CDX4 regulates the progression of neural differentiation in the spinal cord

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Abstract

The progressive maturation of cells down differentiation lineages is controlled by collaborative interactions between networks of extracellular 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 regulates the progression of neural differentiation by controlling the sequential transition of neural progenitors from states of high to low potency. 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.

Introduction

Differentiating cells transit from one temporary state to another as they lose potency and acquire specialized functions. At each step of their differentiation path, the next state is specified by the regulatory state of the cell, as defined by the totality of active transcription factors [1, 2]. During this gradual process, the cell's transcriptome also transits from one state to another, mostly cued by dynamic extra-cellular signaling factors [3, 4]. 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 temporal activity of transcription factors to promote directional acquisition of specialized traits [4-6]. 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.

Vertebrate spinal cord serves as an important accessible system to study the maturation of neural progenitors during their transition from one cellular state to the next. In the embryonic neural tube, the maturation of neural progenitors follows a caudal-to-rostral organization, with undifferentiated cells localizing to caudal regions and more mature cells localizing to more rostral positions [7-10]. In chicken embryo, extensive gene expression analysis has resulted in the identification of distinct embryonic regions corresponding to different neural maturation states (Fig 1A). The most caudal population of cells are the neuromesodermal progenitors (NMPs) that can contribute to both neural and mesodermal tissues ([11-13]; reviewed in [14]). In early embryos, these cells reside in the caudal lateral epiblast and the node-streak border surrounding the primitive streak and, in older embryos, in the caudal neural hinge region of the

tailbud [12, 14]. Two key transcription factors are co-expressed in the NMPs, the pan-neural marker *Sox2* and the mesodermal marker *T/Bra*, although they also transcribe the pre-neural identity marker *Nkx1.2* (also known as *Sax1*; [15-17]). Immediately rostral to the NMP domain is the pre-neural tube (PNT; [14, 17]). Cells in the PNT downregulate *T/Bra* but continue to express *Sox2* and *Nkx1.2* [15, 17]. With the use of additional markers, the PNT can be further subdivided into a caudal and rostral PNT: compared to caudal PNT, rostral PNT cells downregulate *Nkx1.2* [15, 18, 19] and activate *Pax6* transcription [18, 20, 21]. Finally, rostral to the PNT is the neural tube (NT; [14, 17]), which lies adjacent to the developing somites. In this region, NT cells are *Nkx1.2*$^-$ and *Pax6*$^+$, and begin to transcribe the neural differentiation genes *Ngn1/2* and *NeuroM* [8]. Thus, from caudal-to-rostral, four spatially distinct populations can be identified that correspond to four maturation states (summarized in Fig 1A): (1) Caudal later epiblast/node-streak border (bipotent state, NMPs: *T/Bra*$^+$, *Sox2*$^+$, *Nkx1.2*$^+$), (2) caudal PNT (acquisition of neural identity state: *Sox2*$^+$, *Nkx1.2*$^+$, *Pax6*$^-$), (3) rostral PNT (neural progenitor state: *Sox2*$^+$, *Nkx1.2*$^-$, *Pax6*$^+$), and (4) NT cells (early neural differentiation state: *Sox2*$^+$, *Pax6*$^+$, *Ngn1/2*$^+$, *NeuroM*$^+$).

The transcriptional signatures observed during neural progenitor maturation have recently been corroborated and expanded using single cell transcriptome analysis of *in vitro* differentiating NMPs, allowing the assignment of specific gene activities to particular maturation states [16, 22]. By reverse engineering NMP's differentiation pathways *in vivo* and then reconstituting these pathways *in vitro*, this approach has circumvented several of the challenges involved in separating the activities of genes with multiple cell specification and maturation functions. For example, CDX transcription factors have been implicated in NMP maintenance, axial patterning and elongation [23]; but the separation of these activities has been challenging

due to the multiple and partially redundant activities of three functionally similar proteins (CDX1, CDX2 and CDX4; [24]). By studying the differentiation of NMPs derived from mouse Embryonic Stem Cells (mESC) lacking individual or different combination of *Cdx* genes, CDX were shown to regulate the temporal maintenance of *T/Bra* [16]. By maintaining or down regulating *T/Bra* transcription, *Cdx* genes play a pivotal function in regulating NMP's fate choice to become either mesoderm or neural tissues [16]. Although *Cdx* are transcribed in NMPs derived from mESC [16] and in embryos ([25, 26]; Fig 1B), the functional significance of this gene to neural progenitor maturation remains unknown.

The transition from NMP to pre-neural to neural transcriptional states is under the control three signaling factors: FGF, Wnt and Retinoic Acid (RA; [8, 27]). At the caudal end of the embryo, FGF8 and Wnts (Wnt3a and Wnt8c) are transcribed in a caudal to rostral gradient that promotes multipotency by maintaining *T/Bra*, *Sox2* and *Nkx1.2* transcription while simultaneously preventing *Pax6* and *Irx3* transcription [8, 15, 21, 28]. In contrast, RA secreted from somites establishes a rostral to caudal signaling gradient that promotes differentiation by first inducing cells to exit the proliferation program by promoting transcription of neural identity genes *Pax6* and *Irx3* [8, 29, 30], and subsequently, by promoting transcription of downstream neurogenic genes *Ngn1/2* and *NeuroM* [8]. 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 [27, 28, 31-33]. In turn, RA inhibits *Fgf8* transcription rostrally, creating a zone where cells can exit the cell cycle and differentiate [8, 34]. These interactions have been proposed to function as the signaling switch that drives the transition of cellular states in the

caudal neural tube [9, 27]. 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 remains poorly understood.

FGF, Wnt and RA signals are known regulators of *Cdx* transcription [35, 36], making CDX transcription factors good candidates to regulate pre-neural tube cell maturation. In chicken embryos, *Cdx* genes are transcribed in nested domains, at levels that are high in NMPs and that taper rostrally towards the NT. These high-to-low levels of transcription have also been observed in differentiating NMPs *in vitro* [16]. In the spinal cord, CDX factors are essential for tissue specification and rostro-caudal patterning [37-41], controlling the initial specification of post-occipital tissues [24], and the subsequent patterned transcription of *Hox* expression domains [37, 42]. Thus, *Cdx* are attractive candidates to integrate multiple signals into coherent cell maturation states.

Here we show that chicken CDX4 controls the progression of PNT cells towards more mature states without allowing 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*$^{-}$) to a neural progenitor state (*Sox2*$^{+}$*, Nkx1.2*$^{-}$*, Pax6*$^{+}$). Significantly, *Pax6* activation by CDX4 is dependent on RA secreted by somites, which restricts the cell's transition to a neural progenitor state to the rostral PNT. Furthermore, we show that CDX4 prevents *Ngn2* activation even in the presence of the *Ngn2* activator PAX6, thus preventing the premature terminal differentiation of cells. 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 FGF, Wnt, and RA

signaling network. Our results support a previously unreported role for CDX factors in regulating the onset and progression of neural precursor maturation.

Results

Cdx4 is transcribed in a dorsal-ventral gradient in the caudal neural tube where it regulates *Pax6* transcription

CDX4 neural function in chicken embryos was first analyzed by correlating its transcription domain to distinct progenitor cell maturation zones of the caudal neural plate (Fig 1A; [27]). As reported [43], in whole embryos, *Cdx4* is transcribed in the neural plate and nascent neural tube 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 neural plate (dorsal-ventral extent after neural plate closure), 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 couldn't examine the CDX4 protein profile in the neural tube. However, a similar dorsally restricted expression profile has been reported for CDX4 in mice [25], suggesting evolutionary conserved transcriptional mechanisms, and a potential function for CDX4 in the specification of DV neural cell identities.

Fig 1. CDX4 activates transcription of the neurogenesis gene *Pax6*.

