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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: TAATACGACTCACTATAGGGCCTCGGGGCTCTTCTACCC, 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*⁺ cells among the GFP⁺ 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* in
687 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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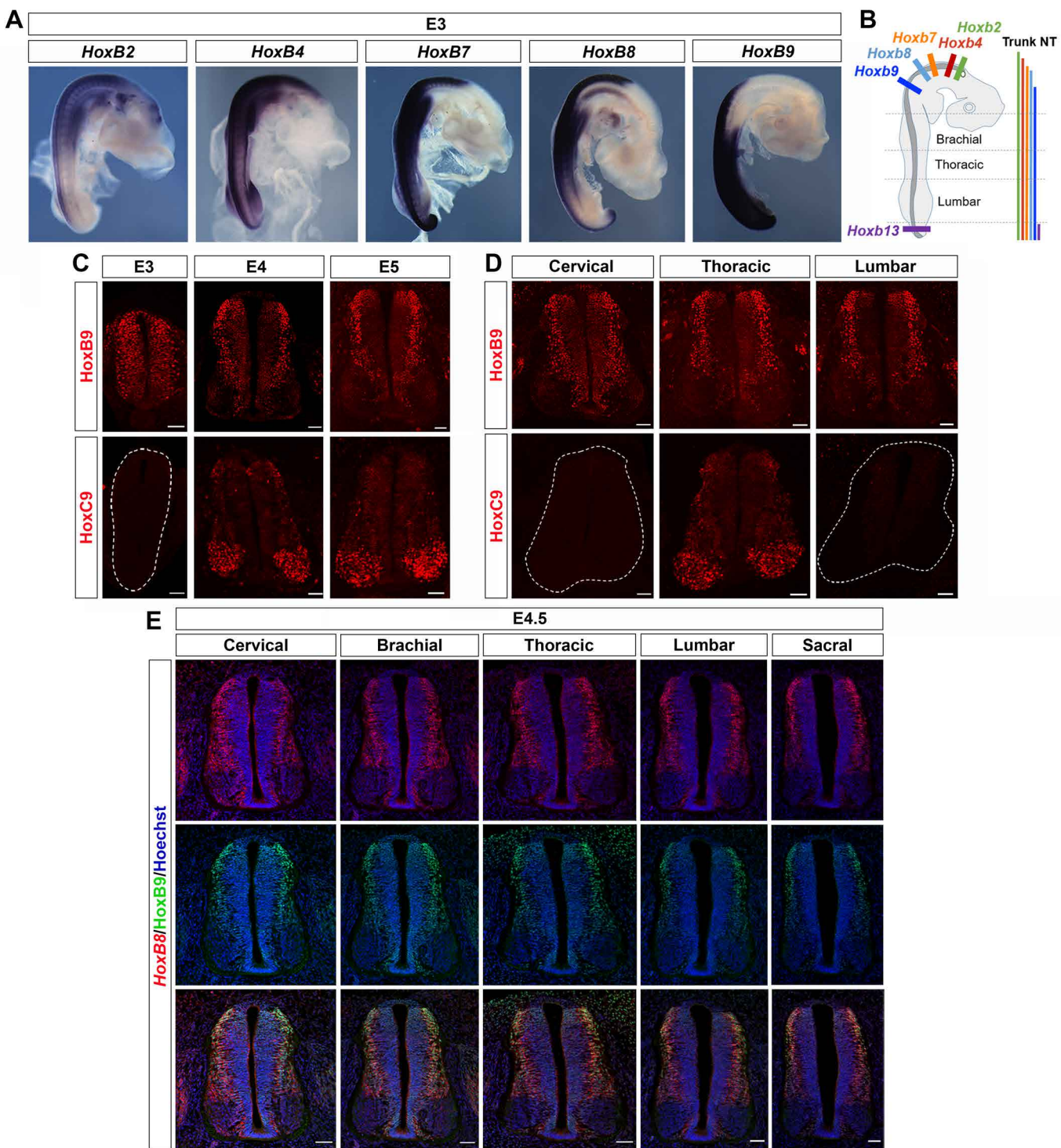


