Cdx4 regulates the onset of spinal cord neurogenesis

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ABSTRACT

The transition of cells from one developmental state to the next is driven by signaling cues interpreted by intracellular networks of transcription factors. In the vertebrate spinal cord, the progressive caudal-to-rostral maturation of cells is controlled by the signaling activities of FGF/Wnt antagonizing Retinoic Acid (RA): FGF/Wnt secreted from the caudal stem zone promote stem cell identities, whereas RA secreted from the somites promotes neural differentiation. It is unclear how intracellular transcription factor networks interpret these extracellular. Using transient gene manipulation techniques in chicken, we show that Cdx4 is at the core of the transcription factor network that integrates upstream signaling information to regulate the sequential maturation of spinal cord neural progenitor cells. We show that Cdx4 represses the stem cell marker *Sax1* and promotes expression of the neural identity gene *Pax6* while simultaneously preventing the activation of the Pax6-dependent, neural-differentiation gene *Ngn2*. Our results suggest a novel role for Cdx4 in regulating the sequential maturation of neural cell states during early spinal cord development. Given Cdx known role in the transcriptional regulation of *Hox* patterning genes, we propose that Cdx factors coordinate the axial specification and maturation of cells 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 transcription factor composition 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 promote 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). In later stages, 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, 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 localize to 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 begin their further maturation in the neural tube that is surrounded by somites, where they begin to express the neural differentiation genes *Ngn1/2* and *NeuroM* (Diez del Corral et al., 2003). Thus, temporally distinct steps in the maturation and differentiation of 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 promote 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 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 respectively segregated to caudal and rostral regions of the nascent spinal cord through positive and negative interactions: FGF indirectly promotes differentiation by inducing RA production rostrally, through a Wnt8c-mediated mechanism (Olivera-Martinez and Storey, 2007), but FGF also maintains RA production away from 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 can exit proliferation and differentiate (Diez del Corral et al., 2003; Kumar and Duester, 2014). These interactions are 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 is poorly understood.

In the NMP domain and the transition zone, Cdx stand out as a transcription factor that is under the simultaneous control of FGF, Wnt and RA signals (Deschamps and van Nes, 2005; Lohnes, 2003). Cdx 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 hematopoiesis (Davidson et al., 2003; Wang et al., 2008). In the spinal cord, Cdx factors are essential for tissue specification and rostrocaudal pattering (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, Cdx family is a potential candidate 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 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 result 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 dorso-ventral gradient of transcription in the caudal neural tube does not specify dorso-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). Upon sectioning, however, it was also revealed that *Cdx4* is transcribed in a highly dynamic dorso-ventral (DV) gradient: caudally, *Cdx4* transcription was ubiquitous throughout the medio-lateral extent of the neural plate (dorso-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 Cdx4 role 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 correspondence 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 *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 *Cdx4* transcription domain (Fig. 1B, C). 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 down regulated. We predicted that if Cdx4 regulates DV cell specification, increasing *Cdx4* levels would result in change in DV marker genes localization. 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 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 hypothesize that Cdx4 may regulate *Pax6* transcription during neural progenitor cell maturation. To test this hypothesis we asked whether Cdx4 was sufficient to activate *Pax6* in the nascent neural tube, in a region where *Pax6* is not yet 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 replicates 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 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 in the transition zone for *Pax6* activation, 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, together with the gain of function experiments, these results also suggest that Cdx4 activation of *Pax6* transcription requires 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 could 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 unable to promote *Pax6* transcription (n=6/6; Fig. 2Ce, D), despite that in a wild type embryos Cdx4 was able to do so (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 provide 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 cell's 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 prevents Pax6 premature activation of 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. All the constructs caused Ngn2 down regulation (Fig. 3B, C; n=6/6 for all conditions), despite that *Cdx4* and *VP16Cdx4* also induced *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). Cdx4 is a transcriptional activator (Isaacs et al., 1998), suggesting that Cdx4 repression of *Ngn2* is indirect. Taken together our results suggest that Cdx4 promote cell's entry into the differentiation program by inducing *Pax6* transcription, but prevents cell's further differentiation by inhibiting *Ngn2* activation (Fig. 3D).

