Cdx4 regulates the onset of spinal cord neurogenesis

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Keywords: Cdx, neurogenesis, spinal cord, signaling network, transcription network

Running title: Cdx in spinal cord neurogenesis

SUMMARY STATEMENT

Cdx4 is at the core of a gene regulatory network the coordinates the sequential transition of neural cell states during early spinal cord development, from pluripotency to early differentiation.

ABSTRACT

The integration of extracellular signals by intracellular transcription factor networks is a critical determinant of cell fate decisions during embryonic development. In the vertebrate spinal cord, the progressive caudal-to-rostral maturation of neural progenitors (NPs) is controlled by the signaling activities of FGF/Wnt antagonizing retinoic acid. The incompleteness of transcriptional gene regulatory network (GRN) involved hinders proper understanding of early spinal cord development. Using transient gene manipulation technique in chicken, we show that Cdx4 is at the core of the GRN that integrates upstream signaling information to regulate the sequential maturation of NPs. We show that Cdx4 represses the pluripotency gene *Sax1* and promotes expression of the neural gene *Pax6* while simultaneously preventing the activation of the Pax6-dependent neurogenic gene *Ngn2*. Our results suggest a novel role for Cdx4 in regulating the sequential transition of neural cell states during early spinal cord development. We propose that Cdx factors broadly coordinate axial specification and maturation of NPs during spinal cord development.

INTRODUCTION

Cells transit from one temporary state to another during their gradual differentiation towards acquiring 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 (Davidson, 2006; Royo et al., 2011). During this gradual process, the cell's transcriptome also transits from one state to another, 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 temporal 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.

Vertebrate spinal cord serves as an important accessible system to study the transition of cellular states during neural differentiation due to its characteristic head to tail development (Diez del Corral and Storey, 2004). The progressively differentiated cells are arranged in caudal-to-rostral direction in a configuration mirroring their temporal progression in differentiation: cells born earlier in development are more advanced in the differentiation program and localize to more rostral regions than the later born, more immature cells in caudal regions (Butler and Bronner, 2015; Diez del Corral et al., 2003; Diez del Corral and Storey, 2004; Wilson et al., 2009). In chicken and mouse embryos, four distinct regions containing cells at different states of differentiation can be identified rostro-caudally based on the expression of marker genes (Fig. 1A). At the caudal most end, regions in the stem zone act as reservoir of bipotent neuromesodermal progenitors (NMPs; Henrique et al., 2015). In the early embryos, NMPs are located in the caudal lateral epiblast (CLE) and the node-streak border (NSB) regions that surrounds the primitive streak (Brown and Storey, 2000; Cambray and Wilson, 2007; Henrique et al., 2015). At later stages, the caudal neural hinge (CNH) region (derivative of CLE and NSB) in the tail bud is the source of the NMPs (Cambray and Wilson, 2007). NMPs located in the CLE and NSB regions are defined molecularly by the expression of the genes *T (Bra)*, *Sox2* and *Sax1*

(Nkx1.2) (Delfino-Machin et al., 2005), and have been shown by fate mapping studies to contribute not only to the neural tube, but also to the pre-somitic mesoderm (Brown and Storey, 2000; Cambray and Wilson, 2007; Tzouanacou et al., 2009). Immediately rostral to the NMP domain is the transition zone, that can be divided into caudal and rostral regions. While in the caudal transition zone cells begin to downregulate *T (Bra)* (Delfino-Machin et al., 2005) , it is only until the cells migrate into the rostral transition zone that they down regulate *Sax1* and begin to express neural identity markers *Pax6* and *Irx3* (Bertrand et al., 2000; Delfino-Machin et al., 2005; Diez del Corral et al., 2003). Cells continue to mature in the neural tube that is surrounded by somites, where they start expressing the neural differentiation genes *Ngn1/2* and *NeuroM* (Diez del Corral et al., 2003). Thus, temporally distinct steps in the maturation and differentiation of cells within the spinal cord cells can be spatially distinguished by the combinatorial transcription of pluripotency and differentiation factors.

The spatio-temporal gradient of neural differentiation states in the spinal cord is generated and maintained by opposing gradients of the signaling factors FGF/Wnt, and retinoic acid (RA) (Diez del Corral et al., 2003; Olivera-Martinez and Storey, 2007). FGF8 and Wnts (Wnt3a and Wnt8c) establish a caudal to dorsal gradient that promotes pluripotency by directly promoting *T (Bra)*, *Sox2* and *Sax1;* and preventing *Pax6* and *Irx3* transcription (Bertrand et al., 2000; Delfino-Machin et al., 2005; Diez del Corral et al., 2003; Olivera-Martinez et al., 2012). In contrast, RA secreted from somites establishes a rostral to caudal signaling gradient that promotes differentiation by inducing cells to exit the proliferation program, first by promoting transcription of neural identity genes *Pax6* and *Irx3* (Diez del Corral et al., 2003; Novitch et al., 2003; Pituello et al., 1999), and then by promoting transcription of downstream neurogenic genes *Ngn1/2* and *NeuroM* (Diez del Corral et al., 2003). Opposing signaling activities of FGF/Wnt and RA are segregated to caudal and rostral regions of the nascent spinal cord, respectively, through positive and negative interactions: FGF indirectly promotes differentiation by inducing RA production rostrally, through a Wnt8c-mediated mechanism (Olivera-Martinez and Storey, 2007), while FGF also maintains RA production distal to the pluripotency zone by directly inhibiting RA synthesis (Olivera-Martinez et al., 2012) and promoting RA degradation caudally (Boulet and Capecchi, 2012; Sakai et al., 2001; White et al., 2007). RA, in turn, inhibits *Fgf8* transcription rostrally, creating a zone where cells 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 signaling aspect of the differentiation switch have been extensively investigated, the underlying transcription factor network driving the cell transition states in the nascent spinal cord remains poorly understood.

In the NMP domain and the transition zone, Cdx stands out as a transcription factors family that is under the simultaneous control of FGF, Wnt and RA signals (Deschamps and van Nes, 2005; Lohnes, 2003). Cdx factors have been extensively characterized and shown to be necessary for the development of several embryonic tissues including trophectoderm (Sritanaudomchai et al., 2009; Strumpf et al., 2005), post-occipital tissues (van Rooijen et al., 2012), intestinal cell (Beck et al., 1999; Flores et al., 2008) and during hematopoiesis (Davidson et al., 2003; Wang et al., 2008). In the spinal cord, Cdx factors are essential for tissue specification and rostrocaudal 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 initiation, establishment and maintenance of *Hox* gene transcription domains (Deschamps et al., 1999; Hayward et al., 2015). Thus, members of the Cdx family are attractive candidates to integrate FGF, Wnt and RA signals in the caudal neural plate and regulate spinal cord neurogenesis.

