Notch signaling is a critical initiator of roof plate formation as revealed by the use of RNA profiling of the dorsal neural tube

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Abstract

Background: The dorsal domain of the neural tube is an excellent model to investigate the generation of complexity during embryonic development. It is a highly dynamic and multifaceted region being first transiently populated by prospective neural crest (NC) cells that sequentially emigrate to generate most of the peripheral nervous system. Subsequently, it becomes the definitive roof plate (RP) of the central nervous system. The RP, in turn, constitutes a patterning center for dorsal interneuron development. The factors underlying establishment of the definitive RP and its segregation from NC and dorsal interneurons are currently unknown.

Results: We performed a transcriptome analysis at trunk levels of quail embryos comparing the dorsal neural tube at premigratory NC and RP stages. This unraveled molecular heterogeneity between NC and RP stages, and within the RP itself. By implementing these genes, we asked whether Notch signaling is involved in RP development. First, we observed that Notch is active at the RP-interneuron interface. Furthermore, gain and loss of Notch function in quail and mouse embryos, respectively, revealed no effect on early NC behavior. Constitutive Notch activation caused a local downregulation of RP markers with a concomitant development of dI1 interneurons, as well as an ectopic upregulation of RP markers in the interneuron domain. Reciprocally, in mice lacking Notch activity, both the RP and dI1 interneurons failed to form and this was associated with expansion of the dI2 population.

Conclusions: Collectively, our results offer a new resource for defining specific cell types, and provide evidence that Notch is required to establish the definitive RP, and to determine the choice between RP and interneuron fates, but not the segregation of RP from NC.

Keywords: BMP, Quail embryos, delta1, Dorsal interneurons, Fate segregation, Mib1, Mouse embryos, Neural crest, Notch
Summary statement
A new set of genes involved in Notch-dependent roof plate formation is unraveled by transcriptome analysis.

Background
Defining the mechanisms that generate a sequence of specialized cell types and further coordinate their functions is critical for understanding organ development. The dorsal neural tube (NT) epithelium is an excellent system to tackle these questions as it is a highly dynamic structure in both time and space [1]. Initially, it is transiently populated by premigratory neural crest (NC) cells. The latter exit the NT to form the peripheral nervous system as well as a rich assortment of additional derivatives that vary along the neuraxis [2–4]. Subsequently, the dorsal NT midline domain becomes the definitive roof plate (RP) of the spinal cord which is flanked ventrally by dorsal interneuron populations [1, 3, 5, 6].

The RP acts as a neural patterning center that secretes growth factors of the bone morphogenetic protein (BMP) and Wnt families, both crucial for dorsal interneuron development [7–14], and for proliferation of ependymal cells at later stages [15, 16].

The morphology of the RP differs along the axis. In the hindbrain, it is composed of a large sheet of cells, whereas more caudally, it is a relatively thin, wedge-shaped strip of cells located along the midline of the spinal cord [17, 18]. Fate mapping analysis revealed that the RP generates the choroid plexus at cranial levels of the axis [17, 18]. In addition, the RP is transformed into radial glia-like cells [16, 19–21], recently found to support growth of spinal cord axons [22]. These radial glial cells eventually generate the stem cell-containing dorsal ependymal zone in the adult spinal cord of humans and rodents [15, 16, 23].

In spite of its pivotal significance, the origin of the RP in the NT and how it segregates from the earlier NC remained elusive for many years. In fact, the closing dorsal NT was classically termed “RP,” yet, essentially, it included the premigratory cohort of NC [1]. To note, several differences exist between these two populations, whereas NC cells actively proliferate and RP progenitors withdraw from the cell cycle [5, 24]. Moreover, BMP signaling is needed for NC emigration and for initial RP formation, yet the consolodating RP becomes refractory to BMP activity even if it continues synthesizing the protein [5].

To begin understanding the relationship between NC and RP, we previously implemented spatio-temporally controlled lineage analysis in the avian trunk. This revealed first, that the dorsal NT is sequentially transited by distinct cell populations that emigrate to yield NC derivatives [25, 26]; second, that RP progenitors originate ventral to the premigratory NC; and third, that these RP precursors relocate ventro-dorsally to reach their final position in the dorsal midline of the NT upon completion of NC exit [25]. Furthermore, tracing the dynamics of the NC marker Foxd3 using a specific reporter revealed that NC and RP progenitors are initially part of the Foxd3 lineage yet RP precursors downregulate Foxd3 while relocating into the dorsal midline, thus segregating from the NC [26]. The dorsal NT is, therefore, a dynamic area in which progressive NC emigration takes place until replacement by the definitive RP, resulting in the separation between central and peripheral branches of the nervous system [1, 25].

The precedent results provided the basis for understanding the transition between NC and RP stages. Nevertheless, a serious limitation for further mechanistic understanding of this process was the lack of differential markers expressed in either population. Whereas several NC-specific genes are known (e.g., Foxd3, Sox9, Snail2), many of them (e.g., Lmx1a, Lmx1b, Msx, BMP4, Wnt1) continue to be expressed in RP, and only few are known to be expressed uniquely in the latter (e.g., cHes1/Hairy1, R-spo1) [5, 27]. For elucidating RP formation, availability of such unique genes is of utmost significance to serve both for identification purposes as well as for functional studies.

In addition to its separation from the NC lineage, a boundary separates the ventral domain of the nascent RP and prospective dorsal interneurons along the spinal cord. Although the RP secretes BMP and Wnt proteins responsible for specification and differentiation of selected interneuron cell types (see above), the factors that determine the establishment of the RP and/or the formation of the boundary between RP and interneurons remain unknown. At cranial levels, maintenance of the boundary between RP and the rhombic lip neuroepithelium was shown to be mediated by Delta (DII)-Notch signaling but its initial formation was not investigated, neither at cranial levels, nor in the trunk [18]. Notch and its ligands are ubiquitously used for coordination of differentiation between neighboring cells in processes such as boundary formation, lateral inhibition, and cell fate determination [28–30].

In the present study, we performed a transcriptome analysis at trunk levels of quail embryos comparing the dorsal NT domain at premigratory NC and RP stages. This analysis yielded genes that were differentially and specifically expressed in either NC or RP. Furthermore, we unraveled a molecular heterogeneity within the trunk-level RP, with transcripts expressed mainly in its lateral or medial domains, respectively. Collectively, our results provide a new resource for investigating a fundamental fate transition during neural development. By implementing the newly described genes in both quail and mouse embryos, we further examined the hypothesis that Notch signaling is involved in de novo RP
development in the trunk. First, we observed that Notch is active in the dorsal NT except for the dorsal midline region and a similar dorsal limit of mRNA expression was observed for Dll1, Serrate/Jagged, and Lfng, altogether suggesting that Dll-Notch signaling operates at the boundary between the prospective RP and dorsal interneurons. Gain of Notch function in quails revealed a cell-autonomous inhibition of RP markers and their replacement by d11 interneurons. Additionally, an ectopic induction of the expression of RP traits was apparent ventral to the endogenous RP and this was associated with reduced expression of BarHL1, a d11 interneuron marker. Reciprocally, loss of Notch function was performed in mice by tissue specific knockout of the E3 ubiquitin ligase mindbomb1 (Mib1) to remove the function of all Notch ligands in the dorsal NT and its derivatives. In Wnt1-Cre; Mib1fl/fl embryos, no RP or d11 interneurons developed. Concomitantly, d12-dorsal interneurons expanded dorsal-ward. In contrast, both gain and loss of Notch function revealed no effect on emigration/migration of NC cells. Together, we provide evidence that Notch signaling is both sufficient and necessary for the choice between RP and interneuron fates without affecting early stages of NC ontogeny.

**Results**

**Transcriptional profiling of the dorsal NT at early premigratory NC and RP stages**

To conduct a genome-wide transcriptome analysis of dorsal NT development during the transition from NC to RP stages, we electroporated a GFP-encoding plasmid into the dorsal domain of quail NTs at either stage (18ss for NC and 43ss for RP, respectively, Additional file 1: Fig. S1A,B,E,F). Six to 8 h later, NTs were isolated and dissociated into single cells, and fluorescent cells were FAC-sorted. No GFP+ cells were apparent in control, non-electroporated samples (Additional file 1: Fig. S1C, G). In contrast, and as expected from focal transfections, the percentage of live, GFP+ cells was 0.32% and 0.28% of the total input for NC and RP, respectively. In both control and electroporated cases, very low proportions of propidium iodide (PI)+ dead cells were found; these were excluded by gating and only 1000–6000 GFP+/PI− cells/sample were collected (Additional file 1: Fig. S1D, H).

Next, harvested cells were processed for RNA-sequencing (RNA-seq) in triplicates for each stage (Fig. 1a–c). Principal component analysis (PCA) showed that NC and RP samples cluster into two distinct groups (Fig. 1a). Differential gene expression analysis revealed significant upregulation of 1243 and downregulation of 1068 genes in RP when compared to NC, respectively, providing a rich data source for further functional analysis (Fig. 1b, c and Additional file 2: Table S1 and Additional file 3: Table S2).

Further examination by ingenuity pathway analysis (IPA), revealed functional categories containing genes with significant expression changes. Those which were enriched in RP compared to NC (Fig. 1d) included “development of the body trunk,” “development of central nervous system,” and “development of the spinal cord (SC), reflecting that the RP is a part of the central nervous system. Furthermore, among the RP enriched categories was “epithelial tissue development,” consistent with our previous findings showing that during the transition between NC and RP, the latter regains epithelial characteristics [5]. Enrichment of additional categories such as “guidance of axons” and “midline defects” are also consistent with the established role of the RP as a guidance center for adjacent interneuron projections and that RP abnormalities are a source of NT closure defects, such as spina bifida [31, 32]. The presence of “differentiation of neurons” in this analysis may possibly arise because the RP secretes necessary factors for interneuron differentiation [12, 33] and/or due to the inclusion of interneuron progenitors located adjacent to the RP in the sorted cell suspensions.

