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## **HoxB genes regulate neuronal delamination in the trunk neural tube by controlling the expression of Lzts1**

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1 ***HoxB* genes regulate neuronal delamination in the trunk neural tube by controlling the**  
2 **expression of *Lzts1***

3

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17

18 **Running title:** *HoxB* control neuronal delamination

19

20 **Key words:** Hox transcription factors, *Lzts1*, Spinal Cord Development, Neurogenesis,  
21 Delamination, Chicken embryo

22

23 **Summary statement:**

24 Atypical function of *HoxB* genes during spinal cord development: instead of giving positional  
25 information, HoxB regulate the delamination of the neuronally differentiating cells by  
26 controlling the expression of *Lzts1*.

27

28 **ABSTRACT**

29 Differential *Hox* gene expression is central for specification of axial neuronal diversity in the  
30 spinal cord. Here, we uncover an additional function of Hox proteins in the developing spinal  
31 cord, restricted to B cluster Hox genes. We found that members of the HoxB cluster are  
32 expressed in the trunk neural tube of chicken embryo earlier than Hox from the other clusters,  
33 with poor antero-posterior axial specificity and with overlapping expression in the  
34 intermediate zone (IZ). Gain-of-function experiments of HoxB4, HoxB8 and HoxB9,  
35 respectively representative of anterior, central, and posterior *HoxB* genes, resulted in ectopic  
36 progenitor cells in the mantle zone. The search for HoxB8 downstream targets in the early  
37 neural tube identified the Leucine Zipper Tumor Suppressor 1 gene (*Lzts1*), whose expression  
38 is also activated by HoxB4 and HoxB9. Gain and loss of function experiments showed that  
39 *Lzts1*, expressed endogenously in the IZ, controls neuronal delamination. These data  
40 collectively indicate that *HoxB* genes have a generic function in the developing spinal cord,  
41 controlling the expression of *Lzts1* and neuronal delamination.

42

43

## 44 INTRODUCTION

45

46 *Hox* genes encode highly conserved homeodomain (HD) transcription factors essential to  
47 promote morphological diversification of the bilaterian body (Rezsohazy et al., 2015). In  
48 higher vertebrates including humans, mice and chicken, 39 *Hox* genes specify the regional  
49 identity of body structures including the axial skeleton, nervous system, limbs, genitalia, and  
50 the intestinal and reproductive tracts (Crawford, 2003). *Hox* genes are organized in 4 clusters  
51 located on different chromosomes named *HoxA* to *HoxD*, and in 13 paralog groups. Members  
52 of each paralog group, further classified in anterior, central and posterior classes, are deployed  
53 in ordered spatial and temporal patterns along the antero-posterior (AP) axis (Duboule, 2007;  
54 Duboule and Dollé, 1989; Iimura and Pourquié, 2006; Pearson et al., 2005). Genes located 3'  
55 in a cluster are expressed earlier and more rostral than genes located more 5'. The correlation  
56 between the genomic organization and the spatio-temporal characteristics of Hox gene  
57 expression along the AP body axis is referred to as "collinearity". Importantly, genes of the  
58 same paralog group, including in distant species, display higher sequence conservation and  
59 regulatory properties than different paralogs within the same species. Together with the  
60 collinear expression, this results in the deployment of distinct regulatory activities in distinct  
61 spatial territories, allowing for morphological diversification.

62 The expression and function of Hox genes in the developing spinal cord of vertebrates  
63 (the trunk neural tube) illustrate how Hox collinear expression generates morphological  
64 diversification. From a functional point of view, experimental evidence showed that Hox  
65 genes define the identity and synaptic pattern of neurons, setting distinctive features necessary  
66 for the building of locally distinct motor circuits ultimately controlling diverse functions such  
67 as locomotion or respiration (Dasen et al., 2003, 2008; Lacombe et al., 2013; Sweeney et al.,  
68 2018). Hox gene functions in the trunk neural tube include the segregation of motor neurons  
69 columns: LMC (lateral motor column) in a ventrolateral position at limb levels (brachial and  
70 lumbar levels), PGCs (preganglionic motor column) and HMCs (hypaxial motor neurons) at  
71 the thoracic level. This neuron segregation according to their final functions is essential for  
72 subsequent functional organization of the spinal cord.

73 Previous studies provided an extensive view of Hox gene expression in the chicken  
74 and mouse trunk neural tube (Dasen et al., 2005; Jung et al., 2010; Lacombe et al., 2013). The  
75 expression of HoxA, HoxC and HoxD genes display a pronounced axial collinearity, with in  
76 most cases a preferential accumulation in motor neuron territories after the onset of neuronal

77 differentiation, which suits the described pattern of activity of Hox genes in promoting motor  
78 neuronal diversification.

79 Available data on HoxB genes suggest expression with distinct levels of axial  
80 collinearity. HoxB genes start to be expressed very early in the embryo (in the  
81 epiblast/tailbud), and present a temporal collinear onset of expression: HoxB1 and HoxB2 at  
82 HH4, HoxB3 to HoxB6 at HH5, HoxB7 at HH6, HoxB8 and HoxB9 at HH7, and HoxB13,  
83 only expressed in the tail bud, at HH20 (Denans et al., 2015). In the trunk neural tube of  
84 chicken embryo from HH4 to HH17, HoxB genes can be split into two groups: HoxB1 to  
85 HoxB5 are expressed up to the otic vesicle but not in the caudal part, while HoxB6 to HoxB9  
86 are expressed in the caudal part of the neural tube (Bel-Vialar et al., 2002). The exhaustive  
87 view of the expression of Hox genes and proteins much later (at E6) in the trunk neural tube  
88 shows in contrast that at later stages the expression of all HoxB genes is highly overlapping  
89 along the antero-posterior axis : HoxB3 to HoxB9 genes are all expressed at the brachial,  
90 thoracic and lumbar level (Dasen et al., 2005). In addition, at E6, Hox from the B cluster  
91 display an almost complete absence of expression in motor neurons, where Hox from the  
92 other clusters (HoxA, HoxC and HoxD) display a strong collinear expression to specify  
93 columnar and pool subtypes (Dasen et al., 2005). Although less comprehensive, data in mouse  
94 are also consistent with B cluster Hox genes displaying characteristics of expression distinct  
95 from non-B cluster Hox genes (Graham et al., 1991; Jung et al., 2010; Lacombe et al., 2013).  
96 These observations indicate a different spatial deployment of B cluster Hox genes.  
97 Interestingly, HoxB8 protein is present in chicken neural tube progenitors (Asli and Kessel,  
98 2010), thus, long before the expression of non-B cluster Hox proteins. Early transcription at  
99 the progenitor stage of non-B cluster Hox genes were described, but their proteins are either  
100 weakly expressed or undetectable, with proteins observed only in postmitotic neurons (Dasen  
101 et al., 2003). Therefore, HoxB genes may have earlier functions than non-B Hox genes in the  
102 trunk neural tube development.

103 The trunk neural tube is a pseudostratified epithelium that will sequentially give rise to  
104 a large variety of neurons and glial cells of the spinal cord. After an initial phase of  
105 proliferation resulting in the expansion of progenitors by symmetrical divisions (P-P),  
106 neurogenesis and then gliogenesis are achieved via a succession of steps that follow a  
107 stereotypic temporal order. Concomitantly, progenitors become committed to differentiate  
108 into a specific neuronal (and later on glial) subtype according to their dorso-ventral position  
109 (Le Dréau and Martí, 2012). Postmitotic neurons (N) are produced by asymmetric (P-N) or  
110 symmetric terminal (N-N) divisions of progenitor cells (Götz and Huttner, 2005). As neural

111 tube cells progress through the cell cycle, they undergo interkinetic nuclear migration with  
112 nuclei undergoing mitosis at the ventricular surface of the neural tube (at the apical part of the  
113 cells, close to the lumen of the neural tube) while their daughter cells reach the G1/S  
114 checkpoint as nuclei reach the basal limit of the progenitor zone, where they either re-enter or  
115 exit the cell cycle (Lee and Norden, 2013). Post-mitotic cells remain at the lateral face of the  
116 neural tube where they contribute to the mantle zone (MZ) and acquire further differentiated  
117 features. Therefore, as neurogenesis progresses, the MZ thickens. The intermediate layer  
118 between the progenitor area (or ventricular zone (VZ) and the MZ, called intermediate zone  
119 (IZ), contains the newly born neurons on their way to their final position (Corral and Storey,  
120 2001).

121 The progenitors/neurons ratio is controlled by the proliferation properties of  
122 progenitors (length and rounds of cell cycles), by the survival of progenitors and  
123 differentiating neurons, and by progenitor cell fate decisions (to remain a progenitor or to  
124 differentiate). Progenitor cell fate decisions are based on the activation of a cascade of  
125 transcription factors triggered by proneural genes (Bertrand et al., 2002; Lacomme et al.,  
126 2012; Ma et al., 1996), whose expression is largely controlled by the Notch signaling pathway  
127 (Formosa-Jordan et al., 2013; Hatakeyama, 2004; Hatakeyama et al., 2006). The mediolateral  
128 spatial organization of the differentiating neural tube into the three layers (VZ, IZ, MZ) is  
129 important for ensuring a proper differentiation rate since in the nascent IZ neurons, proneural  
130 genes induce the expression of Notch ligands such as Delta1 and Jagged which in turn  
131 activate Notch1 that down-regulates proneural gene expression and inhibits neurogenesis in  
132 neighboring precursors. Correct spatial organization of the neural tube along the medio-lateral  
133 axis requires timely detachment of newborn neurons from the apical surface in order to exit  
134 this proliferative zone and begin the morphological reorganization that underlies neuronal  
135 differentiation. This apical detachment process is known as delamination (Kasioulis and  
136 Storey, 2018). While it has been recently shown that the synchronization of the delamination  
137 is controlled by Notch pathway (Baek et al., 2018), molecular mechanisms controlling the  
138 timing of the delamination are not fully understood.

139 In this study, we aimed at investigating the function of B cluster Hox genes at early  
140 steps of trunk neural tube development using the chicken embryo as a model from E2 stage  
141 onwards when non-B Hox genes are not yet expressed, and prior to the well-documented role  
142 of Hox genes in motor neuron differentiation.

143

144

## 145 RESULTS

146

### 147 ***HoxB* genes are expressed early in the trunk neural tube during neurogenesis with little** 148 **antero-posterior axial specificity**

149 Numerous studies have described *Hox* gene expression in the trunk neural tube of chicken  
150 embryo. The lack of marked axial specificity of the *HoxB* genes within the brachial, lumbar  
151 and sacral territories of the trunk neural tube at E6, with an expression profile very different  
152 from the *Hox* genes of other clusters (Dasen et al., 2005) prompted us to re-investigate the  
153 expression and function of B cluster Hox genes during early spinal cord development.