Early differentiation of neural tube cells 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 overexpression of *EnRCdx4* did not affect *Hes5* transcription (Fig. 3E; n=6/6 for all conditions). Given that Hes5 is required for neural cell proliferation (Fior and Henrique, 2005), our result suggests that Cdx4 could indirectly control cell division. Previously, Pax6 has been shown to activate *Hes5* in mouse cortical stem cells (Sansom et al., 2009) raising the possibility that Cdx4's regulates *Hes5* via Pax6. To test this scenario, we analyzed the effect of increasing or decreasing Pax6 over *Hes5* transcription. Unfortunately, the results were inconclusive; both wild type and dominant negative *Pax6*, *EnRPax6*, reduced *Hes5* transcription 8 hpe (data not shown, n=6/6 embryos for each condition), preventing further analysis. Despite this setback, our results suggest that Cdx4 represses cell proliferation (*Hes5*) and differentiation (*Ngn2*) to promote cells to enter and remain in an early differentiation state.

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, the neuromesomdermal progenitor

(NMP) cells that express *T (Bra) and Sax1* (*Nkx1.2* in mouse) (Henrique et al., 2015; Yamaguchi et al., 1999), and the early neural progenitor cells that only express *Sax1* (Delfino-Machin et al., 2005; Sasai et al., 2014). If, as our results suggest, Cdx4 promotes 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), 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 also indirectly involved in *Sax1* activation (Fig. 4Bc). In contrast, the observation that Cdx4 and constitutively active VP16Cdx4 also repress *Sax1* suggests the existence of an indirect mechanism of regulation that fine tune *Sax1* transcription levels (Fig. 4Ba, b). One likely candidate to mediate this indirect activity 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 mediate Cdx4 indirect repression of *Sax1*. As Cdx4 mediated Sax1 downregulation does not alter *T (Bra)* transcriptional domain, 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 that suggest 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 state and prevent their premature differentiation. 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). Together, these results suggest that Pax6 represses *Cdx4* in the rostral transition zone through indirect mechanisms. This indirect 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). 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

Role of Cdx4 in neurogenesis as a differentiation switch

Cdx4 dynamic RC-DV gradient at the caudal end of the embryo overlaps with various cellular states involved in early spinal cord neurogenesis (Fig. 1A, B, C). At the caudal most end, *Cdx4* is transcribed in NMPs that are self-renewing and pluripotent, and can give rise to both mesoderm and neuroectoderm (Henrique et al., 2015). NMPs are principally defined by the co-expression of genes *T (Bra)* and *Sox2* (Henrique et al., 2015). In contrast, at the rostral end of the expression domain, *Cdx4* is transcribed in cells expressing early neural identity markers such *Pax6*. Rostral to the *Cdx4* expression domain, cells express the differentiation marker *Ngn2* (Fig. 1B; Fig. 3A). Our results support the idea that across its domain of expression, *Cdx4* interact with factors involved in onset of spinal cord neurogenesis.

At the caudal end of the embryo, NMPs differentiate to give rise to growing spinal cord tissue and surrounding mesoderm. NMPs express the mesodermal marker *T (Bra)* and the neural marker *Sox2*. According to current models, *T (Bra)* and *Sox2* cross regulation maintains NMP status in dynamic equilibrium (Henrique et al., 2015; Martin and Kimelman, 2012). A tilt in the equilibrium leading to accumulation of *T (Bra)* would lead to cells acquiring mesodermal fate, whereas a tilt favoring *Sox2* transcription would lead the cells to acquire a neural fate (Gouti et al., 2015; Henrique et al., 2015).

Recent evidence suggests that Cdx factors might also be involved in maintaining the balance of NMPs (Amin et al., 2016; Gouti et al., 2017). *Cdx2* mutant mice, compared to wild type, exhibit early axis truncation with deficiencies in mesodermal and ectodermal tissue after somite 5 (Chawengsaksophak et al., 2004; Savory et al., 2009). In this respect, *Cdx2* null mutants are

similar to *T (Bra)* null mutants (Chawengsaksophak et al., 2004). Indeed, loss of *Cdx2* lead to reduction in *T (Bra)* expression domain post E9.5 (Savory et al., 2009). Conversely, *Cdx* triple mutants exhibit a complete loss of *T (Bra)* in the stem zone, suggesting a redundant function of Cdx in maintaining *T (Bra)* expression (van Rooijen et al., 2012). Even though Cdx2 binding sites were found on *T (Bra)* locus, Cdx dependent *T (Bra)* regulation has been suggested to occur via Cdx2 modulation of *Wnt3a* expression (Savory et al., 2009). In the current study, however, Cdx4 activity manipulation did not change the expression domain of *T (Bra)*. Overexpression of *Cdx4* or *VP16Cdx4* did not upregulate *T (Bra)*, suggesting Cdx4 is not sufficient to induce *T (Bra)* (Fig. 4A). Conversely, constitutive repressive *EnRCdx4* did not lead to downregulation of *T (Bra)* expression. This could be a result of Cdx functional redundancy as other Cdx members were not altered. Also, as the Cdx dependent regulation of *T (Bra)* is suggested via modulation of Wnt3a signaling (Savory et al., 2009), given the observation that cells at the caudal end were sparsely electroporated, any change in *Wnt3a* expression wouldn't have been sufficient to downregulate *T (Bra)*.