Figure 1

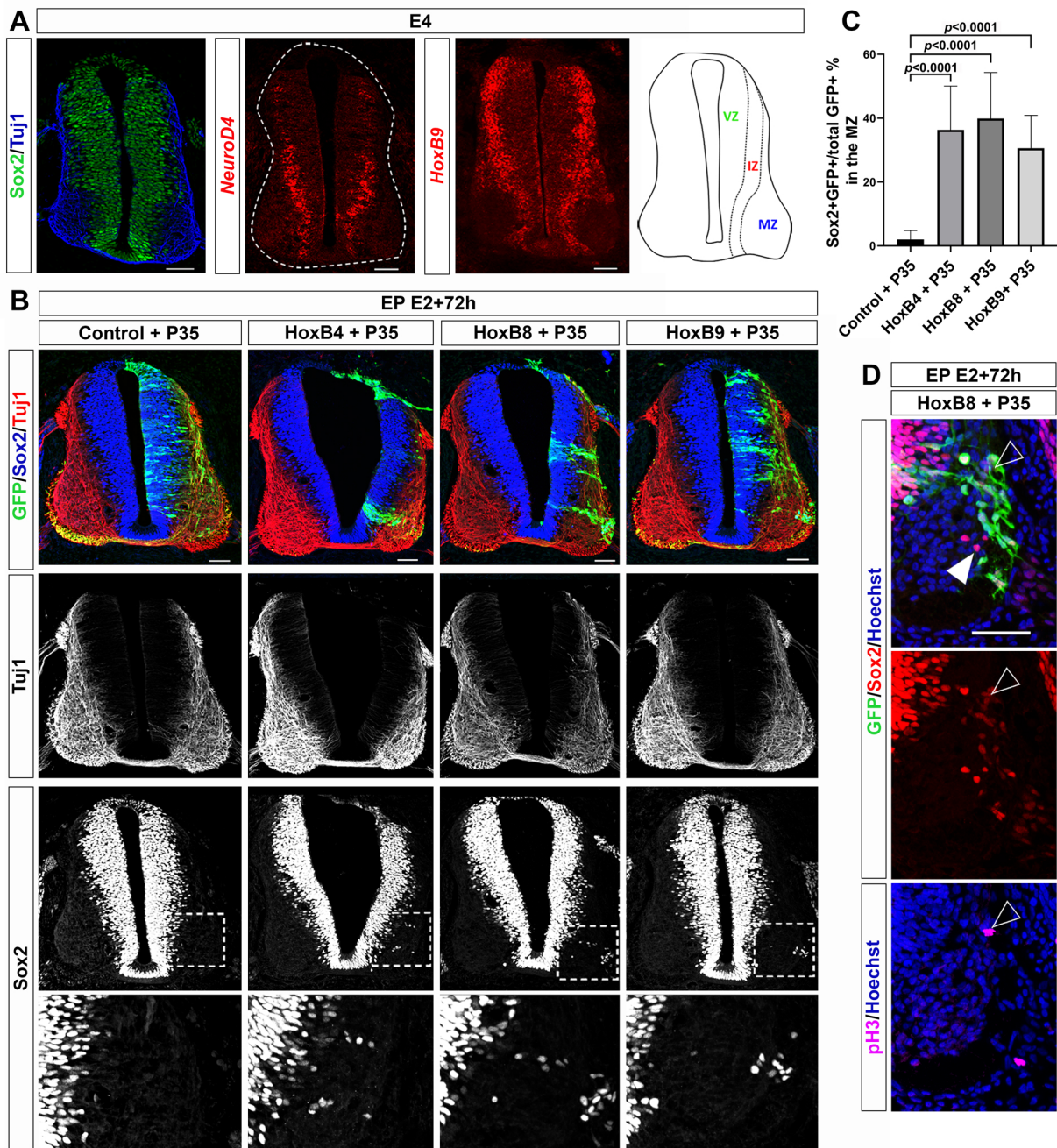
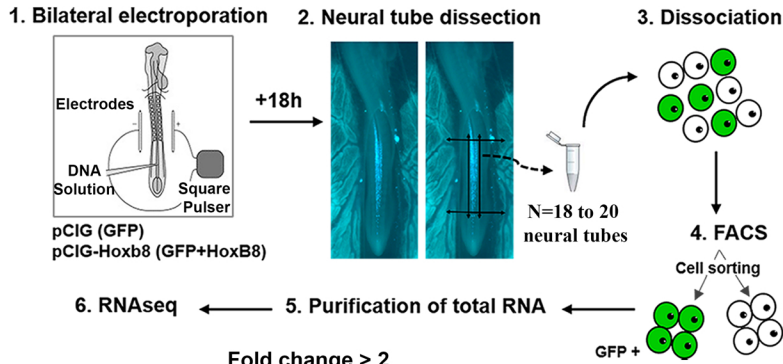