(**A**) Schematic representation of the caudal end of HH10 chicken embryo showing primary subdivisions (central diagram; adapted from [27]), 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 at HH11 stage. 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 a and red in b, c; *Pax6* expression in purple in b and d). 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 8 or 24 hours post electroporation (hpe), when electroporated cells are localized to the rostral portion of the PNT or the caudal portion of the NT, 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 regulate DV patterning in the neural tube. Ectopic *Cdx4* did not change the distribution of PAX7 or NKX6.1 proteins (n=6/6 for both), but caused ectopic PAX6 accumulation outside its normal domain (arrowheads, n=6/6). Marker proteins are in red and electroporated cells are in green (nuclear GFP tag). Embryos were electroporated at HH10-11 and analyzed 24 hpe. (**E**) Summary of results. Scale bar is 200µm for whole mount and 40µm for transverse sections.

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* [8, 44], the dorsal-to-intermediate cell marker *Pax6* [29, 44], and the ventral cell marker *Nkx6.1* [8, 44, 45]. We did not observe any clear correlation between the transcriptional domain of *Cdx4* and any of these markers. For example, in the caudal PNT, PAX7 domain was nested within, and NKX6.1 domain was complementary to *Cdx4* domain of transcription (Fig 1B, C). However, in the rostral PNT and caudal NT, PAX7 domain was broader than, and NKX6.1 domain no longer complemented the *Cdx4* transcription domain (Fig 1B, C). Similar results were observed for *Cdx4* and PAX6 protein in the NT (Fig 1B, C). Only in the PNT we observed a correlation between *Cdx4* and *Pax6* transcription, with *Cdx4* levels decaying and *Pax6* levels increasing in the caudal to rostral direction (Fig 1D).

To formally test *Cdx4* involvement in DV cell fate specification, we artificially maintained high levels of *Cdx4* in the neural tube at times when *Cdx4* would normally be downregulated. We predicted that if CDX4 regulates DV cell specification, increasing *Cdx4* levels would result in 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 at 24-hours post-electroporation (hpe; HH16-17), at time when electroporated cells would not be transcribing endogenous *Cdx4* (Fig 1E). While high levels of *Cdx4* expression did not change NKX6.1 and PAX7 protein distribution (Fig 1F; n=6/6 for both conditions), *Cdx4* caused ectopic production of PAX6 protein outside its normal domain, both ventrally and dorsally (Fig 1F; n=6/6). In this and all other experiments, electroporation of a control reporter

GFP vector did not result in changes in protein distribution/gene transcription (Fig S1). Together, these results do not support a general role for CDX4 in regulating DV identities in the NT. Instead, these results suggest that CDX4 is involved in the specific regulation of *Pax6* (Fig 1G).

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 [20]. Given that our results do not support a function for CDX4 in DV cell specification (Fig 1), we hypothesized that CDX4 might regulate *Pax6* transcription during neural progenitor cell maturation. To test this hypothesis, we asked whether the presence of CDX4 was sufficient to activate *Pax6* in the PNT, in a region where *Pax6* is not normally transcribed. Embryos were electroporated in PNT with different *Cdx4* constructs, grown for 8 hours only, 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 faithfully recapitulates Cdx functions in *Hox* gene regulation assays (VP16CDX4; [46, 47]). Overexpression of *VP16Cdx4*, but not wild type *Cdx4*, resulted in the premature, high-level activation of *Pax6* transcription (Fig 2A; n=4/6 by ISH. Fig 2B; n=3/4 by IHC). Several explanations could account for the lack of wild type CDX4 activity on *Pax6* including the requirement for additional cofactors, or its inability to overcome the presence of repressors. VP16CDX4 was able to overcome these hurdles, suggesting that, under the right conditions, activated CDX4 can induce *Pax6* transcription during neural progenitor cell maturation.

Fig 2. CDX4 activation of *Pax6* transcription is RA-dependent.

(**A**) CDX4 is not sufficient to activate *Pax6* transcription in the PNT. In the PNT, *Cdx4* has no effect (a, a'; n=6/6), *VP16Cdx4* induces (arrowheads in b, b'; n=4/6), and *EnRCdx4* downregulates (c, c', box; n=6/6) *Pax6* transcription (purple signal by ISH; green GFP tag labeled by IHC). Embryos were electroporated at HH10-11 and analyzed 8 hpe. (**B**) Ectopic *VP16Cdx4* (n=3/4) but not *Cdx4* (n=0/4), causes ectopic PAX6 protein accumulation (arrowheads). (**C**) In the NT, CDX4 requires Retinoic Acid (RA) to activate *Pax6* transcription. In the NT, both *Cdx4* and *VP16Cdx4* overexpression result in ectopic PAX6 protein accumulation (a, a', b, b'; arrowheads; n=6/6 for both), whereas *EnRCdx4* overexpression causes the loss of PAX6 (c, c'; box; n=6/6). Inhibition of RA signaling using a dominant negative RA receptor (*dnRAR*) causes the loss of PAX6 (d, d'; box; n=6/6). In the absence of RA signaling, *Cdx4* overexpression is unable to induce ectopic PAX6 (e, e'; box; n=6/6). Under similar conditions, *VP16Cdx4* overexpression induces ectopic PAX6 (f, f'; arrowheads; n=6/6). Embryos were electroporated at HH10-11 and analyzed 24 hpe. (**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 [48]). Cells were counted using ImageJ. Significance is shown with a bar and a star (two tailed t-test analysis, $p<0.05$). (**E**) Summary of results. Scale bar is 200µm for whole mount and 40µm for transverse sections.

To test if CDX4 activation is necessary for *Pax6* activation in the PNT, we outcompeted endogenous CDX4 by overexpressing 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 (49). This chimeric form of CDX4 has been shown to repress transcription of downstream CDX targets (e.g., *Hox* genes; [46, 50]). Overexpression of *EnRCdx4* resulted in loss of *Pax6* in the PNT as early as 8 hpe (Fig 2Ac; n=6/6), indicating that CDX4 indeed regulates *Pax6* transcription. The observations that (1) the caudal PNT transcribes *Cdx4* but not *Pax6* and that (2) VP16CDX4 but not CDX4 can induce *Pax6* suggest that CDX4 activation of *Pax6* transcription is permissive and requires additional factors.

CDX4 activation of *Pax6* is dependent on Retinoic Acid signaling

Somite-derived RA regulates spinal cord neurogenesis by activating numerous target genes in the rostral PNT, including *Pax6* [29, 30]. Given that RA and CDX4 interact during zebrafish spinal cord cell specification [51, 52], we hypothesized that RA and CDX4 might also interact during spinal cord cell maturation. To test this hypothesis, we electroporated PNT cells with dominant negative RA receptors (dnRAR) to block RA signaling [29], and then analyzed the transcription of *Pax6* 24-hpe, at a time when electroporated cells would be undergoing maturation. As previously shown [29], overexpression of *dnRAR* blocked RA signaling and caused *Pax6* down regulation (Fig 2Cd, D), even as *dnRAR* enhanced *Cdx4* transcription (Fig S2). To test if the induction of *Pax6* by CDX4 is RA dependent, we co-electroporated different *Cdx4* constructs together with *dnRAR*. In these RA-deficient cells, CDX4 was unable induce *Pax6* in the NT (Fig 2Ce, D; n=6/6), despite its ability to do so in RA-responsive cells (Fig 1D). Significantly, however, VP16CDX4 was able to induce *Pax6* transcription even in the absence of RA (Fig 2Cb, Cf, D; n=6/6). Together, these results suggest that *Pax6* activation by CDX4 is dependent on RA signaling, and illuminates a mechanism for the restricted transcription of *Pax6* to the rostral portion of the PNT (Fig 2E).

