Posterior neural tube closure depends on
*Dlx5/Dlx6* expression at the neural plate border

Nicolas Narboux-Neme¹, Marc Ekker², Giovanni Levi¹ and Églantine Heude¹*

¹ Évolution des Régulations Endocriniennes, Centre National de la Recherche Scientifique, UMR-7221, Muséum National d'Histoire Naturelle, Paris, France
² Centre for Advanced Research in Environmental Genomics, Department of Biology, University of Ottawa, Ottawa, Ontario, Canada

* Correspondence should be addressed to:
  Églantine Heude
  UMR7221 CNRS/MNHN
  7, rue Cuvier
  75231 Paris, Cedex 05, FRANCE
  Tel. : +33 1 40 79 80 29
  Fax. : +33 1 40 79 36 18
  Email : eglantineheude@hotmail.fr
ABSTRACT

Neural tube defects (NTDs), one of the most common birth defects in human, present a multifactorial etiology with a poorly defined genetic component. The Dlx5 and Dlx6 bigenic cluster encodes two evolutionary conserved homeodomain transcription factors, which are necessary for proper vertebrate development. It has been shown that Dlx5/6 genes are essential for anterior neural tube closure, however their role in the formation of the posterior neural tube has never been described. Here, we show that Dlx5/6 expression is required during vertebrate posterior neural tube closure. Dlx5 presents a similar expression pattern in neural plate border cells during zebrafish and mouse posterior neurulation. Dlx5/6-inactivation in mouse results in a phenotype reminiscent of NTDs characterized by open thoracic and lumbar vertebral arches and failure of epaxial muscle formation at the dorsal midline. Similarly, dlx5a/6a zebrafish morphants show defects of posterior neural tube closure accompanied by aberrant delamination of neural crest cells with altered expression of cell adhesion molecules and defects of motoneuron formation. Our findings provide new molecular leads to decipher the mechanisms involved during vertebrate posterior neurulation for a better understanding of the etiology of human congenital NTDs and other midline field defects.
INTRODUCTION

Neural tube defects (NTDs) correspond to a wide spectrum of common congenital disorders resulting from total or partial failure of neural tube closure during early embryogenesis. NTDs affect from 0.3 to 200 per 10 000 births worldwide and vary in type and severity depending on the neural tube levels affected along the antero-posterior axis. Anterior and posterior neural tube closure defects lead respectively to brain (ie. exencephaly, anencephaly) or spinal cord malformations (ie. spina bifida); complete antero-posterior defect in neural tube closure is at the origin of a more severe form of NTD termed craniorachischisis (reviewed in 2,3). The origins of NTDs have been associated to genetic and/or environmental factors and more than 200 mutant mice have been reported to present different forms of neural tube malformations 4,5. However, given the complexity of the NTD spectrum, there has been limited progress in defining the molecular basis of these conditions.

In vertebrates, neural tube closure defects originate from a failure in morphogenetic events taking place during the neurulation process. In mammalian embryos, neurulation involves two distinct morphogenetic processes along the rostro-caudal axis, known as primary and secondary neurulations. Primary neurulation refers to neural tube formation originating from folding of an open neural plate that forms the central lumen in the anterior part of the embryo. In contrast, secondary neurulation is characterized by mesenchymal condensation and cavitation in the posterior axis caudal to the tail bud 6,7. In zebrafish, neurulation occurs homogeneously along the rostro-caudal axis by epithelial condensation forming the neural plate followed by cavitation as observed during secondary neurulation in mammals 8,9. Zebrafish neurulation has been linked either to primary or to secondary neurulation of higher vertebrates 6,7. However, the morphogenetic similarities observed between neurulation in teleosts and other vertebrates indicate that zebrafish neural tube formation rather correspond to primary neurulation and constitutes a viable model to study vertebrate neural tube development 6,10.

The general primary neurulation dynamic seems conserved among vertebrates and is characterized by convergent movement of the neural plate borders (NPB) toward the dorsal midline to generate the neural tube with a central lumen 6,9. NPB cells constitute a competence domain, established between neural and non-neural ectoderm, that delineates the presumptive domain at the origin of migratory neural crest cells (NCCs) and responsible for neural tube closure 11,12.
Dlx genes, the vertebrate homologues of distal-less (dll) in arthropods, code for an evolutionary conserved group of homeodomain transcription factors. The mouse and human Dlx gene system is constituted by three closely associated bigenic clusters located on the same chromosomes as Hox genes clusters. In teleost, the dlx clusters are arranged on chromosomes similarly to their tetrapod Dlx counterparts. The most probable scenario suggests that Dlx genes have arisen from an ancestral dll gene as a result of gene duplication events. Data indicate that Dlx genes from a same cluster, such as Dlx5 and Dlx6 paralogs, present redundant functions during vertebrate development.

It has been shown that Dlx5 is one of the earliest NPB marker defining the limit of the neural plate during neurulation of mouse, chick, frog and zebrafish. Inactivation of Dlx5 in mouse results in a frequent exencephalic phenotype suggesting defects of anterior neural tube closure, however the mice do not present obvious posterior neural tube malformations. As Dlx5 and Dlx6 have partially redundant functions, it has been necessary to simultaneously inactivate both genes to fully reveal their roles during development. Functional analyses of Dlx5/Dlx6 inactivation in mice, avians and fish have demonstrated evolutionary conserved roles in appendage morphogenesis, in neurogenesis, in the development of the face and of the reproductive system. Dlx5/6−/− mice also present midline-fusion abnormalities including hypospadias, failure of anterior neuropore closure and posterior axis malformations. However, the origin of the latter phenotype has never been described.

