

# CDX4 regulates the progression of neural maturation in the spinal cord

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## ABSTRACT

The progression of cells down different lineage pathways is a collaborative effort between networks of extra-cellular signals and intracellular transcription factors. In the vertebrate spinal cord, FGF, Wnt and Retinoic Acid signaling pathways regulate the progressive caudal-to-rostral maturation of neural progenitors by regulating a poorly understood gene regulatory network of transcription factors. We have mapped out this gene regulatory network in the chicken pre-neural tube, identifying CDX4 as a dual-function core component that simultaneously regulates gradual loss of cell potency and acquisition of differentiation states: in a caudal-to-rostral direction, CDX4 represses the early neural differentiation marker *Nkx1.2* and promotes the late neural differentiation marker *Pax6*. Significantly, CDX4 prevents premature PAX6-dependent neural differentiation by blocking *Ngn2* activation. This regulation of CDX4 over *Pax6* is restricted to the rostral pre-neural tube by Retinoic Acid signaling. Together, our results show that in the spinal cord, CDX4 is part of the gene regulatory network controlling the sequential and progressive transition of states from high to low potency during neural progenitor maturation. Given CDX well-known involvement in *Hox* gene regulation, we propose that CDX factors coordinate the maturation and axial specification of neural progenitor cells during spinal cord development.

## 1. Introduction

Differentiating cells transition from one temporary state to another, losing potency and acquiring specialized functions in the process. Each step along the differentiation pathway is defined by a unique assortment of active transcription factors (Davidson, 2006; Royo et al., 2011). This transcriptome can change over time, mostly cued by dynamic extra-cellular signaling factors (Peter and Davidson, 2013; Sandmann et al., 2007). It is the cross-regulation between transcription and signaling components that promotes the progressive acquisition of specialized functions while preventing dedifferentiation: transcription factors specify the cell's identity and ability to respond to signaling factors (competence), and signaling factors control the sequential activity of transcription factors to promote directional acquisition of specialized traits (Davidson and Levine, 2008; Levine and Davidson, 2005; Sandmann et al., 2007). These interactions between transcription factors and signaling pathways form complex networks that have been challenging to dissect, hindering our understanding of the mechanisms regulating cellular state transitions.

The vertebrate spinal cord serves as an important accessible model to

study the maturation of neural progenitors during their transition from one cellular state to the next. Maturation of spinal cord progenitors at the caudal end of the embryo follows a caudal-to-rostral organization, with undifferentiated cells localizing to the caudal region and more mature cells localizing to the more rostral positions (Butler and Bronner, 2015; Diez del Corral et al., 2003; Diez del Corral and Storey, 2004; Wilson et al., 2009). During the early segmentation stages in chick embryos up to the point of tailbud formation (0–16 somites equivalent to Hamburger and Hamilton (HH) stages 6–12; Hamburger and Hamilton, 1951), extensive fate mapping and gene expression analysis has resulted in the identification of four distinct embryonic regions corresponding to four different neural maturation states (reviewed in Gouti et al., 2015 and Henrique et al., 2015; summarized in Fig. 1A). The most caudal region is the caudal lateral epiblast and node-streak border region containing bipotent neuromesodermal progenitors (NMPs) cells that contribute to both neural and mesodermal tissues (region 1; Brown and Storey, 2000; Cambray and Wilson, 2007; Tzouanacou et al., 2009; reviewed in Henrique et al., 2015). NMPs are defined molecularly by the co-expression of two key transcription factors, the pan-neural marker *Sox2* and the mesodermal marker *T/Bra*, although NMPs also transcribe the pre-neural

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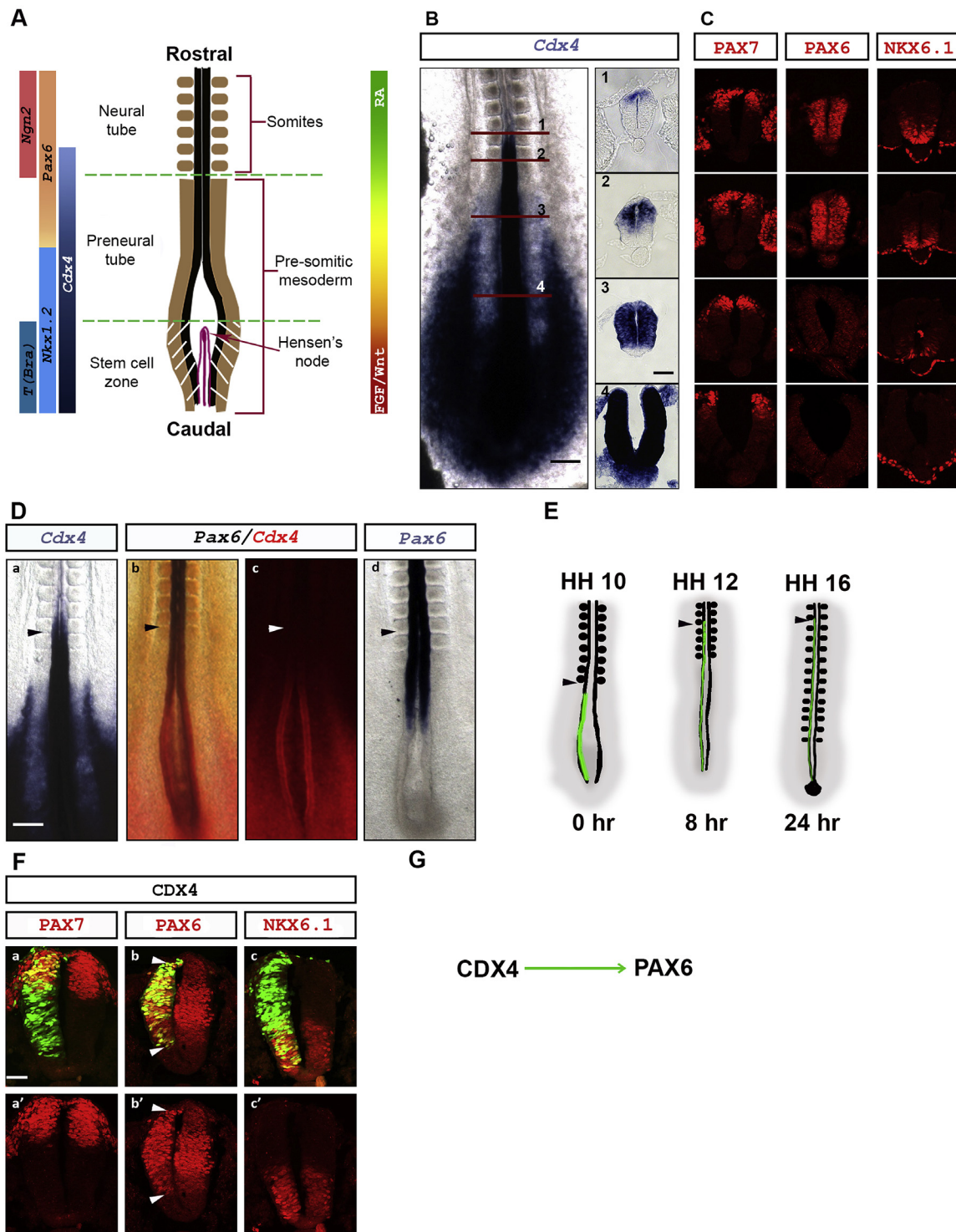
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**Fig. 1.** CDX4 activates *Pax6* gene transcription. (A) Schematic representation of the caudal end of HH10 chicken embryo showing primary subdivisions (central diagram; adapted from [Olivera-Martinez and Storey, 2007](#)), and expression domains of key transcription and signaling factors (left and right of diagram, respectively). (B) *Cdx4* is transcribed in a dynamic dorsal-ventral (DV) gradient along the rostro-caudal (RC) axis of embryos (HH11). Red lines indicate position of transverse sections shown on right. (C) Distribution of PAX7 (dorsal), PAX6 (dorsal-to-intermediate), and NKX6.1 (ventral) proteins relative to *Cdx4* transcription domain. (D) *Cdx4* and *Pax6* transcription domains overlap in the rostral pre-neural tube (PNT) at stage HH 11-11+ (ISH; *Cdx4* expression in purple in Da, and red in Db and Dc; *Pax6* expression in purple in Db and Dd). Arrow shows position of somite 13. (E) Graphical representation of the experimental approach used throughout this work. The PNT of HH10-11 stage embryos were electroporated on the left side with appropriate constructs carrying a GFP-reporter gene. Embryos were processed for analysis after 8 (HH 12–13) or 24 (HH16-16+) hours post electroporation (hpe), when electroporated cells are localized to the rostral portion of the PNT (HH 12–13) or the caudal portion of the NT (thoracic level; HH16-16+), respectively. Control experiments demonstrate that electroporation alone has not affect gene transcription, and that overexpression of electroporated constructs is long lasting ([Fig. S1](#) for all experiments). Arrowhead shows the position the last somite formed at the time of electroporation. (F) CDX4 does not affect the distribution of PAX7 or NKX6.1 proteins, but caused ectopic PAX6 accumulation outside its normal domain (arrowheads). Marker proteins are in red and electroporated cells are in green (nuclear GFP tag). Embryos were electroporated at HH10-11 and analyzed 24 hpe (HH16). (E) Summary of results. Scale bar is 200  $\mu$ m for whole mount and 40  $\mu$ m for transverse sections.