CDX4 promotes early differentiation states by simultaneously inhibiting cell proliferation and late differentiation programs

Despite PAX6 being present in both the rostral PNT and the NT, PAX6 only activates neural differentiation genes in the NT [20]. What prevents PAX6 from prematurely activating neural differentiation genes in the PNT? We addressed this question by analyzing the transcription of the neural differentiation gene *Ngn2*, a downstream target of PAX6 [53]. *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 analyzed NGN2 distribution after *Cdx4*, *VP16Cdx4* and *EnRCdx4* overexpression. Each construct independently caused *Ngn2* down regulation (Fig 3B, C; n=6/6 for all conditions), despite that CDX4 and VP16CDX4 inducing *Pax6* transcription in the NT. These results suggest that CDX4 represses *Ngn2*. In this experiment, ENRCDX4 repressed *Ngn2* because ENRCDX4 represses *Pax6* (Fig 2Cc), and PAX6 is essential for ventral *Ngn2* transcription [53]. To confirm that CDX4 represses *Ngn2* even in the presence of PAX6, we co-expressed *Cdx4* and *Pax6* simultaneously. While PAX6 on its own can ectopically activate *Ngn2* (Fig 3Bd, C; n=6/6; [20]), it is unable do so in the presence of CDX4 (Fig 3Be, C; n=6/6). Given that CDX4 has been shown to be a transcriptional activator [50], the repression of *Ngn2* by CDX4 is likely to be indirect. Taken together, our results suggest that CDX4 promotes different neural cell maturation steps by promoting the cell's entry into the differentiation program by inducing *Pax6* transcription, while prevents further differentiation by inhibiting *Ngn2* activation (Fig 3D).

Fig3. CDX4 inhibits early cell maturation by repressing the neural differentiation gene *Ngn2* and the cell cycle gene *Hes5*.

(**A**) *Ngn2* expression in the NT of wild type HH11 embryos. Expression is first observed caudally at the site of NT closure. (**B**) *Cdx4* and *VP16Cdx4* overexpression results in the loss of NGN2 (a, a', b, b'; boxes; n=6/6 for both conditions), despite both inducing the *Ngn2*-activator *Pax6* (Fig 2A, C). *EnRCdx4* overexpression also causes the loss of NGN2 (c, c'; box; n=6/6), similar to its effect on PAX6 (Fig 2Cc). *Pax6* overexpression results in ectopic NGN2 (d, d'; arrowhead; n=6/6), but not in the presence of *Cdx4* (e, e'; box; n=6/6). (**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. Cells were counted using ImageJ. Significance is shown with a bar and a star (two tailed t-test analysis, $p<0.05$). (**D**) Figure summarizing CDX4-PAX6-NGN2 interactions. (**E**) *Cdx4* overexpression represses the cell cycle marker *Hes5*. Overexpression of *Cdx4* and *VP16Cdx4* (a, a', b, b'; boxes), but not *EnRCdx4* (c, c') results in downregulation of *Hes5* transcription (n=6/6 for all conditions). Scale bar is 200µm for whole mount and 40µm for transverse sections.

Early differentiation of cells within the neural tube is accompanied by their withdrawal from the cell cycle [54, 55]. To test whether CDX4 also regulates cell cycle exit, we analyzed the expression of *Hes5*, a Notch-pathway component that is a marker cell proliferation in the ventricular zone [56]. Overexpression of either *Cdx4* or *VP16Cdx4* repressed *Hes5*, whereas *EnRCdx4* had no effect on *Hes5* transcription (Fig 3E; n=6/6 for all conditions). Given that *Hes5* controls neuronal cell cycle progression [56], this result suggest that CDX4 can also regulate neural progenitor cell proliferation.

CDX4 initiates maturation of early neural progenitor cells

Cdx4 transcription domain in the caudal neural plate also includes the region containing *T/Bra*$^-$, *Sox2*$^+$, *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 analyzed the transcription of *Nkx1.2*, an early PNT marker [15-18] in cells electroporated with different *Cdx4* constructs. Unexpectedly, both gain and loss of CDX4 function caused downregulation of *Nkx1.2* transcription (*Cdx4*, *VP16Cdx4,* and *EnRCdx4* overexpression; Fig 4A; n=6/6 for all conditions), suggesting that *Nkx1.2* is under tight positive and negative CDX4 regulation. Given that CDX4 is a transcriptional activator and not a repressor [50], the repression of *Nkx1.2* transcription by CDX4 is likely to be indirect.

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; n=6/6 for all conditions). (**B**) Overexpression of *Pax6* has no effect on *Nkx1.2* transcription (n=6/6). (**C**) Figure summarizing CDX4-NKX1.2 interactions. Scale bar is 200µm for whole mount and 40µm for transverse sections.

One gene candidate that could mediate CDX4 indirect effect on *Nkx1.2* is PAX6, as PAX6 promotes neural differentiation [20] and its transcription is under CDX4 control (Fig 2). We tested this possibility by overexpressing *Pax6* in the caudal PNT. Under these conditions, we did not observe any changes in *Nkx1.2* transcription (Fig 4B; n=6/6), suggesting other

unidentified gene(s) as mediators of CDX4 indirect activity over *Nkx1.2* (Fig 4C). Notwithstanding, our results suggest that CDX4 regulates neural progenitor cell maturation from their earliest time of specification.

PAX6 promotes late neural cell differentiation states by repressing *Cdx4* transcription

Cdx4, *Nkx1.2* and *Pax6* have clearly defined transcription domains, indicative of the existence of cross-regulatory interactions. For example, the expression domain of *Nkx1.2* and *Pax6* in the pre-neural tube are mutually exclusive (summarized in Fig 1A). To determine the cross-regulatory interactions between CDX4, NKX1.2 and PAX6, we analyzed whether overexpression of *Nkx1.2* and *Pax6* causes changes in *Cdx4* transcription. To analyze NKX1.2 function, we overexpressed the mouse version of *Nkx1.2 (mNkx1.2)*, which has been shown to act as a repressor in mouse cell lines [57] and chicken embryos [18]. Consistent with previous report [18], overexpression of mouse *Nkx1.2* represses *Pax6* (Fig 5Ac, n=6/6). In addition, we found that mouse NKX1.2 also repressed chicken *Nkx1.2* transcription (Fig 5Aa, n=6/6), suggesting that NKX1.2 levels must be tightly controlled to maintain precursors in a stem cell like state. However, mouse *Nkx1.2* overexpression did not affect *Cdx4* transcription (Fig 5Ab; n=6/6). Using similar strategies, we analyzed PAX6 activity on *Cdx4*. Overexpression of *Pax6* downregulated and *EnRPax6* upregulated *Cdx4* (Fig 5B; n=6/6 for all conditions). The observation that the repressor form of PAX6 causes the transcriptional activation of *Cdx4* suggests that PAX6 regulation of *Cdx4* is likely to be indirect through mechanisms that are currently unknown. These results suggest that PAX6 functions to represses *Cdx4* in the rostral PNT. Taken together, these results support a network of feed forward and feedback regulatory

interactions between NKX1.2, CDX4 and PAX6 that promotes the gradual and sequential maturation of neuronal progenitor cells at the caudal end of the spinal cord (Fig 5C).

Fig 5. NKX1.2 and PAX6 contribution 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 *Nkx1.2* (a, a') and *Pax6* (c, c'), without altering *Cdx4* transcription (b, b'; boxed regions; n=6/6 for all conditions). (**B**) PAX6 represses *Cdx4*. Ectopic *Pax6* downregulates (a, a'; boxed region), and *EnRPax6* upregulates (b, b'; arrowhead) *Cdx4* transcription (n=6/6 for both conditions). (C) Figure summarizing NKX1.2-CDX4-PAX6 interactions. Scale bar is 200µm for whole mount and 40µm for transverse sections.