Here we show that simultaneous invalidation of Dlx5 and Dlx6 in zebrafish and mouse results in similar defects of posterior neural tube closure. Our data indicate a conserved role for Dlx5/6 in posterior neurulation in vertebrates and suggest that genetic pathways involving these genes might be implicated in syndromic forms of human midline defects.
RESULTS

Dlx5/6 invalidation induces posterior axis malformations in zebrafish and mouse

To analyse the effect of Dlx5/6 invalidation on axis formation, we generated dlx5a/6a zebrafish morphants and Dlx5/6−/− mouse embryos and compared the resulting axial phenotypes. The disruption of Dlx5/6 function led to similar early posterior malformations characterized by curly-shaped tails in both species (Fig. 1 A-D, white arrowheads). In Dlx5/6 mutant mice, 80% of embryos and foetuses presented a curly-shaped tail associated with varying degrees of exencephaly (Fig. 1, blue arrowhead), the latter phenotype known to result from defect of anterior neural tube closure \(^{17,19,25,34}\). Moreover, E18.5 mutant mice displayed medio-dorsal split in the thoracic/lumbar region (Fig. 2A, B, red arrowheads). Skeletal preparations and immunostainings on sections revealed lack of vertebral arches fusion dorsally at both thoracic and lumbar levels and failure of epaxial muscle formation at the dorsal midline (Fig. 2 C-F, red arrowheads).

Expression of Dlx5 during zebrafish and mouse posterior axis formation

To understand the origin of the axial phenotype observed in Dlx5/6-invalidated specimens, we then compared the spatio-temporal expression of Dlx5 during zebrafish and mouse posterior neurulation. We previously showed in zebrafish that dlx5a-expressing ectodermal cells are laterally connected to the neural ectoderm to form the presumptive median fin fold \(^{18}\). At 15.5 hpf, dlx5a-expressing NPB cells along the neural keel follow a medial convergence toward the dorsal midline during neural rod formation at 16 hpf (Fig. 3A-B, black arrowheads). At later stages, dlx5a expression is limited to median fin fold ectodermal cells at 24 hpf and 48 hpf, and gradually decreased until 72 hpf \(^{18}\).

Similarly, in mouse embryos, Dlx5 transcripts were detected in NPB cells surrounding the posterior neuropore and at the dorsal midline after neural tube closure at E8.25 and E9.5, with gradual decrease of expression in a rostro-caudal manner (Fig. 3C-F, black arrowheads). At E10.5 and E12.5, Dlx5 expression was maintained in the caudal dorsal neural tube after posterior neuropore closure (Fig. 3G-H, black arrowheads). In both models, we also observed Dlx5 expression in the ventral ectodermal ridge (VER) of the tail bud and at the cloacal level (Fig. 3B, D; Supp. Fig. 1A, grey and blue arrowheads respectively). The expression and functional analyses in zebrafish and mouse suggest a conserved role of Dlx5/6 genes in posterior neural tube closure.
**Disruption of dlx5a/6a results in defect of neural tube closure**

To further investigate the role of Dlx5/6 genes during posterior neurulation, we next performed molecular analyses in early dlx5a/6a zebrafish morphants during neural keel-rod transition at 16 hpf. Given the key role for cell adhesion molecules (CAM) in neural tube morphogenesis, neural tube closure and epithelial-to-mesenchymal transition\textsuperscript{10,35-38}, we analysed the expression of ncad (cdh2) and ncam3, members of the CAM family involved in cell-cell adhesion. We also analysed expression of msx1b, marker of NPB cells and premigratory NCCs\textsuperscript{39,40} and expression of foxd3, marker of premigratory and early migratory neural crest cells (NCCs)\textsuperscript{41}.

In 16 hpf control embryos, ncad is constitutively expressed in the neural keel and the presomitic/somitic mesoderm. In contrast, ncam3 expression is limited to the dorsal part of the neural keel (Fig. 4A-A’, C-C’). In dlx5a/6a morphants, we observed a loss of ncad and ncam3 expression in aberrant protruding cells at the dorsal midline of the neural keel (Fig. 4B’, D’, black arrowheads). Moreover, whole-mount expression pattern of msx1b and foxd3 in morphants revealed a defect of neural tube formation compared to controls, with bifid stripes of expression in the caudal-most part of the axis, characteristic of a delay in neural keel-rod transition (Fig. 4F-H, grey arrowheads). On sections, msx1b showed a decrease of expression at the midline where protruding cells were detected in morphants (Fig. 4E’, F’, black arrowhead). At 16 hpf, foxd3 expression in the dorsal neural tube labelled premigratory NCCs (Fig. 4G’). The analysis of dlx5a/6a morphants revealed that the aberrant protruding cells at the dorsal midline of the neural keel were positive for foxd3 expression (Fig. 4H’, black arrowhead). The results indicated that disrupted dlx5a/6a function affects msx1b and CAM (ncad/ncam3) expression at the roof plate of the neural keel in aberrant delaminating foxd3-positive NCCs.

We also studied the effect of dlx5a/6a invalidation at later stages when the neural tube is formed. In 24 hpf controls, dorsal foxd3 expression in migratory NCCs was observed in the caudal neural tube (Fig. 5A-A’). In dlx5a/6a morphants, foxd3-positive cells showed abnormal asymmetric profile (Fig. 5B’, black arrowhead) associated with a reduced neural tube and defect of lumen formation (Fig. 5A’, B’, dashed lines), the latter phenotype being well revealed by the constitutive ncad expression in the neural tissue (Fig. 5C’, D’). The foxd3 and ncad transcripts were also detected in the somitic mesoderm of controls, that later
develops into myotomes arranged into V-shaped chevrons (Fig. 5A, C, Supp. Fig. 2C). In dlx5a/6a morphants, foxd3 and ncad expression patterns revealed defect of somitic segmentation at 16 hpf and abnormal U-shaped chevrons at 24 hpf (Fig. 4A-B, G-H; Fig. 5A-D). The expression profile of msx1b along the neural tube was also altered in dlx5a/6a MO embryos at 24 hpf (Fig. 5E-F, black arrowhead), while expression in the median fin fold compartment seemed unaffected.