Ingenuity pathway analysis uncovered cell cycle regulation as the major functional group downregulated in RP compared to NC (Fig. 1e). This is consistent with the marked change in proliferative properties, as revealed by BrdU incorporation, between mitotically active premigratory NC compared to the definitive RP, that progressively turns into a post-mitotic structure (45.9% ± 4% vs. 4.8% ± 0.5%, respectively, Fig. 1f–h) (see also [5]). Along this line, MycN mRNA, a marker of proliferative cells [34], was present in the dorsal NT at the NC stage, yet was downregulated in RP (Fig. 1i, j). Additional cell cycle associated genes were reduced in RP by at least threefold, such as Cdk2-Ap2, olfactomedin4, S100A11, Dlg1, cyclin D3, and Rgce (Supplementary Table S1), whereas cell cycle inhibitors were upregulated (Cdkn2c, Cdkn1c, Dach1 and 2, Btg11, Ndrg1 (Additional file 3: Table S2).

Taken together, enrichment of the above categories substantiate the validity of our molecular analysis in exposing well-known differential traits of premigratory NC versus RP populations.

**The expression patterns of differential genes**

**NC versus RP markers**

To further validate our RNAseq results, we examined by in situ hybridization (ISH) the localization of genes known to be differentially expressed in either NC or RP, as well as that of transcripts never tested before in this context. We present a subset of them alongside graphical representations of their normalized expression based on triplicate samples at either stage. Consistent with our previous results [25, 26, 35], the NC markers FoxD3 and Snail2 were downregulated at the RP stage
Fig. 1. (See legend on next page.)
Inducers of RP formation were also selected based on their expression profiles in the RP. These included the Wnt inhibitors Dact2, Sfrp2, and Dkk1 (Additional file 4: Fig. S2G-O). In all cases, there was a positive correlation between the transcriptome data and the ISH patterns. Additional transcripts significantly reduced in RP when compared to NC were Ltk, Mesp1, Runx1, Calponin2, Ets1, Ets2, Glipr2, Ptn, Ripply1, Lmo4, Mylk, Sall4, syndecan4, Cdx2, Cdx4-like, Spry2, Nrp2, Tjap2c, Tspan1, Sax2, Me2c, and Gata5 (Additional file 2: Table S1). Cell adhesion molecules also changed between the stages, with Cad1, Cad6, Cad11, Cad20, Pcdh1, Pcdh19, and B-cadherin-like, being downregulated in RP. Among upregulated cell adhesion genes were Pcdh8, Pcdh9, Pcdh11, Pcdh18, Cad4, Cad8, Cad22, and SDK2 (Supplementary Table S2). However, N-Cad (Cdh2) mRNA levels did not change, consistent with previous results showing that the dynamics of N-cad function in the dorsal NT is regulated by post-transcriptional processing [36].

Importantly, we found a selection of genes expressed in RP but not premigratory NC (Fig. 2). These included the RP-specific Spondin Rspl [27], and HES4, the quail ortholog of chick Hairy1 that exhibits 94% identity at the protein level. In addition, the BMP member Gdf7, and the BMP antagonists BAMBI and Gremlin, the retinoic acid-associated genes Raldh2 and CRABP1, and the chemorepellents of commissural axons Draxin and Slit1 are also included (Fig. 2).

In the category of genes enriched in RP compared to NC, there were additional transcripts that exhibited a wider expression pattern that included, but was not restricted to the RP. Examples were Norrin (NDP), LRP8, Znf536, and Zic4 (Additional file 5: Fig. S3). In all cases, there was also a good correlation between the differential expression patterns and transcriptome results. Together, our RNAseq provided us with a set of spatiotemporal-specific genes appropriate for cell type identification and for functional studies.

Expression of “non-RP” markers is complementary to the RP-specific genes

While attempting to validate by ISH the localization of differential transcripts, a subset of genes showing a significant upregulation in the RP (Additional file 6: Fig. S4, right panels) yielded a surprising localization pattern. Dach1, Lfng, Cdkn2c, Bcl11b, Wnt4, Olfm1, and Ins1 mRNAs were all expressed in different cell subsets along the NT, except for the RP itself which remained negative (Additional file 6: Fig. S4). This observation suggests that results of RNAseq cannot be taken as the sole criterion for marker expression in a given cell type, and further spatial confirmation by ISH is warranted.

We predicted that the precedent genes could prove to be useful in defining the extent of the nascent RP, if delimiting the ventral boundary of this structure. To this end, we performed ISH of adjacent sections at the RP stage, combining the Notch-related gene Lfng with a set of RP-specific transcripts. In all cases examined, the dorsal limit of Lfng expression corresponded to the ventral limit of localization of Rspl, Hes4, Bambi, Draxin, Raldh2, Gdf7, and Crabp1, respectively (Additional file 7: Fig. S5). Reciprocally, the RP-specific Rspl gene was hybridized in combination with various “non-RP” genes such as Bcl11b, Dach1, Olfm1, Ins1, and Wnt4, exhibiting a comparable complementary pattern (Additional file 8: Fig. S6, A-O). Hence, the expression of RP and “non-RP” genes enabled us to define the precise size of the RP territory. Measurement of the number of Hoechst-positive nuclei within the RP territory delimited by Lfng, revealed that the flank-level RP at E3.5 of quail development consists of 61 ± 1.92 cells per 10 μm section (N = 4, Additional file 8: Fig. S6P-R).

Molecular heterogeneity within the RP

Careful inspection of the patterns of expression of RP genes, revealed that Gremlin1, Bambi, Raldh2, Hes4, and Draxin were homogeneously distributed throughout the RP (Fig. 2 and Additional file 7: Fig. S5). In contrast, the downstream Wnt target Axin2, the Wnt-associated ubiquitin ligase Rnf43, and the mRNA encoding the Rspl receptor Lgr4 were confined to the medial domain of the RP (Additional file 9: Fig. S7).
Fig. 2. (See legend on next page.)
A reciprocal pattern was observed for Rspos1 that was primarily transcribed in two peripheral stripes of the RP delimiting the central domains of expression of Axis2, Rnf43, and Lgr4 (Additional file 9: Fig. S7) and abutting the ventral limits of the RP (Additional file 7: Fig. S5A-C and Additional file 8: Fig. S6). A similar pattern was observed for Gdf7 (Additional file 7: Fig. S5P-R, Fig. 2, see also [18]) and CRABP1 (Additional file 7: Fig. S5S-U, and Fig. 2). Hence, similar to the RP at the hindbrain level [17], the flank-level RP also comprises at least two distinct domains, a medial and two flanking lateral regions (Additional file 9: Fig. S7).

**The role of Notch signaling in RP development**

Notch activity was found to be involved in maintenance of the hindbrain RP [18]. By implementing a subset of the newly identified genes described above, we investigated whether this signaling pathway plays a role in de novo RP formation at spinal cord levels.

**Expression and activity of Notch ligands and receptors in the dorsal NT**

Expression ofDll1, Jagged1, and Jagged2 was evident in various domains of the quail NT at E4, except for the RP; thus, localization of ligand mRNAs was complementary to Rspos1 (Additional file 10: Fig. S8). Likewise, LFNG, that facilitates Notch signaling, exhibited a similar non-RP pattern (Additional file 9: Fig. S7). Notch1 was widespread throughout the ventricular layer of the NT, exhibiting an apical localization in RP, and Notch2 was transcribed in RP (Additional file 10: Fig. S8).

To examine the activity of Notch, we used an antibody specific to its active intracellular domain (ICD) that recognizes the mouse but not the quail protein. Whereas immunolabeling was almost undetectable during the NC stage at E9.75 (Fig. 3a, b), expression of N1ICD was evident at both E10.5 and E11.5 throughout the NT except for the dorsal-most domain corresponding to the nascent and definitive RP, respectively (Fig. 3e, f, i, j). To inactivate Notch signaling in the dorsal NT, we conditionally removed the ubiquitin ligase Mindbomb1 (Mib1) that is required for the functional internalization of all Notch ligands, by intercrossing Mib1f/f and Wnt1-Cre mice [37]. As the Wnt1 promoter drives expression of Cre in the dorsal third of the NT prior to the NC stage, this cross abolishes Notch activity prior to and during RP formation. Indeed, N1ICD immunostaining was specifically absent in the dorsal NT of Wnt1-Cre; Mib1f/f, while still present in more ventral domains (Fig. 3c, d, g, h, k, l), consistent with N1ICD specifically representing Notch activity.

Expression of Dll1 protein exhibited a similar pattern in the wildtype embryos to that observed in quails, with no expression in the dorsal NT and later RP (Fig. 3m, n, q, r, u, v). As expected, Wnt1-Cre; Mib1f/f embryos revealed enhanced Dll1 signal, consistent with the role of Mib1 in targeting Notch ligands for ubiquitination and their accumulation at the cell membrane as inactive ligands in its absence [38]. Most importantly, by E10.5, Dll1 expression shifted dorsally with significantly fewer Dll1-negative cells in the dorsal midline region corresponding to the RP, and Dll1 expression reached the dorsal midline by E11.5 with almost no Dll1-negative cells remaining in Wnt1-Cre; Mib1f/f embryos (Fig. 3o, p, s, t, w–z).