154 We started by exploring *HoxB* expression patterns between E3 and E5 in the trunk  
155 neural tube using whole mount and transverse section *in situ* hybridizations for anterior  
156 (*HoxB2*, *HoxB4*), central (*HoxB5*, *HoxB7* and *HoxB8*) and posterior (*HoxB9*) classes of *Hox*  
157 genes. As early as E3, *HoxB* genes are expressed in largely overlapping territories, from the  
158 neck (in which, however, there is still a weak spatial collinearity) to the tail (Fig. 1A and B).  
159 In addition to highlighting the large overlap in the spatial expression domains of *HoxB* genes  
160 (except *HoxB13* which is only expressed in the tail bud after stage HH20 (Denans et al.,  
161 2015)), our results confirm that *HoxB* transcripts are present in the trunk neural tube before  
162 the onset of neurogenesis (Fig. 1A and B). To assess the presence of HoxB proteins in the  
163 trunk neural tube, including at early stages, we raised an antibody specific to the posterior  
164 HoxB9 protein (Supplementary Fig. 1 displays the specificity of the HoxB9 antibody).  
165 Immunostainings with this antibody on transverse sections show that HoxB9 protein is  
166 present in the trunk neural tube as early as E3 (Fig. 1C) and is broadly expressed from the  
167 neck to the tail (Fig. 1D) (coincident with *HoxB9* transcripts (Fig. 1A)). This contrasts with its  
168 paralog HoxC9 protein, not yet expressed at E3 in the neural tube (Fig. 1C), expressed only  
169 later and only at the thoracic level (Fig. 1D). *In situ* hybridization with the *HoxB8* probe (a  
170 similar pattern was described for the HoxB8 protein (Asli and Kessel, 2010)), and  
171 immunostaining with the HoxB9 antibody on the same transverse sections at E4.5 highlight  
172 the strong overlap in the expression of these “central” and “posterior” HoxB members, along  
173 the antero-posterior axis of the chicken embryo, from cervical to sacral level (Fig. 1E).

174 Altogether, our expression data define a temporal and spatial time window that differs  
175 from non-B cluster *Hox* genes. The lack of clear axial specificity does not favor a role for  
176 *HoxB* genes in an antero-posterior axial diversification of the neural tube, but HoxB early and

177 broad expression rather suggest a generic function during neurogenesis of the developing  
178 trunk neural tube.

179

180 ***HoxB* gene expression in the trunk neural tube resolves in the IZ and controls early**  
181 **neurogenesis**

182 The comparison of the expression pattern of *HoxB* genes with markers of the three layers of  
183 the trunk neural tube (*Sox2* for the VZ, *NeuroD4* for the IZ, and *Tuj1* for the MZ) at E4 (Fig.  
184 2A), shows that in addition to disappearing from the differentiating motor neuron domain  
185 where non-B cluster *Hox* genes are expressed, *HoxB* gene and protein expression at that stage  
186 is mainly restricted in the IZ, although weak expression is observed in the VZ (Fig. 1C-E,  
187 Fig. 2A and Supplementary Fig. 2). These expression dynamics suggest that *HoxB* genes,  
188 although not exclusively, may control neurogenesis progression by controlling the expression  
189 of genes expressed in the IZ. As *HoxB* genes are expressed in the IZ all along the dorso-  
190 ventral axis, and from the neck to the tail (Fig. 1C-E), this function would apply to all  
191 neuronal subtypes, i.e. motor neurons and all interneurons, and this, irrespective of the antero-  
192 posterior axial position (except in the neck) and of the paralog identity of the *HoxB* gene.

193 The largely overlapping *HoxB* expression patterns suggest *HoxB* gene functional  
194 redundancy which compromises loss of function approaches. We thus probed the function of  
195 *HoxB* genes in neural tube development by gain-of-function experiments. *HoxB4*, *HoxB8*  
196 and *HoxB9* were chosen as representative of anterior, central, and posterior *HoxB* genes  
197 respectively. Neural tubes of E2 embryos were unilaterally electroporated with a control  
198 plasmid encoding GFP or with each *HoxB* expression vector co-expressing GFP (to report  
199 transfected cells). Immunostainings were performed with antibodies against the progenitor  
200 marker *Sox2* and the pan-neuronal marker *Tuj1*. Results show the presence of ectopic *Sox2*  
201 positive cells in the MZ on the electroporated side for all three *HoxB* gene gain-of-function  
202 experiments at either two (Supplementary Fig. 3) or three days (Supplementary Fig. 4) after  
203 electroporation. We observed the ectopic *Sox2* phenotype at all dorso-ventral positions in the  
204 spinal cord (Supplementary Fig. 4). The phenotype obtained is however modest, with only a  
205 few ectopic *Sox2* positive cells in the MZ. The low penetrance of the phenotype could result  
206 from elimination of ectopic *Sox2* cells by apoptosis, a hypothesis consistent with increased  
207 apoptosis following *HoxB8* electroporation (Supplementary Fig. 5). The hypothesis was  
208 probed by analyzing embryos co-transfected with *HoxB* (*HoxB4*, *HoxB8* or *HoxB9*) and *P35*  
209 (an inhibitor of apoptosis (Sahdev et al., 2010)) expression vectors. Quantification of *Sox2*



210 ectopic cells in the MZ 72 hours after HoxB4, HoxB8 and HoxB9 gain-of-function in the  
211 context of P35 expression shows a strong phenotype (around 35% of transfected cells (GFP+)  
212 in the MZ are Sox2 positive after the overexpression of any of the three HoxB). Ectopic Sox2  
213 cells in the MZ are rarely seen under control condition (control vector + P35) (Fig. 2C). These  
214 results indicate that cell elimination through apoptosis contributes to the modest phenotype  
215 observed in HoxB gain of function experiments, and that the full range of HoxB induced  
216 phenotype can only be observed when suppressing apoptosis.

217 The phenotypes triggered by each of the three HoxB genes' overexpression are  
218 similar, with no marked differences in their potential to induce ectopic Sox2 positive cells  
219 (Fig. 2C) The phenotype of HoxB gain-of-function is not strictly cell-autonomous since Sox2  
220 positive GFP negative can be found in the MZ (Fig. 2D). In addition, Sox2/pH3 and  
221 Sox2/EdU double-staining following HoxB8 gain-of-function shows that Sox2 ectopic cells in  
222 the MZ are still mitotic (Fig. 2D and Supplementary Fig. 6), a characteristic of progenitor  
223 cells.

224 We conclude from this set of experiments that HoxB genes, irrespective of their  
225 paralog identity, induce when overexpressed a similar phenotype consisting of the appearance  
226 of ectopic progenitors (Sox2 positive) cells in the MZ. Taken together with expression pattern  
227 data, this suggests a generic role for HoxB genes in the control of early neural tube  
228 differentiation (neurogenesis and/or neuronal delamination). We next questioned if non-B  
229 Hox genes, while not expressed at these early stages, also have the capacity to induce ectopic  
230 Sox2 cells in the MZ. The hypothesis was probed by forcing the premature expression of  
231 HoxA7, HoxC8 and HoxD8 (one representative of each non-HoxB cluster Hox) from E2  
232 (these experiments were done in a P35 context). Results showed that such an expression leads  
233 to a phenotype similar (ectopic Sox2 cells in the MZ) to those exhibited by B cluster Hox  
234 genes (Supplementary Fig. 7), supporting that the induction of ectopic Sox2 cells in the MZ is  
235 a regulatory property also embedded in non-B Hox proteins.

236

### 237 **Transcriptomic data identifies *Lzts1* as a target of HoxB8**

238 To get molecular insights into HoxB gene function in the early neural tube, we aimed to  
239 identify downstream target genes of the HoxB transcription factors, focusing on the central  
240 class HoxB8 protein. E2 neural tubes were bilaterally electroporated with either a control  
241 vector encoding nuclear GFP (pCIG) or a HoxB8 expression vector co-expressing nuclear  
242 GFP (pCIG-HoxB8) (Fig. 3A). The regions of the neural tube expressing the GFP were

243 dissected 18 hours after electroporation and dissociated. GFP-expressing cells were isolated  
244 by FACS with the use of a dead cell exclusion (DCE)/discrimination dye (DAPI) to eliminate  
245 dying cells (Supplementary Fig. 8). Two independent RNAs samples were extracted, reverse  
246 transcribed, and cDNAs were amplified using a linear amplification system and used for  
247 sequencing library building. Qualitative analysis of RNA-seq data from the two biological  
248 replicates shows a high pearson correlation score ( $>0,98$ ) indicative of the experimental  
249 reproducibility (Supplementary Fig. 9). RNA-seq data from alignment to the Galgal4 genome  
250 assembly identified 1913 genes with significantly changed expression (Fig. 3B FDR5 (False  
251 Discovery Rate 5), Table 1 and Table 2; see also Material and Methods section), of which  
252 1,097 were up-regulated (57%) (Table 1) and 816 down-regulated (43%) (Table 2) (Fig. 3C  
253 left panel). This tendency of HoxB8 to act as activator rather than repressor is amplified when  
254 selecting genes differentially expressed by more than two-fold, with 251 being up-regulated  
255 (90%) and only 25 down-regulated (10%) (Fig. 3C right panel). Gene ontology enrichment  
256 analysis (GOEA) of the biological processes suggests pleiotropic functions of HoxB8 during  
257 spinal cord development (Fig. 3D and Table 3) including neuron differentiation, apoptotic  
258 process, cell cycle and cell migration (Fig. 3D). In particular, the Notch signaling pathway, a  
259 key regulator of neurogenesis (Formosa-Jordan et al., 2013; Hatakeyama, 2004; Hatakeyama  
260 et al., 2006), stands out from the GOEA (Fig. 3D – downregulated genes), suggesting that  
261 HoxB8 controls neurogenesis. This is illustrated by Hes5.1, a Notch pathway effector  
262 expressed in the VZ and known to keep neural tube cells in a progenitor state (Fior and  
263 Henrique, 2005), for which transcripts *in situ* hybridization shows strong transcriptional  
264 downregulation (Supplementary Fig. 10, Table 1).

265         Among all deregulated genes, the Leucine zipper tumor suppressor 1 (*Lzts1*) gene  
266 (also known as FEZ1 and PSD-Zip70) (Baffa et al., 2008; Ishii et al., 2001; Vecchione et al.,  
267 2007) caught our attention for two reasons. First, *Lzts1*, upregulated by HoxB8 (Fig. 3B,E  
268 and Table 1) is, as HoxB, preferentially expressed in the IZ in the trunk neural tube of chicken  
269 and mouse embryos (Kropp and Wilson, 2012). Second, *Lzts1* has been recently shown to  
270 control neuronal delamination during mammalian cerebral development (Kawaue et al.,  
271 2019), a function that if conserved in the neural tube, could account for the HoxB-induced  
272 ectopic Sox2 positive cells found in the MZ.