We next analysed in dlx5a/6a morphants the primary motoneuron (PMN) population, originating from NCCs and known to be affected in NTDs. In 24 hpf controls, the PMNs express the synaptic vesicle SV2 and axonal projections start to elongate from the neural tube to reach their myotomal targets, forming the neuromuscular junctions at 30 hpf and 36 hpf (Fig. 5G, I, K). In contrast, dlx5a/6a MO showed defect of motoneuronal outgrowth at 24 hpf and PMNs failed to connect the myotomes at 30 hpf (Fig. 5H, J). At 36 hpf, the axonal projection was completed but PMNs showed defect of neuromuscular junction with aberrant synaptic arborescence (Fig. 5L). Moreover, defect of PMN formation was associated with accumulation of SV2 protein in the dorsal neural tube at 30 hpf and 36 hpf (Fig. 5J, L). The defect of neuromuscular innervation was also observed in Dlx5/6 mutant mice as shown by immunostainings on sections for the neuronal marker Tuj1 in trunk epaxial muscles positive for Tnnt3 (Supp. Fig. 3A-B, white arrowheads).

We also studied the expression of shha and bmp4 that are organizers of tail development. In 24 hpf controls, shha is expressed in the notochord, the neural tube floor plate and in the tail stem cell pool, namely the chordoneural hinge (Supp. Fig. 2A). At 48 hpf, shha expression is limited to the floor plate (Supp. Fig. 2C). In morphants, shha expression was maintained but well reveals the undulating phenotype of axial structures (Supp. Fig. 2A-D). While bmp4 did not show obvious defect of expression at 16 hpf and 24 hpf, expression in the spinal cord was altered at 48 hpf (Supp. Fig. 2E-F).

Altogether, our data indicated that disrupted dlx5a/6a function in zebrafish led to loss of expression of cell adhesion molecules in protruding NCCs at the dorsal midline, resulting in defects of posterior neural tube closure and of primary motoneuron formation.
DISCUSSION

It has been described previously that *Dlx5* is one of the earliest NPB marker during gastrulation. Particular attention has been paid on its role during anterior neural tube formation in defining the border between non-neural and neural plate territories. However, the role of *Dlx5/6* during posterior neurulation has never been reported.

Our analysis in zebrafish and mice shows that *Dlx5* is expressed in NPB cells during posterior neurulation (Fig. 3). *Dlx5* expression is also detected in the VER, a source of midline ventral ectoderm known to act as a signalling centre during tail morphogenesis. VER cells undergo epithelial-to-mesenchymal transition during tail formation as observed dorsally in NCCs. The continuous ectodermal *Dlx5*-positive domain might reflect that the VER represents a ventral extension of dorsal ectodermal cells during tail morphogenesis. Expression analyses for *Dlx* homologs in various models including chick, xenopus, lamprey and amphioxus suggest that *Dlx* expression in NPB cells has been established early during chordate evolution. However, we can still notice differences in *Dlx5* expression along the rostro-caudal axis of zebrafish and mouse. In mouse, *Dlx5* is expressed in NPB cells during both anterior and posterior neurulation (Fig. 3). In amphioxus, *amphiDll* is expressed in NPB cells all along the rostro-caudal axis, suggesting a conserved role of Distal-less-related genes in anterior and posterior neurulation in chordates. In contrast, in zebrafish, the anterior limit of *dlx5a*-expressing NPB cells is located at the 8th somite level and appears closely related to the establishment of the presumptive median fin fold. According to our expression analysis, we did not find evidence of anterior neural tube closure defects in *dlx5a/6a* morphants. In teleosts, neurulation is characterized by uniform epithelial condensation and cavitation, which give rise to both anterior and posterior neural tube. It has been suggested that zebrafish might present primitive mechanisms of neurulation, however basal chordates show primary and secondary neurulation as observed in higher vertebrates. Our results indicate that, while the cellular morphogenetic basis of neural tube formation is uniform along the rostro-caudal axis of zebrafish, the anterior and posterior neurulation processes do not involve same molecular mechanisms, suggesting evolutionary divergence of *Dlx5/6* function during anterior neural tube formation in teleosts. Our data bring new insights into the genetic and evolutionary origins of neural tube formation in chordates. Special attention should be paid in future studies to elucidate the genetic requirement differences between anterior and posterior neurulation in teleosts.
Our analysis reveals that disrupted Dlx5/6 function in zebrafish and mouse leads to early curly-shaped tail phenotypes in both models (Fig. 1). In Dlx5/6<sup>-/-</sup> mice, the tail phenotype is associated with dorsal axis defects and brain malformations characteristic of NTDs (Figs. 1-2) <sup>17,19,25</sup>. Intriguingly, the axis defects observed in Dlx5/6<sup>+/−</sup> mice was similar to the phenotype of CT “curly tail” mutants, a historical model of NTDs that show neural tube closure defects <sup>5,56,57</sup>. Our results suggested that the axis phenotype observed in Dlx5/6-invalidated zebrafish and mice resulted from defects of posterior neural tube closure, an aspect that we confirm through our functional analysis in zebrafish.