NMPs also express pluripotency marker *Sax1 (Nkx1.2* in mice*). Sax1* expression is maintained in the neural committed cells as they leave the NMP domain and become part of the neural plate. By contrast, newly differentiated mesodermal fated cells do not express *Sax1* (Delfino-Machin et al., 2005; Gouti et al., 2017), suggesting *Sax1* is a neural specific marker. While *Sax1* has been consistently used as a marker for pluripotent cells (Bertrand et al., 2000; Delfino-Machin et al., 2005; Sasai et al., 2014), its function in promoting pro-neural fate in the caudal neural tube has not been investigated. However, a recent study suggested that Sax1 acts a repressor and is involved in mediating upstream FGF signaling in inhibiting differentiation and promoting floor plate competence and induction (Sasai et al., 2014). In the caudal transition zone, *Sax1*

expression is nested within the *Cdx4* expression domain. Our data showed that Cdx4 negatively regulates *Sax1* (Fig. 4B). Significantly, Cdx4 repression of *Sax1* was indirect, as VP16Cdx4, the constitutively active form of Cdx4 also repressed *Sax1*. Surprisingly EnRCdx4, the constitutive repressor form of Cdx4 also repressed *Sax1* suggesting that loss of function of Cdx4 also downregulated *Sax1*. One possibility that will explain both constitutive active and repressive forms of Cdx4 resulting in the same phenotype is that Cdx4 regulates *Sax1* by activating its activator (for example *Wnt3a* (van Rooijen et al., 2012)) and also its inhibitor (currently unknown). Another possibility is that Cdx4 inhibits activator of *Sax1* while promoting its repressor. Our speculation is that in the caudal stem zone Cdx4 dependent repression is not dominant due to high concentration of signaling factors FGF/Wnt that induces both *Sax1* and *Cdx4* (Bel-Vialar et al., 2002; Bertrand et al., 2000; Diez del Corral et al., 2002; Nordstrom et al., 2006; Tamashiro et al., 2012). High levels of FGF/Wnt signaling are either able to repress the Cdx4 dependent repressor of *Sax1* or outcompete its activity. However, in the caudal transition zone Cdx4 dependent repression is able to outcompete FGF/Wnt dependent *Sax1* activation leading to *Sax1* downregulation in the transition zone.

It is important to mention that despite the downregulation of *Sax1* in neural plate in *Cdx4* overexpression experiments, there was no change in the expression domain of *T (Bra)*, as discussed earlier. This suggest that Cdx4 is not directly involved in determining the size of NMP domain and might be playing an indirect role in balancing neural and mesodermal specification by regulating levels of *T(Bra)*. Further research would explicate if Cdx promotes neural or mesodermal fates in the NMPs or both.

Cdx4 dependent downregulation of *Sax1* in the pro-neural cells located in the caudal transition zone has two implications: downregulating pluripotency and promoting differentiation. Sax1 has

been shown to inhibit *Pax6* and *Irx3* expression in caudal neural tube (Sasai et al., 2014), thereby indirectly promoting expression of floor plate specification factors. By downregulating *Sax1*, Cdx4 primes the neural progenitor cells to begin their differentiation by getting rid of the repression, thus rendering the neural tube competent to respond to differentiating signals. Cdx4's role in furthering differentiation of pro-neural cells also involves the activation of *Pax6* (Fig. 2). This activity only takes place in the rostral portion of the transition zone because Cdx4 activation of *Pax6* transcription is dependent on RA signaling. Hence, Cdx4 promotes acquisition of neural identity by two mechanisms, by downregulating pluripotency by indirectly repressing *Sax1*, which otherwise inhibits *Pax6*; and by directly activating *Pax6*.