Next, we assessed the dorsal boundary of expression of Dll1 relative to N1ICD. As shown in Fig. 3aa, both markers display a similar dorsal border of expression. To further explore whether the dorsal domain of Notch activity overlaps with the RP, N1ICD and Gdf7 ISH were performed in adjacent sections. The domains of Gdf7 expression and Notch activity were juxtaposed with no apparent overlap (Fig. 3bb, cc, dd), consistent with the complementary expression of Notch ligands and RP markers in the quail (Additional file 10: Fig. S8). Taken together, this indicates that the ventral border of the prospective RP, where both ligands and receptors meet, is an area of Notch activity. Furthermore, the dorsal expansion of Dll1 expression in Wnt1-Cre; Mib1f/f embryos suggests a requirement for Notch signaling in border formation and RP development.

**Gain of Notch function in quail NTs regulates the choice between RP and dI1 interneuron identities without affecting early NC development**

To begin testing the possible involvement of Notch signaling in RP development, we first expressed a constitutively active form of Notch2 (an2) in embryos aged 25ss, prior to RP formation, followed by fixation at E4 (RP stage). Electroporation of an2 prevented, in a cell-autonomous manner, the upregulation of all the RP markers examined, including Rspos1, BAMBI, Raldh2, and Draxin (Fig. 4, arrows). In contrast, control GFP had no effect on the expression of either gene (Fig. 4, arrowheads). Likewise, electroporation of active Notch1 in RP similarly reduced expression of BAMBI, Raldh2,
Fig. 3. (See legend on next page.)
and Draxin (Additional file 11: Fig. S9), suggesting that both Notch1 and Notch2 have similar functions in this context.

Interestingly, when electroporation of aN2 attained areas adjacent to the RP (Fig. 5, arrows), an ectopic up-regulation of Rspo1, BAMBI, Aldh2, and Draxin was often observed more ventrally within the domain populated by dorsal interneurons (Fig. 5, arrowheads). This was not observed upon transfection of control GFP (Fig. 4).

These results suggest that ectopic Notch activation in RP suppresses development of RP-specific traits while inducing them in the adjacent interneuron domain.

To examine the possibility that interneurons developed at the expense of RP cells, we electroporated control GFP or aN2, and sections were subjected to ISH for BAMBI mRNA together with immunolabeling for the dI1 interneuron marker BarHL1. Electroporation of control GFP had no effect on either BAMBI expression in RP or on the distribution of BarHL1+ interneurons immediately ventral to the RP (Fig. 6a–d). In contrast, many dorsal cells misexpressing aN2 lacked BAMBI mRNA and expressed instead the dI1 interneuron marker; as expected, these differentiated neurons localized to the basal domain of the dorsal NT (Fig. 6e–h, arrowheads). Quantification of BarHL1+ dI1 interneurons within the BAMBI+ domain confirmed their ectopic development at the expense of RP (Fig. 6i).

Next, we examined whether gain of Notch activity outside the RP would affect normal dI1 interneuron development. Control GFP did not alter the expression of BarHL1-positive interneurons, whereas aN2-misexpressing cells lacked BarHL1 immunoreactivity (Fig. 6j–o, arrowheads), suggesting a failure of dI1 formation upon excess Notch.

At the time of electroporation (22–25ss), NC cells are already specified in the dorsal NT at the trunk level [39]. To further examine a possible effect of aN2 on NC migration, electroporated embryos were fixed a day later. No significant effect was monitored in the number of GFP-positive NC cells in control vs. aN2-treated embryos (Fig. 6p–t). Altogether, misexpression of active Notch affects the choice between adopting a RP fate or a dI1 interneuron identity, yet has no significant effect on emigration or migration of specified NC progenitors (Figs. 4, 5, and 6).

**Failure of RP formation in mutant mice lacking Notch activity in the dorsal NT**

Next, by implementing various RP-specific markers uncovered in the transcriptome analysis of quail embryos, we asked whether Notch signaling is necessary for RP formation in the mouse. Similar to avians, wildtype mouse embryos expressed Rspo1, GDF7, Raldh2, Slit1, Msx1, and Lmx1A in the trunk RP at E11.5 ((Fig. 7a, e, g, i, k, m, see also [40]). At variance with the quail, BAMBI was expressed at cranial levels of the mouse axis and was weak to undetectable in the trunk (Fig. 7c, and not shown). Similar to avian embryos, Rspo1, Bambi, and Gdf7 were expressed in the lateral part of the RP, suggesting that this structure is heterogeneous in both species. In striking contrast, no expression of the above transcripts was apparent at E11.5 in Wnt1-Cre; Mib1fl/fl mutants (Fig. 7b, d, f, h, j, l, n). Furthermore, co-labeling of adjacent sections with both Lmx1A and Dll1, confirmed the complementary expression of both markers in wildtype embryos, the downregulation of Lmx1A in Wnt1-Cre; Mib1fl/fl embryos and the corresponding dorsalization of Dll1 (Fig. 7m, n). Next, we asked whether the loss of the RP genes was due to a failure in “de novo” RP formation as opposed to its maintenance. To this end, control and mutant embryos were in situ hybridized at E10.5 corresponding to the onset of RP formation, with RP-specific genes not transcribed previously at the NC stage. Expression of Rspo1, Bambi, Gdf7, Raldh2, and Slit1 was undetectable in Wnt1-Cre Mib1fl/fl mutants when compared to wildtype embryos (Fig. 7a–x). Together, our data demonstrate that Notch activity in the dorsal NT is necessary for initiation of RP formation.
Fig. 4. (See legend on next page.)
Loss of Notch results in absence of RP and dI1 interneurons

It has been previously shown that in the absence of a RP, development of dI1, the dorsal-most interneurons, is abnormal [41, 42]. We examined whether Notch-dependent failure of RP formation is associated with a similar phenotype. Math1-positive dI1 interneuron progenitors were located lateral to the RP in E10.5 and E11.5 embryos. In contrast, in Wnt1-Cre; Mib1fl/fl embryos, very few Math1+ progenitors remained at E10.5 and none were detected at later ages (Fig. 8a–d). Hence, in the absence of a RP, the formation of dI1 interneurons is compromised.

Next, we monitored expression of Ngn1+ and Ngn2+ dI2 interneuron progenitors and observed that their localization in the mutants progressively shifted towards the dorsal midline at the expense of the missing dI1 population. Although ISH of these markers prevented us from accurate quantification, a clear increase in the dI2 interneuron subset was apparent at both stages (Fig. 8e–l), suggesting aberrant specification of dorsal progenitors into dI2 neurons. Notably, Isl1+ dI3 interneurons were also shifted dorsal-ward in Wnt1-Cre; Mib1fl/fl embryos, but their total amount was unaffected (Fig. 8m–p, y). Further visualization of AP2α-positive

Fig. 4. Misexpression of active Notch (aN2) in quail neural tubes prevents the upregulation of RP markers. Left column represents electroporation of control GFP (a, g, m, s) (N = 7 for Bambi, and N = 3 for Rspo1, Raldh2, and Draxin) or aN2-GFP (d, j, p, v) (N = 7,15,6,6 for Rspo1, Bambi, Raldh2, and Draxin, respectively). Middle column depicts ISH for the denoted genes in control GFP (b, h, n, t) or aN2 (e, k, q, w), and right column is an overlay of the precedent, respectively. Note that control GFP-treated cells co-express both GFP and the RP genes (arrowheads), whereas aN2-treated cells are devoid of marker expression (arrows). Bar = 50 μm

Fig. 5. Misexpression of active Notch (aN2) ventral to the prospective RP causes an ectopic upregulation of RP markers. a, d, g, j Electroporation of aN2-GFP. b, e, h, k ISH of corresponding sections. c, f, i, l Overlay of the precedent. Arrows indicate the localization of electroporated cells and arrowheads mark the ectopic upregulation of Rspo1 (N = 6/7), Bambi (N = 7/9), Raldh2 (N = 4/6), or Draxin (5/5), respectively, within the interneuron domain. For control GFP, see Fig. 4. Bar = 50 μm
dl2-5 interneurons [40], revealed a similar dorsal shift at both stages, with their total amount being significantly enhanced in Wnt1-Cre; Mib1<sup>fl/fl</sup> embryos at E10.5 and E11.5, respectively (Fig. 8q–t, z). Together, this increase is likely to be accounted for by expansion of the dl2 population at the expense of dl1 interneurons, since the Isl1+ dl3 interneurons were not quantitatively affected. Consistent with the replacement of RP by interneurons, we further documented a remarkable dorsalization of the neuronal marker Tuj1 in Wnt1-Cre; Mib1<sup>fl/fl</sup> embryos that was already apparent at E10.5 and progressively reached the dorsal midline by E11.5 (Fig. 8u–x).

As also previously reported, we observed no effect on specification and emigration of AP2α+ NC cells at E9.5 in Wnt1-Cre; Mib1<sup>fl/fl</sup> mutants when compared to wild-type ((Additional file 12: Fig. S10 A,B, and see [37] for quantification), suggesting that Notch signaling is involved in RP/interneuron development but not in early stages of NC ontogeny, as also seen in our quail experiments.

In addition, we asked whether the loss of RP and dl1 interneurons was due to enhanced cell death. No evidence for cleaved caspase3-positive cells was found in the dorsal NT of Wnt1-Cre; Mib1<sup>fl/fl</sup> embryos at E9.5,
E10.5, or E11.5 when compared to control embryos. In contrast, NC-derived dorsal root ganglia revealed significant cell death at E11.5, as previously documented (Additional file 12: Fig. S10, A-F) [37]. Likewise, we tested for cell proliferation of the Dll1-negative dorsal cell population by pulse labeling E10.5 wildtype and Wnt1-Cre; Mib1fl/fl embryos with EdU. About 40% of prospective RP progenitors still proliferated at this stage in wildtype embryos. A 30% reduction in the proportion of EdU+ nuclei was measured in the mutants compared to controls (Additional file 10: Fig. S10 G-I). Reduced proliferation in the dorsal NT of Wnt1-Cre; Mib1fl/fl embryos at E10.5 coincides with the replacement of RP progenitors with post-mitotic interneurons (Figs. 7 and 8).