We show that dlx5a/6a morphants present defects of neural tube formation associated with aberrant dorsal delamination and migration of NCCs (Figs. 4-5). The protruding NCCs observed at the dorsal midline of the neural keel show loss of cell adhesion molecule transcripts (Fig. 4). It has been shown that these latter are important actors during neural tube formation <sup>7</sup>. In particular, ncad is required for NPB convergence, neural tube closure, maintenance of neural tube integrity and epithelial-to-mesenchymal transition <sup>10,35-38</sup>. In zebrafish, ncad invalidation represses neural tube formation due to defect of convergence and intercalation of NCCs <sup>10,35,36</sup>. The data thus indicate that dlx5a/6a genes act in NPB cells for cell adhesion integrity of NCCs during neural tube closure.

In addition, msxb1 shows a decrease of expression in the protruding NCCs and the expression profile is affected at 24 hpf in dlx5a/6a morphants (Figs. 4-5). The morphants also display severe defects of NCC-derived primary motoneurons (Fig. 5). It has been shown that msx and ncad genes are required during zebrafish neurogenesis <sup>36,39</sup>. The neuromuscular deficiencies observed in dlx5a/6a morphants may result from altered expression of msx1b and cell adhesion molecules in premigratory NCCs. We also noticed that zebrafish morphants present failure of somite segmentation and myotomal morphology (Figs. 4-5) that might indirectly originates from NTDs or defect of signalling from the VER. It has been shown that the VER regulates tail paraxial mesoderm induction and elongation <sup>50,51</sup>. However, Dlx5 expression in the VER does not appear required for tail elongation as Dlx5/6<sup>+/−</sup> mice and dlx5a/6a morphants show equivalent number of axial segments compare to controls.

Taken together, our findings reveal the central role of Dlx5/6 genes in vertebrate posterior neural tube closure. It has been demonstrated previously that Dlx5 acts to specify NPB cells
during cranial neural tube formation in mouse and chick\textsuperscript{20,21,24}. However, $Dlx$ activity in xenopus is not necessary for induction of NCCs\textsuperscript{48}. Our findings confirm that $Dlx$ invalidation does not impact on NCC induction as $foxd3$ expression is maintained in the protruding NCCs observed in $dlx5a/6a$ morphants (Fig. 4). Moreover, $Dlx5$ and $Msx2$ genes regulate anterior neural tube closure through expression of EphrinA5-EphA7 involved in cell adhesion\textsuperscript{34}. This suggests that a common genetic network implicating $Dlx$, $Msx$ and cell adhesion molecules is involved in neural plate border and neural crest cells during mouse and zebrafish neurulation.

These new data give insights for a better understanding of the cellular and molecular processes that could be altered in some human congenital NTDs, such as craniorachischisis that originates from defects of both anterior and posterior neurulation. The anterior and posterior NTDs observed in $Dlx5/6$ mutant mice are also associated with hypospadias, characterized by midline urethral malformations, and limb ectrodactyly\textsuperscript{17,31,33}. Expression of $Dlx5/6$ in the cloacal membrane, linked to the ventral ectodermal ridge\textsuperscript{52}, and in the genital tubercle is necessary for urethral formation. Moreover, it has been shown that $Dlx5/6$ expression in the apical ectodermal ridge is required for proper appendage formation in mouse and zebrafish\textsuperscript{17,18,33}. The limb phenotype of $Dlx5/6^{-/-}$ mice resembles that of patients with congenital split hand-foot malformation type I (SHFM-I), linked to genomic deletion or rearrangement in the $DLX5/DLX6$ cluster locus. Altogether, the data unveil the role of $Dlx5/6$ in ectodermal cells for the proper development of the neural tube, the urogenital system and limbs. Interestingly, it has been reported that NTDs can be associated with limb malformations and other midline defects, including urogenital and diaphragmatic disorders, as observed in Czeizel-Losonci syndrome\textsuperscript{58-62}. Our data bring new light on common aetiology for a spectrum of idiopathic anomalies characterizing certain human congenital disorders.
METHODS

Ethical statement

All experiments with zebrafish were performed according to the guidelines of the Canadian Council on Animal Care and were approved by the University of Ottawa animal care committee (institutional licence #BL 235 to ME). All efforts were made to minimize suffering; manipulations on animals were performed with the anaesthetic drug tricaine mesylate (ethyl 3-aminobenzoate methanesulfonate; Sigma-Aldrich, Oakville, ON, Canada). Embryos were killed with an overdose of the latter drug.

Procedures involving mice were conducted in accordance with the directives of the European Community (council directive 86/609) and the French Agriculture Ministry (council directive 87–848, 19 October 1987, permissions 00782 to GL).

Animal maintenance

Zebrafish and their embryos were maintained at 28.5°C according to methods described in 63. Wild-type adult zebrafish were kept and bred in circulating fish water at 28.5°C with a controlled 14-h light cycle. Wild type, controls and morphant embryos were raised at similar densities in embryo medium in a 28.5°C incubator. Embryos were treated with 0.0015% 1-phenyl 2-thiourea (PTU) to inhibit melanogenesis. Embryos were killed with an overdose of tricaine mesylate for analysis.

Mice were housed in light, temperature (21°C) and humidity controlled conditions; food and water were available ad libitum. WT animals were from Charles River France. The mouse strain Dlx5/6+/− was maintained on a hybrid genetic background resulting from the cross between a C57BL/6N female and a DBA/2JRj male (B6D2N; Janvier Labs, France).

Morpholino-mediated knock down

The morpholino-mediated knock down was performed as previously described 18. To ensure specificity of the morpholinos, rescue of the resulting morphant phenotypes was performed as previously described 18.

Histological analyses

In situ hybridization on whole-mount zebrafish embryos were performed as previously described 18.