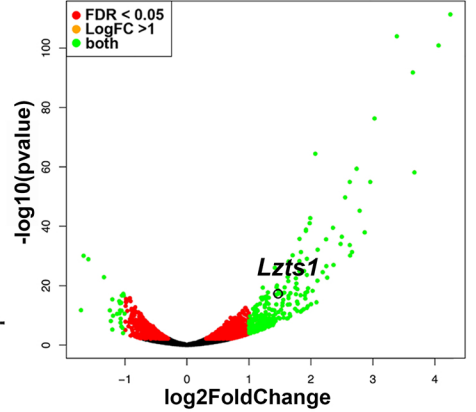
Figure 2

A

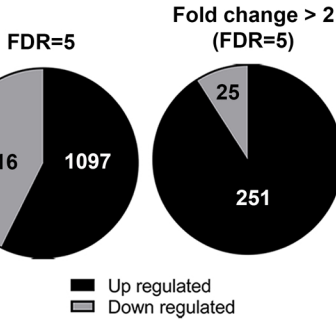


B

Volcano Plot DGE GFP+ pCiG-HoxB8 vs pCiG



C



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

E

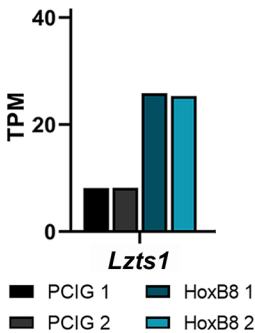