Altogether, in the absence of Notch signaling, initial NC development was normal, yet no RP or dI1 interneurons developed. This loss could not be explained by changes in cell death or proliferation; instead, it is likely to be accounted for by aberrant specification of the dorsal NT with corresponding expansion of the dI2 interneuron population.

Discussion

Understanding the role/s of gene activity in the transition between cellular states and fate decisions is essential
Fig. 8. (See legend on next page.)
for elucidating mechanisms of normal development as well as the origin of embryonic malformations. The dor-
sal NT provides an excellent platform for investigating the sequential formation and delamination of a variety of NC cell types followed by the end of NC production/emigration and the concomitant establishment of the de-
finitive RP [1]. Although the formation and emigration of the NC has been extensively studied [2, 3, 43], how the dorsal NT moves from the early NC all the way into the definitive RP stage remains largely unknown. This is mainly due to the scarcity of RP-specific markers that enable tracking of the shift in cell identity and understanding the molecular pathways involved in the process. Using RNA-seq and ISH, we identify genes differentially expressed at either stage in the trunk level of the axis, their detailed expression patterns, and infer below on their possible functions in the above transition.

The observed coverage patterns correlate well with the known functions of BMP signaling in the proliferation, delamination, and emigration of NC cells [39, 44] and, in particular, with the reported end of BMP activity that leads to the arrest of NC emigration and RP formation [5]. The latter process is illustrated by the upregulation of the BMP inhibitors BAMBI, Grem1, Norrin, Smad3, and PRDM12 and also that of HES4, the quail ortholog of Hairy1/HES1, shown to inhibit BMP signaling in RP [5]. Furthermore, synthesis of factors of the BMP family of Hairy1/HES1, shown to inhibit BMP signaling in RP, such as Dkk1, Frzb, DACT2, a R-spo/Wnt agonist that adds to Lgr5, induction of Norrin, a R-spo/Wnt agonist that additionally inhibits BMP activity [51], and a decrease of Rnf43. Moreover, several Wnt inhibitors are downregulated in RP, such as Dkk1, Frzb, DACT2, and Sfrp2. In contrast, the Wnt receptors Fz3 and Fz10, the downstream target Axin2, and the co-receptors LRP 4,8, and 11 are enhanced. Our data show that R-spo1, Gdf7, and CRBPI are predominantly expressed in the lateral RP along the trunk (our data and see [18, 27]) butting the boundary between the RP and adjacent neuroepithelium. In contrast, Axin2, Lgr4, and Rnf43, all belonging to the R-spo/Wnt module (see above), are expressed in the medial RP, suggesting that the trunk RP is molecularly heterogeneous. Indeed, this molecular heterogeneity also seems to be conserved in mice where R-spo1 also marks the lateral RP domains. Apart from uncovering molecular
components of this heterogeneity in the trunk, we confirm the notion suggested for the hindbrain RP that the latter is formed by molecularly and temporally separable lineages [17, 18, and refs. therein]. Importantly, at cranial levels, only the lateral RP seems to contribute to the formation of the choroid plexus [17]. Whether the observed heterogeneity in the trunk RP predicts a differential fate and/or function of medial vs. lateral domains remains to be elucidated.

Taken together, results of our transcriptome data enabled us first to postulate new roles and interactions between candidate signaling pathways and specific markers of RP domains in the dynamics of dorsal NT development, and second, to plan concrete experiments to put these to the test.

Along this line, we presently addressed the role of the highly conserved Notch signaling pathway in RP formation. In both avians and mice, the dorsal limit of Notch ligand expression corresponds to the ventral boundary of the RP. Although Notch mRNA s are widely transcribed, analysis of N1ICD reveals a similar dorsal limit of activity as that of its ligands and of its modulator LNFG. This suggests that the ventral border of the prospective RP, where all the components of the pathway meet, is a region of Notch/Dll signaling.

Mosaic electroporation of active Notch in prospective RP prevented the advent of RP-specific markers in a cell-autonomous manner, while inducing them in ectopic positions immediately ventral to the RP in a non-cell-autonomous manner. Reciprocally, loss of endogenous Notch activity prevented de novo RP formation. Hence, both gain and loss of function approaches unravel a role for Notch signaling in development of the definitive RP.

This interpretation was possible by implementing RP-specific markers, not expressed in the earlier premigratory NC population. Previous studies used BMP4, Wnt1, and Lmx1 as RP markers [52], yet these are transcribed in both populations.

Of importance is the finding that gain of Notch function in the dorsal NT prevented the expression of endogenous RP traits, while upregulating ectopically specific markers of dI1 interneurons. Conversely, loss of RP development in the absence of Notch signaling compromised development of Math1+ dI1 interneurons, a phenotype that was coincident with expansion of the dI2 population (Fig. 9). Likely, this may be associated with our observation that Notch activity in normal mouse embryos corresponds to the dorsal limit of dI1 interneurons. These results show, first, that Notch plays a role in the binary choice between RP and interneuron fates, and second, confirm that the RP is necessary for at least dI1 interneuron development [41, 42]. Alternatively, changes in Notch activity in the boundary between RP and interneurons may affect both populations altogether (Fig. 9f), as previously suggested for the hindbrain where a well-defined boundary zone may act as a bidirectional signaling center [18].

In this regard, Notch may act to create an interface between RP and non-RP cell types. This is consistent with missexpression of active Notch in non-RP domains, where it downregulates interneuron identity cell autonomously and induces RP traits in adjacent cells. Similar effects were reported on the formation of interhomomeric boundaries in zebrafish [53], and in cultured mouse neural progenitors transfected with the Notch target Hes1 [54]. Thus, forced expression of Notch in non-RP cells prevents them from differentiating into neurons while inducing RP properties in neighboring progenitors. Reciprocally, loss of Notch function prevented RP formation and the dorsal NT became instead populated by dI2 interneurons (Fig. 9). Our results are in agreement with the phenotypes observed in triple HesI,3,5 mutants, in which the RP at spinal cord levels was compromised and replaced by ectopic interneurons [54]. Hence, our loss of function data are consistent with our gain of function results. Both can be explained by the lack of formation of a proper border between RP and non-RP cells in combination with the anti-neurogenic role of Notch.

Notably, activities of Notch may be context-dependent as, in the dorsal-most NT, where no endogenous Notch activity is detected, transfection of active Notch prevented the transcription of endogenous RP markers (Fig. 9). This is consistent with misexpression of LNFG that repressed formation of the zona limitans intrathalamic, another boundary structure similar to the RP that is devoid of endogenous LFNG [55]. Together, our gain of function results suggest that cells overexpressing Notch activity cannot become RP, yet are able to induce RP characteristics non-cell autonomously.

The above results further exemplify the complexity of Notch signaling. Our finding showing the non-cell-autonomous induction of RP next to cells transfected with active Notch could also be consistent with non-RP cells inducing a RP fate. Although the RP is known to function as a signaling center, reciprocal interactions could exist between RP and non-RP (Fig. 9). These might not be necessarily mediated by Notch itself, as Notch activity seems to be confined to the non-RP area. In this regard, Dl1-Notch signaling was shown to indirectly induce cranial NC by the activation of BMP. Moreover, Notch simultaneously restricted the lateral margin of the cranial NC territory by cell-autonomously repressing the NC fate [56]. This provides an example of bimodal functions of Notch signaling in the regionalization of distinct domains.
We cannot rule out the possibility of a time-dependent activity of Notch in the present context. It is possible that initially, Notch signal coming from Dll1-expressing cells lateral to the NC/RP domain instructs dorsal NT cells to adopt a RP fate. This would be consistent with our previous results showing that both NC and RP are generated from common Foxd3-positive precursors [26], with the RP cells stemming from the lateral-most domain [25, 26]. Subsequently, Notch signal in non-RP cells may maintain their non-RP identity (e.g., dorsal interneuron fate) and set the interface between RP and non-RP.

Fig. 9. Role/s of Notch signaling in RP/interneuron development. a–c Gain of Notch activity in quail embryos. In controls (a), the RP (red) is flanked by adjacent dorsal interneurons (dI1, yellow). b Misexpression of active Notch (green) in RP prevents expression of RP-specific traits in a cell-autonomous manner while upregulating BarH-L1, a marker of dI1 interneurons. c Misexpression of active Notch ventral to the RP suppresses dI1 interneuron fate while upregulating RP markers in adjacent cells. d, e Wild type (WT) mouse embryo showing the RP flanked bilaterally by dI1 and dI2 interneurons. d Loss of Notch function in Wnt1-Cre; Mib1 mutants prevents the development of RP and of dI1 interneurons, with a corresponding dorsal expansion of the dI2 interneuron population (E, orange). f At the trunk level of the axis, a sharp boundary between non-RP (blue) and RP (red) is demarcated by the dorsal extent of Notch activity (arrowhead). Notch signaling from this domain acts either directly or indirectly to induce RP formation (arrow 1 in left side of the image) and the RP in turn induces dorsal interneurons (arrow 2, IN). Alternatively, this boundary acts bidirectionally to induce both RP and INs (dotted arrows, right side). See text for detailed discussion.
It is noteworthy that different experimental paradigms affecting the advent of a definitive RP have varied outcomes on dorsal interneurons. For instance, ablation of the RP by targeting diphtheria toxin under the regulation of Gdf7 caused the disappearance of both dI1 and dI2 interneurons and the dorsal midline was occupied instead by dI3 cells [42]. In Lmx1a mutants, however, Math1+ dI1 interneurons were generated albeit in reduced numbers and no effect on dI2 was monitored [41]. This difference in phenotype severity could be accounted for by the documented lack of Gdf7, BMP6, Msx1, and Wnt1 in the Gdf7 mutants compared with the Dreher mice in which residual expression of Wnt1 and Msx1/2, factors shown to operate on interneuron development persisted [52, 57].