Discussion

A gene regulatory network controlling spinal cord neuronal maturation

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 NMP into neural and mesodermal precursors by balancing the activity of Wnt3a, Fgf8 and RA signaling [16, 23, 58]. Mouse embryos deficient for all *Cdx* genes fail to develop post-occipital structures due to the premature differentiation of NMP cell [23, 24]. The primary cause for this premature differentiation is the premature activation of the RA pathway, which in cell culture conditions causes NMP's to follow a neural fate by maintaining *Sox2* and *Nkx2.1*, and repressing *T/Bra* transcription [16].

Here we show important additional functions for *Cdx4* roles in the progressive maturation of spinal cord neuronal progenitors from the earliest time in their determination. As NMPs' descendants acquire neural identity, CDX4 promotes their further maturation by repressing *Nkx1.2* transcription (Fig 4). Control of *Nkx1.2* transcription is tightly balanced by Wnt and FGF signaling [21, 57], which in turn are regulated by CDX [16, 24, 58, 59]. Thus, increasing or decreasing CDX4 levels would cause an imbalance in Wnt and FGF and a loss in *Nkx1.2* transcription. Given that NKX1.2 inhibits floor plate cell specification by repressing *Pax6* and *Irx3* [18], we propose that CDX4 downregulation of *Nkx1.2* is the first step in spinal cord neural progenitor maturation.

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

Progression of cells from caudal to the 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 [20]. CDX factors have been observed to increase *Pax6* transcription in embryoid bodies [60]. 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 4), and by the direct activation of *Pax6* transcription (Fig 2, [6]). Importantly, CDX4 activation of *Pax6* is dependent on RA (Fig 2), which is secreted from somites [27, 61]. 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 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; [62]) and CDX4 binding sites ([63] and Fig S3). In addition to DNA binding, RA has been implicated in opening up the *Pax6*

locus by antagonizing FGF signaling [64]. 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 CX4 can further promote neural progenitor cell maturation.

Fig 6. Proposed gene regulatory network controlling spinal cord neuronal cell maturation. Gene regulatory network of the genetic interactions identified in figures 1-5, superimposed to the FGF-Wnt8C-RA signaling network shown by others to regulate cell transitions states during spinal cord neuronal cell maturation [27]. Network map was generated using Biotapestry [65]. In this model, CDX4 is at the core of the gene regulatory network that coordinates upstream signaling information into downstream transcriptional response.

CDX4 prevents premature neural cell differentiation

PAX6 induces neural cell differentiation [20], and yet, despite *Pax6* being induced by CDX4 in the rostral PNT (Fig 2), neural cell differentiation does not begin until after the neural tube has formed and *Cdx4* has been down regulated (Fig 1). One mechanism by which PAX6 promotes differentiation is by activating *Ngn2* [20, 53], a gene that promotes cell cycle exit and further differentiation [54]. 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 CDX are known transcriptional activators [50]. 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 [66, 67] and hematopoietic [60, 68] tissues.

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

In current models of spinal cord neurogenesis, cells progressively lose potency and acquire neural characteristics under the control of FGF, Wnt and RA signaling [9, 17]. Mutual interactions among these signaling factors restrict the activity of respective pathways to specific domains within the caudal neural plate to direct cell fate decisions. In the posterior, high levels of FGF promote *Wnt* transcription while repressing RA pathway activity through a variety of mechanisms [28, 31-33]. In turn, WNT8c promotes RA synthesis in rostral regions, away from the caudal domain of FGF activity. RA secreted from the anterior source represses FGF synthesis, helping establish and refine the high-posterior to low-anterior gradients of FGF, and indirectly, Wnts [8, 34]. This cross-repressive and balanced activities of FGF/Wnts and RA create a posterior-to-anterior gradient of potency signals and an anterior-to-posterior gradient of pro-differentiation signaling that promote the gradual maturation of spinal cord cells (Fig 1, 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*, *Irx3*, and *Ngn1/2*. In contrast, RA promotes differentiation by repressing *T/Bra* and *Nkx1.2* and inducing *Pax6*, *Irx3* 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 [8].

We have expanded the model of spinal cord neurogenesis by integrating signaling and transcription network models (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 [38, 51, 52, 69, 70]. FGF and Wnt promote potency by directly activating *Nkx1.2* [57, 71], 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 [21] and activating (via CDX4) *Pax6* transcription (Fig 2B). 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 [64]. 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*; [72]). 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.

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 [38-40, 46, 73] and antagonizes RA's ability to induce hindbrain *Hox* genes [40, 52, 73]. 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 the 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 [16, 23], whereas, later in development, CDX activate *Hox13* genes involved in axial termination [74, 75]. *Cdx* mutations and premature *Hox13* activation in mouse impairs elongation and morphogenesis of the spinal cord neuroepithelium, which results in irregular or duplicated neural structures [75]. These neural tube defects are similar to those observed in mutants in the mesoderm specification genes *T/Bra* and *Tbx6* [76, 77], leading to the proposal that caudal spinal cord defects associated with the loss of CDX arise through defects in the specification of NMP descendent [75]. 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.

Materials and methods

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 [78]. Embryos post-electroporation were incubated until stipulated time for further analysis.

DNA constructs and chicken *in ovo* electroporation

Gene over expression 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 [79, 80]. 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 [20]; and mNkx1.2-pEF2 was kindly provided by Dr. Yusuke Marikawa [57]. 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 50ng/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 [81, 82]. 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: pre-neural tube for stage HH12-13 (prospective thoracic level; [83]), and thoracic level for stage HH16-17 (somites 20-25; [84]).

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 [85]. 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*, *Hes5*, *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.

Cryo-sectioning and Immunohistochemistry

Embryos harvested for immunohistochemistry (IHC) analysis were fixed with 4 % PFA for 3 hours 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 [86]. Rabbit polyclonal antibody against GFP Tag was obtained from AnaSpec Inc. Goat anti-mouse Alexa Flour 488, Alexa Flour 556 and goat anti-guinea pig Alexa Flour 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.

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 [87]. Images were processed for figures using Adobe Photoshop (CC2017, Adobe) for size and resolution adjustment, and for figure preparation.

Quantification of IHC data

To quantify changes in the levels of candidate proteins after electroporation, cells positive for PAX6 or NGN2 were counted on both electroporated and control sides at the same dorsal-ventral position, and their relative ratio was determined. Images were processed with ImageJ IHC toolbox plugin [88] before cell counting to select for cells above threshold level as determined by the program algorithm. A total of 6 embryos per conditions were used for determining significance. 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).

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.

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Competing interests

No competing interest declared.

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Supplemental information

Supplemental materials and methods

Table S1. Genes and constructs received from other labs.

Table S2. Genes cloned in the lab with respective primers.

Supplementary figures

Fig S1. GFP overexpression does not affect wild type gene transcription of selected factors.

Fig S2. Loss of RA signaling causes retention of *Cdx4* transcription in the NT.

Fig S3. *Pax6* gene harbors evolutionarily conserved CDX4 binding sites.