273         We studied the dynamics of *Lzts1* expression by *in situ* hybridizations with an *Lzts1*  
274 probe at E2, E3 and E4 stages (Fig. 4A-B and Supplementary Fig. 11). At E3, *Lzts1*  
275 transcripts are already found in the IZ, which, due to the lack of differentiated neurons that  
276 will form the MZ at that stage, is in the most lateral region of the neural tube (Fig. 4A). At E4,

277 as previously described (Kropp and Wilson, 2012), *Lzts1* transcripts are still associated with  
278 the IZ, located between progenitors of the VZ and differentiated neurons of the MZ  
279 (Supplementary Fig. 11B), with an expression pattern very similar to HoxB genes (Fig. 4B  
280 and Supplementary Fig. 12). Indeed, while not completely overlapping since HoxB are still  
281 expressed in the VZ at low level, *Lzts1* transcripts are enriched where the HoxB9 protein level  
282 is the highest (Supplementary Fig. 12). The expression of *Lzts1* gene is not restricted to a  
283 specific antero-posterior region of the neural tube (Supplementary Fig. 11A and  
284 Supplementary Fig. 12). The *Lzts1* expression pattern is thus compatible with a regulation by  
285 HoxB proteins in the IZ. Consistent with its identification as a HoxB8 target in the  
286 transcriptomic approach, *in situ* hybridization with an *Lzts1* probe following HoxB8 gain-of-  
287 function shows ectopic *Lzts1* expression in the trunk neural tube (Fig. 4C). If *Lzts1* regulation  
288 illustrates at the level of a single target the generic control of early neurogenesis documented  
289 in Fig. 2, HoxB4 and HoxB9 should also induce ectopic *Lzts1* expression, which is indeed  
290 observed (Fig. 4C). *Lzts1* transcriptional activation is faint, consistent with the 2.7-fold  
291 transcript enrichment seen in the RNA-seq data, and is mainly observed in the ventricular  
292 zone (Fig. 4). Co-expressing HoxB4, HoxB8 or HoxB9 with the P35 apoptotic inhibitor does  
293 not allow for stronger and more frequent *Lzts1* induction, in particular in the MZ, where in  
294 such conditions the frequency of Sox2 positive cells in the MZ is high. This indicates that the  
295 lack of *Lzts1* induction in cells of the MZ, is not due to cell elimination by apoptosis,  
296 suggesting that HoxB proteins can only transcriptionally control *Lzts1* expression within a  
297 sharp time window, when cells are still in the VZ or IZ.

298 We also found that HoxA7, HoxC8 and HoxD8 gain-of-functions induce *Lzts1*  
299 expression (Supplementary Fig. 14), showing that as in the case of Sox2 ectopic cells  
300 induction in the MZ, *Lzts1* transcriptional activation by Hox proteins relies on regulatory  
301 properties embedded in B and non-B Hox proteins. These results show that *Lzts1*, identified  
302 as a HoxB8 target, is a generic Hox target. However, only B cluster Hox genes are expressed  
303 at the proper time and space for assuming that function.

304

### 305 **Lzts1 controls the delamination of newborn neurons in the trunk neural tube**

306 Premature delamination of neural progenitors may explain the presence of ectopic Sox2 cells  
307 in the MZ after the HoxB gain-of-functions. The function of *Lzts1* in neuronal development  
308 within the trunk neural tube is not known, but it has been described to positively control  
309 neuronal delamination in brain development in mammalian (Kawaue et al., 2019). Due to its

310 expression in the IZ, where progenitors switch to neurons and lose their apical attachment,  
311 *Lzts1* may also control delamination during spinal cord neurogenesis.

312 To examine this, we analyzed the consequences of *Lzts1* gain-of-function, obtained  
313 through unilateral electroporation of an *Lzts1* expression vector in the neural tube at E2  
314 (Supplementary Fig. 15). The tracking of the cytoplasmic GFP demonstrated massive cell  
315 delamination with nearly all electroporated cells losing their attachment to the lumen and  
316 found in the MZ (Fig. 5A). This phenotype is seen both at two and three days after  
317 electroporation (Fig. 5A). Under normal conditions, only newborn neurons lose their apical  
318 attachment (Kasioulis and Storey, 2018) suggesting that *Lzts1* in the IZ is involved in the  
319 control of newborn neuron delamination. Immunostainings with Sox2 and Tuj1 antibodies  
320 (progenitor and neuronal markers, respectively) (Fig. 5 and Supplementary Fig. 16) showed  
321 that nearly half (47,7%) of the *Lzts1* gain-of-function cells in the MZ ectopically express  
322 Sox2 (Fig. 5C). This suggests that *Lzts1* gain-of-function forces the delamination but not  
323 neural differentiation, since cells which prematurely delaminate stay in a progenitor state (Fig.  
324 5B-C). This is distinct from Neurogenin2 gain-of-function where electroporated cells  
325 massively delaminate but also prematurely differentiate. (Garcia-Gutierrez et al., 2014). The  
326 *Lzts1* delamination “only” phenotype is confirmed by the finding that the MZ ectopic Sox2  
327 positive cells keep progenitor characteristics: they express CCND1/CyclinD1 (Fig. 5D) and  
328 the pH3 mitotic marker (Fig. 5E), and Hes5.1 and NeuroD4 genes respectively markers of the  
329 VZ and IZ (Fig. 5F, G). The phenotype induced by *Lzts1* gain-of-function is independent of  
330 the dorso-ventral and antero-posterior position within the trunk neural tube (Fig. 5B and  
331 Supplementary Fig. 16) and is not strictly cell-autonomous (ectopic Sox2 positive GFP  
332 negative cells can be found following *Lzts1* gain of function; Supplementary Fig. 16).

333 To support conclusion from *Lzts1* gain-of-function experiments, we analyzed the  
334 effects of *Lzts1* loss-of-function. Knockdown was obtained through unilateral electroporation  
335 of a ShRNA expressing plasmid at E2. The efficiency of the ShRNA was assessed by  
336 following *Lzts1* transcripts (Fig. 6A), showing a strong effect illustrated by the absence of the  
337 typical *Lzts1* expression in the IZ. The effects of *Lzts1* knockdown were studied using the  
338 Sox2 progenitor (Fig. 6B) and Tuj1 or HuC/D (Fig. 6C-F, Supplementary Fig. 17) neuronal  
339 markers. Results show that *Lzts1* knockdown does not lead to ectopic Sox2 cells as induced  
340 by *Lzts1* gain-of-function, but instead leads to an ectopic expression of Tuj1 or HuCD  
341 neuronal markers in the VZ with neurons keeping their apical attachment (Fig. 6C-F). The  
342 loss-of-function of *Lzts1* thus results in neuronal delamination inhibition, a phenotype that

343 mirrors the promotion of neuronal delamination seen in *Lzts1* gain-of-function experiments  
344 (Fig. 5 and Supplementary Fig. 16).

345 Collectively, *Lzts1* gain and loss of function experiments demonstrate a role for *Lzts1*  
346 in controlling neural delamination in the trunk neural tube. As impaired delamination is a  
347 plausible explanation for the generic HoxB-induced MZ ectopic Sox2 cells, and as *Lzts1*  
348 transcripts are induced by HoxB proteins, *Lzts1* is likely to be a key HoxB effector leading to  
349 the MZ ectopic Sox2 phenotype. To probe this hypothesis, we performed epistatic  
350 experiments by co-expressing HoxB8 and the ShRNA-*Lzts1* ShRNA, in the P35 context so to  
351 start with a stronger HoxB induced phenotype. Results show that *Lzts1* gene inactivation  
352 lowers significantly the occurrence of Sox2 ectopic cells in the MZ (Fig. 6 G-I), supporting  
353 that *Lzts1* is a key effector in the HoxB induced delamination phenotype.

354

## 355 **DISCUSSION**

356

### 357 **A broad B-cluster specific function for Hox genes in early spinal cord development**

358 Our work extends the functional contribution of Hox genes to spinal cord development. While  
359 largely shown to act as “choreographers” of neural development in specifying motor neurons  
360 subtypes (Philippidou and Dasen, 2013), this study highlights an unexpected early and  
361 general role in controlling early neurogenesis and neuronal delamination. Previous expression  
362 data delineated that B cluster *Hox* gene expression at E6, a stage when motor neuron subtypes  
363 are defined, does not follow axial collinearity, as non-B cluster Hox genes do, and are  
364 generally excluded from differentiating motor neurons (Dasen et al., 2005; Jung et al., 2010;  
365 Lacombe et al., 2013). Based on the expression analysis of representatives of anterior, central  
366 and posterior Hox paralogs, we propose that B cluster Hox genes (excepted HoxB13) are  
367 expressed in the chicken neural tube earlier than non-B cluster Hox genes, in a largely  
368 ubiquitous pattern that later resolves in preferential expression in the IZ, the region of the  
369 trunk neural tube where neuronal progenitors exit the cell cycle and delaminate to transit  
370 toward the mantle zone. Consistent with the lack of axial collinearity already observed at E6,  
371 B cluster Hox gene expression displays little axial specificity, with most HoxB genes  
372 expressed in largely overlapping expression patterns in the trunk neural tube (Figure 7). These  
373 expression patterns suggest a function distinct from endowing the neural tube with axial  
374 positional information required for proper setting of neuronal subtype along the AP axis, well

375 documented for *Hox* A, C and D genes. It rather suggests that B cluster Hox genes act without  
376 paralog specificity all along the trunk neural tube, in a “generic” manner, giving a temporal  
377 instead of positional information. While long underseen, a recent literature survey indicates  
378 that such generic functions are constitutive of Hox protein function (Saurin et al., 2018), and  
379 may be an intrinsic deeply rooted property of Hox proteins reflecting their phylogenetic  
380 common origin. An illustration of such a function is the generic control of autophagy by Hox  
381 proteins in the *Drosophila* fat body (Banreti et al., 2014), where as seen here in the chicken  
382 neural tube, Hox genes are broadly expressed in the tissue. A difficulty to study such generic  
383 function, which has contributed to its late recognition, is that revealing them can often not be  
384 achieved by conventional loss-of-function approaches, as mutating one or even a few Hox  
385 genes does not alter the shared generic function, due to inter-paralog functional compensation  
386 (Banreti et al., 2014).