In our loss of Notch function experiments, dI1 progenitors, as assessed by Math1 expression, were reduced albeit still apparent at E10.5, and absent by E11.5; dI2, but not dI3 interneurons, were expanded in their absence. It is possible that early specification of interneuron subsets occurs prior to the advent of the definitive RP by dorsal NT-derived BMPs [9, 12, 58]. Additionally, it is possible that BMPs derived later from the ectoderm dorsal to the spinal cord [59] still enable development of selected interneurons of the dI2 subclass, expanded in our mutants. In this context, it would be interesting to examine the relationship between Notch signaling and dorsal NT factors of the BMP and Wnt families.

In both avians and mice, the protocols implemented did not affect early development of NC cells. In avians, epidermal Dll1 signaling was shown to induce cranial NC cells indirectly via BMP activation [56]. In the present study, gene misexpressions were performed following initial NC induction, suggesting that Notch plays no role on specified premigratory NC or during migration. Nevertheless, at later stages, Notch was shown to play significant roles in neuron-glia fate decisions within NC-derived dorsal root ganglia [37, 60].

Conclusions
Taken together, the combination of RP-specific markers with targeted gene misexpression allows us to begin understanding the mechanisms underlying segregation of the three sequential lineages that inhabit the dorsal NT, e.g., NC, RP, and interneurons. Of the precedent lineages, we report that Notch signaling is required for RP formation and for the choice between RP and interneuron fates, yet is dispensable for early stages of NC ontogeny.

Methods
Quail and mouse embryos
Fertilized quail (Coturnix coturnix japonica) eggs were obtained from commercial sources (Moshav Mata) and incubated at 38 °C to the desired developmental stages. All experiments on mice were approved by the SA Pathology and University of South Australia Ethics Committee (project # u26/20). To obtain embryos of defined gestational ages, mice were mated in the evening, and the morning of vaginal plug formation was counted as embryonic day (E) 0.5. Pregnant dams were humanely euthanized at relevant days post vaginal plug detection by CO2 inhalation and cervical dislocation. To delete Mib1 specifically in the dorsal neural tube, we mated Mib1β/β male carrying a heterozygous Wnt1-Cre transgene to Mib1β/β female mice [61]. At least 3 embryos/age/genotype were analyzed for each experiment.

Expression vectors and in ovo electroporation
For transcriptome analysis, pCAGGS-EGFP (2–5 μg/μl) was microinjected into the lumen of the NT opposite the segmental plate region of embryos aged 18ss to label the early premigratory NC or at the flank region of embryos aged 43ss to label the RP. For functional studies, electroporations were directed to the flank level (somites 20–25) of embryos aged 22–25 somites, as detailed in the “Results” section.

An inducible version of the intracellular part of Notch2 (aN2) [62] was sub-cloned into a tetracycline-sensitive plasmid (pBI-TRE-GFP) [63] as previously described [64]. Active cNotch-1 (pMIW-CNICΔ89) [65] was used as described [66]. pBI-TRE-GFP or pCAGGs were used as controls.

To perform focal electroporations directed to the dorsal NT at each stage, a 5-mm tungsten electrode was inserted under the blastoderm (negative electrode) and a fine 2-mm-long electrode was placed dorsal to the neuroepithelium. For hemi-NT transfections, electrodes were placed at both sides of the NT. A square wave electroporator (BTX, San Diego, CA, USA) was used to deliver one to three pulses of current at 10–20 V for 8 ms.

Cell dissociation
Dorsally electroporated NT’s were dissected manually 6–8 h after electroporation before significant emigration of NC cells begun in the early-stage embryos. Prior to NT isolation, embryos were inspected under a fluorescent binocular microscope. Embryos with emigrating cells, or with large electroporated domains were excluded. Single-cell suspensions were obtained by enzymatic digestion with 0.01% Trypsin/0.02% EDTA and 7 μg/ml DNase1 in Ca/Mg-free PBS for 3.5 and 4.5 min for NC and RP stages, respectively. Dissociation was completed by manual pipetting. The resulting cell suspensions were centrifuged at 1000 RPM in an Eppendorf centrifuge, washed once with washing solution (Ca/Mg-free PBS/10%FCS/0.1 units of RNAs inhibitor), and then with fluorescent activated cell sorting (FACS)
buffer (PBS CaMg-free /2% FCS). The final suspension was filtered through a 40-μm pore size Cell strainer.

**FACS sorting**

ARIA III (BD Biosciences) was used for sorting. The cut-off for sorting the electroporated GFP+ cells and for exclusion of dead/damaged PI+ cells was chosen manually. Three samples for each stage containing a total of 1000–6000 GFP+/propidium iodide (PI)-negative cells were collected in Trizol. As expected from very focal transfections, the percentage of GFP+/PI− cells of the total population was about 0.3% at both stages. Total viability of the cells was 99.9% and at least 95% or 87% for the electroporated GFP+ cells at NC and RP stages, respectively.

**Library preparation**

RNA was extracted according to standard Trizol protocol (Invitrogen), using LPA as carrier (Sigma). Amount and quality were checked on Agilent Bioanalyzer using an RNA-pico chip. In total, 120 pg RNA of each sample was taken for library preparation using the CEL-Seq2 protocol [67], with the following modifications: RNA was added to dNTP/ERCC spike-in mix, and 3 different CEL-Seq primers added to each sample as technical replicates. Library was sequenced on HiSeq2500 in rapid mode, 15 bases for read 1, and 50 bases for read 2.

**Bioinformatic analysis**

Demultiplexing was performed according to the pipeline from CEL-Seq2 [67], R2 reads were split into their original samples using the CEL-Seq barcode from R1. The reads were cleaned using trim_galore (uses cutadapt version 1.10) for removal of adaptors, polyA, low-quality sequences (Phred < 20), and short reads (< 25 bp, after trimming). Mapping to the *Coturnix coturnix japonica* genome was conducted using Tophat2 version 2.1.0. The reference and annotation files were downloaded from [https://www.ncbi.nlm.nih.gov/](https://www.ncbi.nlm.nih.gov/), respectively. HTseq-count version 0.6.1 was used for counting. Normalization and differential expression analysis using DESeq2 R package version 1.14.1 was performed after combining reads of the technical replicates.

**Proliferation assays and immunohistochemistry**

**Quail embryos**

Incorporation of BrdU was performed 1 h before fixation and immunolabeling was done as described [5]. For immunostaining, embryos were fixed in 4% formaldehyde overnight at 4 °C, processed for paraffin wax embedding, and sectioned at 8 μM. Antibodies used were rabbit anti BarHL1 (1:100, Sigma, HPA004809), rabbit anti GFP (1:1000, Invitrogen, Thermo-Fisher Scientific, A6455), and rabbit anti RFP (1:000, OriGene, AP09229PU-N). Nuclei were visualized with Hoechst.

**Mouse embryos**

To examine cell proliferation, EdU (5-ethyl-2′-deoxyuridine) (Life technologies) was prepared in 0.9% NaCl solution at 10 mg/ml. EdU was delivered by intraperitoneal injections to pregnant dams at E10.5 at 100 mg/kg of body weight and embryos were collected 1 h after injection.

Embryos were fixed in 4% formaldehyde overnight at 4 °C, cryopreserved in 20% sucrose, and embedded in OCT compound for cryosectioning. Sections were blocked in 10% Dako serum-free blocking reagent in PBS /0.1% TritonX-100, followed by incubation in primary antibody for 2 h at room temperature or overnight at 4 °C. Fluorescent Alexafluor-conjugated secondary antibodies were incubated for 1 h at room temperature. Sections were mounted in Prolong Diamond antifade with DAPI. The following primary antibodies were used: mouse anti-Tuj1, 1:750 (Sigma-Aldrich T5076); mouse anti-Isl1, 1:50 (DSHB 40.3A4); rabbit anti-cleaved Caspase-3, 1:500 (Cell Signaling Technology 9661); mouse anti-AP2α, 1:20 (DSHB 3B5); sheep anti-Dll1, 1:200 (R&D Systems AF5026); rabbit anti-N1ICD, 1:100 (Cell Signaling Technology 4147). Sections of EdU-labeled embryos were incubated with Click-iT EdU Kit Alexa-555 conjugated (Life Technologies) following staining with primary antibodies.

**Antigen retrieval**

Immunostaining for interneuron markers and N1ICD required antigen retrieval. Paraffin-sectioned embryos were immersed in pre-boiled sodium citrate pH 6, 0.05% Tween20, and microwaved for 10 min (20% strength). Blocking was performed with 5% newborn calf serum (Biological Industries 04-102-1A, Israel) in PBS for 1 h at room temperature. Cryosections were incubated in 10 mM sodium citrate pH 6.0 at 90 °C for 20 min, and then cooled to room temperature before proceeding with immunohistochemistry procedure as above.