Figure 1

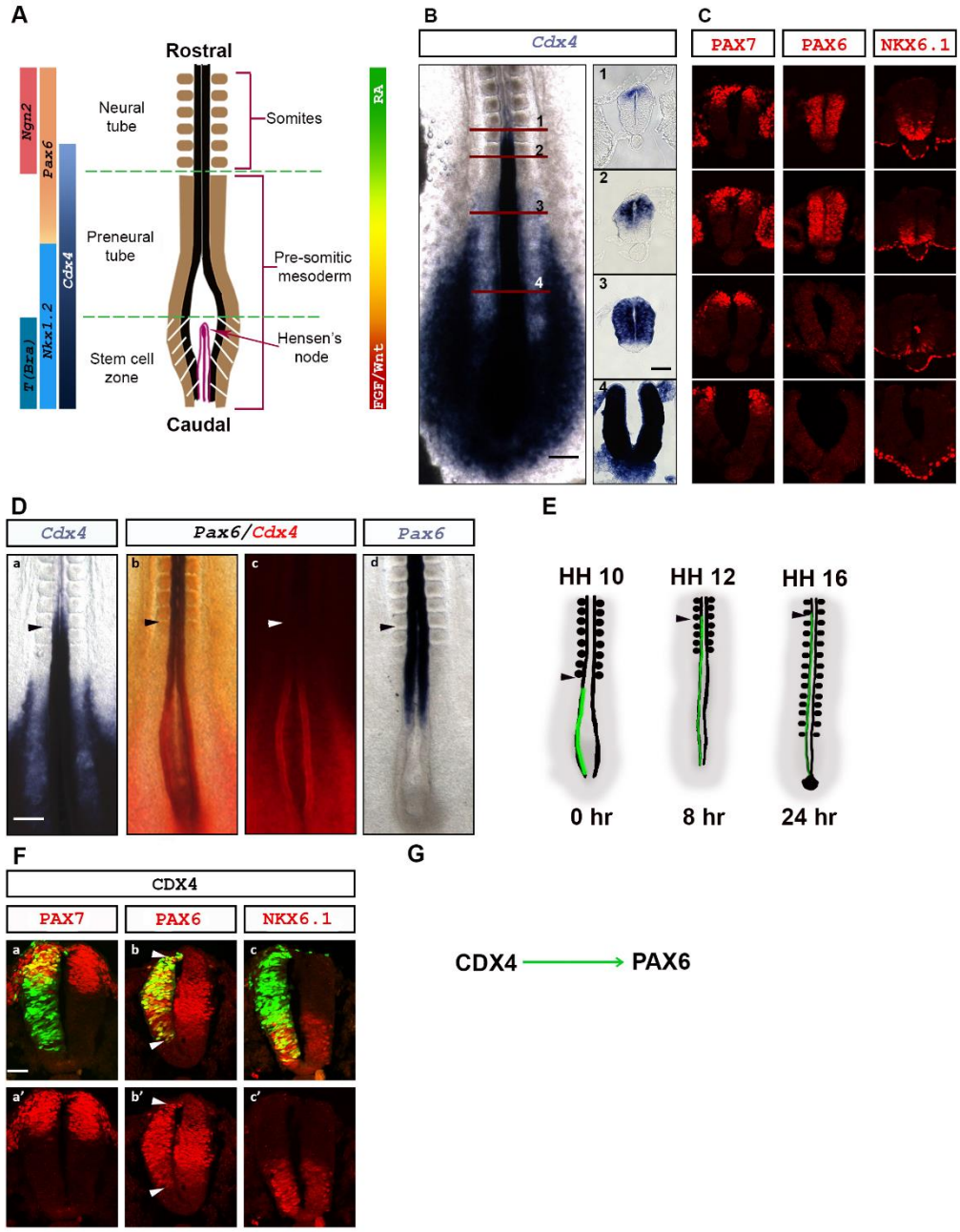

A
Rostral
Neural tube
Somites
Preneural tube
Pre-somitic mesoderm
Stem cell zone
Hensen's node
Caudal
Ngn2
Pax6
Cdx4
T (Bra)
Nkx1.2
RA
FGF/Wnt
B
Cdx4
C
PAX7
PAX6
NKX6.1
D
Cdx4
Pax6/Cdx4
Pax6
E
HH 10
HH 12
HH 16
0 hr
8 hr
24 hr
F
CDX4
PAX7
PAX6
NKX6.1
G
CDX4
PAX6

Figure 2

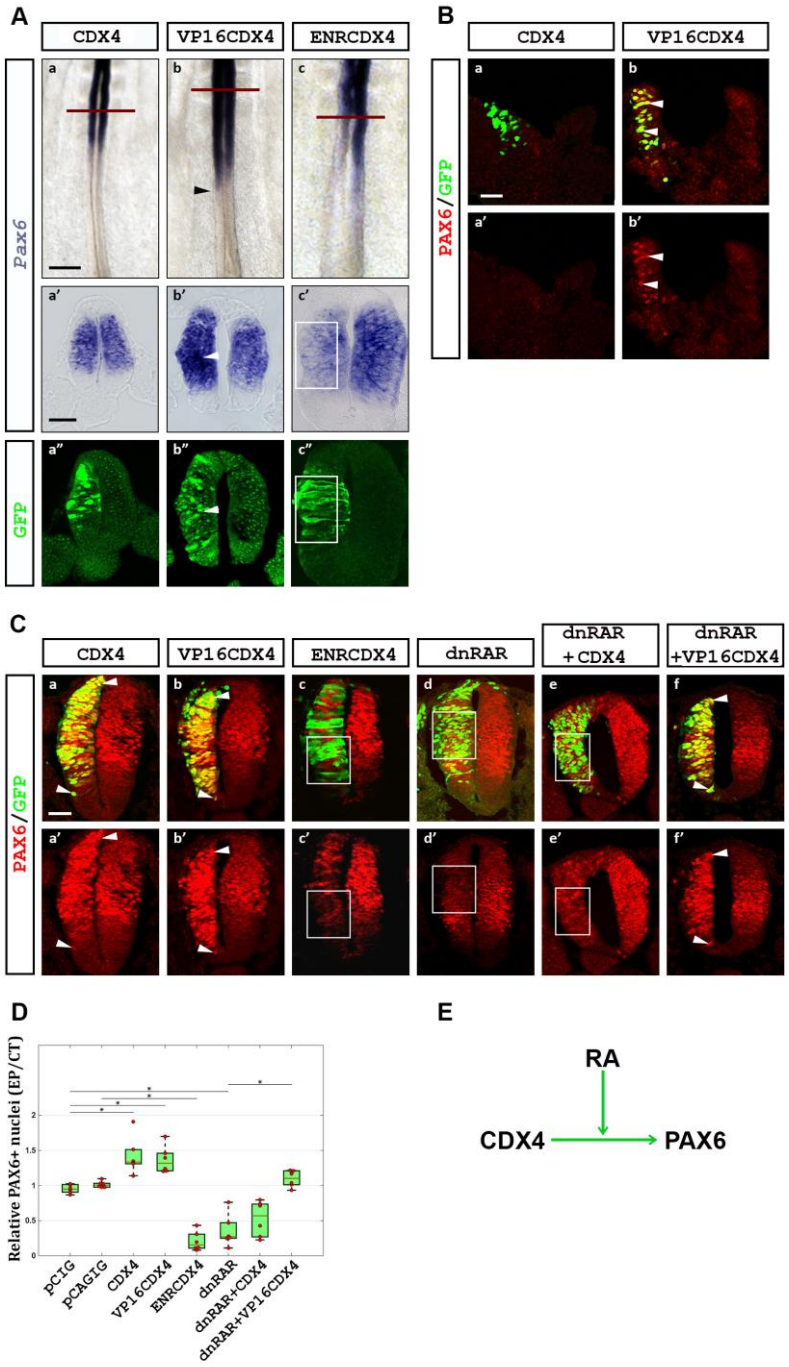

A
CDX4
VP16CDX4
ENRCDX4
Pax6
GFP
B
CDX4
VP16CDX4
PAX6/GFP
C
CDX4
VP16CDX4
ENRCDX4
dnRAR
dnRAR + CDX4
dnRAR +VP16CDX4
PAX6/GFP
D
Relative PAX6+ nuclei (EP/CT)
pCIG
pCAGIG
CDX4
VP16CDX4
ENRCDX4
dnRAR
dnRAR+CDX4
dnRAR+VP16CDX4
E
RA
CDX4
PAX6