Here we show that Cdx4, one of three members of Cdx family, regulates the temporal progression of cellular states in the developing chicken caudal neural tube. Using transient gene expression strategies, we show that Cdx4 regulates the expression of several genes involved in the sequential differentiation of neural precursors, including *Sax1*, *Pax6* and *Ngn2*. Our results show that Cdx4 acts as a dual control switch during neurogenesis, inhibiting pluripotency in NMPs while promoting acquisition of neural identity. Furthermore, we show that timely downregulation of Cdx4 is needed for further neuronal cell differentiation. Our results support a novel role for Cdx factors in regulating the onset and progression of caudal neural tube neurogenesis.

RESULTS

Cdx4 is transcribed in a dorsal-ventral gradient in the caudal neural tube, but it does not specify dorsal-ventral cell identities

Cdx4 neural function was first analyzed by correlating its transcription domain to distinct progenitor cell maturation zones of the caudal neural plate (Fig. 1A; Olivera-Martinez and Storey, 2007). As previously reported (Morales et al., 1996), 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 post HH13), whereas rostrally, *Cdx4* transcription was progressively excluded from ventral regions and the roof plate (Fig. 1B, transverse sections). A similar dorsally restricted transcription profile has been reported for *Cdx4* in mice (Gaunt et al., 2005), suggesting evolutionary conserved gene regulatory 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 that of the known DV markers *Pax7* (dorsal) (Briscoe et al., 2000; Diez del Corral et al., 2003), *Pax6* (dorsal-to-intermediate) (Briscoe et al., 2000; Novitch et al., 2003), and *Nkx6.1* (ventral) (Briscoe et al., 2000; Diez del Corral et al., 2003; Novitch et al., 2001). We did not observe any clear correlation between the transcriptional domain of *Cdx4* and any of these markers. For example, in the caudal transition zone, Pax7 domain was nested within, and Nkx6.1 domain was complementary to the *Cdx4* transcription domain (Fig. 1B, C). However, in the rostral transition zone and neural tube, Pax7 domain was broader than, and Nkx6.1 domain no longer complemented the *Cdx4* transcription domain (Fig. 1B, C). A similar lack of correlation was observed between *Cdx4* and Pax6 domains (Fig. 1B, C).

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 wild type *Cdx4* by electroporating the neural tube of stage HH10-11 embryos, and analyzed the protein distribution of Pax7, Pax6, and Nkx6.1 24-hours post-electroporation (hpe; HH16-17).

While high levels of *Cdx4* expression did not change Nkx6.1 and Pax7 protein distribution (Fig. 1D; n=6/6 for both conditions), ectopic expression of Pax6 protein was detected both ventrally and dorsally outside its normal domain (Fig. 1D; n=6/6). Together, these results suggest that Cdx4 is not involved in the general specification of DV neural identities, but in the specific regulation of *Pax6* (Fig. 1E)

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 nascent neural tube (Bel-Vialar et al., 2007). 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 nascent neural tube, in a region where *Pax6* is not normally transcribed. Embryos were electroporated in the transition zone with different *Cdx4* constructs, grown for 8 hours only, and analyzed for premature *Pax6* activation by *in situ* hybridization. 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; Bel-Vialar et al., 2002; Faas and Isaacs, 2009). 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 co-factors or its inability to overcome the presence of repressors. VP16Cdx4 was able to overcome these hurdles, suggesting that, under the right conditions, activated Cdx4 induces *Pax6* transcription during neural progenitor cell maturation.

To test if Cdx4 activation is necessary for *Pax6* activation in the transition zone, 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 (Han and Manley, 1993). This chimeric form of Cdx4 has been shown to repress transcription of Cdx downstream targets (e.g., *Hox* genes; Bel-Vialar et al., 2002; Isaacs et al., 1998). Overexpression of *EnRCdx4* resulted in loss of *Pax6* in the transition zone as early as 8 hpe (Fig. 2Ac; n=6/6), indicating that Cdx4 indeed regulates *Pax6* transcription. However, when interpreted together with the results of the gain of function

experiments, these results also suggest that Cdx4 activation of *Pax6* transcription requires a permissive state.

Cdx4 activation of *Pax6* is dependent on retinoic acid (RA) signaling

Somite-derived RA regulates spinal cord neurogenesis by activating numerous target genes in the rostral transition zone, including *Pax6* (Novitch 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 Skromne, 2014) , we hypothesized that RA and Cdx4 might also interact during spinal cord cell maturation. To test this hypothesis we analyzed the transcription of *Pax6* in wild type and RA-deficient embryos 24 hpe, at a time when electroporated cells would be undergoing maturation. As previously shown (Novitch et al., 2003), overexpression of a dominant negative RA receptor (dnRAR) blocked RA signaling and caused *Pax6* down regulation (Fig. 2Cd, D). In these RA-deficient embryos, Cdx4 was incapable of inducing *Pax6* transcription (n=6/6; Fig. 2Ce, D), despite its ability to do so in wild type embryos (n=6/6; Fig. 1D; Fig. 2Ca, D). Significantly, however, VP16Cdx4 was able to induce *Pax6* transcription even in the absence of RA (n=6/6; Fig. 2Cb, Cf, D). Together, these results suggest that RA is required for the Cdx4-dependent activation of *Pax6*, and illuminates a mechanism for the restricted transcription of Pax6 to the rostral portion of the transition zone and the caudal neural tube (Fig. 2E).

Cdx4 promotes early differentiation states by simultaneously inhibiting cell proliferation and late differentiation programs

Despite Pax6 being present in both the transition zone and the neural tube, Pax6 only activates neural differentiation genes in the neural tube (Bel-Vialar et al., 2007). What then prevents Pax6 from prematurely activating neural differentiation genes in the transition zone? We addressed this question by analyzing the transcription of the Pax6-dependent differentiation gene *Ngn2* (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 analyzed Ngn2 expression after *Cdx4, VP16Cdx4* and *EnRCdx4* overexpression. Each construct independently resulted in Ngn2 down regulation (Fig. 3B, C; n=6/6 for all conditions), despite Cdx4 and VP16Cdx4 induction of *Pax6* transcription (EnRCdx4 represses *Pax6* that is essential for ventral *Ngn2* transcription; Fig.

2Cc), suggesting that Cdx4 represses *Ngn2*. 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; Bel-Vialar et al., 2007), it is unable do so in the presence of Cdx4 (Fig. 3Be, C; n=6/6). Previous works showed Cdx4 as a transcriptional activator (Isaacs et al., 1998), suggesting that in our experiments Cdx4 repression of *Ngn2* is likely indirect. Taken together, our results suggest that Cdx4 promotes cell's entry into the differentiation program by inducing *Pax6* transcription, but prevents further differentiation by inhibiting *Ngn2* activation (Fig. 3D).