**Tyramide amplification**

Immunostaining for N1ICD required tyramide signal amplification, performed using Invitrogen Alexa Fluor 555 Tyramide SuperBoost Kit (B40923). Following primary antibody incubation, sections were incubated with goat anti-rabbit horseradish peroxidase, then tyramide signal amplification was performed for 30 min, following the manufacturer’s instructions.
In situ hybridization

Quail embryos

ISH was performed as described previously [36]. Embryos were fixed in Fornoy (60% ethanol, 30% formaldehyde, 10% acetic acid), then dehydrated in ethanol/toluene, processed for paraffin wax embedding, and sectioned at 10 μm. Slides were sequentially rehydrated in toluene/ethanol/PBS, treated with proteinase K (1 μg/ml, Sigma-Aldrich P2308) at 37 °C for 7 min, and then fixed in 4% formaldehyde at room temperature for 20 min. Next, slides were washed in PBS followed by 2× SSC and hybridized in hybridization buffer (1× salt solution composed of 2 M NaCl, 0.12 M Tris, 0.04 M NaH₂PO₄·2H₂O, 0.05 M Na₂HPO₄, 0.05 M EDTA, pH 7.5), 50% formamide, 10% dextran sulfate, 1 mg/ml Yeast RNA, and 1× Denhardt solution containing 1 μg/ml DIG labeled RNA probes (prepared with a DIG RNA labeling mix, Roche, 11277073910) for overnight at 65 °C in a humid chamber. Post-hybridization, slides were rinsed in a rotating incubator with 50% formamide, 1× SSC, and 0.1% Tween 20, until coverslips dropped, and then an additional wash for 1 h followed by 2 washes in MABT (10% Maleic acid 1 M pH 7.5, 3% NaCl 5 M, 0.1% Tween 20) and preincubation in MABT/2.5% FCS. Anti-DIG-AP antibody (1/1000, Roche 11903274910) for overnight at room temperature. This was followed by rinsing in MABT and then in NTMT (2% NaCl 5 M, 10% Tris HCl 1 M pH 9.5, 5% MgCl² 1 M, 0.1% Tween20), and then incubation in NTMT + 1:200 NBT/BCIP Stock Solution (Sigma-Aldrich, 11681451001) for overnight at 37 °C until the AP reaction was completed.

The following probes were kindly provided: Foxd3 [68, 69], Snail2 [70], Dact2 [71], Sfrp2, Dkk1, LFNG, and Wnt4 [72]. Additional probes were produced by PCR amplification (using Q5 high fidelity DNA polymerase) with specific primers (see Additional file 13: Table S3 for list of primers). Template for PCR was cDNA, which was synthesized by RNA precipitation followed by reverse transcription PCR. RNAs were produced from 20ss-E4 quail embryos. Tissue samples were homogenized with TriFast reagent, and RNA was separated with chloroform and isopropanol.

Importantly, all hybridizations, whether done in intact embryos at NC vs. RP stages, or in control vs. experimental embryos, were always performed for similar signal developing times per given probe and in the same experiment.

Mouse embryos

Section ISH was performed as described [73]. Riboprobes were transcribed from plasmids containing PCR amplified cDNA sequences (Additional file 13: Table S3). mNgn1, mNgn2, and Msx1 riboprobes were kindly provided by F. Guillemot. These and Math1 were prepared as previously described [37, 74].

Data analysis and statistics

The number of Isl1+ or AP2α + nuclei in the dorsal NT was monitored in at least 5 sections per wildtype or Wnt1-Cre; Mib1fl/fl embryos. The number of BarH1-positive interneurons or of migrating GFP-positive NC cells was measured in at least 10 sections per embryo from control GFP or aN2-electroporated embryos. Graphs represent mean number of positive cells/section ± SEM.

For measurement of BrdU incorporation, cells in 15 sections per embryo were counted and expressed as percentage of BrdU+/total GFP+ cells in the dorsal NT.

For monitoring cell proliferation in mice, the number of Edu-positive cells was counted in nuclei located dorsal to the boundary of Dll1 expression, i.e., Dll1-negative cells, and expressed as a percentage of total Dll1-negative cells. At least 5 sections per embryo were quantified. Graphs represent mean number of positive nuclei ±SEM.

Images were photographed using a DP70 (Olympus) cooled CCD digital camera mounted on a BX51 microscope (Olympus). For figure preparation, images were exported into Photoshop CS6 (Adobe). If necessary, the levels of brightness and contrast were adjusted to the entire image and images were cropped without color correction adjustments or γ adjustments. Data were analyzed using unpaired Student’s t test. A p value ≤ 0.05 was considered significant.

Supplementary Information

Supplementary information accompanies this paper at https://doi.org/10.1186/s12915-021-01014-3.

Additional file 1: Fig. S1. Labeling of the dorsal NT at NC and RP stages followed by FACS analysis. Focal electroporations of GFP-DNA were directed to the dorsal NT at NC and RP stages. Embryos were sacrificed 6-8 h later to isolate premigratory NC progenitors prior to the onset of EMT. (A,E) dorsal views of live embryos showing restricted labeling of the dorsal midline domain at both stages. (B,F) Focal dorsal labelings confirmed in transverse sections. Green cells outside the NT in F represent autofluorescence of blood cells. (C,D,G,H) FACS purification of GFP-labeled cells. Note absence of GFP+ cells in control non-electroporated samples (C and G) and very low proportions of podoplanin positive (Ph+dead cells in both control and electroporated cases. Ph+ cells were excluded by gating and GFP+/Ph- cells were collected (D,H). As expected from focal transfections, the percentage of live, labeled cells (GFP+/Ph-) was 0.32% and 0.28% of the total input for NC and RP, respectively. Abbreviations, EP, electroporation, FITC, fluorescein isothiocyanate. Bar= 50 μm.

Additional file 2: Table S1. A list of transcripts downregulated in RP when compared to NC. Excel file containing the list of transcripts downregulated in RP when compared to the premigratory NC stage. Based on triplicate samples for each stage. See Methods for technical details.

Additional file 3: Table S2. A list of transcripts upregulated in RP when compared to NC. Excel file containing the list of transcripts enriched in...
the RP when compared to the premigratory NC stage. Based on trilicate samples for each stage. See Methods for technical details.

Additional file 4: Fig. S2. In situ hybridization (ISH) for selected genes downregulated in RP compared to NC. (A, D, G, J, M) Transcripts expressed in the dorsal tube at the NC but not RP stage (B, E, H, K, N). (C, F, I, L, O) Quantification of gene expression levels stemming from the transcriptome analysis. NC–1 and RP–1 represent experimental triplicates. Note positive correlation between ISH and transcriptome results. In each chart, the B-H adjusted p value is indicated. Abbreviations, DRG, dorsal root ganglion, E6, epithelial somite, NT, neural tube, No, notochord. Bar = 50 μm.

Additional file 5: Fig. S3. ISH for selected genes expressed in the RP as well as in more ventral domains of the NT. (A, D, G, J) ISH at NC and RP (B, E, H, K, N), respectively. (C,F,I,L) Quantification of gene expression levels stemming from the transcriptome analysis. In each chart, the B-H adjusted p value is indicated. Note at RP stage the extended expression of transcripts beyond the RP domain. Abbreviations, NT, neural tube, No, notochord. Bar = 50μm.

Additional file 6: Fig. S4. ISH for selected “non-RP” genes upregulated at the RP stage but not transcribed in RP. (A, D, G, J, M) ISH at the NC stage. (B, E, H, K, N, O, P, Q, R, S, T, U) Overlay of the precedent showing complementary marker expression. Bar = 50μm.

Additional file 7: Fig. S5. ISH of adjacent sections with selected RP markers in combination with the “non-RP” marker lunatric fringe (LFNG). (A, D, G, J, M, P, S) ISH for non-RP markers. (B, E, H, K) ISH for NRG1 (C, F, I, L, O, R, U) Overlay of the precedent showing complementary marker expression. Bar = 50μm.

Additional file 8: Fig. S6. ISH of adjacent sections with selected “non-RP” genes in combination with Rspo-1. (A, D, G, J, M) ISH for non-RP markers. (B, E, K, N) ISH for Rspo1. (C, F, I, L, O, R, U) Overlay of the precedent showing complementary marker expression. (P, Q, R) Combined ISH for LFNG and Hoechst nuclear staining. Bar = 50 μm.

Additional file 9: Fig. S7. Molecular heterogeneity within the RP. (A, D, G, J, M) Expression of three genes in the central domain of the RP (delimited by dotted lines). (B, E, H, K) Rspo1 is preferentially expressed in the RP periphery. (C, F) Combination of adjacent sections showing complementary expression of the above. (J) Schematic representation of the expression pattern of several genes upregulated at the RP stage to either its medial (M, red), lateral (L, green), to both domains (green+red) or to NT regions except for the RP (blue). Bar= 50μm.

Additional file 10: Fig. S8. Expression patterns of Notch ligands and receptors. (A, D, G, J, M) Expression of three Notch ligands (A, D, G) and the Notch1 and 2 receptors (J, M) at the RP stage. (B, E, H, K) Rspo1 expression in adjacent sections. (C, F, I, L, O) Overlay of the above. Note that the dorsal limit of Dll1, Jagged1 and Jagged2 mRNA expression corresponds to the ventral limit of the RP. Notch1 and Notch2 mRNA are expressed apically in the RP domain. Bar = 50μm.

Additional file 11: Fig. S9. Misexpression of active Notch1 (CNIC) in quail neural tubes prevents the upregulation of RP markers. Left column represents electroporation of CNIC–GFP (A, D, G). Middle column depicts ISH for BAMB1 (N= 3), Rakd2 (N=5) or Dravx (N= 4), and right column is an overlay of the precedent, respectively. Note that CNIC-treated cells are devoid or have reduced marker expression (arrows). For control GFP see Fig. 4. Bar= 50μm.