387 To get insights into early B cluster Hox gene function in the chicken neural tube, we  
388 thus took a gain-of-function approach. Results obtained indicate that Hox gain-of-function  
389 results in the appearance of progenitor Sox2 positive cells in the MZ, a region of the neural  
390 tube that normally hosts differentiated post-mitotic neurons. Consistent with a shared  
391 “generic” function suggested by the expression patterns, we found that anterior, central and  
392 posterior HoxB genes induce similar defects, all resulting in ectopic Sox2 positive cells in the  
393 MZ. This phenotype is observed all along the trunk neural tube and occurs at all dorso-ventral  
394 positions within the tube, indicating that this generic Hox function may be relevant to  
395 neurogenesis progression in general, irrespective of the final antero-posterior or dorso-ventral  
396 driven final neuronal identity. We also found that gain-of-function experiments conducted  
397 with the non-B proteins HoxA7, HoxC8 and HoxD8, not expressed at early stage in the neural  
398 tube, also result in ectopic Sox2 positive cells in the MZ. This suggests that the control of the  
399 process leading on the Sox2 positive cells in the MZ is a regulatory property likely embedded  
400 into Hox proteins in general, and may rely on the similar biochemical characteristics of Hox  
401 proteins, with most Hox proteins displaying similar DNA binding properties (Hayashi and  
402 Scott, 1990; Mann and Chan, 1996; Mann et al., 2009; Merabet and Mann, 2016; Zandvakili  
403 and Gebelein, 2016). The B cluster specificity would thus arise strictly from the temporal and  
404 spatial deployment of B cluster proteins, and not from intrinsic properties specific to B cluster  
405 Hox proteins. In agreement with this hypothesis, in silico survey of sequence conservation in  
406 Hox proteins, including short linear motifs (SLiMs), does not reveal any characteristics  
407 specific to the B cluster Hox proteins (Rinaldi et al., 2018).

408

409

410 **Insights into HoxB generic function from the study of the *Lzts1* downstream target**

411 To circumvent the difficulty of gaining functional insights into HoxB gene function in the  
412 early chicken neural tube from loss-of-function approaches, we reasoned that identifying and  
413 studying HoxB downstream targets, including through loss-of-function approaches, would  
414 allow assessing better how HoxB genes influence early spinal cord development.  
415 Transcriptomic data obtained one day after HoxB8 overexpression highlights genes and  
416 pathways well known to control multiple aspects of neurogenesis including Notch and IGF  
417 pathway effectors (Fior and Henrique, 2005; Fishwick et al., 2010; Vilas-Boas and Henrique,  
418 2010), suggesting that HoxB gene influence on early neurogenesis is diverse. For this study  
419 we focused on *Lzts1*, which shares with HoxB genes a preferential expression in the IZ, the  
420 region of the trunk neural tube containing the newly born neurons on their way to their final  
421 position (the MZ), and thus may account for the main phenotype (Sox2 ectopic cells in the  
422 MZ) seen in HoxB overexpression experiments.

423         The study of *Lzts1* gain and loss-of-function experiments showed that *Lzts1* controls  
424 the delamination of newborn neurons: gain-of-function induces massive cell delamination  
425 with nearly all electroporated cells losing their attachment to the lumen and found in the MZ,  
426 while loss-of-function leads to differentiated neurons keeping their apical attachment. The  
427 promotion of delamination by *Lzts1* further suggests that the appearance of Sox2 positive  
428 cells in the MZ, seen in *Lzts1* and Hox gain-of-function experiments, results from loss of  
429 apical attachment and subsequent migration of progenitor Sox2 positive cells in the MZ.  
430 Consistent with the view that *Lzts1* mediates HoxB generic function in the chicken early  
431 neural tube, we observed that *Lzts1* expression is influenced not only by HoxB8, but also by  
432 all other HoxB cluster genes probed (the anterior HoxB4 and posterior HoxB9 class genes),  
433 and that *Lzts1* gene knockdown in a HoxB8 gain-of-function experiment significantly lowers  
434 the HoxB8-induced ectopic Sox2 phenotype. Although both HoxB and *Lzts1* overexpression  
435 result in ectopic Sox2 positive cells in the MZ, the HoxB overexpression phenotypes are less  
436 pronounced than *Lzts1* gain-of-function (including in a P35 context which inhibits cell death),  
437 with fewer ectopic Sox2 positive cells seen in the MZ. This weaker phenotype is in line with  
438 the limited capacity of HoxB genes to induce *Lzts1* expression in gain-of-function  
439 experiments, which likely reflects a sharp time window within which *Lzts1* transcriptional  
440 activation by HoxB proteins is possible.

441

442 **HoxB and Lzts1 function in the IZ might be conserved in higher vertebrates**

443 HoxB control of neural delamination via the regulation of *Lzts1* in the IZ uncovered by this  
444 study might be shared by higher vertebrates. Expression patterns of the *HoxB* and *Lzts1* genes  
445 in the trunk neural tube of mouse embryo are highly reminiscent of chicken embryo: *Lzts1* is  
446 also expressed in the IZ (Kropp and Wilson, 2012); *HoxB* genes, also expressed earlier than  
447 non-B cluster *Hox* genes, also have a broadly overlapping expression in the neural tube and  
448 are also excluded from the motor neuron area as in chicken (Graham et al., 1991; Jung et al.,  
449 2010; Lacombe et al., 2013). However, *HoxB* gene expression does not seem to resolve in the  
450 IZ as sharply as in the chicken. HoxB activity might be restrained to the IZ through the  
451 expression and action in the VZ of Geminin, a pleiotropic cell-cycle regulator also known to  
452 inhibit Hox protein (Luo et al., 2004; Patterson et al., 2014). In this situation, only cells that  
453 express *HoxB* genes laterally to the limit of Geminin expression, which corresponds to the IZ,  
454 would be free of the Geminin inhibitor and allow HoxB-mediated *Lzts1* transcriptional  
455 activation.

456 Lzts1 function in the control of neural delamination has already been described in  
457 mammals, in the context of the brain (cephalic neural tube) of mouse and ferret (Kawaue  
458 et al., 2019). Kawaue and colleagues demonstrate that *Lzts1*, which associates with  
459 microtubule components and is involved in microtubule assembly (Ishii et al., 2001), controls  
460 apical delamination of neuronally committed cells of the brain by altering apical junctional  
461 organization (Kawaue et al., 2019). Indeed, in neuronally differentiating cells of the brain,  
462 *Lzts1* modulates the microtubule-actin-AJ system at the apical endfeet to evoke apical  
463 contraction and reduce N-cadherin expression (Kawaue et al., 2019). Molecular mechanisms  
464 by which *Lzts1* controls delamination in the trunk neural tube might be the same as in the  
465 mammalian brain. However, *Lzts1* upstream regulation has to be distinct, as the brain is  
466 known as a Hox-free territory.

467 In humans, expression of the *LZTS1* gene (also named *FEZ1*) is altered in multiple  
468 tumors (Ishii et al., 1999). *LZTS1* tumor suppressor function has been attributed at least in  
469 part to its role in the control of mitosis progression (Vecchione et al., 2007) and in regulating  
470 the Pi3k/AKT pathway (He and Liu, 2015; Zhou et al., 2015). Since the Pi3k/AKT pathway is  
471 required for neuron production in the trunk neural tube in both mouse and chicken embryos  
472 (Fishwick et al., 2010), *Lzts1* might also regulate neuronal production in the trunk neural tube  
473 by regulating the Pi3k/AKT pathway in addition to controlling delamination.

474



475

476

477

## 478 **MATERIALS AND METHODS**

479

### 480 *Ethics statement*

481 Experiments performed with non-hatched avian embryos in the first two thirds of embryonic  
482 development time are not considered animal experiments according to the Directive  
483 2010/63/EU.

484

### 485 *Chicken embryos*

486 Fertilized chicken eggs were obtained from EARL les Bruyeres (Dangers, France) and  
487 incubated horizontally at 38°C in a humidified incubator. Embryos were staged according to  
488 the developmental table of Hamburger and Hamilton (HH) (Hamburger and Hamilton, 1992)  
489 or according to days of incubation (E).

### 490 *In ovo electroporation and plasmids*

491 Neural tube *in ovo* electroporations were performed around HH12. Eggs were windowed, and  
492 the DNA solution was injected in neural tube lumen. Needle L-shape platinum electrodes  
493 (CUI613P5) were placed on both sides of the embryo at trunk level (5 mm apart), with the  
494 cathode always at its right. Five 50 ms pulses of 25 volts were given unilateral (or bilateral for  
495 RNAseq experiments) at 50 ms intervals with an electroporator NEPA21 (Nepagene).

496 The plasmids used for the gain-of-function experiments co-express a cytoplasmic or  
497 nuclear GFP (pCAGGS and pCIG respectively, used alone as controls) and the coding  
498 sequence (CDS) of the gene of interest. Vector used are: pCIZ-HoxB4, pCIG-HoxB8, pCIG-  
499 HoxB9 and pCIG-HoxC8 (gifted by Olivier Pourquié), pCAGGS-P35 (gifted by Xavier  
500 Morin) and pCAGGS-Lzts1, pCAGGS-HoxA7, pCAGGS-HoxD8, pCAGGS-HoxB4  
501 pCAGGS-HoxB8 and pCAGGS-HoxB9 (this study). The CDS of HoxA7 and HoxD8 (second  
502 isoform, 567 bp) were PCR amplified from chicken neural tube cDNA; the CDS of HoxB4,  
503 HoxB8 and HoxB9 were PCR amplified from the plasmids described above and the CDS of  
504 Lzt1 was amplified from pGEMT-Lzts1 (gifted by Dr. S. Wilson). All sequences were  
505 subcloned in the pCAGGS plasmid using In-Fusion HD Cloning Kit (Takara). RNA  
506 interference technology was used to inhibit *Lzts1*, with the pRFPRNAiC vector, which

507 contains an RFP reporter gene (Das et al., 2006) and insertion sites for two siRNAs in tandem.  
508 The two 22 nucleotide-long target sequences for the ShLzts1 plasmid were chosen using the  
509 design tool “siRNA Target Finder” (AAGGTCAACCTGTTAGAGCAGG and  
510 AACATCATGCAGTGTGCCATCA). A shscrambled-Lzts1 plasmid was designed as control  
511 (AGAAGAGTGTACGGTCGCAGTC and GCATGTTGAACCGCAATACT).  
512 All the plasmids used for electroporation were purified using the Nucleobond Xtra Midi kit  
513 (Macherey-Nagel). Final concentration of DNA delivered by embryo for electroporation is  
514 between 1 to 2µg/µl except for epistatic experiment performed with DNA solution at 2,5µg/µl  
515 due to technical constraints (Table 4).

#### 516 *Immunofluorescence and fluorescent in situ hybridization*

517 Embryos were fixed in 4% buffered formaldehyde in PBS then treated with a sucrose gradient  
518 (15% and 30% in PBS), embedded in OCT medium and stored at -80°C. Embryos were  
519 sectioned into 16 µm sections with a Leica cryostat and the slides were conserved at - 80°C  
520 or directly used for FISH and/or immunofluorescence.