For whole-mount immunostaining on zebrafish embryos, dechorionated embryos were fixed in 4% paraformaldehyde (PFA) in 1X phosphate buffered saline (PBS) overnight at
4°C, washed in PBST (PBS 0.1% Tween), dehydrated in methanol and stored in methanol 100% at -20°C. The samples were then rehydrated in a graded methanol-PBST series and treated with PBDT (PBS 1% DMSO 0.1% Tween). Cells were immunodetected on wholemount embryos with mouse anti-SV2 monoclonal antibody diluted in PBDT an incubated overnight at 4°C (1:100, DHBS). After 5 rounds of 30 min washes in PBST, embryos were incubated overnight at 4°C with secondary anti-mouse HRP-conjugated antibody diluted in PBST (1:200, Jackson Immuno), washed with 5 times 30 min in PBST and revealed with DAB chromogenic substrate.

For immunostaining on cryosections, foetuses were fixed 3h in 4% PFA 0.5 % Triton X-100 at 4°C, washed overnight at 4°C in PBST, cryopreserved in 30% sucrose in PBS and embedded in OCT for 12-16µm sectioning with a Leica cryostat. Cryosections were dried for 30 min and washed in PBS. Rehydrated sections were blocked for 1h in 10% normal goat serum, 3% BSA, 0.5% Triton X-100 in PBS. Primary antibodies were diluted in blocking solution and incubated overnight at 4°C (Tnnt3 antibody, 1/200, T6277, Sigma; Tuj1, 1/1000, BLE801202, Ozyme). After 3 rounds of 15 min washes in PBST, secondary antibodies were incubated in blocking solution 2h at RT together with 1µg/ml Hoechst 33342 to visualize nuclei. Secondary antibodies consisted of Alexa 488 or 555 goat anti-rabbit or anti-mouse isotype specific (1/500, Jackson Immunoresearch). After 3 rounds of 15 min washes in PBST, slides were mounted in 70% glycerol for analysis.

Skeletal preparations on mouse foetuses were performed as previously described 64.

**DATA AVAILABILITY**

Availability of materials and data upon request
REFERENCES

1 Zaganjor, I. et al. Describing the Prevalence of Neural Tube Defects Worldwide: A Systematic Literature Review. *PloS one* 11, e0151586, doi:10.1371/journal.pone.0151586 (2016).
2 Copp, A. J. & Greene, N. D. Neural tube defects--disorders of neurulation and related embryonic processes. *Wiley interdisciplinary reviews. Developmental biology* 2, 213-227, doi:10.1002/wdev.71 (2013).
3 Copp, A. J., Stanier, P. & Greene, N. D. Neural tube defects: recent advances, unsolved questions, and controversies. *The Lancet. Neurology* 12, 799-810, doi:10.1016/S1474-4422(13)70110-8 (2013).
4 Greene, N. D., Stanier, P. & Copp, A. J. Genetics of human neural tube defects. *Human molecular genetics* 18, R113-129, doi:10.1093/hmg/ddp347 (2009).
5 Harris, M. J. & Juriloff, D. M. An update to the list of mouse mutants with neural tube closure defects and advances toward a complete genetic perspective of neural tube closure. *Birth defects research. Part A, Clinical and molecular teratology* 88, 653-669, doi:10.1002/bdra.20676 (2010).
6 Lowery, L. A. & Sive, H. Strategies of vertebrate neurulation and a re-evaluation of teleost neural tube formation. *Mechanisms of development* 121, 1189-1197, doi:10.1016/j.mod.2004.04.022 (2004).
7 Nikolopoulou, E., Galea, G. L., Rolo, A., Greene, N. D. & Copp, A. J. Neural tube closure: cellular, molecular and biomechanical mechanisms. *Development* 144, 552-566, doi:10.1242/dev.145904 (2017).
8 Harrington, M. J., Chalasani, K. & Brewster, R. Cellular mechanisms of posterior neural tube morphogenesis in the zebrafish. *Developmental dynamics : an official publication of the American Association of Anatomists* 239, 747-762, doi:10.1002/dvdy.22184 (2010).
9 Araya, C., Ward, L. C., Girdler, G. C. & Miranda, M. Coordinating cell and tissue behavior during zebrafish neural tube morphogenesis. *Developmental dynamics : an official publication of the American Association of Anatomists* 245, 197-208, doi:10.1002/dvdy.24304 (2016).
10 Hong, E. & Brewster, R. N-cadherin is required for the polarized cell behaviors that drive neurulation in the zebrafish. *Development* 133, 3895-3905, doi:10.1242/dev.02560 (2006).
11 Milet, C. & Monsoro-Burq, A. H. Neural crest induction at the neural plate border in vertebrates. *Developmental biology* **366**, 22-33, doi:10.1016/j.ydbio.2012.01.013 (2012).

12 Kimura-Yoshida, C., Mochida, K., Ellwanger, K., Niehrs, C. & Matsuo, I. Fate Specification of Neural Plate Border by Canonical Wnt Signaling and Grhl3 is Crucial for Neural Tube Closure. *EBioMedicine* **2**, 513-527, doi:10.1016/j.ebiom.2015.04.012 (2015).

13 Zerucha, T. & Ekker, M. Distal-less-related homeobox genes of vertebrates: evolution, function, and regulation. *Biochem Cell Biol* **78**, 593-601 (2000).

14 Stock, D. W. *et al.* The evolution of the vertebrate Dlx gene family. *Proc Natl Acad Sci U S A* **93**, 10858-10863 (1996).

15 Kraus, P. & Lufkin, T. Dlx homeobox gene control of mammalian limb and craniofacial development. *American journal of medical genetics. Part A* **140**, 1366-1374, doi:10.1002/ajmg.a.31252 (2006).