As mentioned above, Cdx4's regulation of *Pax6* is dependent on RA and thereby restricted at the rostral transition zone. Multiple mechanisms have been proposed to explain lack of *Pax6* expression at the caudal end. Firstly, FGF signaling has been suggested to inhibit *Pax6* via higher-order chromosomal modification resulting in the *Pax6* locus being inaccessible to activators (Patel et al., 2013). This could explain Cdx4's inability to turn on *Pax6* in the caudal stem zone as it is unable to bind to the *Pax6* locus. However, as observed, VP16Cdx4 was able to prematurely activate *Pax6* in the caudal transition zone suggesting that locus inaccessibility is not the only reason for *Pax6* inhibition. Secondly, Sax1 is a known inhibitor of *Pax6* in the newly differentiated pro-neural cells. However, our results showed that while *Cdx4* overexpression downregulates *Sax1*, this downregulation is not concomitant with *Pax6* activation in *Sax1*- cells. The discrepancy between Cdx4 and VP16Cdx4 ability to activate *Pax6* suggest that in addition to open chromatin and absence of Sax1, Cdx4 also requires a co-factor in order to activate *Pax6*.

In the presence of RA, Cdx4 was able to activate *Pax6* similar to VP16Cdx4 (Fig. 2C). RA has been implicated in opening up the *Pax6* locus by antagonizing FGF signaling (Patel et al., 2013). In addition, the *Pax6* locus also contain retinoic acid response elements (RAREs) (Cunningham et al., 2016), suggesting RA-RAR and Cdx4 cooperate at the promoter levels to activate *Pax6*. Cdx factors are known to regulate *Hox* gene activation via chromatin remodeling (Mazzoni et al., 2013), suggesting another possibility that Cdx4, in the presence of RA, can open repressive chromatin structure, thus opening the *Pax6* locus for regulation. Thus, while it is unclear how RA modulates Cdx4 activity in the transition zone, epigenetic modification is the most probable candidate.

The final step in our model proposes that Cdx4 prevents the further differentiation of cells promoted by Pax6. Pax6 promotes cell differentiation by activating several downstream targets, including *Ngn2*. In the neural tube surrounded by somites, *Ngn2* is expressed in a subset of *Pax6*+ cells where it promotes cell cycle exit and further differentiation (Lacomme et al., 2012). This expression of *Ngn2* in the intermediate domain of neural tube is under direct Pax6 regulation (Scardigli et al., 2003), as suggested by lowered *Ngn2* expression in *Pax6* mutants compared to wild type mice (Bel-Vialar et al., 2007). In the neural tube domain where *Cdx4* and *Pax6* overlap, *Ngn2* is not transcribed (Fig 3A). Our experiments demonstrated that Cdx4 can repress *Ngn2* transcription (Fig. 3B), suggesting that Cdx4 primes the cells for differentiation but does not let them differentiates just yet. As *Cdx4* expression gets dorsally restricted, Pax6 is now able to activate *Ngn2* in the ventral neural tube regions. This is evident from ventral expression of *Ngn2* in the early neural tube (Fig. 3A). As Cdx factors are known to act as transcriptional activators, the negative regulation of *Ngn2* seems to be indirect, but does not involve promoting

proliferation (Fig. 3E). Indirect regulation could also provide a time delay between *Cdx4* elimination and *Ngn2* activation.

Integration of signaling and transcription factor models during spinal cord neurogenesis

The proposed GRN (Fig. 6), with Cdx4 at its core, involved in progression of cellular states in caudal neural tube could be acting under the signaling switch proposed by Diez del Corral (2003) and Olivera-Martinez and Storey (2007). High concentration of FGF/Wnt signaling in the caudal stem zone leads to activation of *T (Bra)*, Sax1 and *Cdx4*. With the decrease in FGF/Wnt signaling strength in the transition zone Cdx4 is able to downregulate *Sax1*. Wnt8c is responsible for activating *Raldh2* in the somites, where Wnt8c is able to overcome FGF repression of *Raldh2*. RA synthesized in somites then diffuses caudally into the neural tube, and promotes differentiation of competent cells in the rostral transition zone. Cdx4 is responsible for initial activation of *Pax6* in combination with RA; however, in the neural tube surrounded by the somites, RA can maintain *Pax6* expression in a Cdx4 independent manner. In the rostral transition zone, Pax6 activation drive cells to acquire neural identity. However, the presence of Cdx4 prevents them from turning on *Ngn2* and further differentiating. Once *Cdx4* starts clearing from the ventral neural tube, *Pax6*+ cells start upregulating *Ngn2* and acquire neurogenic identity.