521

#### 522 *Immunofluorescence*

523 Slides were rehydrated in PBS then blocked with 10% goat serum, 3% BSA, 0,4% Triton X-  
524 100 in PBS for one hour. Primary antibodies were incubated over-night diluted in the same  
525 solution at 4°C. The following primary antibodies were used in this study: chicken anti-GFP  
526 1:1000 (1020 AVES), rabbit anti-SOX2 1:500 (AB5603 Merck Millipore), mouse anti-Tuj1  
527 1:500 (801202 Ozyme), mouse anti-HuC/D 1: 200 (Thermofischer 16A11), rat anti-pH3 1:  
528 250 (S28, abcam ab10543), rabbit anti-Caspase 3 1:500 (Asp175, CST 9661), guinea pig anti-  
529 HoxC9 antibody 1:1000 (NY1638, gifted by Jeremy Dasen) and rabbit anti-LZTS1 1:250  
530 (Sigma HPA006294). Polyclonal HoxB9 antibodies were raised in guinea pig using the  
531 peptide “143-158 GIVSNQRPSFEDNKVC” and used at 1:500. The secondary antibodies  
532 used were: anti-chicken, anti-rabbit, anti-mouse, anti-rat or anti-guinea pig conjugated with  
533 fluorochromes (488, 568 or 647) at 1:500. They were incubated for one hour in the blocking  
534 solution containing Hoechst (1:1000). Slides were washed, mounted (Thermo Scientific  
535 Shandon Immu-Mount) and imaged with a Zeiss microscope Z1 equipped with Apotome or a  
536 confocal LSM 780.

537

#### 538 *EdU labelling and detection*

539 Proliferative cells were labelled with EdU using the Click it EdU Alexa Fluor 647 kit  
540 (Thermofisher). 400µl of a 0.5mM EdU solution (in PBS) was applied on top of the embryo  
541 and incubated at 38°C for 30mn. Cryostat sections were stained for EdU using manufacturer  
542 instructions.

543

#### 544 *Fluorescent in situ hybridization*

545 The slides were treated with proteinase K 10 µg/ml (3 minutes at 37°C) in a solution of  
546 TrisHCl 50 mM pH 7.5, then in triethanolamine 0.1M and 0.25% acetic anhydride. They were  
547 pre-incubated with hybridization buffer (50% formamide, SCC 5X, Denharts 5X, yeast tRNA  
548 250 µg/ml and herring sperm DNA 500 µg/ml) for 3h at room temperature, and incubated in  
549 the same buffer with DIG-labelled RNA probes over-night at 55°C in a wet chamber. The  
550 slides were then washed twice with 0.2X SCC for 30 minutes at 65°C. After 5 minutes in  
551 TNT buffer (100 mM Tris pH7.5, 150mM NaCl and 0.1% Tween-20), they were then blocked  
552 for 1h in buffer containing TNT 1X, 1% Blocking reagent (Roche) and 10% goat serum, then  
553 incubated in the same buffer for 3h with anti-DIG-POD antibodies (1:500, Roche) and  
554 revealed using the kit TSA-Plus Cyanin-3 (Perkin Elmer). RNA probes used for *in situ*  
555 hybridization were: *Lzts1*, *Hes5.1*, *Ccnd1*, *NeuroD4*, *HoxB4*, *HoxB5*, *HoxB7*, *HoxB8* and  
556 *HoxB9*. The plasmids used to generate the *Hox* RNA probes were gifts from Jeremy Dasen  
557 and Olivier Pourquié (except *HoxB8* –PCR primer forward: CCAGCTCCCCTTACCAACAG and T7  
558 reverse: TAATACGACTCACTATAGGGCCTCGGGGGCTCTTCTACCC, transcription from neural tube  
559 cDNA). The vector for the *Lzts1* probe (pGEMT-Lzts1) was a kind gift from Dr. S. Wilson  
560 and the vector for the *Hes5.1* probe gifted by Dr. X. Morin.

561

#### 562 *Whole mount in situ hybridization*

563 Embryos were fixed 2h at RT in 4% formaldehyde in PBS. Embryos were dehydrated with  
564 sequential washes in 50% ethanol/ PBS+ 0.1% Tween20 and 100% ethanol and conserved at -  
565 20°C. Embryos were bleached for 45mn in 80% ethanol + 20% H2O2-30% and then  
566 rehydrated. They were treated with proteinase K 10µg/ml at RT and refixed with 4%  
567 formaldehyde, 0.2% glutaraldehyde. After 1h of blocking in the hybridization buffer (50%  
568 formamide, SSC 5X, 50µg/mL Heparine, yeast tRNA 50µg/mL, SDS 1%) hybridization with  
569 DIG-labelled RNA probes (*HoxB2*, *HoxB4*, *HoxB7*, *HoxB8*, *HoxB9* and *Lzts1*) was performed  
570 at 68°C overnight. The next day, embryos were washed (3 times 30 minutes) in hybridization  
571 buffer and 1 time in TBS (25 mM Tris, 150 mM NaCl, 2 mM KCl, pH 7.4) +0.1% Tween 20.

572 They were incubated 1h at RT in a blocking buffer (20% Blocking reagent + 20% Goat  
573 serum) and then overnight with an anti-DIG-AP antibody (1:2000, Roche) in the blocking  
574 buffer. After 3 washes (1 hour) in TBS+0.1% Tween 20, embryos were equilibrated (2 times  
575 10 minutes) in NTMT buffer (NaCl 100mM, TrisHCl 100mM pH9,5, MgCl2 50mM,  
576 2%Tween20) and incubated in NBT/BCIP (Promega) at RT in the dark until color  
577 development. Pictures of whole embryos were made using a BinoFluo MZFLIII and a color  
578 camera.

579

### 580 ***RNA-seq analysis***

581 Electroporations were carried out as described in a previous section but with 5 bilateral  
582 pulses. Plasmids DNA concentrations were for the control mix: pCIG 2µg/µl and for the  
583 HoxB8 mix: HoxB8-pCIG 1 µg/µl + pCIG 1µg/µl. Part of the neural tube expressing the GFP  
584 were dissected 18 hours after electroporation and dissociated (Trypsin-EDTA 0,25%). GFP  
585 and CDS take around 3 hours to be expressed after electroporation. As a consequence, 18  
586 hours post-electroporation means that HoxB8 is overexpressed in neural tube cells for about  
587 15 hours. We have chosen this timing as it is the earliest at which the size of the neural tube  
588 allows for rapid dissection, a condition required for collecting sufficient starting material for  
589 FACS in a minimum timeframe. A highly enriched population of GFP-expressing cells was  
590 isolated by FACS with the use of a dead cell exclusion (DCE)/discrimination dye (DAPI) to  
591 eliminate dying cells (Supplementary Fig. 8). RNA was extracted (RNeasy Mini Kit) and  
592 reverse transcribed and cDNA was amplified using a linear amplification system and used for  
593 sequencing library building (GATC): Random primed cDNA library, purification of poly-A  
594 containing mRNA molecules, mRNA fragmentation, random primed cDNA synthesis, adapter  
595 ligation and adapter specific PCR amplification, Illumina technology, 50 000 000 reads paired  
596 end with 2 x 50 bp read length. Bioinformatics analysis were done using the galgal4.0 chicken  
597 genome. Qualitative analysis of RNA-seq data from the two biological replicates shows a  
598 high Pearson Correlation score (>0,98) indicative of the experimental reproducibility  
599 (Supplementary Fig. 9).

600 RNA-seq data have been deposited in NCBI's Gene Expression Omnibus and are accessible  
601 through GEO Series access number GSE162665.

602

### 603 ***Quantifications and statistical significance***

604 The number of embryos and number sections analyzed are indicated in the figure legends. A  
605 minimum of 3 embryos and 6 sections per embryos were used to quantify. All quantifications

606 were made using the cell counter tool of Fiji software. The results were analyzed and plotted  
607 using Prism 8 software (GraphPad software). Statistical analyses were performed using a two-  
608 tailed Mann Whitney test and considered significant when p-value < 0,05. All p-values are  
609 indicated on the graphs. The error bars represent the standard deviation (SD).

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614 manuscript. FACS experiments were done at the CRCM (Marseille, France).

615

#### 616 **Competing interests**

617 The authors declare no competing or financial interests.

618

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638 **FIGURE LEGENDS**

639

640 **Figure 1: *HoxB* genes are expressed in the trunk neural tube during early neurogenesis**  
641 **with little antero-posterior axial specificity**

642 **A-** *HoxB2*, *HoxB4*, *HoxB7*, *HoxB8* and *HoxB9* gene expression patterns of E3 chicken  
643 embryos by whole mount *in situ* hybridization. **B-** Schematic of *HoxB* gene (except *HoxB13*)  
644 expression patterns at E3 in the trunk neural tube showing overlapping patterns from the neck  
645 (with little spatial collinearity) to the tail. **C-** Brachial and thoracic *HoxB9* and *HoxC9* protein  
646 expression patterns at E3, E4 and E5 by immunofluorescence on transversal sections. In the  
647 trunk neural tube, *HoxB9* protein is expressed as early as E3 and is excluded from the motor  
648 neuron territories at E4. Its paralog protein *HoxC9* is expressed from E4 in the motor neuron  
649 territories. **D-** *HoxB9* and *HoxC9* protein expression patterns along the neural tube at E5.  
650 *HoxB9* protein is expressed all along the trunk neural tube. Its paralog *HoxC9* is only  
651 expressed at the thoracic level. **E-** Immunofluorescences and fluorescent *in situ* hybridizations  
652 (FISH) of *HoxB9* protein (green), *HoxB8* gene (red) and Hoechst (nuclear staining, blue)  
653 show a strong expression overlap in the neural tube at E4.5 between a posterior and a central  
654 *HoxB* gene (Scale bar: 50µm).

655

656 **Figure 2: *HoxB* genes are expressed in the IZ and their gain-of-function leads to ectopic**  
657 **progenitor cells in the MZ**

658 **A-** Immunofluorescences and FISH on transversal sections of trunk neural tube at E4. The  
659 expression of *Sox2* and *Tuj1* (respectively markers of the VZ (progenitors) and the MZ  
660 (neurons) in green and blue) and expression of the *NeuroD4* gene (marker of the IZ, in red)  
661 illustrate that *HoxB9* is mainly expressed in the IZ. **B-** Immunofluorescences on transversal  
662 sections three days after electroporation of the chicken neural tube with a control (pCAGGS),  
663 *HoxB4*, *HoxB8* or *HoxB9* expression vectors (in the pCAGGS vector), co-transfected with a  
664 vector expressing the cell death inhibitor P35, stained with GFP (green), *Tuj1* (red) and *Sox2*  
665 (blue) antibodies. The gain-of-function in all three cases leads to the appearance of ectopic  
666 positive *Sox2* cells in the MZ. **C-** Percentage of ectopic *Sox2*<sup>+</sup> cells among the GFP<sup>+</sup> cells in  
667 the MZ per section, three days after co-electroporation of a vector expressing P35 and the

668 control pCAGGS (n= 3 animals / 18 sections) or Hox expressing pCAGGS vectors ( HoxB4  
669 (n= 3 animals/ 21 sections), HoxB8 (n= 3 animals/ 21 sections) and HoxB9 (n= 3 animals/ 19  
670 sections) (in +P35 condition). The quantifications showed a significant increase of ectopic  
671 Sox2 cells in the MZ after any HoxB gain-of-function. (Two-tailed Mann-Whitney test, error  
672 bars represent SD). **D-** High magnification of immunofluorescences on transversal sections in  
673 the MZ three days after electroporation of the chicken neural tube with HoxB8 +P35 vectors,  
674 stained with GFP (green), Sox2 (red), and Hoechst (blue), illustrating that while most Sox2  
675 ectopic cells are GFP+, some ectopic Sox2+ cells in the MZ are also GFP- (white arrow, top  
676 panel). Staining with a mitotic marker pH3 (s28) (magenta) (in the bottom panel) identifies  
677 HoxB8-induced Sox2+ cells in the MZ, indicating that these cells are still mitotic (Sox2+ and  
678 pH3+, white border arrow on all three panels) (Scale bar: 50µm).