Figure 3

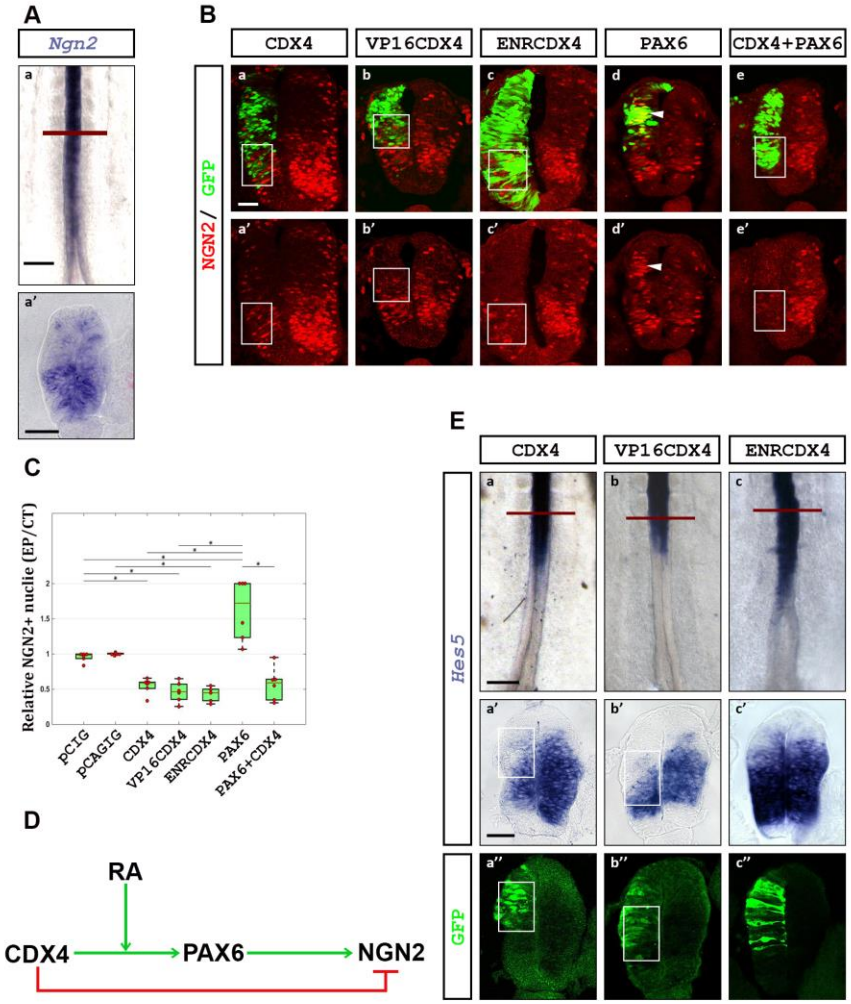

A
Ngn2
a
a'
B
CDX4
VP16CDX4
ENRCDX4
PAX6
CDX4+PAX6
NGN2 / GFP
a
b
c
d
e
a'
b'
c'
d'
e'
C
Relative NGN2+ nuclie (EP/CT)
pCIG
pCAGIG
CDX4
VP16CDX4
ENRCDX4
PAX6
PAX6+CDX4
D
RA
CDX4
PAX6
NGN2
E
CDX4
VP16CDX4
ENRCDX4
Hes5
GFP
a
b
c
a'
b'
c'
a''
b''
c''

Figure 4

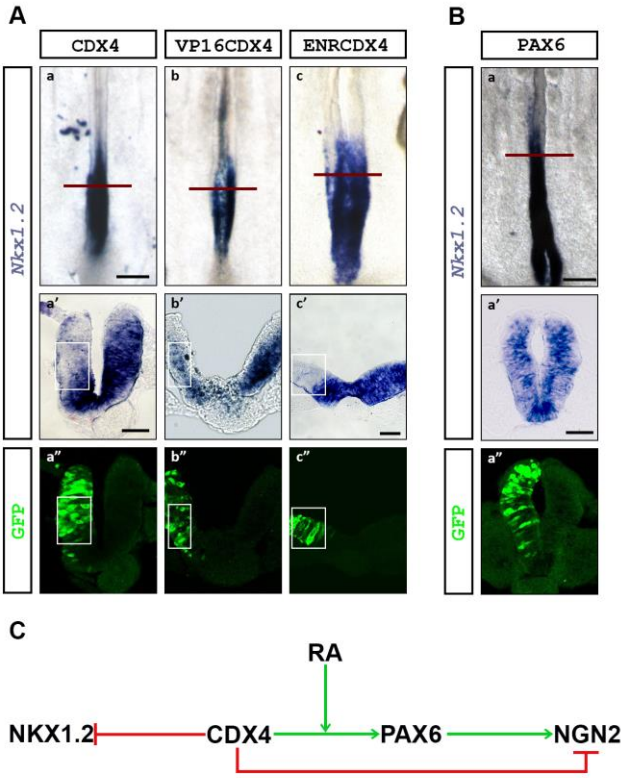

A
CDX4
VP16CDX4
ENRCDX4
B
PAX6
Nkx1.2
GFP
a
b
c
a'
b'
c'
a''
b''
c''
C
RA
NKX1.2
CDX4
PAX6
NGN2

Figure 5

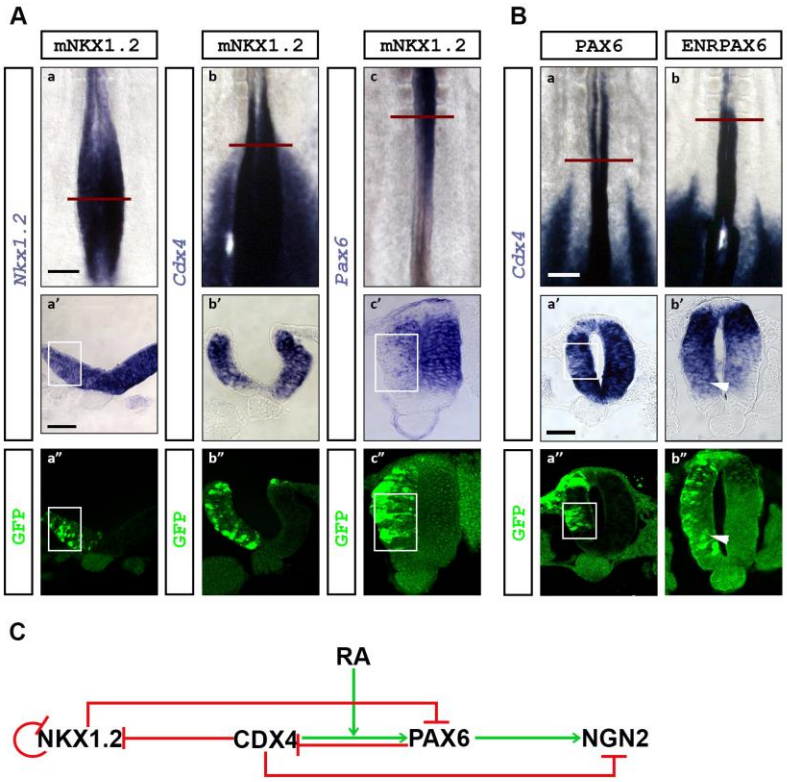
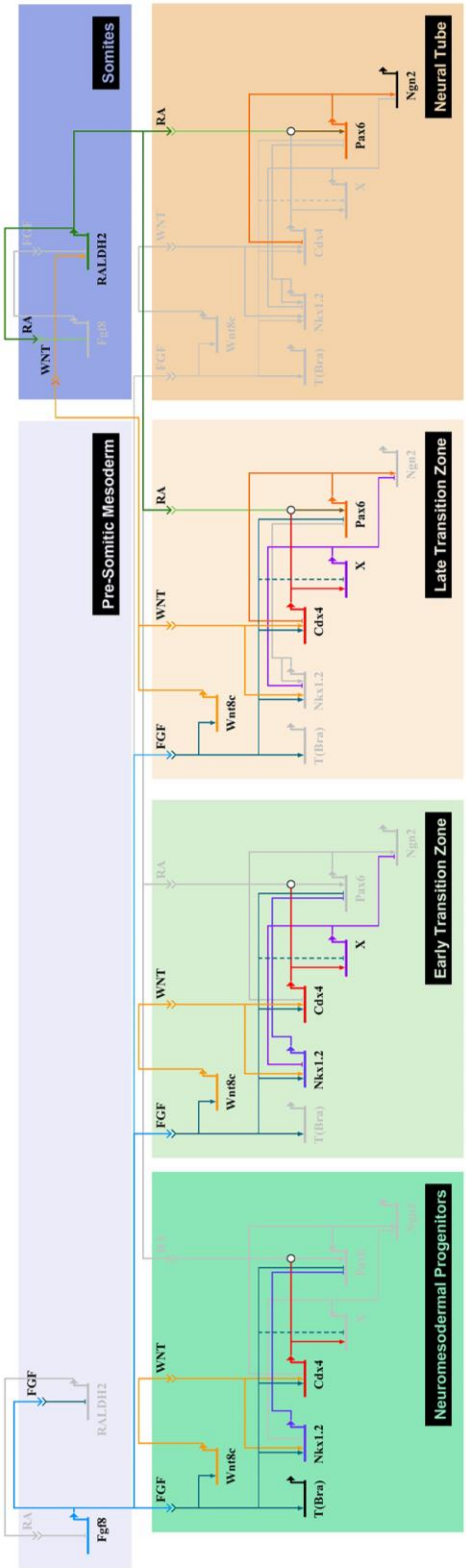