16 Acampora, D. *et al.* Craniofacial, vestibular and bone defects in mice lacking the Distal-less-related gene Dlx5. *Development* **126**, 3795-3809 (1999).

17 Robledo, R. F., Rajan, L., Li, X. & Lufkin, T. The Dlx5 and Dlx6 homeobox genes are essential for craniofacial, axial, and appendicular skeletal development. *Genes & development* **16**, 1089-1101, doi:10.1101/gad.988402 (2002).

18 Heude, E., Shaikho, S. & Ekker, M. The dlx5a/dlx6a genes play essential roles in the early development of zebrafish median fin and pectoral structures. *PloS one* **9**, e98505, doi:10.1371/journal.pone.0098505 (2014).

19 Depew, M. J. *et al.* Dlx5 regulates regional development of the branchial arches and sensory capsules. *Development* **126**, 3831-3846 (1999).

20 Yang, L. *et al.* An early phase of embryonic Dlx5 expression defines the rostral boundary of the neural plate. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **18**, 8322-8330 (1998).

21 Pera, E., Stein, S. & Kessel, M. Ectodermal patterning in the avian embryo: epidermis versus neural plate. *Development* **126**, 63-73 (1999).

22 Luo, T., Matsuo-Takasaki, M., Lim, J. H. & Sargent, T. D. Differential regulation of Dlx gene expression by a BMP morphogenetic gradient. *The International journal of developmental biology* **45**, 681-684 (2001).

23 Fernandez-Garre, P., Rodriguez-Gallardo, L., Gallego-Diaz, V., Alvarez, I. S. & Puelles, L. Fate map of the chicken neural plate at stage 4. *Development* **129**, 2807-2822 (2002).
24 McLarren, K. W., Litsiou, A. & Streit, A. DLX5 positions the neural crest and preplacode region at the border of the neural plate. Developmental biology 259, 34-47 (2003).
25 Beverdam, A. et al. Jaw transformation with gain of symmetry after Dlx5/Dlx6 inactivation: mirror of the past? Genesis 34, 221-227 (2002).
26 Panganiban, G. & Rubenstein, J. L. Developmental functions of the Distal-less/Dlx homeobox genes. Development 129, 4371-4386 (2002).
27 Heude, E. et al. Jaw muscularization requires Dlx expression by cranial neural crest cells. Proc Natl Acad Sci U S A 107, 11441-11446 (2010).
28 Vieux-Rochas, M. et al. BMP-mediated functional cooperation between Dlx5;Dlx6 and Msx1;Msx2 during mammalian limb development. PloS one 8, e51700, doi:10.1371/journal.pone.0051700 (2013).
29 Macdonald, R. B. et al. The ascl1a and dlx genes have a regulatory role in the development of GABAergic interneurons in the zebrafish diencephalon. Developmental biology 381, 276-285, doi:10.1016/j.ydbio.2013.05.025 (2013).
30 Nishida, H. et al. Positive regulation of steroidogenic acute regulatory protein gene expression through the interaction between Dlx and GATA-4 for testicular steroidogenesis. Endocrinology 149, 2090-2097, doi:10.1210/en.2007-1265 (2008).
31 Suzuki, K. et al. Abnormal urethra formation in mouse models of split-hand/split-foot malformation type 1 and type 4. European journal of human genetics : EJHG 16, 36-44, doi:10.1038/sj.ejhg.5201925 (2008).
32 Kitazawa, T. et al. Developmental genetic bases behind the independent origin of the tympanic membrane in mammals and diapsids. Nat Commun 6, 6853, doi:10.1038/ncomms7853 (2015).
33 Merlo, G. R. et al. Mouse model of split hand/foot malformation type I. Genesis 33, 97-101, doi:10.1002/gene.10098 (2002).
34 Lee, J., Corcoran, A., Han, M., Gardiner, D. M. & Muneoka, K. Dlx5 and Msx2 regulate mouse anterior neural tube closure through ephrinA5-EphA7. Development, growth & differentiation 55, 341-349, doi:10.1111/dgd.12044 (2013).
35 Bronner-Fraser, M., Wolf, J. J. & Murray, B. A. Effects of antibodies against N-cadherin and N-CAM on the cranial neural crest and neural tube. Developmental biology 153, 291-301 (1992).
36 Lele, Z. et al. parachute/n-cadherin is required for morphogenesis and maintained integrity of the zebrafish neural tube. Development 129, 3281-3294 (2002).
37 Harrington, M. J., Hong, E., Fasanmi, O. & Brewster, R. Cadherin-mediated adhesion regulates posterior body formation. *BMC Dev Biol* **7**, 130, doi:10.1186/1471-213X-7-130 (2007).

38 Shoval, I., Ludwig, A. & Kalcheim, C. Antagonistic roles of full-length N-cadherin and its soluble BMP cleavage product in neural crest delamination. *Development* **134**, 491-501, doi:10.1242/dev.02742 (2007).

39 Phillips, B. T. *et al.* Zebrafish msxB, msxC and msxE function together to refine the neural-nonneural border and regulate cranial placodes and neural crest development. *Developmental biology* **294**, 376-390, doi:10.1016/j.ydbio.2006.03.001 (2006).

40 Cox, S. G. *et al.* An essential role of variant histone H3.3 for ectomesenchyme potential of the cranial neural crest. *PLoS genetics* **8**, e1002938, doi:10.1371/journal.pgen.1002938 (2012).

41 Stewart, R. A. *et al.* Zebrafish foxd3 is selectively required for neural crest specification, migration and survival. *Developmental biology* **292**, 174-188, doi:10.1016/j.ydbio.2005.12.035 (2006).

