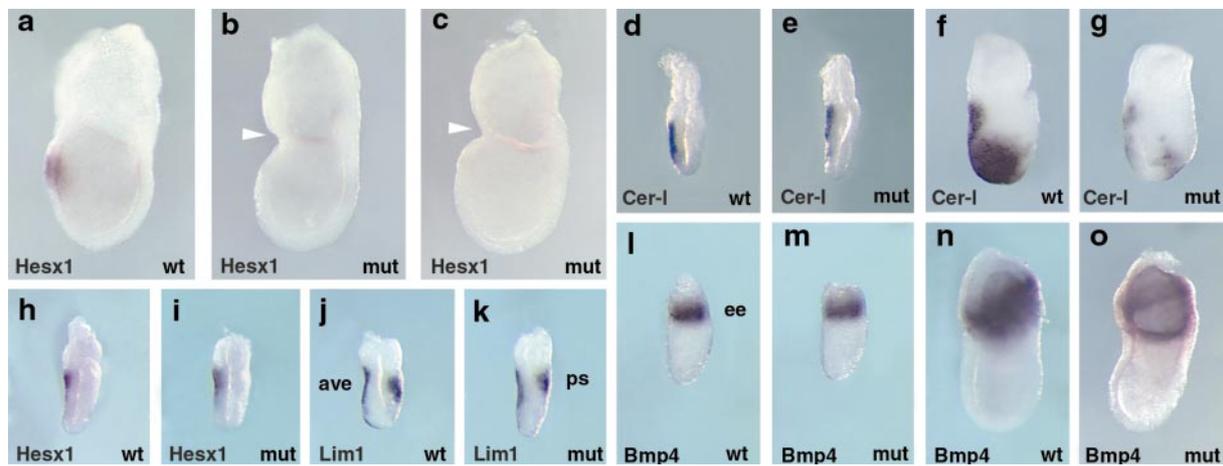
Figure 3 Molecular marker analyses of double *chordin;noggin* mutants during early development. **a**, *In situ* hybridization with *Hesx1* probe at the early neural-plate stage (E7.5) in a wild-type (wt) embryo; the probe stains both the endoderm underlying the future forebrain and the overlying epiblast in which the forebrain is forming. **b** and **c**, Two *Chd⁻/⁻;Nog⁻/⁻* embryos (mut) showing absence of *Hesx1* expression. Note the presence of a mild constriction (arrowhead) at the anterior embryonic–extraembryonic border. **d, e** and **h, k**, Wild-type and double-mutant embryos hybridized at primitive streak stages with *Hesx1*, *Lim1* and *Cer-like*; anterior visceral endoderm (ave) and primitive streak (ps) are formed normally in the mutants. **f, g,** At the late streak stage *Cer-like* expression fails to be maintained in the anterior endoderm of double-mutant embryos. **l, m,** Expression of *Bmp-4* in extraembryonic ectoderm (ee) in early/midstreak embryos. **n, o,** *Bmp-4* expression at node stage. In both wild-type and mutant embryos expression remains confined to the extraembryonic region. The wild-type embryos shown here may be heterozygous for one of the genes; the mutants were in all cases genotyped as *Chd⁻/⁻;Nog⁻/⁻*. At least two *Chd⁻/⁻;Nog⁻/⁻* embryos were documented for each probe and developmental stage, with the exception of *Hesx1* at early gastrula for which only one double-mutant embryo was recovered. Embryos at these early stages were genotyped by polymerase chain reaction after *in situ* hybridization and photography (*n* = 310); double-homozygous mutant embryos were recovered at mendelian ratios (*n* = 21).

clearly discerned, four had inversions, indicating a randomization of the heart situs (Fig. 2e, f, e′ and f′). We conclude that the anti-BMP factors Chordin and Noggin are required for development and maintenance of dorsal mesendoderm (notochord, sclerotome, foregut), as well as for left–right patterning. Mutations in either *Hnf3β* or *Shh*, genes downregulated in *Chd⁻/⁻;Nog⁻/⁻* embryos, cause similar mesodermal phenotypes[17,22].