A
mNKX1.2
mNKX1.2
mNKX1.2
Nkx1.2
Cdx4
Pax6
GFP
GFP
GFP
B
PAX6
ENRPAX6
Cdx4
GFP
C
RA
NKX1.2
CDX4
PAX6
NGN2

Figure 6


Somites
Pre-Somitic Mesoderm
Neuromesodermal Progenitors
Early Transition Zone
Late Transition Zone
Neural Tube
RA
WNT
Fgf8
RALDH2
FGF
Wnt8c
T(Bra)
Nkx1.2
Cdx4
X
Pax6
Ngn2

Supplemental information

Supplemental materials and methods

Gene and gene constructs employed in this work where either obtained from other laboratories, or generated by us using standard molecular biology techniques and publicly available annotated sequences. A list of genes and constructs obtained from other laboratories is summarized in Table S1, and a list of primers for genes and constructs generated by us is summarized in Table S2.

Full length Cdx4 for in situ hybridization and sub-cloning (Cdx4-pGEM-T-Easy). Full length *Cdx4* (NM_204614.1) was cloned from reverse transcribed, total mRNA from chicken embryos at different stages of development (HH4-HH12; qScript cDNA Synthesis kit, Quantabio), using primers designed with the online program Primer BLAST (Table S2). Fragment product of the correct size was then cloned using pGem-T Easy Plasmid (Promega,). Cloning of the correct gene was confirmed by sequencing. This construct was used to generate *in situ* hybridization probe and as a template for additional construct.

Full length Cdx4 for chicken electroporation (Cdx4-pCIG). Full-length chicken *Cdx4* was digested with SpeI and blunt ended with Mung Bean nuclease (NEB). Purified, linear *Cdx4* was then digested with EcoRI. The purified *Cdx4* fragment was then subcloned into pCIG previously digested with EcoRI-SmaI. This construct was used for overexpressing wild type *Cdx4* in chicken embryos by electroporation. pCIG contains nuclear GFP under IRES promoter for concomitant expression of GFP in electroporated cells [1].

Constitutively active Cdx4 for chicken electroporation (VP16Cdx4-pCIG). The transactivator domain of the *VP16* was amplified from *VP16*-pCS2+ and fused to the C-terminal domain of Cdx4 containing the DNA binding homeodomain (corresponding to amino acids 119-364; renamed Cdx4-HD). Primers used for these amplifications are described in Table S2. Chimeric *VP16Cdx4* was then generated by PCR amplification from a mixture containing *VP16* and *Cdx4*-HD fragments and VP16 forward and Cdx4-HD reverse primer. The segment was cloned into pGEM-T-easy and open reading frame confirmed by sequencing. *VP16Cdx4* was then digested using ClaI-EcoRI and inserted into ClaI-EcoRI sites of pCIG.

Dominant negative Cdx4 for chicken electroporation (EnRCdx4-pCAGIG). Engrailed (*EnR*) repressor domain from *EnR*-pCS2+ was digested with XhoI and blunt ended with Mung Bean nuclease. After purification, the fragment was digested with EcoRI and re-purified. This EcoRI-blunt *EnR* product was ligated to a blunt ended Cdx4 fragment generated using SmaI (nucleotide site 328). As a final step, the chimeric construct was ligated to pCAGIG vector digested with EcoRI-EcoRV. Several clones were analyzed by sequencing to confirm correct orientation of the *EnR* and *Cdx4* fragments, and the continuity of the open reading frame. pCAGIG contains GFP under IRES promoter for concomitant expression of cytoplasmic GFP in electroporated cells. pCIG is derived from pCAGIG backbone, with addition of nuclear localization signal in from of GFP, making to GFP concentrate in nucleus [2].

Full length mNkx1.2 for chicken electroporation (mNkx1.2 pCIG). Mouse *Nkx1*.2 was PCR amplified from the *mNkx1*.2-myctag pEf2 construct (gift from Y. Marikawa), using the primers shown in Table S2. The cloned segment was digested with ClaI and EcoRI included in the

forward and reverse primers, respectively. Purified segment was then cloned into ClaI-EcoRI site of pCIG.

Table S1. Genes and constructs received from other labs.			
Gene construct	**Purpose**	**Sources**	**References**
Pax6-pBS	*In situ* hybridization	Dr. Martyn Gouldin (Salk Institute, USA)	
Ngn2-pBS	*In situ* hybridization	Dr. Francois Medevielle (CBI, Toulouse, France)	[3]
Pax6-pCIG	Electroporation		
EnRPax6-pCIG	Electroporation		
Chick-*T(Bra)*- pBS	*In situ* hybridization	Dr. Susan Mackem (NCI, USA)	[4]
mNkx1.2-pEF2	For generating mNkx1.2-pCIG	Dr. Yusuke Marikawa (U of Hawaii, USA)	[5]
dnRAR-pCIG	Electroporation	Dr. Axia V. Morales (Cajal Institute, Spain)	[6]

Table S2. Genes cloned in the lab with respective primers.		
Gene construct	**Primers**	**Annealing T (°C)**
Cdx4	Forward: ACATGTATGTGAGTTCTCTCTTGG	55
	Reverse: TGATCATTCTGAAACTATGAC	
VP16	Forward: ATCGATATGTCAAGGCCTCTCGAGTCGAC (ClaI site underlined.)	50
	Reverse: TGTGTGCCAACCCCACCGTACTCGTCAATT	
Cdx4-HD	Forward: GAGTACGGTGGGGTTGGCACACAGCAGGTC	55
	Reverse: TGATCATTCTGAAACTATGAC	
mNkx1.2	Forward: ATATCGAT*CCACCATG*TTGGCATGGCAGG (ClaI site underlined, Kozack sequence italicized.)	60
	Reverse: GAGAATTCTCATAGGTGTGGAGCATAG (EcoRI site underlined.)	