Early differentiation of cells within the neural tube is accompanied by their withdrawal from the cell cycle (Lacomme et al., 2012; Shimojo et al., 2008). 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 (Fior and Henrique, 2005). Overexpression of either *Cdx4* or *VP16Cdx4* repressed *Hes5*, whereas *Hes5* transcription was unaffected by *EnRCdx4* overexpression (Fig. 3E; n=6/6 for all conditions). Given that Hes5 is required for neural cell proliferation (Fior and Henrique, 2005), our results suggest that Cdx4 might indirectly control cell division. Pax6 has been shown to activate *Hes5* in mouse cortical stem cells (Sansom et al., 2009), raising the possibility that Cdx4 regulates *Hes5* via Pax6. To test this scenario, we analyzed the effect of modulating Pax6 activity on *Hes5* transcription. Unfortunately, the results were ambiguous, as both wild type and dominant negative *Pax6* reduced *Hes5* transcription (data not shown, 8 hpe, n=6/6 embryos for each condition), which prevented further analysis. Nonetheless, our results suggest that Cdx4 promotes entry and sustains cells in an early differentiation state by repressing both proliferation (*Hes5*) and differentiation (*Ngn2*) processes.

Cdx4 initiates maturation of early pluripotent neural progenitor cells

Cdx4 transcription domain in the caudal neural plate is not restricted to the transition zone, but encompasses two additional pluripotent cell populations, NMPs that express *T (Bra) and Sax1* (*Nkx1.2* in mouse) (Henrique et al., 2015; Yamaguchi et al., 1999), and early neural progenitor cells that only express *Sax1* (Delfino-Machin et al., 2005; Sasai et al., 2014). If, as our results suggest, Cdx4 drives cells to enter an early neural differentiation state, then, what is Cdx4 function in the two progenitor cell populations? To answer this question we analyzed *T* (*Bra*) and *Sax1* expression in embryos electroporated with different *Cdx4* constructs. While neither

gain nor loss of Cdx4 function altered *T (Bra)* transcription (Fig. 4A; n=6/6 for all conditions), both conditions caused loss of *Sax1* expression (Fig. 4B, n=6/6 for all). These results suggest that Cdx4 can regulate early neural progenitor cell fate decisions. The observation that dominant negative EnRCdx4 downregulates *Sax1* transcription suggests that Cdx4 activity is somehow involved in *Sax1* activation (Fig. 4Bc). This regulation is likely to be indirect, as constitutively active VP16Cdx4 also repress *Sax1* (Fig. 4Ba, b). A likely candidate to mediate such a mechanism is Pax6, as VP16Cdx4 induced this gene in the transition zone (Fig. 2A, B), and Pax6 directs cells towards differentiation (Bel-Vialar et al., 2007). We tested this possibility even though *Cdx4* overexpression didn't expand *Pax6* expression in caudal neural tube (Fig. 2). Overexpression of *Pax6* did not change *Sax1* transcription in the caudal neural tube (Fig. 4C), suggesting that other as yet unidentified gene(s) mediate Cdx4 indirect repression of *Sax1*. As Cdx4 only regulates *Sax1* but not *T (Bra)* transcription, these results suggest that Cdx4 does not regulate NMP's neural versus mesodermal cell decisions, but instead, Cdx4 regulates neural progenitor cell maturation from their earliest time of specification.

Pax6 promotes late neural cell differentiation states by repressing *Cdx4* transcription

Cdx4, *Sax1* and *Pax6* have clearly defined transcription domains, indicative of the existence of cross-regulatory interactions. For example, the expression domain of *Sax1* and *Pax6* in the transition zone are mutually exclusive. To determine the cross-regulatory interactions between Cdx4, Sax1 and Pax6, we analyzed whether overexpression of *Sax1* and *Pax6* causes changes in *Cdx4* transcription. To analyze Sax1 function, we overexpressed a mouse version of *Sax1 (mSax1, Nkx1.2)*, which has been shown to act as a repressor in mouse cell lines (Tamashiro et al., 2012) and chicken embryos (Sasai et al., 2014). As previously suggested (Sasai et al., 2014), overexpression of mouse *Sax1* represses *Pax6* (Fig. 5Ac, n=6/6). Furthermore, mouse Sax1 repressed chicken *Sax1* transcription (Fig. 5Aa, n=6/6), suggesting that Sax1 levels must be tightly controlled to maintain cells in a stem cell like state. However, mouse *Sax1* overexpression didn't affect *Cdx4* transcription (Fig. 5Ab; n=6/6). Similar strategies were used to analyze Pax6 regulation of *Cdx4*. Overexpression of *Pax6* downregulated and *EnRPax6* upregulated *Cdx4* transcription levels (Fig 5B; n=6/6 for all conditions). These results suggest that Pax6 represses *Cdx4* in the rostral transition zone through indirect mechanisms. This regulation is unlikely to be mediated by the Pax6-target *Ngn2*, as the expression domain of *Cdx4*

and *Ngn2* do not overlap (Fig. 1B; Fig. 3C). Taken together, these results support a network of feed forward and feedback regulatory interactions between Sax1, Cdx4 and Pax6 that promotes the gradual and sequential maturation of neuronal progenitor cells at the caudal end of the embryo (Fig. 5C).

DISCUSSION

A gene regulatory network controlling spinal cord neuronal maturation

Acquisition of neural fate by neuromesodermal progenitors (NMPs) is independent of Cdx4

Most of the trunk and tail spinal cord originates from NMPs that follow a neural path over a mesodermal fate (Gouti et al., 2015; Henrique et al., 2015). Recent evidences in mouse implicate Cdx family members in this specification event (Amin et al., 2016; Gouti et al., 2017), however, our results do not support an involvement of Cdx4 in neuromesodermal cell fate decision (Fig. 4A). According to current models, the bipotent NMP cell state is dynamically maintained through cross regulatory interactions between *T/Bra* and *Sox2*, and that a tilt in favor of *Sox2* through the reduction of *T/Bra* levels leads to the acquisition of neural identities (Gouti et al., 2014; Henrique et al., 2015; Martin and Kimelman, 2012). We found that manipulation of Cdx4 activity levels did not modulate *T/Bra* transcription (Fig. 4A). This contrasts with the observation that the loss of *Cdx2* in mice leads to a Wnt3a-dependent reduction in *T/Bra* transcription (Savory et al., 2009), a phenotype exacerbated in *Cdx1/2/4* triple mutants (Gouti et al., 2017; van Rooijen et al., 2012). It is possible that the sparse electroporation of NMPs in our experiments is not sufficient to change Wnt3a levels (or the level of other signaling factors) in the NMP domain, which in mouse is a prerequisite for the Cdx2-dependent modulation of *T/Bra* expression (Savory et al., 2009). Further work is required to determine the specific function of Cdx4 in NMP cell fate decisions.