679

680 **Figure 3: Identification of *Lzts1*, expressed in the IZ, as a HoxB8 downstream target by**  
681 **RNAseq analysis**

682 **A-** 18h after bilateral electroporation of trunk neural tube at the stage HH12 with the pCIG  
683 control vector (expression of GFP only) or the pCIG-HoxB8 (expression of GFP and HoxB8),  
684 the electroporated region of the neural tube was dissected (18 to 20 embryos per condition in  
685 duplicates) and the GFP+ cells were sorted by FACS. **B-** Volcano plot of Differential Gene  
686 Expression (DGE) for the HoxB8 versus control (pCIG) conditions. The position of *Lzts1*  
687 in the volcano plot is circled. (FDR: False Discovery Rate; FC: Fold Change). **C-** Circle graphs  
688 representing the number of HoxB8 up-regulated and down-regulated genes for a FDR=5 (all  
689 the genes) or for a FDR=5 and a FC>2. This illustrates that HoxB8 acts more as an activator  
690 than a repressor of transcription. **D-** Gene ontology enrichment analysis (GOEA) of the  
691 biological processes for up (top table) and downregulated genes (low table). This analysis  
692 suggests a HoxB8 pleiotropic function during spinal cord development **E-** The graph of the  
693 number of *Lzts1* TPM (Transcripts Per Kilobase Million) obtained for the two replicates of  
694 the control (pCIG1, pCIG2) and HoxB8 (HoxB8-1 and HoxB8-2) expressing samples,  
695 illustrates the reproducibility between replicates.

696

697 **Figure 4: *Lzts1* expression in the IZ is controlled by *HoxB* genes**

698 **A-** FISH on trunk transversal sections of chicken embryo at E3 with *Lzts1* probe. **B-** FISH and  
699 immunofluorescences on trunk transversal sections of chicken embryo at E4 showing an  
700 overlapping expression of *Lzts1* gene (red) and HoxB9 protein (green) in the IZ. **C-** The gain-  
701 of-function of HoxB4, HoxB8 or HoxB9 two days after electroporation (GFP, green) induces

702 the ectopic expression of *Lzts1* (red) in the VZ. Blue is Hoechst staining. Arrows point *Lzts1*  
703 ectopic expression. (Scale bar: 50µm).

704

705 **Figure 5: *Lzts1* gain-of-function triggers neuronal delamination and leads to ectopic**  
706 **progenitor cells in the MZ**

707 **A-** *Lzts1* gain-of-function induces massive delamination of the electroporated cells two and  
708 three days following electroporation. Cells electroporated with a control vector (pCAGGS)  
709 do not display this phenotype. **B-** The gain-of-function of *Lzts1*, two and three days after  
710 electroporation induces ectopic Sox2 positive cells in the MZ. **C-** Percentage of ectopic  
711 Sox2+ cells among the GFP+ cells in the MZ per section. Counts performed three days after  
712 the electroporation for control plasmids pCIG or pCAGGS (n= 3 animals / 18 sections) or  
713 *Lzts1* expressing plasmid (n= 3 animals / 18 sections) showed a significant and strong  
714 increase of ectopic Sox2 cells in the MZ (Two-tailed Mann-Whitney test, error bars represent  
715 SD). **D-E-F-G-** FISH and/or immunofluorescences on transversal sections of trunk neural  
716 tube two or three days after the *Lzts1* expressing vector electroporation. *Lzts1* induces the  
717 presence of ectopic *CCND1*, pH3, *Hes5.1* and *NeuroD4* expressing cells in the MZ. (GFP,  
718 green and Hoechst, blue, scale bar: 50µm).

719

720 **Figure 6: *Lzts1* loss-of-function inhibits neuronal delamination downstream of HoxB8**

721 **A-** FISH on trunk transversal sections stained for *Lzts1* transcripts (green) two days after  
722 electroporation of a ShRNA-*Lzts1* expression plasmid (co-expressing RFP) shows a reduction  
723 in the quantity of *Lzts1* transcripts. **B-** Electroporation of ShRNA-*Lzts1* does not lead to Sox2  
724 ectopic expression. **C-F-** Knock-down of *Lzts1* (ShRNA-*Lzts1*) while inhibiting cell death  
725 (co-electroporation with a P35 expressing vector) leads to ectopic Tuj1 (membrane) and  
726 Huc/D (cytoplasmic) expression, neuronal markers in the VZ, with neurons still attached to  
727 the apical surface (RFP, red and Hoechst, blue). The number of Tuj1 protrusions (**D**) and  
728 ectopic Huc/D cells (**F**) in the VZ per section were quantified two days after the  
729 electroporation (n=3 animals / 41 sections-Tuj1 and 35 sections-HuC/D), and compared to a  
730 control experiment (ShRNA-scramble (scr) + P35; n=3 animals / 42 slides-Tuj1 and 24 slides-  
731 HuC/D). (Two-tailed Mann-Whitney test, error bars represent SD). **G-H-I-** Co-expression of  
732 HoxB8 with ShRNA-*Lzts1* (in the P35 context) leads to less Sox2 ectopic cells in the MZ  
733 compared to HoxB8 co-expressed with the scramble ShRNA (RFP, red, GFP, green and  
734 Hoechst, blue, scale bar 50µm). The percentage of ectopic Sox2+ cells among the GFP+ cells  
735 in the MZ was counted two days after the electroporation of ShRNA-scramble + HoxB8 +



736 P35 (n= 3 animals / 32 sections) and shRNA-Lzts1 + HoxB8 + P35 (n= 3 animals / 38  
737 sections) (Two-tailed Mann-Whitney test, error bars represent SD).

738

739 **Figure 7: Model**

740 **A-** During the developing spinal cord, non-HoxB proteins start to be expressed in the trunk  
741 neural tube at E4 and are expressed in a clear antero-posterior spatial collinear manner, and  
742 mainly in the motor neurons territories. **B-** HoxB proteins (except HoxB13) are expressed  
743 during early neurogenesis (from E3), present poor antero-posterior spatial collinearity (largely  
744 overlapping expression from neck to tail. They are not expressed in motor neuron territories  
745 but preferentially expressed in the IZ at E4. The data presented in this study show that HoxB  
746 proteins control *Lzts1* expression in the IZ which controls neuronal delamination (B).

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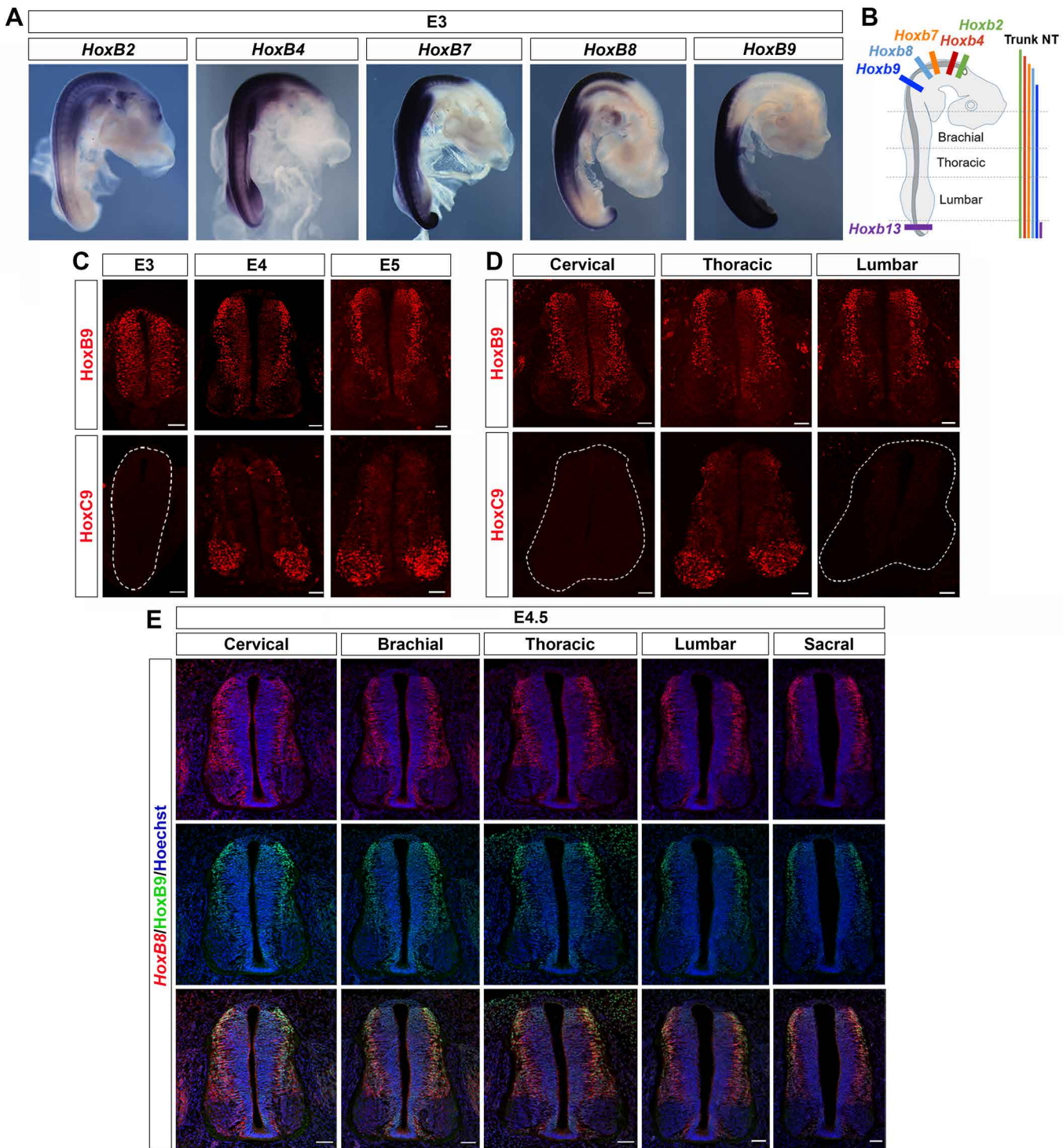