identity marker *Nkx1.2* (also known as *Sax1*; Delfino-Machin et al., 2005; Gouti et al., 2015; Gouti et al., 2017). Immediately rostral to the NMP domain is the pre-neural tube (PNT; Gouti et al., 2015; Henrique et al., 2015). Cells in the PNT downregulate *T/Bra* but continue to express *Sox2* (Delfino-Machin et al., 2005; Gouti et al., 2015). PNT can be further subdivided into a caudal PNT that continues to express *Nkx1.2* (region 2) and the rostral PNT which downregulates *Nkx1.2* and activates *Pax6* transcription (region 3; Bel-Vialar et al., 2007; Bertrand et al., 2000; Delfino-Machin et al., 2005; Sasai et al., 2014; Spann et al., 1994). Finally, rostral to the PNT and situated adjacent to the developing somites is the neural tube (NT; region 4; Gouti et al., 2015; Henrique et al., 2015). NT cells are *Nkx1.2*-negative and *Pax6*-positive and begin to transcribe the neural differentiation genes *Ngn1/2* and *NeuroM* (Diez del Corral et al., 2003). Thus, from caudal-to-rostral, four spatially distinct populations can be identified that correspond to four maturation states (summarized in Fig. 1A).

Recently, single cell transcriptome analysis of *in vitro* differentiating NMPs has confirmed and expanded the known transcriptional signatures observed throughout NMPs initial cell fate choice decision, allowing the more accurate assignment of gene activities to particular specification states (Gouti et al., 2014, 2017). For example, CDX transcription factors have been implicated in NMP maintenance, and also axial patterning and elongation (Amin et al., 2016), however the separation of these activities has been challenging to dissect due to the multiple and partially redundant activities of the three functionally similar CDX proteins (CDX1, CDX2 and CDX4; van Rooijen et al., 2012). By studying the differentiation of NMPs derived from mouse Embryonic Stem Cells (mESC) lacking individual or different combination of *Cdx* genes, CDX proteins were shown to regulate the temporal maintenance of *T/Bra* (Gouti et al., 2017). By maintaining or down regulating *T/Bra* transcription, *Cdx* genes regulate the fate decision of NMP cell to become either mesoderm or neural tissues (Gouti et al., 2017). In addition to the NMPs, *Cdx* are also transcribed in NMP descendants in the PNT and NT (Gaunt et al., 2005; Marom et al., 1997, Fig. 1B), where their function remains largely unknown.

The transition from NMP to pre-neural to neural transcriptional states is under the control of three signaling factors: FGF, Wnt and Retinoic Acid (RA; Diez del Corral et al., 2003; Olivera-Martinez and Storey, 2007). At the caudal end of the embryo, *Fgf8* and *Wnts* (*Wnt3a* and *Wnt8c*) are transcribed in a caudal to rostral gradient that promotes potency by maintaining *T/Bra*, *Sox2* and *Nkx1.2* expression while simultaneously preventing *Pax6* transcription (Bertrand et al., 2000; Delfino-Machin et al., 2005; Diez del Corral et al., 2003; Olivera-Martinez et al., 2012). FGF also maintains tissue proliferation by limiting precocious cell cycle exit (Akai et al., 2005). In contrast, RA secreted from somites establishes a rostral to caudal signaling gradient that promotes differentiation: first by promoting transcription of neural identity genes *Pax6* (Diez del Corral et al., 2003; Novitch et al., 2001; Pituello et al., 1999), and subsequently, by promoting transcription of downstream neurogenic genes *Ngn1/2* and *NeuroM* (Diez del Corral et al., 2003). By inducing *Pax6* and *Ngn2*, RA induces cells to exit the proliferation program (Bel-Vialar et al., 2007; Lacomme et al., 2012). The signaling activities of FGF/Wnt and RA are segregated to opposite caudal and rostral regions of the nascent spinal cord through positive and negative interactions: caudally, high FGF directly prevents RA synthesis and stimulates its degradation, while rostrally, low FGF indirectly promotes RA production through a Wnt8c-dependent mechanism (Boulet and Capecchi, 2012; Olivera-Martinez et al., 2012; Olivera-Martinez and Storey, 2007; Sakai et al., 2001; White et al., 2007). In turn, RA inhibits *Fgf8* transcription rostrally, creating a zone where cells can exit the cell cycle and differentiate (Diez del Corral et al., 2003; Kumar and Duester, 2014). These interactions have been proposed to function as the signaling switch that drives the transition of cellular states in the caudal neural tube (Diez del Corral and Storey, 2004; Olivera-Martinez and Storey, 2007). While the signal interactions regulating the transition from NMP to pre-neural to neural states have been extensively investigated, the underlying transcription

factor network driving the cell transitions are incomplete.

FGF, Wnt and RA signals are known regulators of *Cdx* transcription (Deschamps and van Nes, 2005; Lohnes, 2003), making CDX transcription factors good candidates to regulate PNT cell maturation. In chicken embryos, *Cdx* genes are transcribed in nested domains, at levels that are high in NMPs and low in the NT (Marom et al., 1997). These high-to-low levels of transcription have also been observed in differentiating NMPs *in vitro* (Gouti et al., 2017). In the spinal cord, CDX factors are essential for tissue specification and rostro-caudal patterning (Deschamps et al., 1999; Nordstrom et al., 2006; Shimizu et al., 2006; Skromne et al., 2007; van den Akker et al., 2002), controlling the initial specification of post-occipital tissues (van Rooijen et al., 2012), and the subsequent patterned transcription of *Hox* expression domains (Deschamps et al., 1999; Hayward et al., 2015). Thus, *Cdx* genes are attractive candidates to integrate multiple signals into coherent cell maturation states.

Here we show that chicken CDX4, the only CDX present in the chick PNT and NT during early segmentation stages (HH10-16; Marom et al., 1997), controls the progression of PNT cells towards more mature states without promoting their terminal differentiation. In the PNT, transient CDX4 results in *Nkx1.2* downregulation and *Pax6* activation, which drives cells with recently acquired neural identity (*Sox2+*, *Nkx1.2+*, *Pax6-*) toward a more restricted neural progenitor state (*Sox2+*, *Nkx1.2-*, *Pax6+*). Significantly, *Pax6* activation by CDX4 is dependent on RA secreted by somites, which restricts the maturation of cells to the rostral PNT. Furthermore, we show that CDX4 prevents *Ngn2* transcription even in the presence of the *Ngn2*-activator PAX6, thus preventing the premature cell's terminal differentiation. Our results support a model in which CDX4 is an integral component of a gene regulatory network that functions to simultaneously reduce the potency and increase the differentiation state of cells. We propose that this gene regulatory network operates under the control of previously described signaling network involving FGF, Wnt, and RA.