Altogether, FGF-Wnt-RA provides spatial information to the maturing neural cells, in addition to driving transition in signaling factors. The transcription network in the NMPs responds to this spatio-temporal information and regulates the pace of differentiation in the maturing neural cells.

Cdx role in coordinating FGF-Wnt-RA signaling information

In regulating spinal cord neurogenesis, Cdx coordinates FGF, Wnt and RA signaling information that other studies have shown to direct spinal cord cell maturation (Diez del Corral et al., 2003; Olivera-Martinez and Storey, 2007). Our results showed that with respect to maturation of spinal cord progenitors, Cdx acted antagonistically to FGF-Wnt signaling in the caudal end. FGF-Wnt signaling directs activation of pluripotency marker *Sax1* (Diez del Corral et al., 2002; Tamashiro et al., 2012), in addition to activating *Cdx4* that, our results showed, represses *Sax1* (Fig. 4B). Hence, while FGF-Wnt signaling promotes pluripotency, the downstream target Cdx4 antagonizes pluripotency and primes the cells towards their differentiation pathway. Thus, in controlling *Sax1* transcription, Cdx4 provides a feedforward that is antagonistic to FGF-Wnt activity. A similar antagonism was observed in the regulation of *Pax6*, where FGF represses *Pax6* (Bertrand et al., 2000) while Cdx4 promoted its induction (Fig. 2) In contrast, Cdx4 cooperates with RA in promoting *Pax6* transcription and differentiation. In all, Cdx4 acts antagonistically to the inducing FGF-Wnt signaling and synergistic to RA signaling in promoting sequential maturation of cells.

Interestingly, however, Cdx factors cooperate with FGF-Wnt but not RA during spinal cord specification and patterning, which is the opposite of what our results suggest for spinal cord cell maturation. In regulating spinal cord patterning, Cdx factors act downstream of FGF and Wnt signaling in activating caudal 5' *Hox* genes to bestow identity to brachial and thoracic regions of the spinal cord (Bel-Vialar et al., 2002; Marletaz et al., 2015; Nordstrom et al., 2006; Shimizu et al., 2006). Cdx factors also inhibit rostral identities by repressing 3' *Hox* genes that are in turn activated by RA signaling (Lee and Skromne, 2014; Marletaz et al., 2015; Skromne et al., 2007).

Hence, Cdx factor cooperate with FGF-Wnt and antagonize RA signaling during patterning of the hindbrain and spinal cord.

Thus, Cdx factors coordinate the signaling information from the FGF-Wnt-RA factors in a contrasting manner depending upon the functional context during spinal cord development. One possible explanation could be that the access of Cdx factors to the regulatory regions is controlled by the signaling information. This is evident in the case of *Pax6* transcription, where FGF signaling leads to higher order chromosomal silencing of *Pax6* (Patel et al., 2013). Recent evidence shows that FGF signaling leads to translocation of the *Pax6* locus to nuclear boundary, which has been associated with chromatin inactivity (Patel et al., 2013). Downregulation of FGF signaling leads to removal of higher order chromatin modifications that were previously blocking access of regulators to the *Pax6* locus. The other possible speculation could be the availability of cofactors that are regulated by signaling information, as in the case of Cdx regulation of intestinal differentiation. During intestinal stem cell maturation, CDX2 has been shown to associate with Wnt signaling transcription factor TCF4, to achieve tissue specific Wnt function (Verzi et al., 2010). Thus while Cdx factors would act as an activator, the epigenetic state of the DNA, the signaling state of the cell and the availability of other transcription factors in the cell, will determine whether Cdx target genes are activated or not.

In conclusion, Cdx factors are at the core of gene regulatory network that regulate patterning and differentiation across tissue during vertebrate embryonic development. Importantly, the role of Cdx factors as a coordinator of upstream signaling make them indispensable for proper embryonic development.

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 vectors (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 dorso-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.

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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 dorso-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). 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). Overexpression of dominant negative *EnRCdx4* downregulates *Pax6* transcription (c, c', box; n=6/6). 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* at 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), despite inducing its activator Pax6 (Fig. 2Ca, b). EnRCdx4 represses Ngn2 (c, c'; box; n=6/6) 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). *EnRCdx4* overexpression also downregulates *Sax1* (c, c') (n=6/6 for all conditions). (**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* up regulates (b, b';

n=6/6) *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.

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.

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)

Full legth 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 TA 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).

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.)	

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.