Figure 1

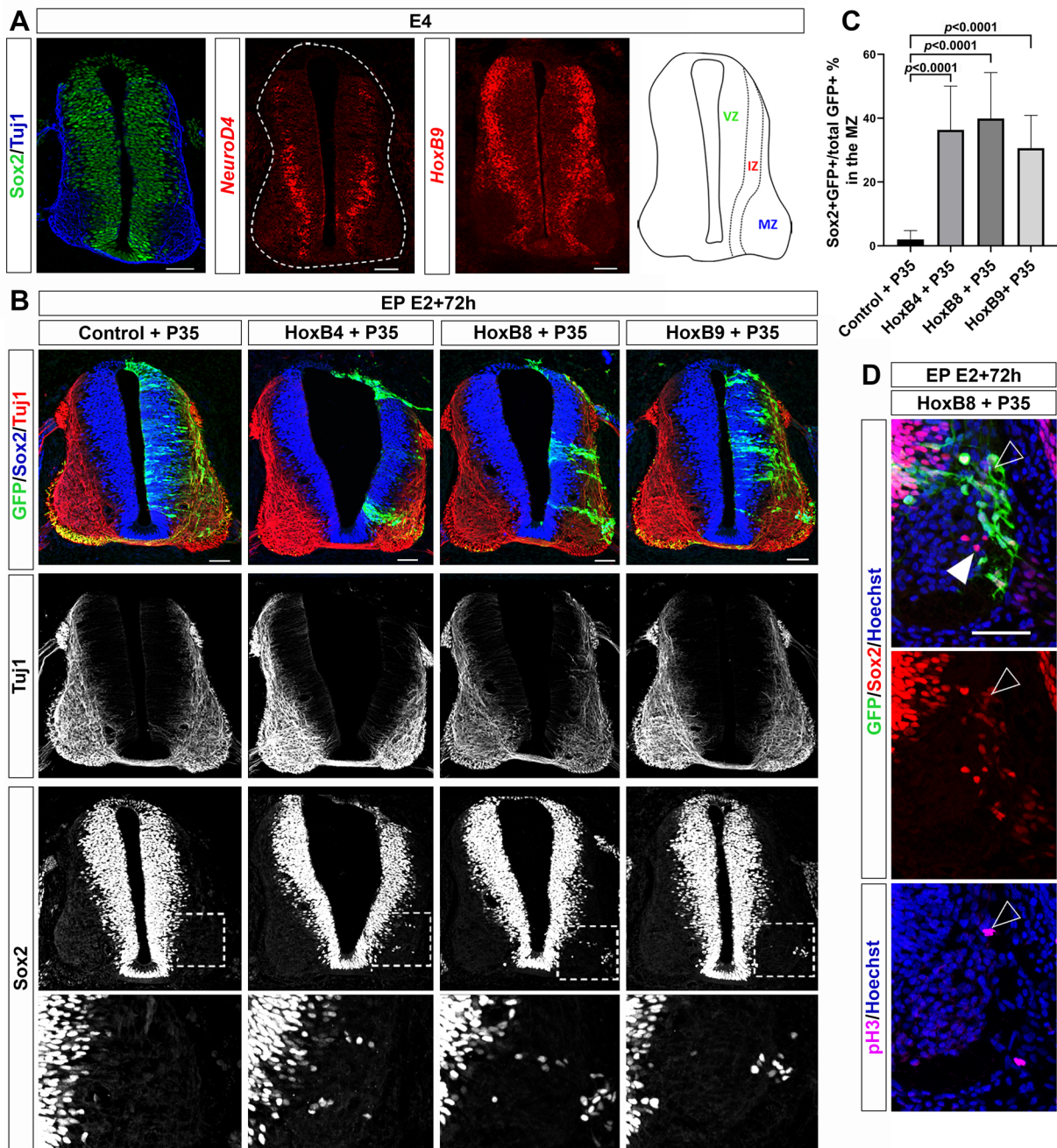
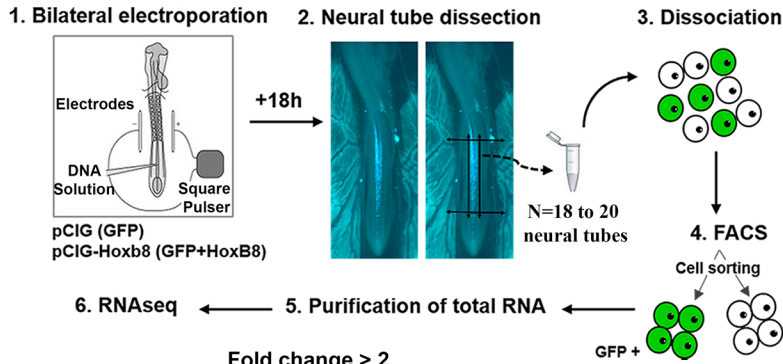


Figure 2

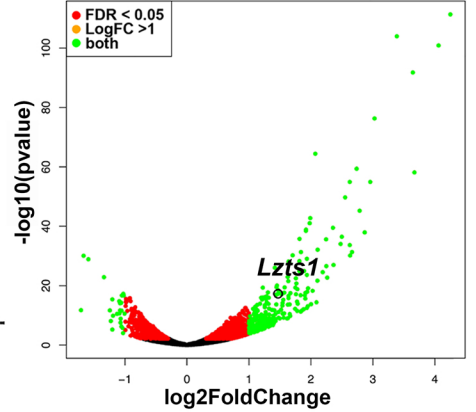


**A**

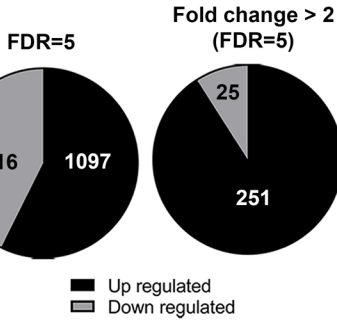


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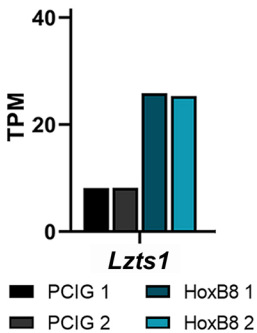
Volcano Plot DGE GFP+ pCiG-HoxB8 vs pCiG



**C**



**E**



**D**

GOEA biological process complete - HoxB8/PCIG upregulated genes	Fold Enrichment	P value
Regulation of signaling	1.64	1.29E-09
Regulation of cell communication	1.61	8.70E-09
Neuron differentiation	1.99	7.40E-07
Regulation of cell migration	2.12	2.00E-06
Transmembrane receptor protein tyrosine kinase signaling pathway	2.54	6.09E-06
Movement of cell or subcellular component	1.75	7.46E-06
Positive regulation of Wnt signaling pathway	3.40	9.73E-05
Positive regulation of catalytic activity	1.65	1.03E-04
Regulation of plasma membrane bounded cell projection organization	2.01	1.25E-04
Regulation of cell adhesion	1.93	4.60E-04

GOEA biological process complete - HoxB8/PCIG downregulated genes	Fold Enrichment	P value
Ribonucleoprotein complex biogenesis	4.18	1.63E-15
Morphogenesis of an epithelium	3.14	8.44E-08
Neural tube formation	5.14	5.10E-06
Anterior/posterior pattern specification	3.30	8.53E-06
Regulation of neuron differentiation	2.36	1.17E-05
Negative regulation of apoptotic process	2.27	1.26E-05
Cell cycle checkpoint	3.72	3.30E-05
Chromatin assembly or disassembly	4.14	1.56E-04
Notch signaling pathway	4.22	2.68E-04
Movement of cell or subcellular component	1.64	5.48E-04