## 2. Results

### 2.1. *Cdx4* regulates *Pax6* transcription in the caudal neural tube

CDX4 neural function in chicken embryos was first analyzed by correlating its transcription domain to distinct progenitor cell maturation zones of the caudal neuroectoderm (Fig. 1A; Olivera-Martinez and Storey, 2007). We focused our attention on CDX4 as this is the only *Cdx* gene transcribed in chick PNT past stage HH12 (tailbud stages; Marom et al., 1997). As previously reported in whole chick embryos (HH10-12; Morales et al., 1996; Marom et al., 1997), *Cdx4* is transcribed in the pre-neural tube (PNT) and nascent neural tube (NT) in a high caudal to low rostral gradient (Fig. 1B). However, transverse sections also revealed that *Cdx4* is transcribed in a highly dynamic dorsal-to-ventral (DV) gradient: caudally, *Cdx4* transcription was ubiquitous throughout the medio-lateral extent of the PNT (dorsal-ventral extent in the NT), whereas rostrally, *Cdx4* transcription was progressively excluded from ventral regions as well as the roof plate (Fig. 1B, transverse sections). Due to the lack of chicken specific CDX4 antibody, we were unable to examine the CDX4 protein profile in the neural tube. However, a similar dorsally restricted expression profile has been reported for *Cdx4* in mouse embryos (Gaunt et al., 2005), suggesting evolutionary conserved transcriptional mechanisms and a potential function for CDX4 in the specification of DV neural cell identities.

To test the role of CDX4 in DV specification, we analyzed *Cdx4* transcriptional domain relative to various DV identity markers including the dorsal cell marker *Pax7* (Briscoe et al., 2000; Diez del Corral et al., 2003), the dorsal-to-intermediate cell marker *Pax6* (Briscoe et al., 2000; Novitch et al., 2003), and the ventral cell marker *Nkx6.1* (Briscoe et al., 2000; Diez del Corral et al., 2003; Novitch et al., 2003). At HH11, we observed a correlation between the transcriptional domain of *Cdx4* and some of these markers. For example, in the caudal NT, PAX7 domain was nested within, and NKX6.1 domain was complementary to *Cdx4* domain

of transcription (Fig. 1B and C). However, more rostrally, there was a loss of correlation between D/V markers and *Cdx4* domain of transcription; PAX7 domain was broader than, and NKX6.1 domain no longer complemented *Cdx4* transcription domain (Fig. 1B and C). The only correlation we observed was between *Cdx4* and *Pax6*, with levels of *Cdx4* transcript decaying as levels of *Pax6* transcript and PAX6 protein increased in a caudal to rostral direction (Fig. 1C and D).

To formally test *Cdx4* involvement in DV cell fate specification, we artificially maintained high levels of *Cdx4* in the NT in a domain where *Cdx4* would normally be downregulated. We reasoned that if CDX4 regulates DV cell specification, increasing *Cdx4* levels would result in a change in the localization of DV marker genes. We overexpressed CDX4 by electroporating wild type *Cdx4* in the PNT of stage HH10–11 embryos, a region that transcribes endogenous *Cdx4*, and analyzed the protein distribution of PAX7, PAX6, and NKX6.1 24-h post-electroporation (hpe; HH16–17), at a time when electroporated cells have become part of the NT and no longer transcribe endogenous *Cdx4* (Fig. 1B, E). While artificially maintained high levels of *Cdx4* expression did not change NKX6.1 and PAX7 protein distribution (Fig. 1F), ectopic *Cdx4* caused production of PAX6 protein outside its normal domain, both ventrally and dorsally (Fig. 1F). In this and all other experiments, electroporation of a control reporter GFP vector had no effect on target gene transcription and protein distribution (Fig. S1). Together, these results suggest that CDX4 can regulate *Pax6* transcription (Fig. 1G), but appears to have no broad impact on the establishment of dorsal and ventral identities.

## 2.2. CDX4 regulates *Pax6* transcription during neural progenitor cell maturation

In addition to its function in DV cell specification, PAX6 promotes the maturation of neural progenitor cells in the PNT (Bel-Vialar et al., 2007). Given that our results do not appear to support a function for CDX4 in global DV cell specification (Fig. 1), we hypothesized that CDX4 might regulate *Pax6* transcription during PNT cell maturation. To test this hypothesis, we asked whether the presence of CDX4 was sufficient to change *Pax6* transcription in the rostral PNT, a region where *Pax6* transcription initiates. Embryos were electroporated in the PNT with different *Cdx4* constructs (HH10–11), grown for 8 h only (HH12–13), and analyzed by *in situ* hybridization for premature *Pax6* activation. Two constructs were used in this assay, a wild type and a constitutive active version of CDX4 that phenocopies CDX functions in *Hox* gene transcription assays (VP16CDX4; Bel-Vialar et al., 2002; Faas and Isaacs, 2009). In these short incubation experiments, VP16CDX4 was able to induce *Pax6* transcription more caudally and at higher levels than CDX4 (Fig. 2A and B), while CDX4 could induce strong *Pax6* transcription after long incubation periods (24 hpe; Fig. 1F). These results suggest that CDX4 has the potential to regulate *Pax6* transcription in the rostral PNT and caudal NT.

To test if CDX4 is required for *Pax6* activation in the PNT, we out-competed endogenous CDX4 with a dominant negative form of CDX4 in which the transcription activation domain of the protein was replaced with the transcriptional repressor domain of the *Drosophila* Engrailed protein (ENRCDX4; Han and Manley, 1993). This chimeric form of CDX4 has been shown to repress transcription of downstream CDX targets (e.g., *Hox* genes; Bel-Vialar et al., 2002; Isaacs et al., 1998). Overexpression of *EnrCDx4* caused *Pax6* downregulation in the rostral PNT (8 hpe; Fig. 2Ac), indicating that in this region, CDX4 protein activity is required for *Pax6* transcription.

## 2.3. CDX4 activation of *Pax6* in the pre-neural tube is dependent on Retinoic Acid signaling

Transcription of *Pax6* is restricted to the rostral PNT despite that *Cdx4* is transcribed in both caudal and rostral PNT regions (Fig. 1D). To investigate the possible mechanisms that restrict *Pax6* transcription to the rostral PNT, we turned our attention to Retinoic Acid (RA). Somite-

derived RA regulates spinal cord neurogenesis by activating numerous target genes in the rostral PNT, including *Pax6* (Novitsch et al., 2003; Pituello et al., 1999). Given that RA and CDX4 interact during zebrafish spinal cord cell specification (Chang et al., 2016; Lee and Skromme, 2014), we hypothesized that RA and CDX4 might also interact during spinal cord maturation. To test this hypothesis, we electroporated PNT with dominant negative RA receptors (dnRAR) to suppress RA-dependent gene transcription (Novitsch et al., 2003), and then analyzed *Pax6* transcription 24-hpe, at a time when electroporated cells would be undergoing maturation. As previously shown (Novitsch et al., 2003), overexpression of *dnRAR* caused *Pax6* down regulation (Fig. 2Cd, D), even as *dnRAR* enhanced *Cdx4* transcription (Fig. S2). To test if induction of *Pax6* by CDX4 is RA-dependent, we co-electroporated *dnRAR* with different *Cdx4* constructs. In the presence of *dnRAR*, CDX4 was unable to induce *Pax6* (Fig. 2Ce, D), despite CDX4 ability to do so in control conditions (Fig. 1D). Significantly, however, VP16CDX4 was able to overcome *dnRAR* repression and induce *Pax6* transcription (Fig. 2Cb, Cf, D). Together, these results suggest that activation of *Pax6* by CDX4 is dependent on RA signaling, illuminating a mechanism for the restricted transcription of *Pax6* to the rostral portion of the PNT (Fig. 2E).