In zebrafish, mutations in *chordin* affect patterning of both neurectoderm and mesoderm, causing reduction of neural and of dorsal mesoderm markers[23,24]. In *chordino* mutants the *Bmp-4* expression domain is expanded into the dorsal region of the gastrula[23]. In contrast, this transcriptional regulation is not seen in mice; in *Chd⁻/⁻;Nog⁻/⁻* embryos *Bmp-4* transcripts remain confined to extraembryonic tissues during gastrulation stages (Fig. 3l–o). This observation illustrates differences in the regulatory mechanisms by which mouse and zebrafish gastrulae are patterned. Despite the unchanged *Bmp-4* expression, removal of the antagonists is expected to cause an increase in BMP signalling, because Chordin and Noggin bind BMPs in the extracellular space, which prevents their binding to BMP receptors[7,8]. In addition to *Bmp-4*, other BMPs are expressed at neural-plate and head-fold stages. *Bmp-2, 5* and *7* are expressed in the anterior mesoderm and neurectoderm adjoining the prospective headfolds[25–27]. *Bmp-7* is also expressed during gastrulation in the node and axial mesoderm, overlapping with the expression domains of *chordin* and *noggin*. Thus, the phenotypes observed in the double mutants may result from increased activity of multiple BMPs. Ectopic application of BMP proteins has been shown to cause holoprosencephaly[28], inhibition of cell proliferation and increased cell death in E10.5 forebrain explants[29], and decreased expression of *Shh* in the CNS (ref. 26). Although loss of Chordin and Noggin leads to defects in forebrain development, the formation of the midbrain and more posterior CNS is relatively normal; therefore, other regulatory mechanisms must come into play.

As neither *chordin* nor *noggin* are expressed in the visceral endoderm (Fig. 1a–c, ref. 14), this study specifically tests the influence of patterning factors derived from the anterior primitive streak and node on the development of prospective forebrain. The AVE is initially formed in *Chd⁻/⁻;Nog⁻/⁻* embryos, as shown by the normal expression of *Hesx1*, *Lim1* and *Cer-like* in this region (Fig. 3d, e, h–k). In *Chd⁻/⁻;Nog⁻/⁻* embryos, anterior defects are apparent as early as the start of neurulation (E7.5), when the anterior endodermal expression domain of *Hesx1* underlying the future forebrain is no longer detectable; these antagonists are therefore first required at, or before, this stage (Fig. 3a–c). Two interpretations of the observed phenotypes are possible. First, the node and mesendodermal cells emerging from it may have an essential head-inducing activity, and this activity is deficient in *Chd⁻/⁻;Nog⁻/⁻* embryos. Second, anti-BMP signals secreted by the node and its derivatives may be required for the maintenance of anterior patterning initiated by the AVE. In *Xenopus*, expression of *cerberus* and *XHex* in the topological equivalent of the mouse anterior endoderm is maintained by signals from the Spemann organizer, and these can be mimicked by *chordin* or *noggin* mRNA microinjections[3–5]. Conversely, the expression of *cerberus* in *Xenopus* during gastrulation is decreased by microinjection of *Xolloid* mRNA encoding a protease that inactivates Chordin[30] (E. Agius, M. Oelgeschläger and E.M.D.R., unpublished observations). The results presented here demonstrate that the BMP antagonists Chordin and Noggin compensate for each other during early mouse development. When both gene products are removed, antero-posterior (forebrain defects), dorso-ventral (holoprosencephaly in milder phenotypes, notochord and sclerotome defects) and left–right (randomization of heart situs) patterning are affected. Thus, the BMP antagonists Chordin and Noggin secreted by the trunk organizer are required for the proper specification of the three body axes in the mouse embryo. □

Methods

Mutant Mice

The *chordin* mutant allele was generated by inserting stop codons in all three possible reading frames of the signal peptide and by introducing a frameshift immediately after this (D.B. *et al.*, manuscript in preparation). Double homozygotes were obtained by crossing

compound heterozygous *noggin*[9] and *chordin* parents in a B6SJLF1 background (Jackson Laboratories).