Figure 3

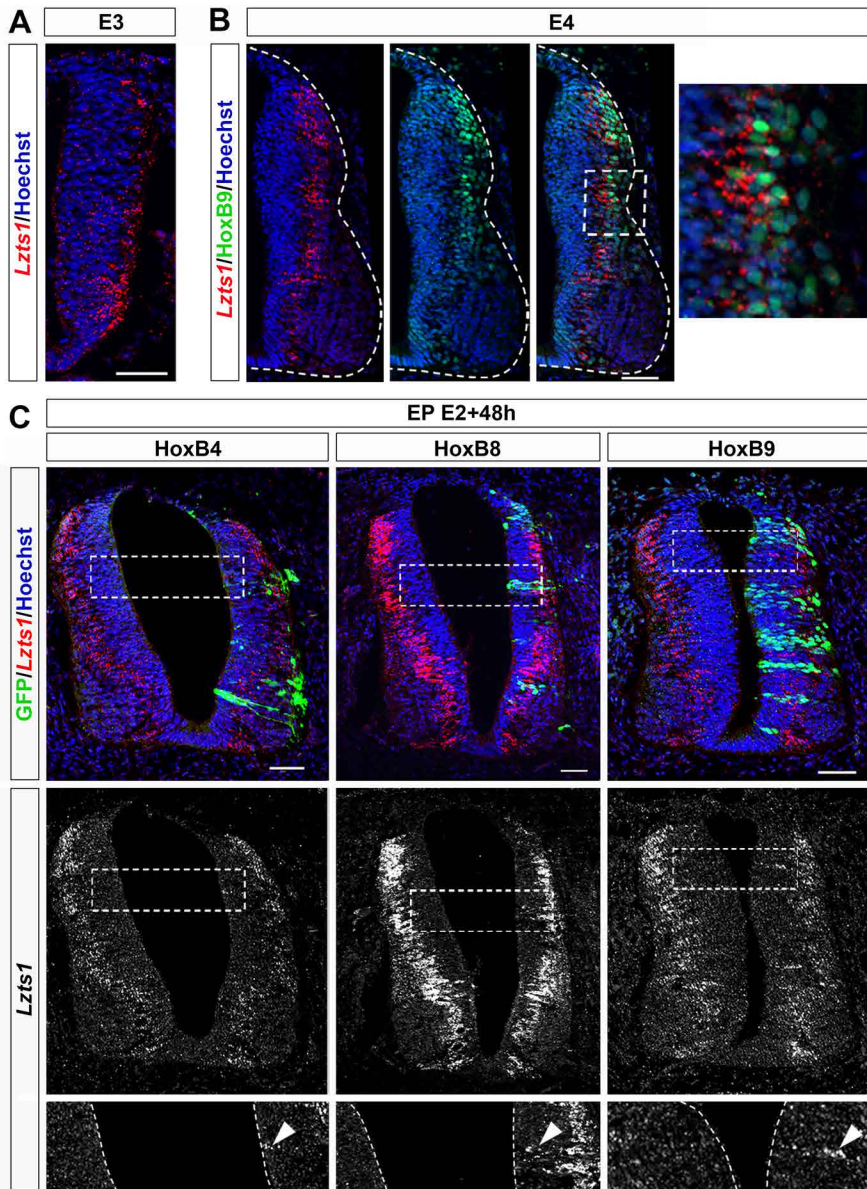


Figure 4

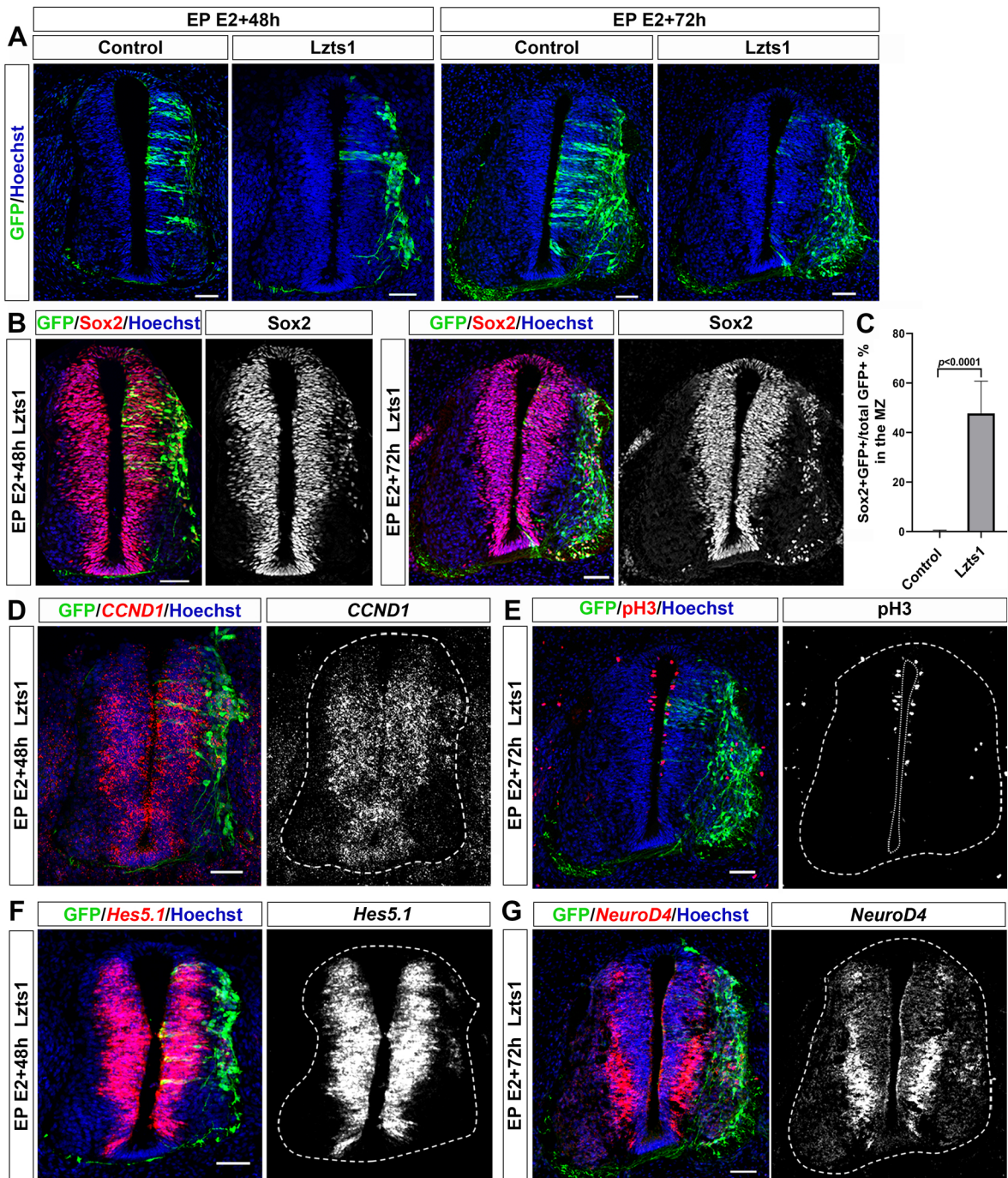


Figure 5

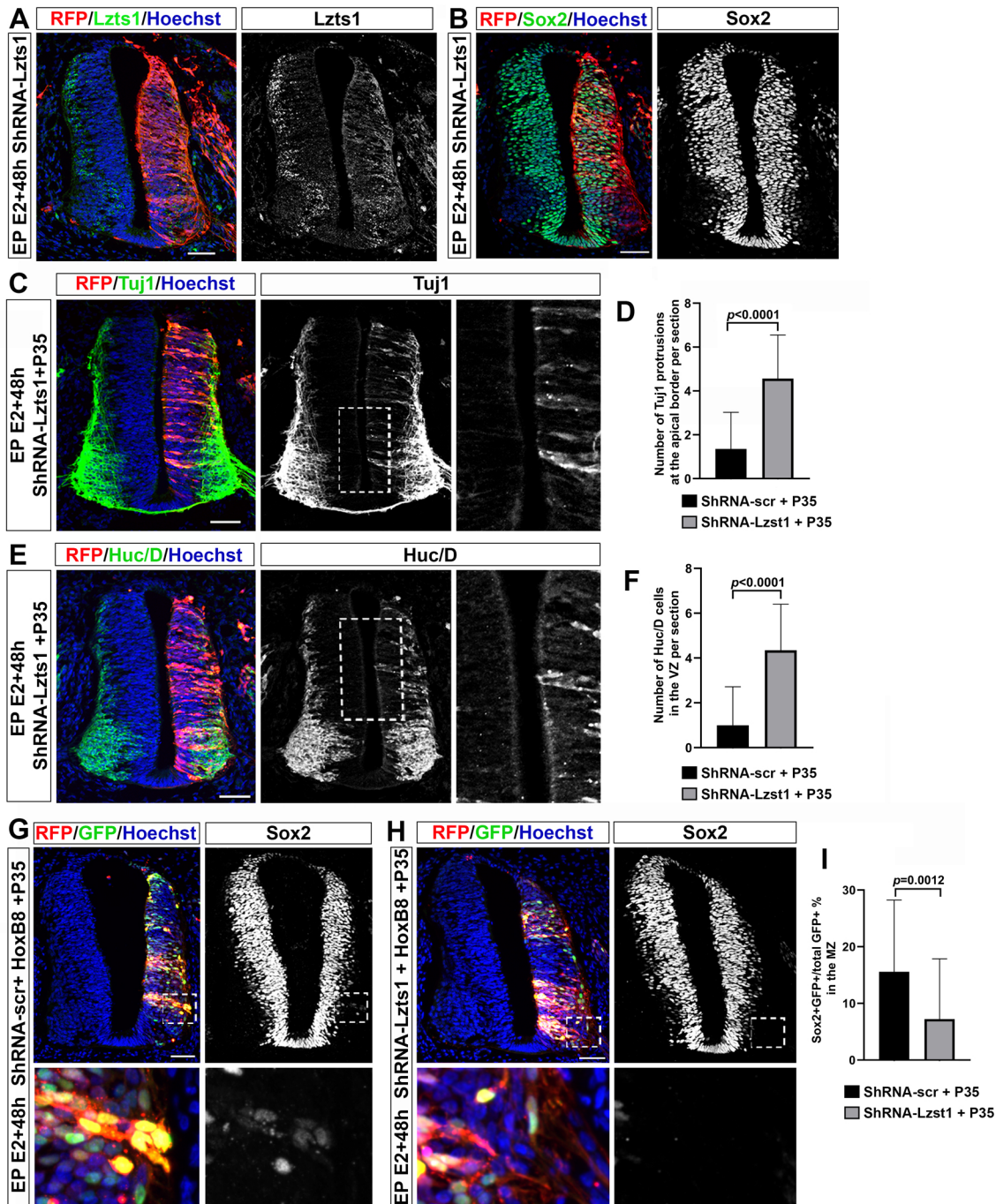


Figure 6

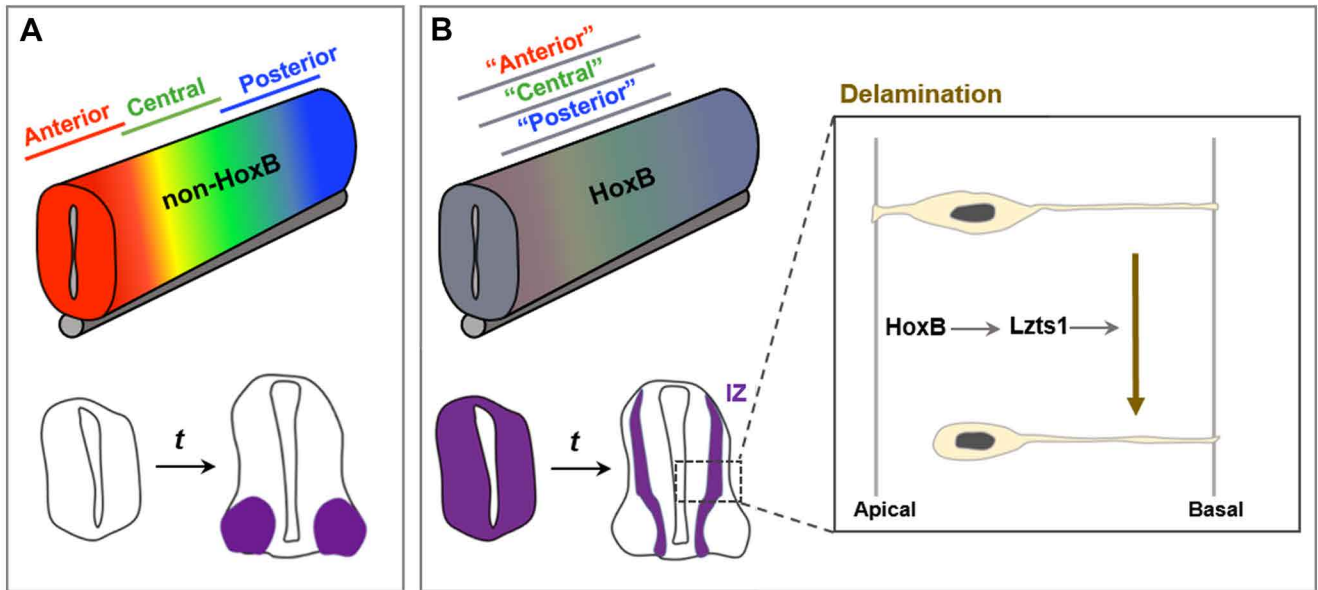


Figure 7