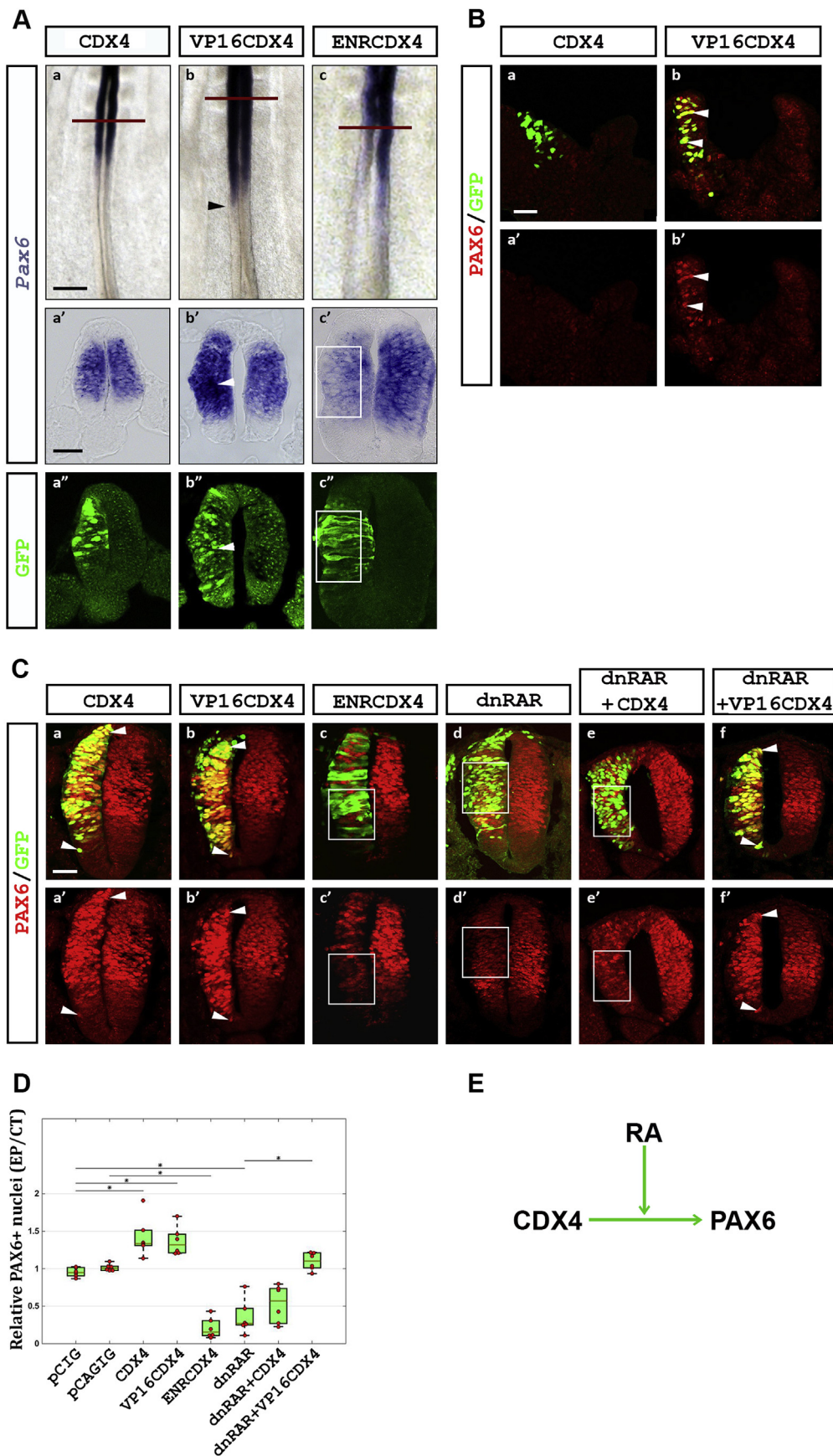
## 2.4. CDX4 inhibits PAX6-dependent activation of *Ngn2* in the neural tube

PAX6 is present in both the rostral PNT and the NT, but it only activates neural differentiation genes in the NT (Bel-Vialar et al., 2007). Then, what prevents PAX6 from prematurely activating neural differentiation genes in the PNT? To address this question we analyzed the transcription of the neural differentiation gene *Ngn2*, a downstream target of PAX6 (Scardigli et al., 2003). *Ngn2* transcription domain is nested within that of *Pax6* and lays immediately rostral to that of *Cdx4* (Fig. 1B; Fig. 3A), raising the possibility that CDX4 activity is incompatible with *Ngn2* transcription. To test this possibility, we overexpressed *Cdx4*, *VP16Cdx4* and *EnrCDx4* in HH10–11 embryos and analyzed their effect on NGN2 distribution at HH16–17 (24 hpe). As expected, ENRCDX4 caused loss of NGN2 (24 hpe; Fig. 3Bc, 3C), as ENRCDX4 also reduced the levels of PAX6 (24 hpe; Fig. 2Cc), and PAX6 is required for *Ngn2* transcription (Scardigli et al., 2003). Surprisingly, CDX4 and VP16CDX4 also caused the loss of NGN2 (24 hpe; Fig. 3Ba-b, 3C), under conditions that resulted in ectopic PAX6 (Fig. 2Ca-b), suggesting that CDX4 represses *Ngn2*. This result is consistent with *Cdx4* and *Ngn2* complementary expression domains (Figs. 1B and 3A). To confirm that CDX4 represses *Ngn2* in the presence of PAX6, we repeated the experiment by simultaneously co-expressing *Cdx4* and *Pax6*. While PAX6 on its own was able to ectopically activate *Ngn2* (Fig. 3Bd, C; Bel-Vialar et al., 2007), it was unable to do so in the presence of CDX4 (Fig. 3Be, C). Given that CDX4 functions as a transcriptional activator in most contexts (Isaacs et al., 1998), these results suggest that CDX4 in the PNT promotes *Pax6* transcription directly and prevents *Ngn2* transcription indirectly (Fig. 3D).

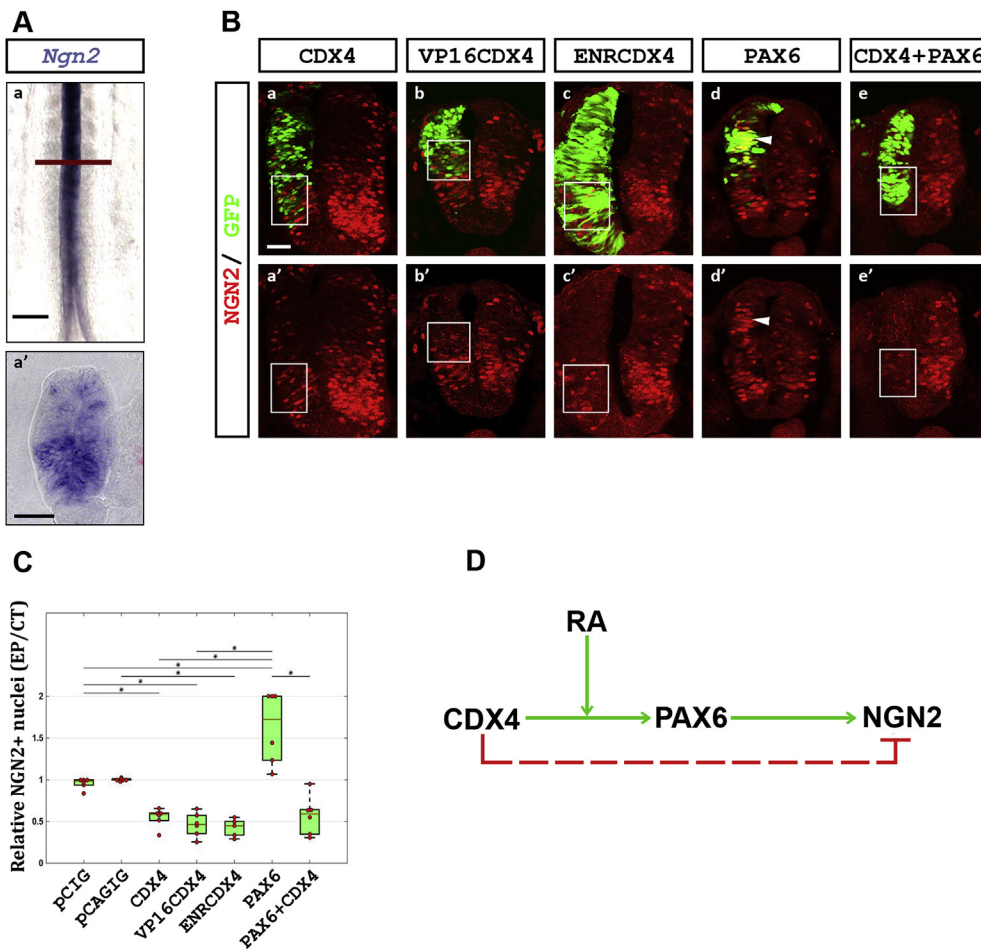
## 2.5. CDX4 inhibits *Nkx1.2* expression in early neural progenitor cells

*Cdx4* transcription domain in the PNT includes the caudal region that contains *T/Bra*-, *Sox2*+ and *Nkx1.2*+ early neural progenitors. This observation prompted us to ask whether CDX4 also regulates aspects of early PNT maturation. To address this question, we electroporated the caudal PNT with different *Cdx4* constructs at HH10–11 and, after growing the embryos for 8 h to HH12–13, we analyzed the transcription of the early PNT marker *Nkx1.2* (Delfino-Machin et al., 2005; Gouti et al., 2015, 2017; Sasai et al., 2014). Overexpression of *Cdx4* and *VP16Cdx4* caused downregulation of *Nkx1.2* transcription (Fig. 4A), suggesting that CDX4 negatively regulates *Nkx1.2*. Unexpectedly, however, *EnrCDx4* overexpression also caused *Nkx1.2* downregulation (Fig. 4A), suggesting that CDX4 activity is also required for *Nkx1.2* transcription. We interpret these results to indicate that *Nkx1.2* is under both positive and negative CDX4 regulation, with high levels of CDX4 repressing *Nkx1.2* (Fig. 4C).