Additional file 12: Fig. S10. Neural crest formation is preserved in the absence of Notch signaling. (A-F) Transverse sections through the dorsal neural tube at the level of the forelimb in wildtype (WT) and Wnt1-Cre; Mib2fl/fl mouse embryos, immunostained for AP2a labeling neural crest and dorsal root ganglion (DRG) neurons (green), and cleaved-caspase 3 labeling apoptotic cells (red). At all stages examined, no cell death is evident in the premigratory neural crest or presumptive roof plate region. At E11.5 (E, F) increased cell death is evident in the DRG of Wnt1-Cre; Mib2fl/fl embryos as previously reported (arrows). Bar = 100 μm. (G-I) Transverse sections at E10.5 immunostained for DLL1 (green) and EdU (red) following a 1 hr EdU pulse to label proliferating cells. The dashed line indicates the boundary of DLL1 expression and defines the region used for quantification in (I). (I) Quantification of the number of EdU-positive proliferating cells in the DLL1-negative domain (presumptive roof plate) at E10.5, expressed as a percentage of the total number of DAPI-positive nuclei. Note the reduction in proliferating cells present in Wnt1-Cre; Mib2fl/fl embryos compared to wildtype. N=6 embryos, *p<0.0018. Bar = 50μm.

Additional file 13: Table S3. List of primers used for both quail and mouse in situ hybridization.

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Authors’ contributions
CK and QS conceived the project and all authors designed the experiments; SO performed the transcriptome analysis and all experiments in quail embryos; SW performed the mouse embryo experiments; SK and MT assisted with probe preparation and ISH analysis; and CK wrote the manuscript. All authors discussed and agreed on the results and approved the manuscript.

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Availability of data and materials
Raw RNAseq files and materials will be provided upon request.

Declarations
Ethics approval and consent to participate
Not applicable.

Consent for publication
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Competing interests
No competing interests declared.

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References
1. Krispin S, Nitzan E, Kalcheim C. The dorsal neural tube: a dynamic setting for cell fate decisions. Dev Neurobiol. 2010;70(12):796–812. https://doi.org/10.1002/dneu.20836.
2. Le Douarin NM, Kalcheim C. The neural crest. 2nd ed. New York: Cambridge University Press; 1999. https://doi.org/10.1017/CBO9780511897948.
3. Kalcheim C, Kumar D. Cell fate decisions during neural crest ontogeny. Int J Dev Biol. 2017;61(3-4-5):195–203. https://doi.org/10.1387/ijdb.160196ck.
4. Martik ML, Bronner ME. Regulatory logic underlying diversification of the neural crest. Trends Genet. 2017;33(10):715–27. https://doi.org/10.1016/j.tig.2017.07.015.
5. Nitzan E, Avraham O, Kahane N, Ofek S, Kumar D, Kalcheim C. Dynamics of BMP and Hes1/Hairy1 signaling in the dorsal neural tube underlies the transition from neural crest to definitive roof plate. BMC Biol. 2016;14(1):23. https://doi.org/10.1186/s12915-016-0245-6.
6. Andrews MG, Kong J, Novitch BG, Butler SJ. New perspectives on the mechanisms establishing the dorsal-ventral axis of the spinal cord. Curr Top Dev Biol. 2019;132:417–50. https://doi.org/10.1016/bs.ctdb.2018.12.010.
7. Chuzhikov VV, Millen KJ. Mechanisms of roof plate formation in the vertebrate CNS. Nat Rev Neurosci. 2004;5(10):808–12. http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt= Citation&list_uids=15378040. https://doi.org/10.1038/nrn1520.
8. Le Dreau G, Marti E. Dorsal-ventral patterning of the neural tube: a tale of three signals. Dev Neurobiol. 2012;72(12):1471–81. https://doi.org/10.1002/dneu.22015.

9. Ile Dréau, Garcia-Campmany L, Angeles Rabadán M, Ferronha T, Tózer S, Briscoe J, et al. Canonical BMP? activity is required for the generation of discrete neural tube populations in the dorsal spinal cord. Development. 2012; 139(2):259–68. https://doi.org/10.1242/dev.074948.

10. Lee KJ, Mendelsohn M, Jessell TM. Neural patterning by BMPs: a requirement for GDF7 in the generation of a discrete class of commissural interneurons in the mouse spinal cord. Genes Dev. 1998;12(21):3394–407. https://doi.org/10.1101/gad.12.21.3394.

11. Duval N, Vasilin C, Barata TC, Frarra Y, Contremoulin V, Bauldin X, et al. BMP4 patterns Smad activity and generates stereotyped cell fate organization in spinal organoids. Development. 2019;146:doi: https://doi.org/10.1242/dev.175430.

12. Timmer JR, Wang C, Niswander L. BMP signaling patterns the dorsal and intermediate neural tube via regulation of homeobox and helix-loop-helix transcription factors. Development. 2002;129(10):2459–72. http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=11973277.

13. Andrews MG, Del Castillo LM, Ochoa-Bolton E, Yamauchi K, Smogorzewski J, Butler SJ. BMPs direct sensory interneuron identity in the developing spinal cord using signal-specific not morphogenic activities. Elife. 2017;6:doi: https://doi.org/10.7554/eLife.30647.

14. Zechnier D, Muller T, Wende H, Walther I, Taketo MM, Crenshaw EB 3rd, et al. Bmp and Wnt/beta-catenin signals control expression of the transcription factor Olig3 and the specification of spinal cord neurons. Dev Biol. 2007;303(1):81–90. https://doi.org/10.1016/j.ydbio.2006.10.045.

15. Shinozuka T, Takada R, Yoshida S, Yonemura S, Takada S. Wnt signaling produced by neural crest cells regulates the dorsal-ventral patterning of the dorsal neural tube. Development. 2009;136(11):1997–2008. https://doi.org/10.1242/dev.029943.

16. Xing L, Anbarbash T, Taji JM, Plant GW, Nusse R. Wnt/beta-catenin signaling regulates epidermal cell development and adult homeostasis. Proc Natl Acad Sci U S A. 2018;115(26):E5954–62. https://doi.org/10.1073/pnas.1803297115.

17. Hunter NL, Dymecki SM. Molecularly and topographically separable lineages form the hindbrain roof plate and contribute differentially to the choroid plexus. Development. 2007;134(19):3449–60. https://doi.org/10.1242/dev.003095.

18. Broome ER, Gilthorpe JD, Butts T, Campo-Paysaa F, Wingate RJT. The roof plate boundary is a bi-directional organiser of dorsal neural tube and plate formation with MSX1(+ )roof-plate-derived cells. Stem Cell Reports. 2018;23(10):2928–39. https://doi.org/10.1016/j.stemcr.2018.05.025.

19. Franzi S, Nitzan E, Krispin S, Pfaltzgraff ER, Klar A, Labosky P, Kalcheim C. A dynamic code of dorsal neural tube genes regulates the segregation between neurogenic and melanogenic neural crest cells. Development. 2013;140(1):2269–79. https://doi.org/10.1242/dev.093294.

20. Karnata T, Katsube K, Miechikawa M, Yamada M, Takada S, Mizusawa H. R-spondin, a novel gene with thrombospondin type 1 domain, was expressed in the dorsal neural tube and affected in Wnt's mutants. Biochem Biophys Acta. 2004;1676:61–62. http://www.ncbi.nlm.nih.gov/pubmed/14732490.

21. Arvatas R, Takoson S, Rand MD, Lake RJ. Notch signaling: cell fate control and signal integration in development. Science. 1999;284:770–6.

22. Lai EC. Notch signaling: control of cell communication and cell fate. Development. 2004;131(3):965–72. http://www.ncbi.nlm.nih.gov/pmc/articles/PMC11497298. https://doi.org/10.1242/dev.01074.

23. Henrik D, Schweigt F. Mechanisms of notch signaling: a simple logic deployed in time and space. Dev 2019;146(11):ev172148. https://doi.org/10.1242/dev.172148.

24. Rolo A, Galea GL, Savery D, Greene NDE, Copp AJ. Novel mouse model of encephalolecipheal: post-neurulation origin and relationship to open neural tube defects. Dis Model Mech. 2019;12:doi: https://doi.org/10.1242/dmm.049683.

25. Chizhikov VM, Millen KJ. Roof plate-dependent patterning of the vertebrate dorsal central nervous system. Dev Biol. 2005;277(2):287–95. http://www.ncbi.nlm.nih.gov/pmc/articles/PMC1151675. https://doi.org/10.1016/j.ydbio.2004.10.011.

26. Muroyama Y, Fujiyama M, Ikeya M, Kondoh H, Takada S. Wnt signaling plays an essential role in neuronal specification of the dorsal spinal cord. Genes Dev. 2002;16(5):548–53. https://doi.org/10.1101/gad.937102.

27. Knoopfer PS, Cheng PF, Eisenman RN. N-myc is essential during neurogenesis for the rapid expansion of progenitor cell populations and the inhibition of neuronal differentiation. Genes Dev. 2002;16(20):2699–712. https://doi.org/10.1101/gad.937102.

28. Nitzan E, Krispin S, Platagof ER, Klar A, Labosky PA, Kalcheim C. A dynamic code of dorsal neural tube genes regulates the segregation between neurogenic and melanogenic neural crest cells. Development. 2012;140:269–79.

29. Shoval I, Ludwig A, Kalcheim C. Antagonistic roles of full-length N-cadherin and its soluble BMP cleavage product in neural crest delamination. Development. 2007;134:491–501.

30. Wisnial S, Schwarz Q. Notch signalling defines dorsal root ganglia neurogial fate choice during early neural crest cell migration. BMC Neurosci. 2010;11:doi: https://doi.org/10.1186/1471-2202-11-50.