Figure 3

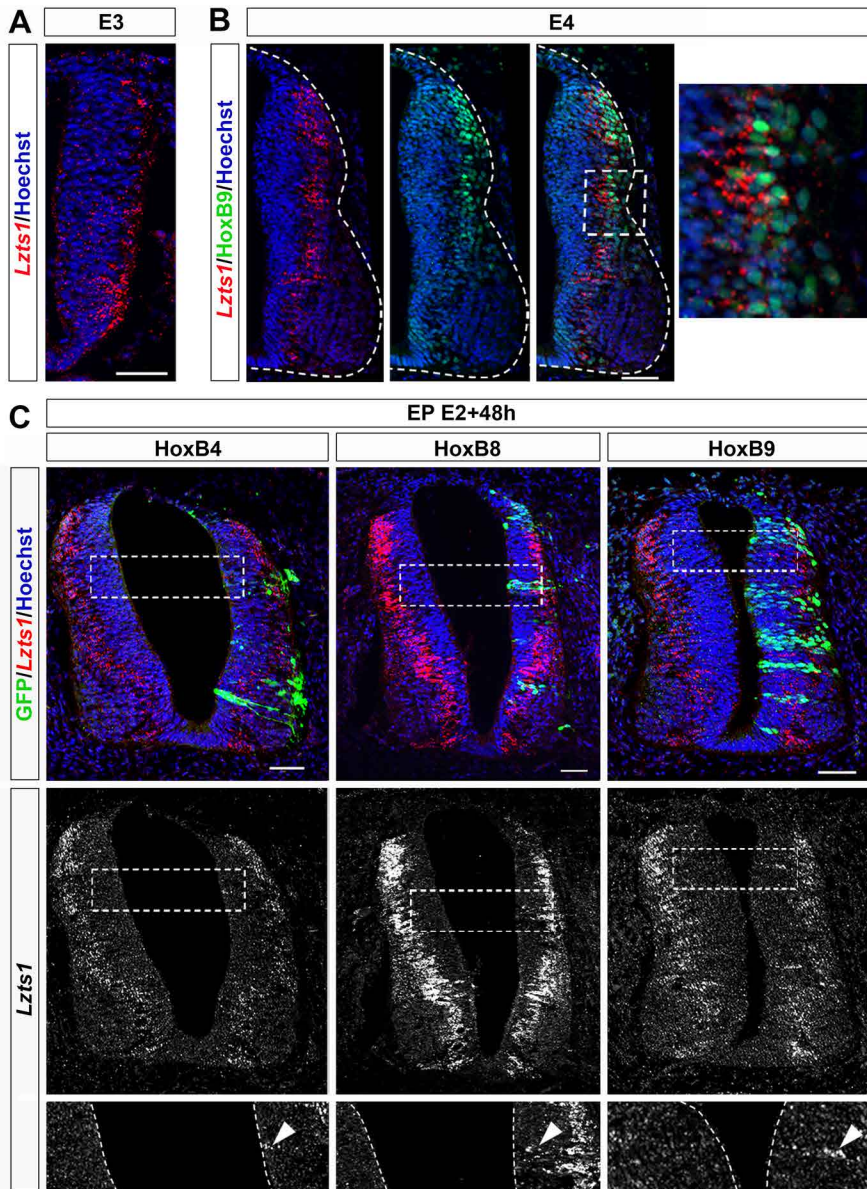


Figure 4

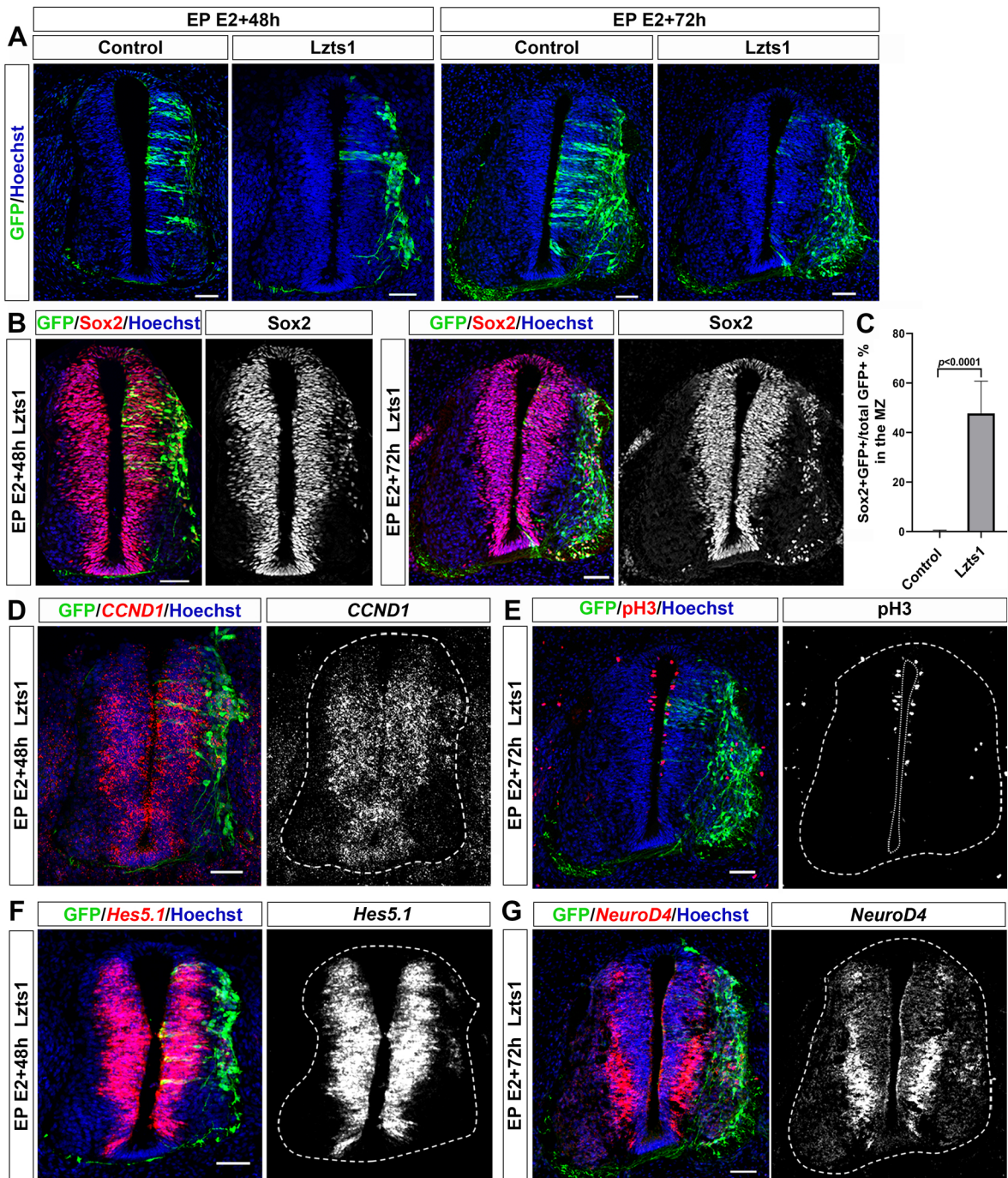