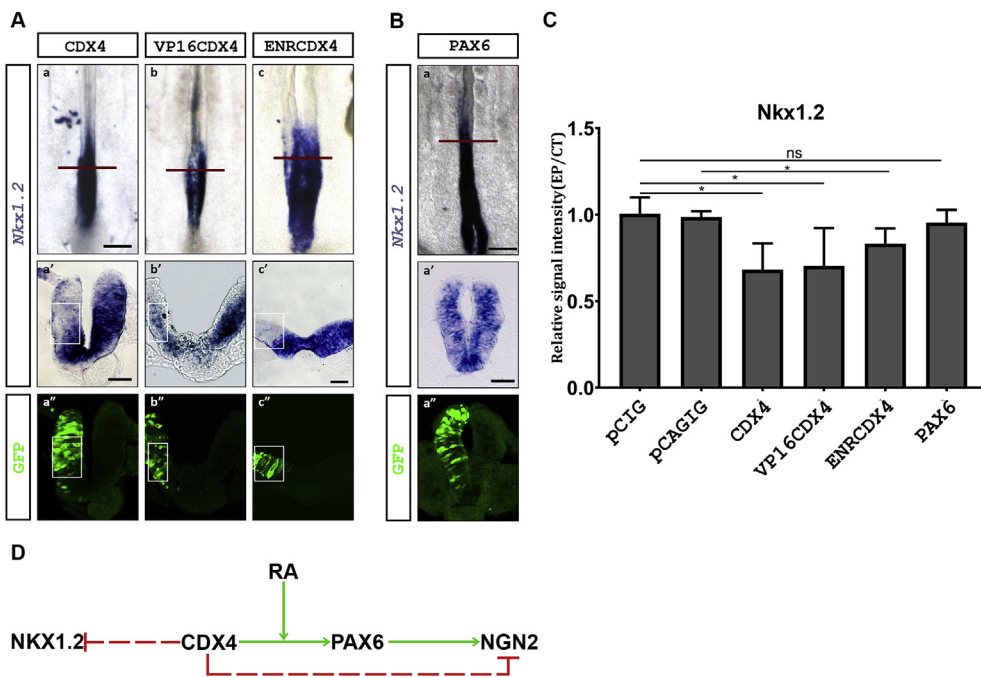
CDX4 is a transcriptional activator (Isaacs et al., 1998) so, in order to



**Fig. 2.** CDX4 activation of *Pax6* transcription is RA-dependent. (A) CDX4 regulates *Pax6* transcription in the rostral PNT. In the PNT, *Cdx4* has no effect (a, a'), *VP16Cdx4* induces (arrowheads in b, b'), and *EnrCdx4* downregulates (c, c') *Pax6* transcription (purple signal by ISH; green GFP tag labeled by IHC). Embryos were electroporated at HH10–11 and analyzed 8 hpe (HH12–13). (B) Similarly, ectopic *VP16Cdx4* but not *Cdx4*, causes ectopic PAX6 protein accumulation (arrowheads). (C) CDX4 requires Retinoic Acid (RA) to activate *Pax6* transcription in rostral regions. In the NT, both *Cdx4* and *VP16Cdx4* overexpression result in ectopic PAX6 protein accumulation (a, a', b, b'; arrowheads), whereas *EnrCdx4* overexpression causes the loss of PAX6 (c, c'; box). Inhibition of RA signaling using a dominant negative RA receptor (*dnRAR*) causes the loss of PAX6 (d, d'). In the absence of RA signaling, *Cdx4* overexpression is unable to induce ectopic PAX6 (e, e'; box). Under similar conditions, *VP16Cdx4* overexpression induces ectopic PAX6 (f, f'; arrowheads). Embryos were electroporated at HH10–11 and analyzed 24 hpe (HH16–17). (D) Quantification of PAX6 positive cells after experiments shown in C. Box-scatter plot representing ratio of PAX6 positive cells on electroporated side to that on the contralateral control side (as per Karaz et al., 2016). Significance is shown with a bar and a star (data from 6 embryos, see Table S3 for cell numbers; two tailed *t*-test analysis, \**p* < 0.05). (E) Summary of results. Scale bar is 200  $\mu$ m for whole mount and 40  $\mu$ m for transverse sections.



**Fig. 3.** CDX4 inhibits early cell maturation by repressing the neural differentiation gene *Ngn2*. (A) *Ngn2* expression in the NT of wild type HH11 embryos. Expression is first observed caudally to the site of NT closure. (B) *Cdx4* and *VP16Cdx4* overexpression results in the loss of NGN2 (a, a', b, b'; boxes), despite both inducing the *Ngn2*-activator *Pax6* (Fig. 2A, C). *EnrCdx4* overexpression also causes the loss of NGN2 (c, c'; box), similar to its effect on PAX6 (Fig. 2Cc). *Pax6* overexpression results in ectopic NGN2 (d, d'; arrowhead), but not in the presence of *Cdx4* (e, e'; box). Embryos were electroporated at HH10-11 and analyzed 24 hpe (HH16-17). (C) Quantification of NGN2 positive cells after experiments shown in B. Box-scatter plot representing ratio of NGN2 positive cells on electroporated side versus contralateral control side. Significance is shown with a bar and a star (data from 6 embryos, see Table S3 for cell numbers; two tailed *t*-test analysis,  $p < 0.05$ ). (D) Figure summarizing CDX4-PAX6-NGN2 interactions, with dashed lines representing indirect interactions. Scale bar is 200  $\mu$ m for whole mount and 40  $\mu$ m for transverse sections.



**Fig. 4.** CDX4 regulates the transcription of *Nkx1.2*, an early neural progenitor cell identity marker. (A) Overexpression of *Cdx4* (a, a'), *VP16Cdx4* (b, b'), and *EnrCdx4* (c, c') downregulate *Nkx1.2* transcription (boxed region). (B) Overexpression of *Pax6* has no effect on *Nkx1.2* transcription. Embryos were electroporated in the caudal PNT at HH10-11 and analyzed 8-hpe (HH12-13). (C) Quantification of *Nkx1.2* transcription levels by relative signal intensity analysis (electroporated (ET)/Control (CT) sides). Experimental conditions were normalized to control pCIG vector (for all conditions,  $n = 6$  from at least 3 bio-replicates,  $*p \leq 0.05$ , ns: not significant). (D) Figure summarizing CDX4-NKX1.2 interactions. Solid lines are direct and dashed lines are indirect interactions. Scale bar is 200  $\mu$ m for whole mount and 40  $\mu$ m for transverse sections.

explain CDX4 negative effect on *Nkx1.2* transcription one has to evoke an indirect mechanism of action. One possible mechanism is that CDX4

activates neural differentiation genes such as *Pax6* that in turn downregulate *Nkx1.2* transcription. To test this possibility, we overexpressed

*Pax6* in the PNT and analyzed the transcription of *Nkx1.2* at HH12-13. Under these conditions, PAX6 did not affect *Nkx1.2* transcription (Fig. 4B). While this result does not shed light on the mechanism by which CDX4 indirectly regulates *Nkx1.2* transcription, it does show that this activity is not mediated by PAX6 (Fig. 4C).