Cdx4 promotes loss of pluripotency in the caudal transition zone

NMPs' acquisition of neural identity and incorporation into the caudal transition zone is concomitant with the loss of pluripotency. Cdx4 participates in this loss of pluripotency by negatively regulating the transcription of *Sax1* (mouse *Nkx1.2*) (Fig. 4), a pluripotency marker whose function is to promote FGF-mediated inhibition of neural differentiation (Bertrand et al., 2000; Delfino-Machin et al., 2005; Sasai et al., 2014). Both *Sax1* and *Cdx4* are transcribed in

NMPs and caudal transition zone cells. Unexpectedly, our experiments show that both activator and repressor forms of Cdx4 downregulated *Sax1* (Fig. 4B), suggesting Cdx4 regulates *Sax1* via combination of activating and repressing mechanisms. Given that Cdx4 is a transcriptional activator (Isaacs et al., 1998), Cdx4-induced *Sax1* downregulation is likely to be indirect and, as our study shows, it is independent of Pax6 (Fig. 4, 6). It is likely, however, that Cdx4 regulation of *Sax1* is mediated by Wnt and FGF, as these pathways are under Cdx control (Chawengsaksophak et al., 2004; Savory et al., 2009; van Rooijen et al., 2012), and they are required for *Sax1* transcription (Bertrand et al., 2000; Tamashiro et al., 2012). In this scenario, dominant-negative Cdx4 would downregulate *Sax1* by blocking Wnt and FGF signaling (Fig. 4B). Given that Sax1 inhibits floor plate cell specification by repressing *Pax6* and *Irx3* (Sasai et al., 2014), we propose that Cdx4 inhibition of *Sax1* primes pluripotent cells for differentiation.

Cdx4 promotes neural cell determination in the rostral transition zone

Progression of cells from caudal to the rostral transition zone is not only marked by the loss of pluripotent characteristics, but also by the acquisition of neural determination markers. Cdx4 promotes this new state by directing *Pax6* activation, a factor involved in neural progenitor maturation (Bel-Vialar et al., 2007). Cdx4 regulation of *Pax6* occurs via two distinct mechanism (Fig. 6): one, by indirect down regulation of the *Pax6* repressor Sax1 (Fig. 4), and the other, by direct activation of *Pax6* (Fig. 2, 6). 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 Cdx4 activity domain to the rostral transition zone. This regulatory mechanisms is likely to be evolutionarily conserved across vertebrates, as in several species examined, the *Pax6* locus contains RA response elements (RAREs; Cunningham et al., 2016), as well as Cdx4 binding sites (Paik et al., 2013). In addition, RA has been implicated in opening up the *Pax6* locus by antagonizing FGF signaling (Patel et al., 2013), thus RA could function to provide locus accessibility to Cdx4. In this scenario, RA provides the context in the rostral transition zone by which Cdx4 promotes neural progenitor cell maturation.

Cdx4 prevents premature neural cell differentiation

Pax6 induces neural cell differentiation (Bel-Vialar et al., 2007), yet, despite Cdx4 inducing *Pax6* in the rostral transition zone (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* (Bel-Vialar et al., 2007; Scardigli et al., 2003), a gene that promotes cell cycle exit and further differentiation (Lacomme et al., 2012). Our data demonstrates that Cdx4 represses *Ngn2* transcription even in the presence of Pax6 (Fig. 3), thus priming but delaying cell differentiation to the neural tube. 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). Ngn2 regulation by Cdx4 is likely to be indirect, as Cdx 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 cells from one state to the next during the early maturation of the spinal cord. The regulation cell transitions by Cdx proteins may be a general property of this family of transcription factors, as Cdx family members have been described to control maturation of intestinal (Hryniuk et al., 2012; Saad et al., 2011) and hematopoietic (McKinney-Freeman et al., 2008; Wang et al., 2008) cells from pluripotent precursors.

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

In current models of spinal cord neurogenesis, cells progressively lose pluripotency and acquire neural characteristics under the control of FGF, Wnt and RA signaling (Diez del Corral and Storey, 2004; Gouti et al., 2015). 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 activate *Wnts* transcription while restricting RA to low levels (Olivera-Martinez et al., 2012; Boulet and Capecchi, 2012; Sakai et al., 2001; White et al., 2007). In turn, Wnt8c promotes RA synthesis in anterior regions, far away from the area 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 (Diez del Corral et al., 2003; Kumar and Duester, 2014). This cross-repressive and balanced activities of FGF/Wnts and RA create a posterior-to-anterior gradient of pluripotency 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 pluripotency genes *T (Bra), Sox2* and *Sax1*, while simultaneously

repressing the differentiation genes *Pax6, Irx3*, and *Ngn1/2*. In contrast, RA promotes differentiation by repressing *T (Bra)* and *Sax1* 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 (Diez del Corral et al., 2003).

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 (Chang et al., 2016; Lee and Skromne, 2014). FGF and Wnt promote pluripotency by directly activating pluripotency marker *Sax1* (Diez del Corral et al., 2002; Tamashiro et al., 2012), but also initiate the loss of pluripotency and primes cells towards differentiation by activating *Cdx4*, an indirect repressor of *Sax1* (Fig 4B). A similar phenomenon is observed in the regulation of *Pax6*, with FGF repressing (Bertrand et al., 2000) and Cdx4 activating in a RA-dependent manner the transcription of *Pax6* (Fig. 2B). While the mechanism of Cdx4-FGF antagonism is unknown, it may involve chromatin modification events. In the case of *Pax6* regulation, 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 (Mazzoni et al., 2013) Regardless of the mechanism, in the context of *Sax1* and *Pax6* regulation, Cdx4 antagonizes FGF and synergizes with RA to provide a time delay mechanism separating pluripotent, early and late differentiation states.

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 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 role in maintaining progenitor population via two distinct mechanisms. Early in spinal cord development Cdx cooperate with T/Bra to promote FGF and Wnt signaling cascades to 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). 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 (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.

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 (Hamburger and Hamilton, 1951). 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 (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 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 (Itasaki et al., 1999; Nakamura and Funahashi, 2001). Only normal-looking embryos with good electroporation in the desired region (e.g., neural tube, transition zone, 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: transition zone for stage HH12-13 (prospective thoracic level; Liu et al., 2001), and thoracic level for stage HH16-17 (somites 20-25; Evans, 2003).