Figure 5

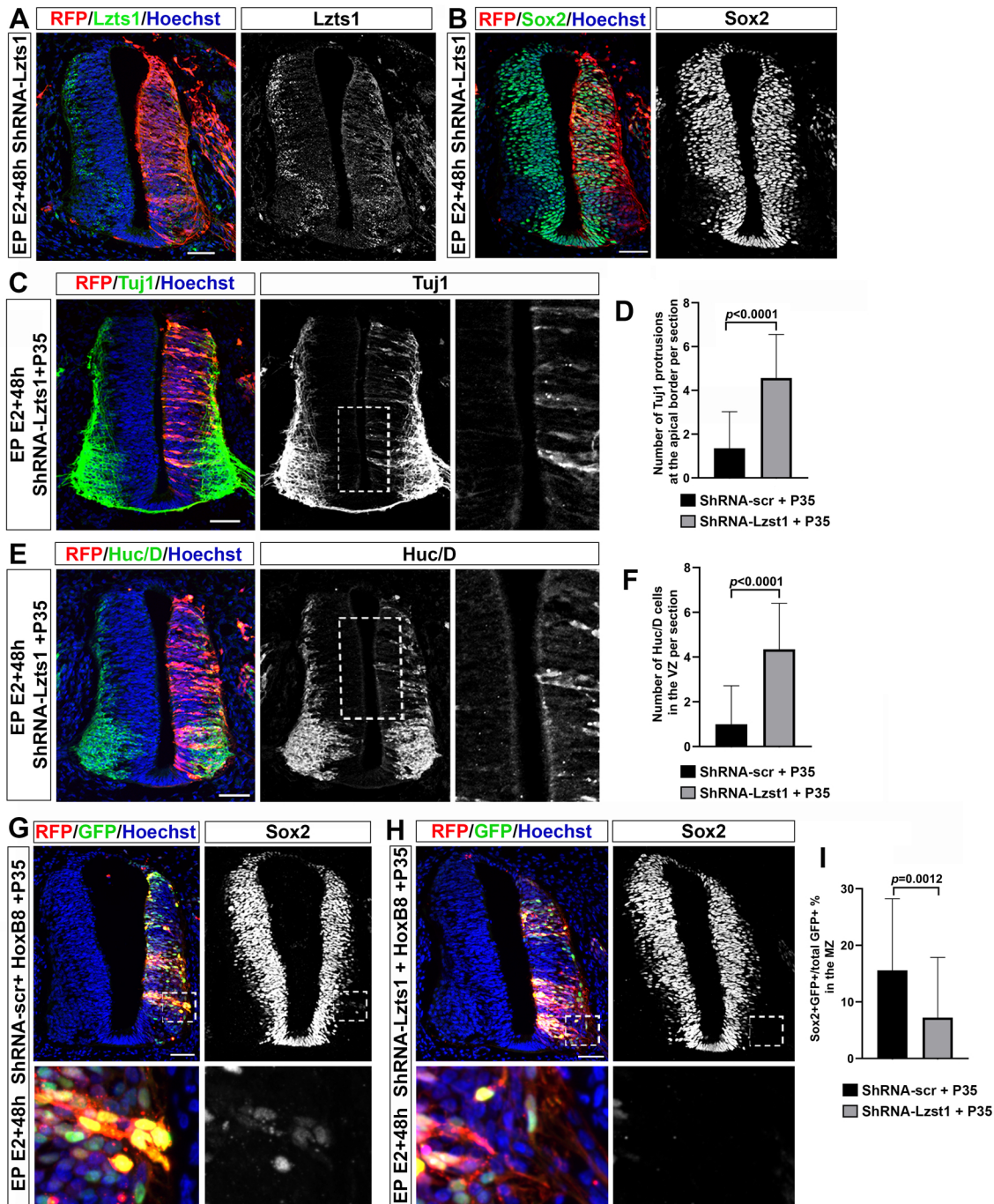


Figure 6

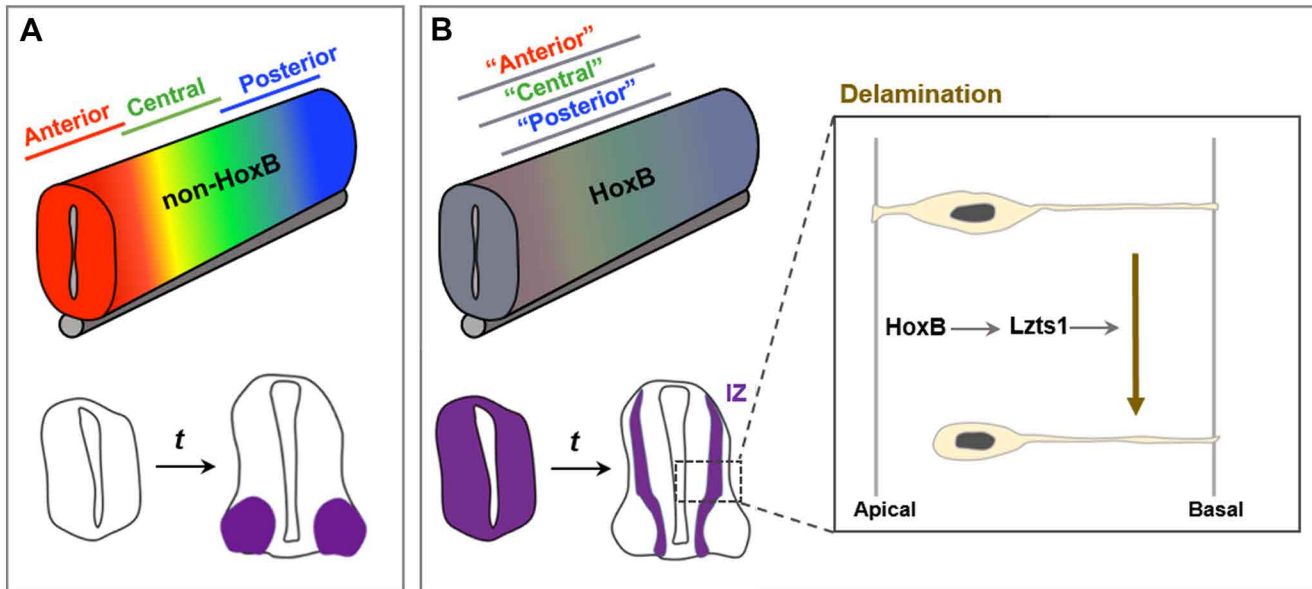


Figure 7