Genotyping

DNA was extracted from extraembryonic membranes of E8.5 and older embryos. For E6.0 to E7.5 embryos the whole animal was digested overnight upon completion of *in situ* analysis and photography of each embryo. Digestion was at 55 °C in 50 mM Tris-HCl, pH 8, 100 mM EDTA, 100 mM NaCl, 1% SDS, 0.5 mg ml⁻¹ Proteinase K. After adding NaCl to 1 M final concentration, the mix was centrifuged for 15 min, the supernatant recovered, and DNA precipitated with an equal volume of isopropanol. The genotype of the specimens was determined by polymerase chain reaction using the following primers: *chordin* primers were a mixture of three oligonucleotides, Chd-F (5′-GAGTTAGGAGG-TGGAGCTCTACAC-3′), Chd-R (5′-GGTAGGAGACAGAGAAGCGTAAACT-3′) and Neo-2 (5′-GTTCCACATACACTTCATTCTCAG-3′), which yielded bands of 417 and 285 base pairs (bp) for the wild-type and mutant alleles respectively. *Noggin* primers were a mixture of three oligonucleotides, Nog-1 (5′-GCATGGAGCGCTGCCCCAGC-3′), Nog-2 (5′-GAGCAGCGAGCGCAGCAGCG-3′) and β-Gal (5′-AAGGGCGATCGGTGC-GGGCC-3′), which yielded bands of 211 and 160 bp for the wild-type and mutant alleles respectively.

In situ hybridization

Whole-mount *in situ* hybridization was performed as described[18]. The *chordin* probe used was a subclone spanning nucleotides 145–2016 of the full-length mouse *chordin* complementary DNA (accession number AF096276), linearized with *Not*I and transcribed with T3 RNA polymerase. The *Krox20* probe was linearized with *Bam*H1 and transcribed with T3 RNA polymerase. *En-1*, *Hnf3β* and *Bmp-4* were linearized with *Cla*I, *Asp*700 and *Bam*H1 respectively, and transcribed with T7 RNA polymerase. Other antisense probes used were: *noggin* (ref. 9), *Shh* (ref. 9), *Six3* (ref. 11), *Hesx1* (ref. 12), *Lim1* (ref. 14) and *Cer-l* (ref. 18).

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Maintenance of functional equivalence during paralogous Hox gene evolution

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Biological diversity is driven mainly by gene duplication followed by mutation and selection. This divergence in either regulatory or protein-coding sequences can result in quite different biological functions for even closely related genes. This concept is exemplified by the mammalian Hox gene complex, a group of 39 genes which are located on 4 linkage groups, dispersed on 4 chromosomes[1–4]. The evolution of this complex began with amplification in *cis* of a primordial Hox gene to produce 13 members, followed by duplications in *trans* of much of the entire unit. As a consequence, Hox genes that occupy the same relative position along the 5′ to 3′ chromosomal coordinate (*trans*-paralogous genes) share more similarity in sequence and expression pattern than do adjacent Hox genes on the same chromosome. Studies in mice indicate that although individual family members may have unique biological roles, they also share overlapping functions with their paralogues[5–12]. Here we show that the proteins encoded by the paralogous genes, *Hoxa3* and *Hoxd3*, can carry out identical biological functions, and that the different roles attributed to these genes are the result of quantitative modulations in gene expression.

The paralogous genes *Hoxa3*, *Hoxb3* and *Hoxd3* show virtually identical expression patterns of messenger RNA and share, in a pairwise comparison, ~50% identity in protein-coding sequences[13–17]. Nevertheless, mice lacking either a functional *Hoxa3* or *Hoxd3* gene show no obvious overlap in phenotype: the former have deficiencies in pharyngeal tissues derived from mesenchymal neural crest[18], the latter in somitic, mesodermally derived tissues of the axial skeleton[19]. Although the uniqueness of the single-mutant phenotypes implies a different qualitative role for each gene product, the analysis of animals carrying different combinations of mutant alleles of the *Hox3* paralogues suggests that there is also functional overlap between the three genes, and that the important parameters that mediate these interactions may be quantitative in nature[5,17,20–22]. For example, in animals that are