42 Wang, M. *et al.* Developmental delay in islet-1-positive motor neurons in chick spina bifida. *The Journal of veterinary medical science* **73**, 447-452 (2011).

43 Geerdink, N. *et al.* Contribution of the corticospinal tract to motor impairment in spina bifida. *Pediatric neurology* **47**, 270-278, doi:10.1016/j.pediatrneurol.2012.06.010 (2012).

44 Patten, I. & Placzek, M. The role of Sonic hedgehog in neural tube patterning. *Cellular and molecular life sciences : CMLS* **57**, 1695-1708, doi:10.1007/PL00000652 (2000).

45 Esterberg, R., Delalande, J. M. & Fritz, A. Tailbud-derived Bmp4 drives proliferation and inhibits maturation of zebrafish chordamesoderm. *Development* **135**, 3891-3901, doi:10.1242/dev.029264 (2008).

46 Reichert, S., Randall, R. A. & Hill, C. S. A BMP regulatory network controls ectodermal cell fate decisions at the neural plate border. *Development* **140**, 4435-4444, doi:10.1242/dev.098707 (2013).

47 Agathon, A., Thisse, C. & Thisse, B. The molecular nature of the zebrafish tail organizer. *Nature* **424**, 448-452, doi:10.1038/nature01822 (2003).

48 Woda, J. M., Pastagia, J., Mercola, M. & Artinger, K. B. Dlx proteins position the neural plate border and determine adjacent cell fates. *Development* **130**, 331-342 (2003).

49 Gammill, L. S. & Bronner-Fraser, M. Neural crest specification: migrating into genomics. *Nature reviews. Neuroscience* **4**, 795-805, doi:10.1038/nrn1219 (2003).
50 Goldman, D. C., Martin, G. R. & Tam, P. P. Fate and function of the ventral ectodermal ridge during mouse tail development. *Development* **127**, 2113-2123 (2000).
51 Liu, C., Knezevic, V. & Mackem, S. Ventral tail bud mesenchyme is a signaling center for tail paraxial mesoderm induction. *Developmental dynamics : an official publication of the American Association of Anatomists* **229**, 600-606, doi:10.1002/dvdy.20017 (2004).
52 Ohta, S., Suzuki, K., Tachibana, K., Tanaka, H. & Yamada, G. Cessation of gastrulation is mediated by suppression of epithelial-mesenchymal transition at the ventral ectodermal ridge. *Development* **134**, 4315-4324, doi:10.1242/dev.008151 (2007).
53 Sauka-Spengler, T., Meulemans, D., Jones, M. & Bronner-Fraser, M. Ancient evolutionary origin of the neural crest gene regulatory network. *Developmental cell* **13**, 405-420, doi:10.1016/j.devcel.2007.08.005 (2007).
54 Holland, N. D., Panganiban, G., Henyey, E. L. & Holland, L. Z. Sequence and developmental expression of AmphiDll, an amphioxus Distal-less gene transcribed in the ectoderm, epidermis and nervous system: insights into evolution of cranial forebrain and neural crest. *Development* **122**, 2911-2920 (1996).
55 Schmitz, B., Papan, C. & Campos-Ortega, J. A. Neurulation in the anterior trunk region of the zebrafish Brachydanio rerio. *Roux's archives of developmental biology : the official organ of the EDBO* **202**, 250-259, doi:10.1007/BF00363214 (1993).
56 Copp, A. J., Seller, M. J. & Polani, P. E. Neural tube development in mutant (curly tail) and normal mouse embryos: the timing of posterior neuropore closure in vivo and in vitro. *Journal of embryology and experimental morphology* **69**, 151-167 (1982).
57 van Straaten, H. W. & Copp, A. J. Curly tail: a 50-year history of the mouse spina bifida model. *Anatomy and embryology* **203**, 225-237 (2001).
58 Martinez-Frias, M. L. Developmental field defects and associations: epidemiological evidence of their relationship. *American journal of medical genetics* **49**, 45-51, doi:10.1002/ajmg.1320490110 (1994).
59 Khoury, M. J., Cordero, J. F., Mulinare, J. & Opitz, J. M. Selected midline defect associations: a population study. *Pediatrics* **84**, 266-272 (1989).
60 Oyen, N., Boyd, H. A., Poulsen, G., Wohlfahrt, J. & Melbye, M. Familial recurrence of midline birth defects—a nationwide danish cohort study. *American journal of epidemiology* **170**, 46-52, doi:10.1093/aje/kwp087 (2009).
61 Deng, C. *et al.* Fibroblast growth factor receptor-1 (FGFR-1) is essential for normal neural tube and limb development. *Developmental biology* **185**, 42-54, doi:10.1006/dbio.1997.8553 (1997).
62 Czeizel, A. & Losonci, A. Split hand, obstructive urinary anomalies and spina bifida or diaphragmatic defect syndrome with autosomal dominant inheritance. *Human genetics* 77, 203-204 (1987).

63 Westerfield, M. The zebrafish book. A guide for the laboratory use of zebrafish (Danio rerio). (4th ed.) University of Oregon Press, Eugene (2000).

64 Wallin, J. *et al.* The role of Pax-1 in axial skeleton development. *Development* **120**, 1109-1121 (1994).
ACKNOWLEDGMENTS

We thank Joachim Wittbrodt for the donation of the zebrafish ncad and ncam3 plasmids. A particular thank goes to the team in charge of animal care, Vishal Saxena, Stéphane Sosinsky and Fabien Uridat, and to Pr. Amaury de Luze in charge of animal well-being. We thank Mss. Aicha Bennana and Lanto Courcelaud for administrative assistance. We also thank Dr. Benoit Robert for helpful discussions.