31. Rolo A, Galea GL, Savery D, Greene NDE, Copp AJ. Novel mouse model of encephalolecipheal: post-neurulation origin and relationship to open neural tube defects. Dis Model Mech. 2019;12:doi: https://doi.org/10.1242/dmm.049683.

32. Kang K, Lee D, Hong S, Park SG, Song MR. The E3 ligase mind bomb-1 (Mib1) modulates delta-notch signaling to control neurogenesis and gliogenesis in the developing spinal cord. J Biol Chem. 2013;288(24):2580–92. https://doi.org/10.1074/jbc.M113.47263.

33. Sela-Donfenfeld D, Kalcheim C. Regulation of the onset of neural crest migration by coordinated activity of BMP9 and Noggin in the dorsal neural tube. Development. 1999;126(21):4749–62.

34. Delile J, Rayon T, Melchionda M, Edwards A, Briscoe J, Sagner A. Single cell transcriptionomics reveals spatial and temporal dynamics of gene expression in the developing mouse spinal cord. Development. 2019;146:doi:https://doi.org/10.1242/dev.173807.1–14.

35. Millen KJ, Millonig HT, Hatten ME. Roof plate and dorsal spinal cord d1 interneuron development in the dreher mutant mouse. Dev Biol. 2004;270(2):382–92. http://www.ncbi.nlm.nih.gov/pmc/articles/PMC1583721. https://doi.org/10.1016/j.ydbio.2004.03.008.

36. Lai EC. Notch signaling: control of cell communication and cell fate. Development. 2004;131(3):965–72. http://www.ncbi.nlm.nih.gov/pmc/articles/PMC11497298. https://doi.org/10.1242/dev.01074.

37. Henrique D, Schweigt F. Mechanisms of notch signaling: a simple logic deployed in time and space. Dev 2019;146(11):ev172148. https://doi.org/10.1242/dev.172148.

38. Rolo A, Galea GL, Savery D, Greene NDE, Copp AJ. Novel mouse model of encephalolecipheal: post-neurulation origin and relationship to open neural tube defects. Dis Model Mech. 2019;12:doi: https://doi.org/10.1242/dmm.049683.

39. Wisnial S, Schwarz Q. Notch signalling defines dorsal root ganglia neurogial fate choice during early neural crest cell migration. BMC Neurosci. 2010;11:doi: https://doi.org/10.1186/1471-2202-11-50.
chick embryo. Dev Biol. 1989;133(1):124–32. https://doi.org/10.1016/0012-1606(89)90162-2.

47. Qin Z, Ren F, Xu X, Ren Y, Li H, Wang Y, Zhai Y, Chang Z. ZNF536, a novel zinc finger protein specifically expressed in the brain, regulates neurogenesis by repressing retinoic acid-induced gene transcription. Mol Cell Biol. 2009;29(13):3633–43. https://doi.org/10.1128/MCB.00362-09.

48. de Lau W, Peng WC, Gross P, Clevers H. The R-spondin/Lgr5/Rnf43 module: regulator of Wnt signal strength. Genes Dev. 2014;28(4):305–16. https://doi.org/10.1101/gad.235473.113.

49. Binnerts ME, Kim KA, Bright JM, Patel SM, Tran K, Zhou M, Leung JM, Liu Y, Binnerts ME, Kim KA, Bright JM, Patel SM, Tran K, Zhou M, Leung JM, Liu Y. The role of bone morphogenetic proteins in vertebral development. Development. 2014;141(8):1726–37. https://doi.org/10.1242/dev.099002.

50. Qin Z, Ren F, Xu X, Ren Y, Li H, Wang Y, Zhai Y, Chang Z. ZNF536, a novel zinc finger protein specifically expressed in the brain, regulates neurogenesis by repressing retinoic acid-induced gene transcription. Mol Cell Biol. 2009;29(13):3633–43. https://doi.org/10.1128/MCB.00362-09.

51. Cheng Y, Amoyel M, Qiu X, Jiang YJ, Xu Q, Wilkinson DG. Notch activation regulates the segregation and differentiation of rhombomeric boundary cells in the zebrafish hindbrain. Dev Cell. 2006(4):539–50. https://doi.org/10.1016/j.devcel.2006.01.010.

52. Deng C, Reddy P, Cheng Y, Luo CW, Hsiao CL, Hsueh AJW. Multi-functional norrin is a ligand for the LGR4 receptor. J Cell Sci. 2013;126(9):2060–8. https://doi.org/10.1242/jcs.123471.

53. Endo Y, Helms AW, Johnson JE. Distinct activities of Msx1 and Msx3 in dorsal neural tube development. Development. 2004;131(5):1017–28. https://doi.org/10.1242/dev.009994.

54. Cheng Y, Amoyel M, Qiu X, Jiang YJ, Xu Q, Wilkinson DG. Notch activation regulates the segregation and differentiation of rhombomeric boundary cells in the zebrafish hindbrain. Dev Cell. 2006(4):539–50. https://doi.org/10.1016/j.devcel.2006.01.010.

55. Hashimshony T, Senderovich N, Avital G, Kohen E, de Leeuw Y, Anany L, Gertsenstein M, Nagy A, et al. Inhibition of internalization of LRP6 regulates Wnt signal strength by a common mechanism. Mol Biol Cell. 2008;19(6):2588–96. https://doi.org/10.1091/mbc.E07-10-0985.

56. Binnerts ME, Kim KA, Bright JM, Patel SM, Tran K, Zhou M, Leung JM, Liu Y, Binnerts ME, Kim KA, Bright JM, Patel SM, Tran K, Zhou M, Leung JM, Liu Y. The role of bone morphogenetic proteins in vertebral development. Development. 2014;141(8):1726–37. https://doi.org/10.1242/dev.099002.

57. Endo Y, Helms AW, Johnson JE. Distinct activities of Msx1 and Msx3 in dorsal neural tube development. Development. 2004;131(5):1017–28. https://doi.org/10.1242/dev.009994.

58. Cheng Y, Amoyel M, Qiu X, Jiang YJ, Xu Q, Wilkinson DG. Notch activation regulates the segregation and differentiation of rhombomeric boundary cells in the zebrafish hindbrain. Dev Cell. 2006(4):539–50. https://doi.org/10.1016/j.devcel.2006.01.010.

59. Endo Y, Helms AW, Johnson JE. Distinct activities of Msx1 and Msx3 in dorsal neural tube development. Development. 2004;131(5):1017–28. https://doi.org/10.1242/dev.009994.

60. Hashimshony T, Senderovich N, Avital G, Kohen E, de Leeuw Y, Anany L, Gertsenstein M, Nagy A, et al. Inhibition of internalization of LRP6 regulates Wnt signal strength by a common mechanism. Mol Biol Cell. 2008;19(6):2588–96. https://doi.org/10.1091/mbc.E07-10-0985.

61. Endo Y, Helms AW, Johnson JE. Distinct activities of Msx1 and Msx3 in dorsal neural tube development. Development. 2004;131(5):1017–28. https://doi.org/10.1242/dev.009994.

62. Cheng Y, Amoyel M, Qiu X, Jiang YJ, Xu Q, Wilkinson DG. Notch activation regulates the segregation and differentiation of rhombomeric boundary cells in the zebrafish hindbrain. Dev Cell. 2006(4):539–50. https://doi.org/10.1016/j.devcel.2006.01.010.

63. Endo Y, Helms AW, Johnson JE. Distinct activities of Msx1 and Msx3 in dorsal neural tube development. Development. 2004;131(5):1017–28. https://doi.org/10.1242/dev.009994.

64. Cheng Y, Amoyel M, Qiu X, Jiang YJ, Xu Q, Wilkinson DG. Notch activation regulates the segregation and differentiation of rhombomeric boundary cells in the zebrafish hindbrain. Dev Cell. 2006(4):539–50. https://doi.org/10.1016/j.devcel.2006.01.010.

65. Endo Y, Helms AW, Johnson JE. Distinct activities of Msx1 and Msx3 in dorsal neural tube development. Development. 2004;131(5):1017–28. https://doi.org/10.1242/dev.009994.

66. Hashimshony T, Senderovich N, Avital G, Kohen E, de Leeuw Y, Anany L, Gertsenstein M, Nagy A, et al. Inhibition of internalization of LRP6 regulates Wnt signal strength by a common mechanism. Mol Biol Cell. 2008;19(6):2588–96. https://doi.org/10.1091/mbc.E07-10-0985.

67. Endo Y, Helms AW, Johnson JE. Distinct activities of Msx1 and Msx3 in dorsal neural tube development. Development. 2004;131(5):1017–28. https://doi.org/10.1242/dev.009994.

68. Cheng Y, Amoyel M, Qiu X, Jiang YJ, Xu Q, Wilkinson DG. Notch activation regulates the segregation and differentiation of rhombomeric boundary cells in the zebrafish hindbrain. Dev Cell. 2006(4):539–50. https://doi.org/10.1016/j.devcel.2006.01.010.

69. Endo Y, Helms AW, Johnson JE. Distinct activities of Msx1 and Msx3 in dorsal neural tube development. Development. 2004;131(5):1017–28. https://doi.org/10.1242/dev.009994.

70. Cheng Y, Amoyel M, Qiu X, Jiang YJ, Xu Q, Wilkinson DG. Notch activation regulates the segregation and differentiation of rhombomeric boundary cells in the zebrafish hindbrain. Dev Cell. 2006(4):539–50. https://doi.org/10.1016/j.devcel.2006.01.010.

71. Cheng Y, Amoyel M, Qiu X, Jiang YJ, Xu Q, Wilkinson DG. Notch activation regulates the segregation and differentiation of rhombomeric boundary cells in the zebrafish hindbrain. Dev Cell. 2006(4):539–50. https://doi.org/10.1016/j.devcel.2006.01.010.

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