2.6. NKX1.2 and PAX6 interactions result in the segregation of their expression domains to different regions of the preneural tube

*Nkx1.2* and *Pax6* transcription domains are mutually exclusive, but both span *Cdx4* transcription domain (summarized in Fig. 1A), suggesting possible cross-regulatory interactions between *Nkx1.2* and *Pax6*, and between these two genes and *Cdx4*. To test for this possibility, we analyzed the expression of these genes after the overexpression of the other two genes in the caudal PNT at HH10-11. To analyze NKX1.2 function, we overexpressed the mouse version of *Nkx1.2* (*mNkx1.2*), which acts as a repressor in mouse cell lines (Tamashiro et al., 2012) and chicken embryos (Sasai et al., 2014). Consistent with previous report (Sasai et al., 2014), overexpression of *mNkx1.2* represses *Pax6* at HH12 (8 hpe; Fig 5Ac). In addition, mNKX1.2 also repressed *cNkx1.2* transcription (Fig 5Aa), suggesting negative autoregulation. However, *mNkx1.2* overexpression had no effect on *Cdx4* transcription (Fig 5Ab). Using the same strategy, we analyzed PAX6 activity on *Cdx4*. In this experiment, overexpression of *Pax6* downregulated and dominant-negative *EnRPax6* upregulated *Cdx4* transcription (Fig. 5B), suggest that PAX6 functions to represses *Cdx4*. Together, these results providing a mechanism to explain the segregation of *Nkx1.2* and *Pax6* transcriptional domains to caudal and rostral PNT, respectively, and the downregulation of *Cdx4* in the caudal NT (Fig. 5C; see model below).

3. Discussion

3.1. A gene regulatory network controlling spinal cord neuronal maturation

3.1.1. CDX4 promotes loss of potency in the caudal pre-neural tube

Previous work has shown that CDX are key in the establishment and subsequent differentiation of NMPs into neural and mesodermal precursors by balancing the activity of WNT3a, FGF8 and RA signaling

(Amin et al., 2016; Chawengsaksophak et al., 2004; Gouti et al., 2017). Mouse embryos deficient for all *Cdx* genes fail to develop post-occipital structures due to the premature differentiation of NMP cell (Amin et al., 2016; van Rooijen et al., 2012). The primary cause for this premature differentiation is the premature activation of the RA pathway, which in cell culture conditions causes NMPs to follow a neural fate by maintaining *Sox2* and *Nkx2.1*, and repressing *T/Bra* transcription (Gouti et al., 2017).

Here we show important additional functions for CDX4, the only CDX member present in chick PNT post HH12 (Marom et al., 1997), in the progressive maturation of spinal cord neuronal progenitors. As NMPs' descendants acquire neural identity (from *T/Bra+*, *Sox2+*, *Nkx1.2+* to *T/Bra-*, *Sox2+*, *Nkx1.2+*), CDX4 promote their further maturation by repressing *Nkx1.2* transcription (Fig. 4). Control of *Nkx1.2* transcription is tightly balanced by Wnt and FGF signaling (Bertrand et al., 2000; Tamashiro et al., 2012), which in turn are regulated by CDX (Chawengsaksophak et al., 2004; Gouti et al., 2017; Savory et al., 2009; van Rooijen et al., 2012). We speculate that increasing or decreasing CDX4 levels could cause an imbalance in Wnt and FGF that could lead to an indirect loss of *Nkx1.2* transcription. Given that NKX1.2 inhibits floor plate cell specification by repressing *Pax6* (Sasai et al., 2014), we propose that CDX4 downregulation of *Nkx1.2* is one of the first steps in PNT cell maturation.

3.1.2. CDX4 promotes neural cell determination in the rostral pre-neural tube

Progression of cells from caudal to rostral PNT is marked by the acquisition of neural determination markers. CDX4 promotes new maturation states by directing *Pax6* activation, a factor involved in neural progenitor maturation (Bel-Vialar et al., 2007). Consistent with this, CDX factors have been observed to increase *Pax6* transcription in embryoid bodies (McKinney-Freeman et al., 2008). We propose that in the PNT, CDX4 regulation of *Pax6* occurs via two distinct mechanism (Fig. 6): by the indirect down regulation of the *Pax6* repressor NKX1.2 (Fig. 5), and by the direct activation of *Pax6* transcription (Fig. 2). Importantly, CDX4 activation of *Pax6* is dependent on RA (Fig. 2), which is secreted from somites (Molotkova et al., 2005; Olivera-Martinez and Storey, 2007). This spatial distribution of RA restricts the *Pax6* inducing activity of CDX4 to the rostral PNT. RA/CDX regulation of *Pax6* is likely to be

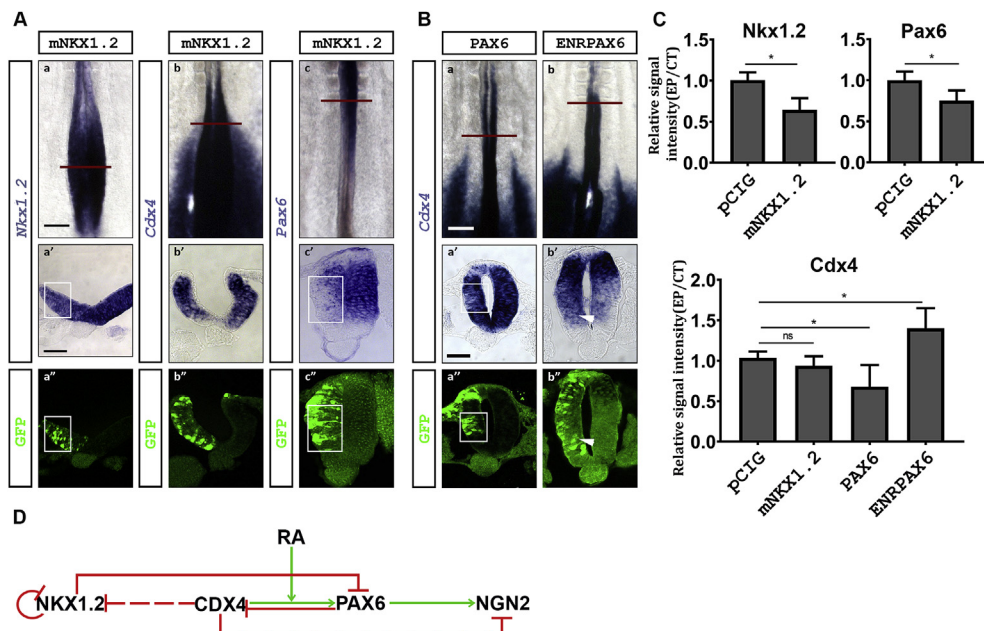
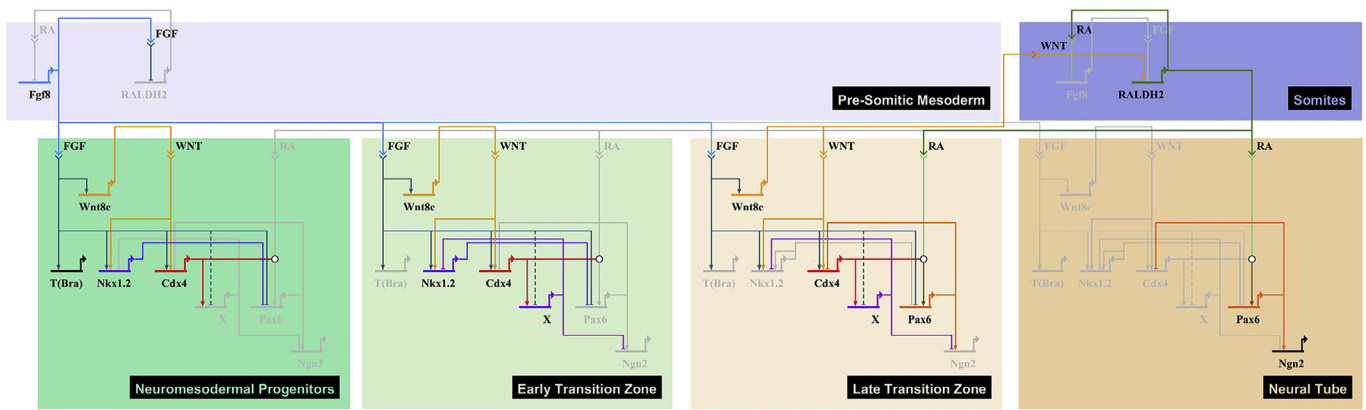


Fig. 5. NKX1.2 and PAX6 contribute to *Cdx4* regulation. (A) NKX1.2 negatively regulates the transcription of its own gene and of *Pax6*, but not *Cdx4*. Overexpression of *mNkx1.2* downregulates *cNkx1.2* (a, a') and *Pax6* (c, c'), without altering *Cdx4* transcription (a', c'; boxed regions). (B) PAX6 represses *Cdx4*. Ectopic *Pax6* downregulates (a, a'; boxed region), and *EnRPax6* upregulates (b, b'; arrowhead) *Cdx4* transcription. Embryos were electroporated at HH10-11 and analyzed 8hpe (HH12-13). (C) Quantification of *Nkx1.2*, *Pax6* and *Cdx4* transcription levels by relative signal intensity analysis (electroporated (ET)/Control (CT) sides). Experimental conditions were normalized to control pCIG vector (for all conditions, n = 6 from at least 3 bio-replicates, \*p ≤ 0.05, ns: not significant). Figure summarizing NKX1.2-CDX4-PAX6 interactions. Solid lines are direct and dashed lines are indirect interactions. Scale bar is 200 μm for whole mount and 40 μm for transverse sections.