This research was partially supported by the EU Consortium HUMAN (EU-FP7-HEALTH-602757), the ANR grants TARGETBONE (ANR-17-CE14-0024) and METACOGNITION (ANR-17-CE37-0007) to NNN/GL and by CIHR grant MOP137082 to ME. EH was a recipient of a postdoctoral fellowship from the government of Canada.

AUTHOR CONTRIBUTIONS

GL and EH conceived and designed the study. NNN and EH carried out the experiments. ME and GL provided materials. EH wrote the original manuscript. All authors discussed the results and contributed to manuscript revision.

COMPETING INTERESTS

The authors declare no competing interests.
FIGURE LEGENDS

Figure 1. Early phenotypes of Dlx5/6-invalidated zebrafish and mouse.
(A-B) Phenotype of a control zebrafish and a dlx5a/6a morphant at 48 hpf. (C-D) Phenotype of a control and a Dlx5/6-/- mutant mouse at E11.5. Invalidation of Dlx5/6 in zebrafish and mouse leads to early defect of axis development characterized by curly-shaped tail phenotype in both models (B, D, white arrowheads). In Dlx5/6-/- embryos, the caudal phenotype is associated with defect of brain formation (D, blue arrowhead). Scale bar in B for A-B 100µm, for C-D 1000µm.

Figure 2. Dorsal midline defects in perinatal Dlx5/6-/- mice
(A-D) Macroscopic dorsal view (A-B) and skeletal preparation (C-D) of the posterior axis of control and Dlx5/6-/- mutant mice at E18.5. (E-F) Immunostaining on coronal cryosections for Tnnt3 in dorsal musculature of control and Dlx5/6-/- E18.5 foetuses. The Dlx5/6-/- mutants display apparent dorsal split associated with defects of thoracic/lumbar vertebrae and of epaxial muscle formation at the midline (B, D, F red arrowheads). Abbreviations: epm, epaxial muscles. Scale bars in B for A-B, in D for C-D 2000µm and in E for E-F 200µm.

Figure 3. Expression analysis for Dlx5 during zebrafish and mouse posterior neurulation.
(A-D) Whole-mount in situ hybridization for dlx5a and Dlx5; (A, C) dorsal and (B, D) lateral views of the posterior axis of 15.5 hpf and 16 hpf zebrafish (A-B) and E8.25 and E9.5 mouse embryos (C-D). (E-F) In situ hybridization for Dlx5 on coronal cryosections at the levels indicated by the dashed lines in (C, D and Supp. Fig. 1). In zebrafish embryos, dlx5a transcripts are detected in NPB cells along neural keel at 15.5 hpf and at the dorsal midline at 16 hpf (A-B, black arrowheads). During mouse posterior neurulation, Dlx5 is expressed in NPB cells surrounding the posterior neuropore and along the dorsal midline of the neural tube after neural tube closure (C-H, black arrowheads). In both species, Dlx5 is also detected in the ventral ectodermal ridge of the tail bud and at the cloacal level (grey and blue arrowheads respectively in B, D). Abbreviations: nc, notochord; nk, neural keel; np, neural plate; nt, neural tube; pnp, posterior neuropore; psm, presomitic mesoderm; tb, tail bud; ys, yolk sac. Scale bar in H for A, H 50µm, for B, E-G 75µm, for C 150µm, for D 200µm.
Figure 4. Defect of neural tube closure in *dlx5a/6a* zebrafish morphants.

(A-H) Dorsal views of whole-mount *in situ* hybridization for *ncad*, *ncam3*, *msx1b* and *foxd3* in controls and *dlx5a/6a* morphants at 16 hpf. (A’-H’) *In situ* hybridization for *ncad*, *ncam3*, *msx1b* and *foxd3* on coronal cryosections of controls and *dlx5a/6a* morphants at 16 hpf at the levels indicated by lines in (A, H). During neural keel-rod transition, the *dlx5a/6a* morphants show a decrease or loss of *ncad*, *ncam3* and *msx1b* in aberrant protruding *foxd3*-positive NCC at the dorsal midline of the neural keel (black arrowhead in B’-H’). Abbreviations: nc, notochord; ncc, neural crest cells; nk, neural keel; psm, presomitic mesoderm; sm; somitic mesoderm. Scale bar in H for A-H 100 µm, for A’-H’ 25 µm.

Figure 5. Defect of neural tube and motoneuron formation in *dlx5a/6a* zebrafish morphants.

(A-F) Lateral views of whole-mount *in situ* hybridization for *foxd3*, *ncad* and *msx1b* in controls and *dlx5a/6a* zebrafish morphants at 24 hpf. (A’-D’) *In situ* hybridization for *foxd3* and *ncad* on coronal cryosections at levels indicated by lines in (A-D). The *dlx5a/6a* morphants show a reduced neural tube associated to a defect of NCC migration, abnormal somitic segmentation and decrease of *msx1b* in the neural tube (A-F). (G-L) Lateral views of whole-mount immunostaining for SV2 in 24 hpf, 30 hpf and 36 hpf control and *dlx5a/6a* morphant embryos. In *dlx5a/6a* morphants at 24 hpf and 30 hpf, axonal projections of primary motoneurons fail to form (G-J). At 36 hpf, primary motoneurons show aberrant synaptic connection to their myotomal targets in *dlx5a/6a* morphants (K, L). Abbreviations: mff, median fin fold; my, myotomes; nc, notochord; ncc, neural crest cells; nt, neural tube; pmn, primary motoneurons; psm, presomitic mesoderm; sm; somitic mesoderm, ys, yolk sac. Scale bar in A for A-F 100 µm, for A’-D’ 25 µm and for G-L 50 µm.
Fig. 1
Fig. 3
Fig. 4