***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*, *Hes5*, *Sax1*, *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. 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 (Skaggs et al., 2011). 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 (Vecta shield) 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 (Schneider et al., 2012). 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 (Shu et al., 2013) 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.

ACKNOWLEDGEMENTS

We thank all members of Skromne lab for intellectual insights, particularly Dr. S. Bandopadhyay. We also thank Dr. K. G. Story (U Dundee, UK), Dr. M. Gouldin (Salk Institute, USA). Dr. F. Medeville (CBI, France), Dr. S. Mackem (NCI, USA), Dr. Y. Marikawa (U Hawaii, USA), Dr. A. V. Morales (Cajal Institute, Spain) and Dr. B. Novitch (UCLA, USA) for generously providing essential constructs and antibodies.

COMPETING INTERESTS

No competing interest declared.

FUNDING

P. J. was supported by Sigma XI GIAR, and the University of Miami College of Art and Science Dean's summer and dissertation grants. I. S. was supported by University of Miami College of Arts and Sciences and the Neuroscience Program, and by the National Science Foundation (IOS-090449).

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FIGURE LEGENDS

Figure 1. Cdx4 activates transcription of the neurogenesis gene *Pax6*. (**A**) Schematic representation of the caudal end of HH10 chicken embryo showing primary subdivisions (adapted from Olivera-Martinez and Storey, 2007), and expression domain 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 the embryo (HH11). Red lines indicate position of transverse sections shown on right. (**C**) Distribution of Pax7 (dorsal), Pax6 (dorsal-to-intermediate), and Nkx6.1 (ventral) protein relative to *Cdx4* transcription domain reveals dynamic but poorly correlated spatial relationships. (**D**) Cdx4 does not regulate DV patterning in the neural tube. Ectopic *Cdx4* did not change the distribution of Pax7 or Nkx6.2 proteins (n=6/6 for both), but caused ectopic Pax6 accumulation outside its normal domain (arrowheads, n=6/6; Control pCIG experiments: Fig.S1). Marker proteins are in red and electroporated cells are in green (nuclear GFP tag). Embryos were electroporated at HH10-11 and analyzed 24 hours post-electroporation (hpe). (**E**) Summary of results. Scale bar 200µm (whole mount); 40µm (transverse section).

Figure 2. Cdx4 activation of *Pax6* transcription is RA-dependent. (**A**) In the transition zone, Cdx4 is not sufficient to activate *Pax6* transcription. Overexpression of constitutive active *VP16Cdx4* (b, b'; n=4/6) but not wild type *Cdx4* (a, a'; n=6/6) results in high levels of *Pax6* transcription (arrowheads; control experiments Fig. S1). Overexpression of dominant negative *EnRCdx4* downregulates *Pax6* transcription (c, c', box; n=6/6; control experiments Fig. S1). Expression of *Pax6* was detected in purple by *in situ* hybridization (ISH), and electroporated cells by anti-GFP immunohistochemistry. Embryos were electroporated at HH10-11 and analyzed 8 hpe. (**B**) In the transition zone, constitutive active Cdx4 (VP16Cdx4; n=3/4), but not wild type Cdx4, leads to Pax6 protein accumulation (arrowheads). (**C**) In the neural tube, Cdx4 requires Retinoic Acid (RA) to activate *Pax6* transcription. *Cdx4* and *VP16Cdx4* overexpression result in ectopic Pax6 protein accumulation dorsally and ventrally (a, a', b, b'; arrowheads; n=6/6 for both). Overexpression of *EnRCdx4* downregulates Pax6 (c, c'; box; n=6/6). RA-signaling inhibition using a dominant negative RA receptor (dnRAR) downregulates Pax6 (d, d'; box; n=6/6). Cdx4 is unable to induce ectopic Pax6 in the absence of RA (e, e'; box; n=6/6). VP16Cdx4 induces ectopic Pax6 even in the absence of RA signaling (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 Karaz et al., 2016). 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 200µm (whole mount); 40µm (transverse section).

Figure 3. Cdx4 inhibits early cell maturation by repressing the neural differentiation *Ngn2* and the cell cycle gene *Hes5*. (**A**) Wild type expression of *Ngn2* in HH11 embryo. Expression of this gene initiates in the ventral neural tube around the most recent formed somite (a, a'). (**B**) Cdx4 represses Ngn2 even in the presence of the Ngn2-activator Pax6. Cdx4 and VP16Cdx4 repress Ngn2 (a, a', b, b'; boxes; n=6/6 for both; control experiments Fig. S1), despite inducing its activator Pax6 (Fig. 2Ca, b). EnRCdx4 represses Ngn2 (c, c'; box; n=6/6; control experiments Fig. S1) and Pax6 (Fig. 2Cc). Pax6 induces 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 represses the cell cycle marker *Hes5*. Cdx4 (a, a') and VP16Cdx4 (b, b') represses (boxes), whereas EnRCdx4 does not alter *Hes5* transcription in the transition zone (n=6/6 for all conditions). Scale bar 200µm (whole mount); 40µm (transverse section).

Figure 4. Cdx4 represses pluripotency in early neural progenitors, but not in neuromesodermal progenitor cell. (A) Cdx4 does not regulate the neuromesodermal progenitor (NMP) marker *T (Bra)*. Neither Cdx4 (a, a'), VP16Cdx4 (b, b') nor EnRCdx4 (c, c') alter *T (Bra)* expression in NMPs (n=6/6 for all conditions). (**B**) Cdx4 represses early neural progenitor marker *Sax1*. Overexpression of *Cdx4* (a, a') or *VP16Cdx4* (b, b') inhibit *Sax1* transcription (boxes; control experiments Fig. S1). *EnRCdx4* overexpression also downregulates *Sax1* (c, c') (n=6/6 for all conditions; control experiments Fig. S1). (**C**) Pax6 does not regulate *Sax1*

transcription (n=6/6). (**D**) Figure summarizing Cdx4-Sax1 interactions. Scale bar 200µm (whole mount); 40µm (transverse section, separate scale bar for Bc', c" shown).

Figure 5. Sax1 and Pax6 feedback interactions regulate Cdx4 transcription. (**A**) Sax1 downregulates the transcription of its own gene and of *Pax6*, but not *Cdx4*. *mSax1* overexpression downregulates *cSax1* and *Pax6* transcription (a, a', c, c'; boxes; n=6/6 for both). However, *mSax1* overexpression does not alter *Cdx4* transcription (b, b'; n=6/6). (**B**) Pax6 represses *Cdx4*. Ectopic *Pax6* downregulates (a, a'; n=6/6), and *EnRPax6* upregulates (b, b'; n=6/6; control experiments Fig. S1) *Cdx4* transcription. (C) Figure summarizing Sax1-Cdx4-Pax6 interactions. Scale bar 200µm (whole mount); 40µm (transverse section).