**Fig. 6.** Proposed gene regulatory network controlling spinal cord neuronal cell maturation. Gene regulatory network of the genetic interactions identified in Figs. 1–5, superimposed to the FGF-Wnt8c-RA signaling network shown by others to regulate cell transitions states during spinal cord neuronal cell maturation (Olivera-Martinez and Storey, 2007). Network map was generated using Biopapestry (Longabaugh et al., 2005). In this model, CDX4 is at the core of the gene regulatory network that coordinates upstream signaling information into downstream transcriptional response.

evolutionarily conserved across vertebrates, as in all species examined the second intron of *Pax6* contains an ultraconserved non-coding region that harbors both RA response elements (RAREs; Cunningham et al., 2016) and CDX4 binding sites (Paik et al., 2013 and Fig. S3). In addition to DNA binding, RA has been implicated in opening up the *Pax6* locus by antagonizing FGF signaling (Patel et al., 2013). In this scenario, RA could function to provide locus accessibility to CDX4 and other factors. Thus, in the PNT, RA provides the context in which CDX4 can further promote neural progenitor cell maturation.

### 3.1.3. CDX4 prevents premature neural cell differentiation

PAX6 induces neural cell differentiation (Bel-Vialar et al., 2007), and yet, despite *Pax6* being induced by CDX4 in the rostral PNT (Fig. 2), neural cell differentiation does not begin until after the NT has formed and *Cdx4* has been down regulated (Fig. 1). Two mechanisms by which PAX6 promotes differentiation is by downregulating *Cdx4* (Fig. 5B) and by activating *Ngn2* (Bel-Vialar et al., 2007; Scardigli et al., 2003), a gene that promotes cell cycle exit and further cell differentiation (Lacomme et al., 2012). Our data shows that CDX4 represses *Ngn2* transcription even in the presence of PAX6 (Fig. 3), thus priming but delaying spinal cord terminal cell differentiation. Along the caudal-to-rostral axis of the neural tube, CDX4 transcription is gradually restricted to the dorsal neural tube (Fig. 1), at a time when *Ngn2* transcription initiates ventrally (Fig. 3). At the moment, it is unclear how CDX4 represses *Ngn2*, as CDX4 are known transcriptional activators (Isaacs et al., 1998). In sum, by regulating the activation of specification, determination and differentiation genes, CDX4 controls the transition of neural cells from one state to the next during the early maturation of the spinal cord. The regulation of cell transitions by CDX proteins may be a general property of this family of transcription factors, as CDX family members have also been described to control maturation of multipotent cell precursors in intestinal (Hryniuk et al., 2012; Saad et al., 2011) and hematopoietic (McKinney-Freeman et al., 2008; Wang et al., 2008) tissues.

## 3.2. A model of spinal cord neuronal maturation that integrates transcription and signaling networks

In current models of spinal cord development, cells progressively lose potency and acquire neural characteristics under the control of FGF, Wnt and RA signaling (Diez del Corral and Storey, 2004; Gouti et al., 2014). Mutual interactions among these signaling factors restrict the activity of respective pathways to specific domains within the caudal and rostral PNT to direct cell fate decisions. In the caudal end, high levels of FGF promote *Wnt* transcription while repressing RA pathway activity through a variety of mechanisms (Boulet and Capecchi, 2012; Olivera-Martinez

et al., 2012; Sakai et al., 2001; White et al., 2007). In turn, WNT8c promotes RA synthesis in anterior pre-somitic mesoderm and somites, away from the caudal domain of FGF activity. RA secreted from these anterior sources represses FGF synthesis, helping establish and refine the high-caudal to low-rostral gradients of FGF, and indirectly, Wnts (Diez del Corral et al., 2003; Kumar and Duester, 2014). This cross-repressive activities of FGF/Wnts and RA create a caudal-to-rostral gradient of potency signals and a rostral-to-caudal gradient of pro-differentiation signaling that promote the gradual maturation of spinal cord cells (Figs. 1 and 2). Molecularly, FGF and Wnt maintain NMPs cells by promoting the transcription of multipotency genes *T/Bra*, *Sox2* and *Nkx1.2*, while simultaneously repressing the differentiation genes *Pax6*, and *Ngn1/2*. In contrast, RA promotes differentiation by repressing *T/Bra* and *Nkx1.2* and inducing *Pax6* and *Ngn1/2* transcription. Thus, the switch from NMP to pre-neural to neurogenic identities is the response of cells to change in extracellular signals (Diez del Corral et al., 2003).

We have expanded the model of spinal cord neurogenesis by integrating this signaling network with a new transcription network into a single model (Fig. 6). The FGF-Wnt-RA network model provides a series of interactions that result in the spatiotemporal separation of regulatory inputs without providing intracellular mechanisms for the specification and separation of cells states, whereas the transcription factor network provides a molecular mechanism for the specification of different cellular states, but lacks the inputs necessary to drive the system forward. CDX4, at the core of the transcription factor network, provides an integration point for the inputs to regulate effector genes, as *Cdx4* transcription is directly regulated by FGF, Wnt and RA (Chang et al., 2016; Keenan et al., 2006; Lee and Skromne, 2014; Nordstrom et al., 2006; Tamashiro et al., 2012). FGF and Wnt promote potency by directly activating *Nkx1.2* (Diez del Corral et al., 2003; Tamashiro et al., 2012), but also initiate the loss of potency by sustaining *Cdx4* transcription that indirectly represses *Nkx1.2* (Fig. 4B). A similar “dual-activity” phenomenon is observed in the regulation of *Pax6*, with FGF both repressing (Bertrand et al., 2000) and activating (via CDX4; Fig. 2B) *Pax6* transcription. While the mechanism by which CDX4 antagonizes FGF activity at the *Pax6* locus is unknown, it may involve a change in *Pax6* chromatin state. FGF signaling has been shown to cause the translocation of the *Pax6* locus to the nuclear boundary associated with inactive chromatin (Patel et al., 2013). CDX4 could antagonize this activity, as CDX family members have been associated with the clearance of repressive histone modifications in other loci (e. g., *Hox*; Mazzoni et al., 2013). Regardless of the mechanism, we observe that for two genes, *Nkx1.2* and *Pax6*, CDX4 antagonizes FGF and synergizes with RA. We propose this FGF-CDX/RA antagonism provide a time delay mechanism to separating early, intermediate and late states of cell differentiation. Experiments are under way to test the interactions



between the signaling and transcription factors discussed in this model.

### 3.3. CDX and the coordinated control of spinal cord neuronal maturation, patterning and growth

In addition to regulating spinal cord neuronal maturation, CDX factors are key regulators of axial patterning and elongation. In the context of patterning, CDX4 work together with FGF (and Wnts) to activate transcription of branchial and thoracic *Hox* genes (Bel-Vialar et al., 2002; Marletaz et al., 2015; Nordstrom et al., 2006; Shimizu et al., 2006; Skromne et al., 2007) and antagonizes RA's ability to induce hindbrain *Hox* genes (Lee and Skromne, 2014; Marletaz et al., 2015; Skromne et al., 2007). Significantly, this interaction is in contrast to the CDX4-FGF antagonism and CDX4-RA cooperation that we observed during spinal cord neuronal maturation (Fig. 6). The molecular mechanism underlying this context-dependent switch in CDX4 activities is currently unknown. However, CDX4 involvement in both processes is significant as it provides a mechanism for coordinating the maturation and anterior-posterior identity specification of spinal cord neurons.