Supplementary figures

Figure S1

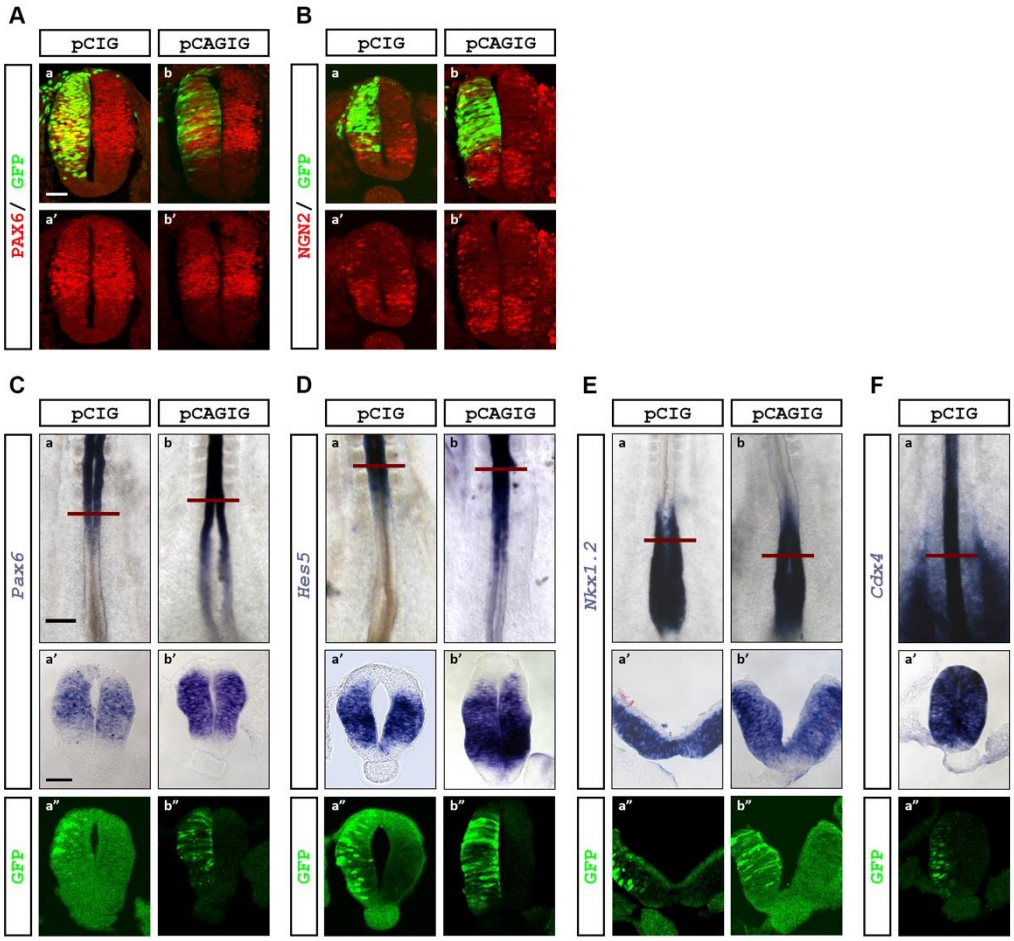


Fig S1. GFP overexpression does not affect wild type gene transcription of selected factors. pCIG (nuclear GFP) and pCAGIG (cytoplasmic GFP) ectopic expression did not change *Pax6* (A: IHC analysis, C: ISH analysis), NGN2 (B), *Nkx1.2* (D) and *Cdx4* (E) expression compared to contralateral control side (n=6/6).*Cdx4* expression analysis for pCAGIG over-expression was not done as none of the pCAGIG backbone construct were analyzed for *Cdx4* expression. Red bar shows RC level of transverse section. Scale bar is 200µm for whole mount and 40µm for transverse sections.

Figure S2

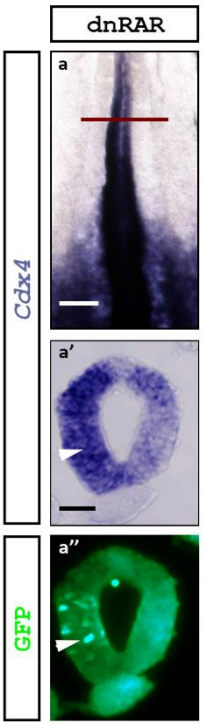


Fig S2. Loss of RA signaling causes retention of *Cdx4* transcription in the NT. Expression analysis of Cdx4 in embryos over expressing dnRAR. Arrowhead indicates electroporated size. Control non-electroporated side shows *Cdx4* downregulation (as seen in Fig. 1B). Scale bar is 200µm for whole mount and 40µm for transverse section.

Figure S3

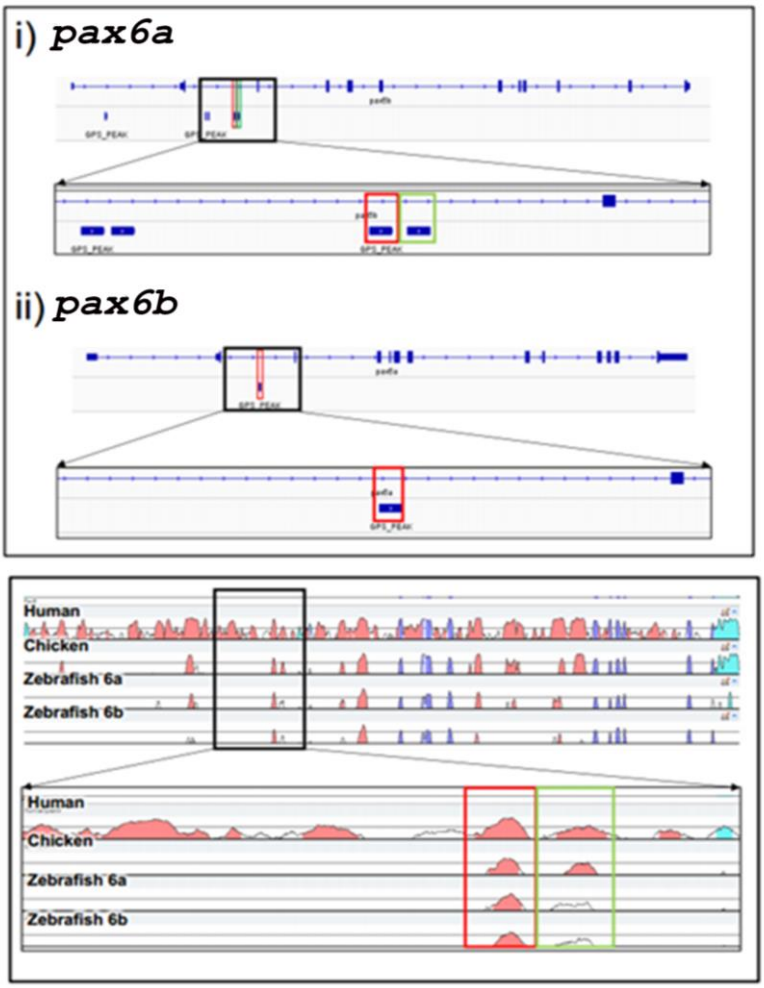


Fig S3. *Pax6* gene harbors evolutionarily conserved CDX4 binding sites. (Top) CDX4 ChIP-seq data showing CDX4 binding regions in zebrafish *pax6a* and *pax6b* genes (data obtained from [7]). (Bottom) Non-coding regions of zebrafish *pax6a* containing Cdx4 binding sites (green and red boxes from top panel) are conserved in human and chicken *Pax6*.

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