Figure 6. Proposed gene regulatory network controlling spinal cord neurogenesis. 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 neurogenesis (Olivera-Martinez and Storey, 2007). Network map was generated using Biotapestry (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.

FIGURE 1

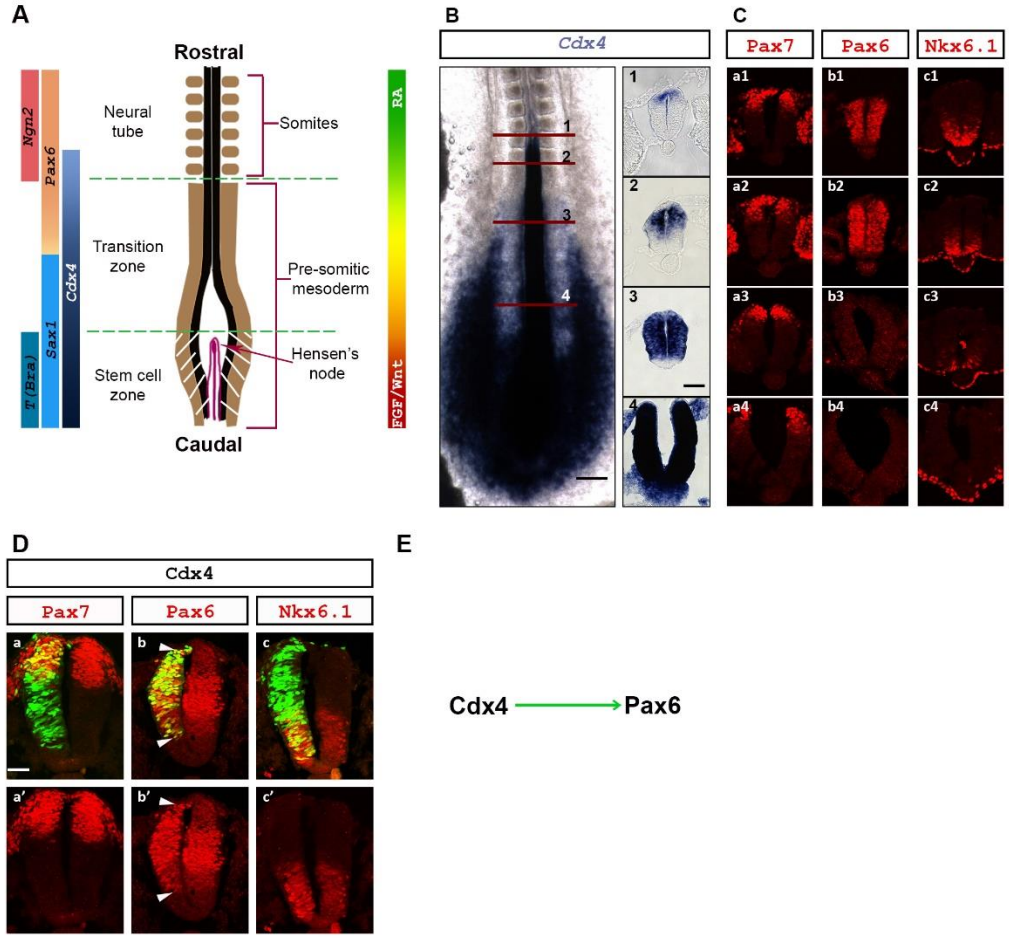


FIGURE 2

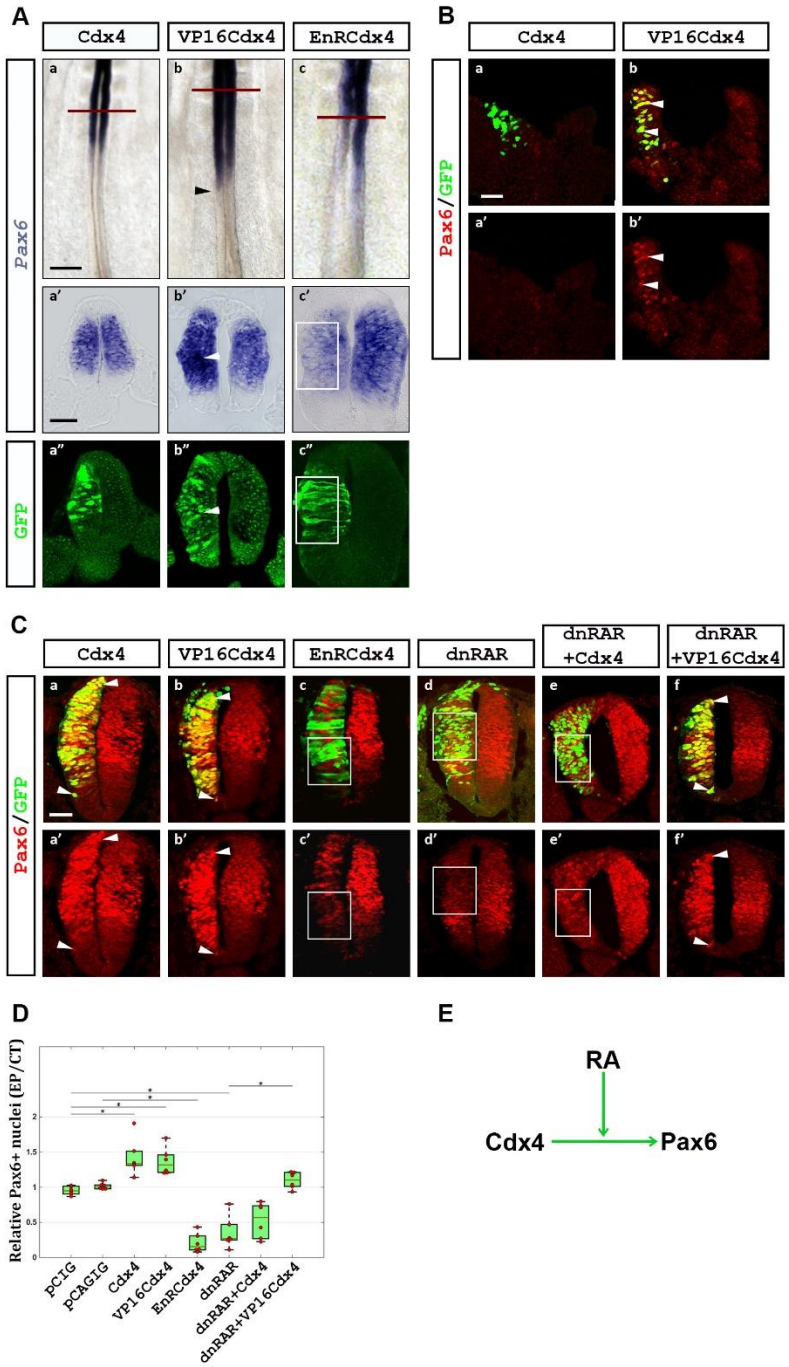


FIGURE 3

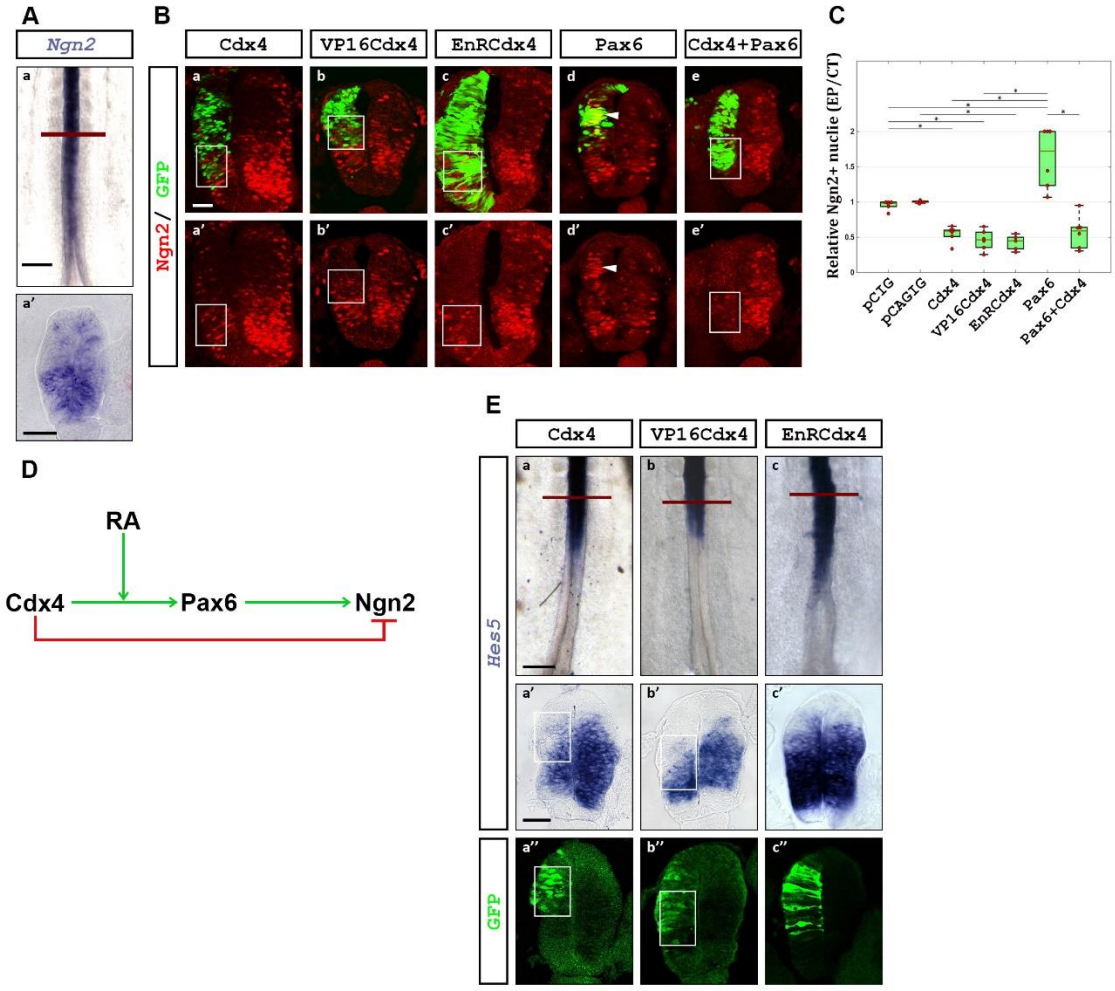


FIGURE 4

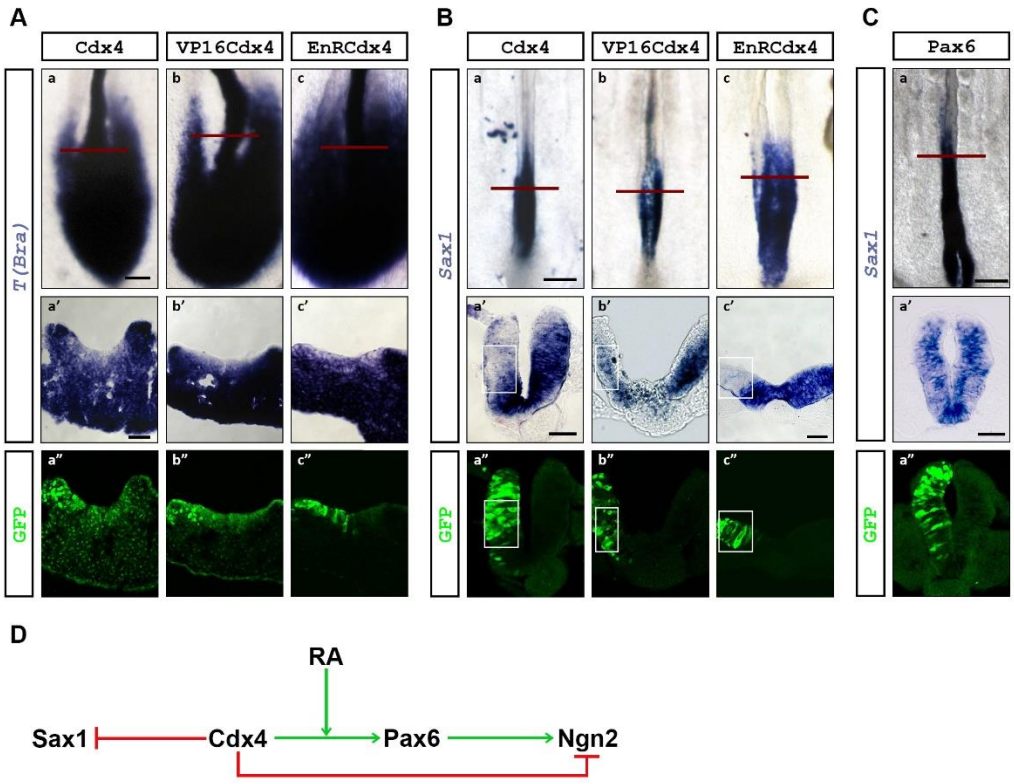


FIGURE 5

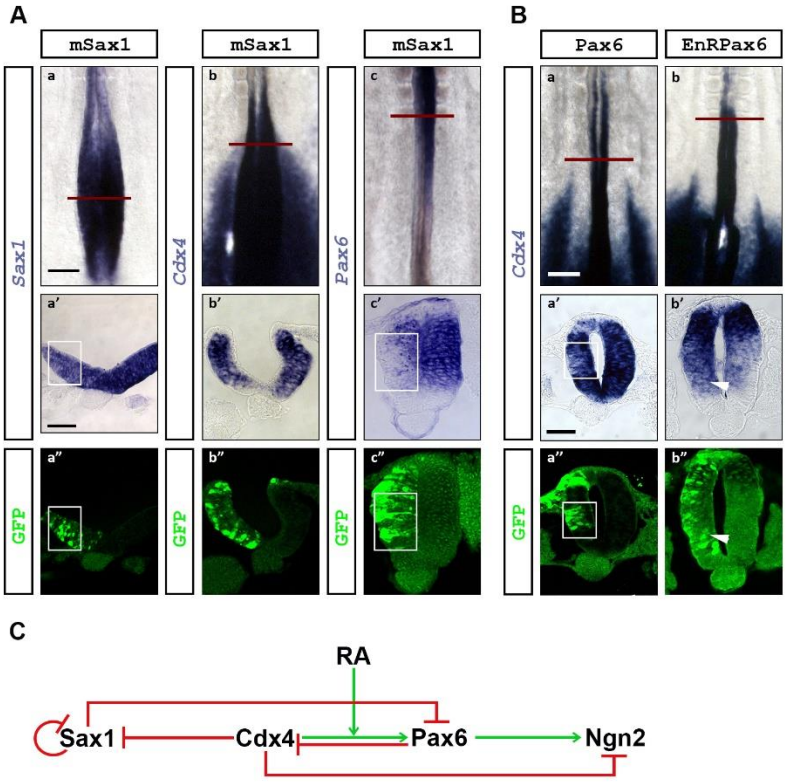
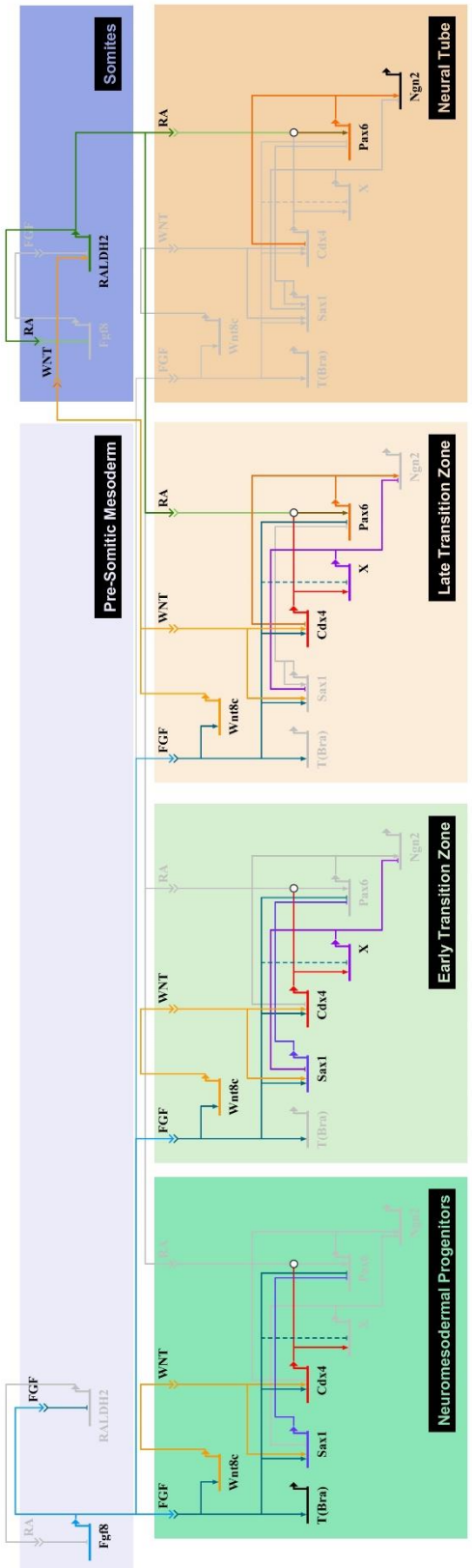


FIGURE 6



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 (Megason and McMahon, 2002).

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 (Matsuda and Cepko, 2004).

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.