CDX role in vertebrate body extension involves maintaining progenitor population via two distinct mechanisms. Early in spinal cord development CDX cooperate with T/BRA to promote FGF and Wnt signaling cascades and sustain NMP proliferation (Amin et al., 2016; Gouti et al., 2017), whereas, later in development, CDX activate *Hox13* genes involved in axial termination (van de Ven et al., 2011; Young et al., 2009). Mutations in mouse that inactive *Cdx* or prematurely activate *Hox13* impairs elongation and morphogenesis of the spinal cord neuroepithelium, which results in irregular or duplicated neural structures (van de Ven et al., 2011). These neural tube defects are similar to those observed in mutants in the mesoderm specification genes *T/Bra* and *Tbx6* (Chapman and Papaioannou, 1998; Yamaguchi et al., 1999), leading to the proposal that caudal spinal cord defects associated with the loss of CDX arise through defects in the specification of NMP descendent (van de Ven et al., 2011). In light of our results, however, the neural tube abnormalities associated with CDX loss could also be explained, at least in part, to defects in spinal cord neuronal maturation. Future work will need to determine the contextual contribution of CDX in coordinating spinal cord cell maturation, differentiation and axial identity specification.

## 4. Materials and methods

### 4.1. Chicken embryo incubation and harvesting

Fertilized broiler chicken eggs (Morris Hatchery, Inc.; Miami, FL) were incubated at 38.2 °C in a humid chamber until reaching the appropriate stage of development. The embryos were staged according to Hamburger and Hamilton normal table of development (Hamburger and Hamilton, 1951). Embryos post-electroporation were incubated until stipulated time for further analysis.

### 4.2. DNA constructs and chicken in ovo electroporation

Gene overexpression studies were done using standard cloning and electroporation techniques. To achieve high level of gene expression and to track electroporated cells, gene of interest was cloned either into pCIG or pCAGIG vector (Matsuda and Cepko, 2004; Megason and McMahon, 2002). These vectors use the chicken *Actin* promoter to drive high gene expression levels, and carry a *GFP* gene as a reporter for transcription. Genes of interest were either cloned into vectors in the laboratory (*Cdx4*, *VP16Cdx4*, *EnRCdx4*, *mNkx1.2*; for details see supplementary material), or obtained already in the appropriate vector from other laboratories (*Pax6*-pCIG and *EnRPax6*-pCIG were kindly provided by Dr. Francois Medevielle (Bel-Vialar et al., 2007); and *mNkx1.2*-pEF2 was kindly provided by Dr. Yusuke Marikawa (Tamashiro et al., 2012). Plasmids for electroporation were purified using QIAGEN maxi-prep kit, and diluted to a final concentration of 0.5 µg/µl in 1X PBS, with 50 ng/ml Fast Green

dye to aid in the visualization of the cocktail mix during the procedure. Neural tube of chicken embryos stage HH10-11 were injected with the DNA cocktail mix and immediately electroporated unilaterally following standard protocols (Itasaki et al., 1999; Nakamura and Funahashi, 2001). Only normal-looking embryos with good electroporation in the desired region (e.g., neural tube, pre-neural tube, or caudal neural plate depending on experimental requirements) were selected for further processing by *in situ* hybridization or immunohistochemistry. Analysis was focused on same axial level in all stage: PNT for stage HH12-13 (prospective thoracic level; Liu et al., 2001), and NT for stage HH16-17 (thoracic level between somites 20–25; Evans, 2003).

### 4.3. In situ hybridization

Analysis of gene transcription by *in situ* hybridization was done using digoxigenin (DIG)-labeled antisense RNA probes synthesized and hybridized using standard protocol (Wilkinson and Nieto, 1993). Briefly, embryos were harvested at the appropriate stage and fixed with 4% paraformaldehyde diluted in 1x PBS at 4 °C overnight, before processing for *in situ* hybridization. After a series of washes, embryos were exposed overnight in hybridization solution to DIG-labeled antisense RNA probes against *Pax6*, *Nkx1.2*, *T/Bra*, or *Cdx4*. mRNA expression was detected using an Alkaline Phosphatase coupled Anti-DIG antibody (Roche) and developing embryos with nitro-blue tetrazolium salt (NBT, Thermo Scientific) and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP, Biosynth) at room temperature until dark purple precipitate deposited revealing the areas of gene transcription. For double *in situ* hybridization, one of the probes was labeled with FITC and developed with Fast Red (Sigma-Aldrich). Post-development, embryos were washed with 1x TBST and then fixed in 4% PFA.

### 4.4. Cryo-sectioning and immunohistochemistry

Embryos harvested for immunohistochemistry (IHC) analysis were fixed with 4% PFA for 3 h at room temperature. Embryos were then embedded in Shandon M1 embedding matrix media (Thermo Scientific) and quickly frozen over dry ice. Mounted embryos were sectioned on Leica CM1850 cryostat and consecutive 20 µm thick sections were collected on positive-charged glass slides (Globe scientific). Antibody staining was performed following standard protocols on slides stacked in Shandon Sequenza slide rack (Thermo Scientific) and supported by Shandon cover plates.

Primary antibodies against anti-mouse PAX6, PAX7 and NKX6.1 were obtained from development Studies Hybridoma Bank. Anti-chicken NGN2 antibody was a kind gift from Dr. Bennett Novitch (Skaggs et al., 2011). Rabbit polyclonal antibody against GFP Tag was obtained from AnaSpec Inc. Goat anti-mouse Alexa Fluor 488, Alexa Fluor 556 and goat anti-guinea pig Alexa Fluor 568 secondary antibodies (Invitrogen) were used for detecting primary antibodies. Sections were covered with DAPI-containing mounting media (Vectashield) and a cover slip, and sealed with nail polish.

### 4.5. Microscopy

Whole embryo images were taken on Zeiss V20 Stereo microscope with an AxioCam MRc digital color camera (Carl Zeiss). Images of transverse section of neural tube were taken on AXIO Examiner Z1 compound microscope with an AxioCam MRc color camera (Carl Zeiss), or on a Leica SP5 confocal microscope (Leica). Confocal images, thickness 2.304 µm, were processed with ImageJ (Schneider et al., 2012). Images were processed for figures using Adobe Photoshop (CC2017, Adobe) for size and resolution adjustment, and for figure preparation.

### 4.6. Data quantification and statistical analysis

For all experiments, a total of six embryos containing fluorescently-

labeled cells at the appropriate anterior-posterior and dorsoventral axes were used for statistical analysis. The only exception was the IHC experiment shown in Fig. 2B, in which four embryos were used to confirm the results obtained by ISH in Fig. 2A. The use of six embryos was based on a priori statistical power of analysis test done using the program GraphPad Statmate 2.00 to determine appropriate sample size. To obtain a 99% power, the program indicated that six embryos per group were needed. The power of analysis result was confirmed by running a hypothetical two-tailed t-tests (Microsoft Excel). In all experiments, 6/6 embryos showed the indicated phenotype, except Fig. 2A, in which 4/6 embryos showed the indicated phenotype. Embryos for analysis were obtained from at least three independent bio-replicates.

Changes in PAX6 or NGN2 levels after electroporation was done by comparing the number of positive cells on control and electroporated side of the spinal cord at the same dorsal-ventral position, as described by others (Karaz et al., 2016). Before cell counting, image threshold levels were processed with ImageJ IHC toolbox plugin (Shu et al., 2013). Quantifications were performed on sections collected from six embryos per condition; the precise number of cells counted indicated in Supplemental Table S3. Significance of difference between mean values of compared pairs was evaluated using two-tailed t-test (Microsoft Excel). Data for each condition was graphed into a box-plus-scatter plot using MATLAB (2014b, The MathWorks Inc., Natick, MA, 2014).

Analysis of relative signal intensity was done using ImageJ. Briefly, ISH image was converted to an 8-bit black and white image, and the colors inverted. Mean signal intensities were obtained by separately defining regions of interests for the control and electroporated sides of the neural tube. Intensity values were then adjusted by subtracting the mean background intensity from each sample. Changes in expression were obtained by dividing the intensity of the signal on the electroporated side by the intensity of the signal in the control side.

#### Author contributions

P.J. and I.S. designed the experiments. P.J. performed the experiments. A. J. D. provided intellectual contributions towards designing and troubleshooting experiments. P.J. and I.S. analyzed the results. P.J., A.J.D and I.S. wrote the manuscript.

#### Competing interests

No competing interest declared.

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#### Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ydbio.2019.02.014>.

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