SUPPLEMENTAL FIGURE

Figure S1. GFP overexpression doesn't affect wild type expression of factors investigated. pCIG (nuclear GFP) and pCAGIG (cytoplasmic GFP) ectopic expression didn't change *Pax6* (A: IHC analysis, C: ISH analysis), *Ngn2* (B), *Sax1* (D) and *Cdx4* (E) expression compared to contralateral control side (n=6/6). *Cdx4* expression analysis for pCAGIG over-expression wasn't done as none of the pCAGIG backbone construct were analyzed for *Cdx4* expression. Red bar shows RC level of transverse section. Scale bar 200µm (whole mount); 40µm (transverse section).

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)	(Bel-Vialar et al., 2007)
Pax6-pCIG	Electroporation		
EnRPax6-pCIG	Electroporation		
Chick-*T(Bra)*- pBS	*In situ* hybridization	Dr. Susan Mackem (NCI, USA)	(Liu et al., 2003)
mNkx1.2-pEF2	For generating mNkx1.2-pCIG	Dr. Yusuke Marikawa (U of Hawaii, USA)	(Tamashiro et al., 2012)
dnRAR-pCIG	Electroporation	Dr. Axia V. Morales (Cajal Institute, Spain)	(Martinez-Morales et al., 2011)

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

Gene construct	Primers	Notes
Cdx4	Forward: ACATGTATGTGAGTTCTCTCTTGG	Ta: 55^0 C
	Reverse: TGATCATTCTGAAACTATGAC	
VP16	Forward: ATCGATATGTCAAGGCCTCTCGAGTCGAC (ClaI site underlined.)	Ta: 50^0 C
	Reverse: TGTGTGCCAACCCCACCGTACTCGTCAATT	
Cdx4-HD	Forward: GAGTACGGTGGGGTTGGCACACAGCAGGTC	Ta: 55^0 C
	Reverse: TGATCATTCTGAAACTATGAC	
mNkx1.2	Forward: ATATCGAT*CCACCATG*TTGGCATGGCAGG (ClaI site underlined, Kozack sequence italicized.)	Ta: 60^0 C
	Reverse: GAGAATTCTCATAGGTGTGGAGCATAG (EcoRI site underlined.)	

FIGURE S1