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Figure 1

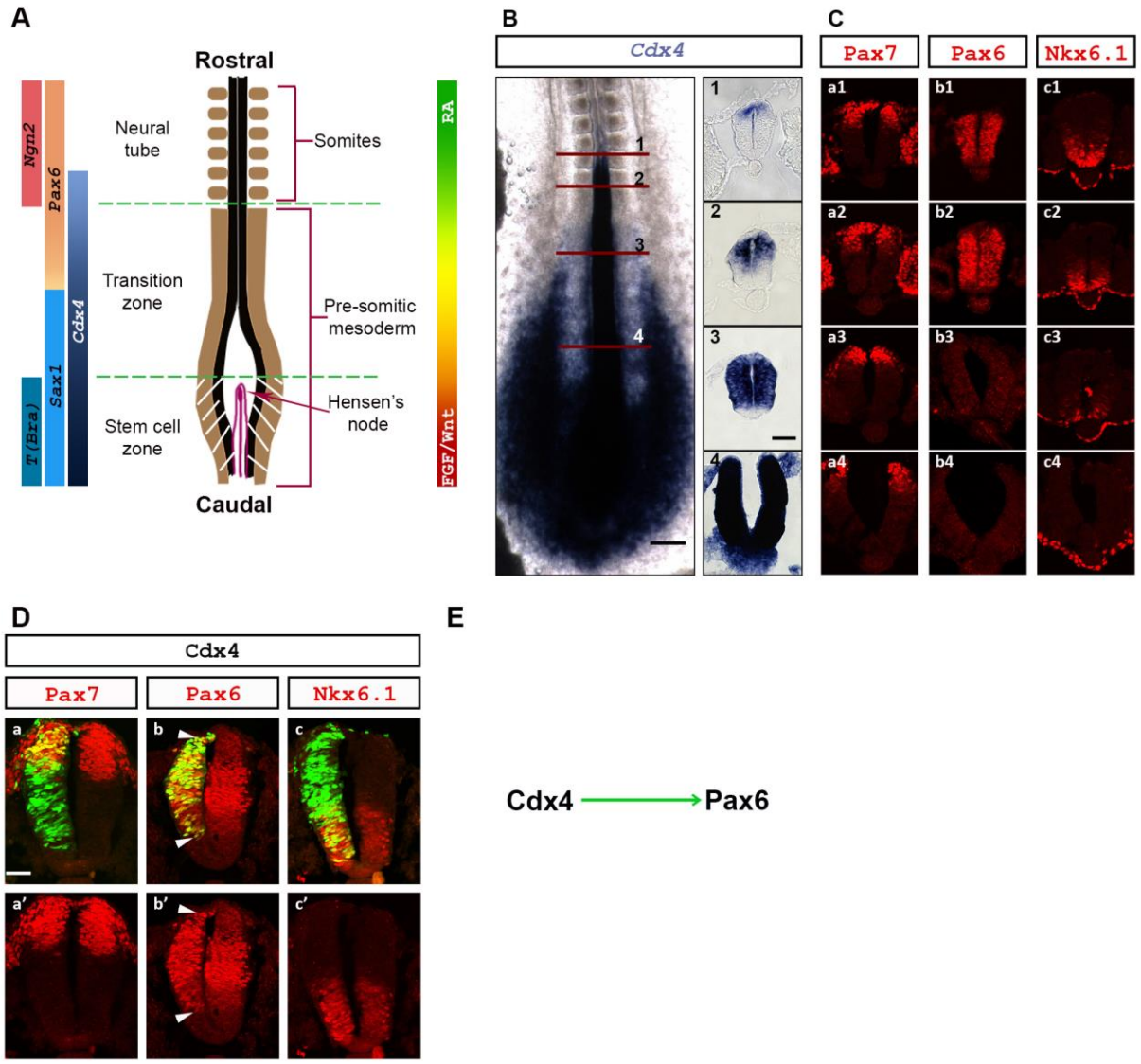

A
Rostral
Neural tube
Somites
Transition zone
Pre-somitic mesoderm
Stem cell zone
Hensen's node
Caudal
Ngn2
Pax6
Cdx4
Sax1
T (Bra)
RA
FGF/Wnt
B
Cdx4
1
2
3
4
C
Pax7
Pax6
Nkx6.1
a1
b1
c1
a2
b2
c2
a3
b3
c3
a4
b4
c4
D
Cdx4
Pax7
Pax6
Nkx6.1
a
b
c
a'
b'
c'
E
Cdx4
Pax6

Figure 2

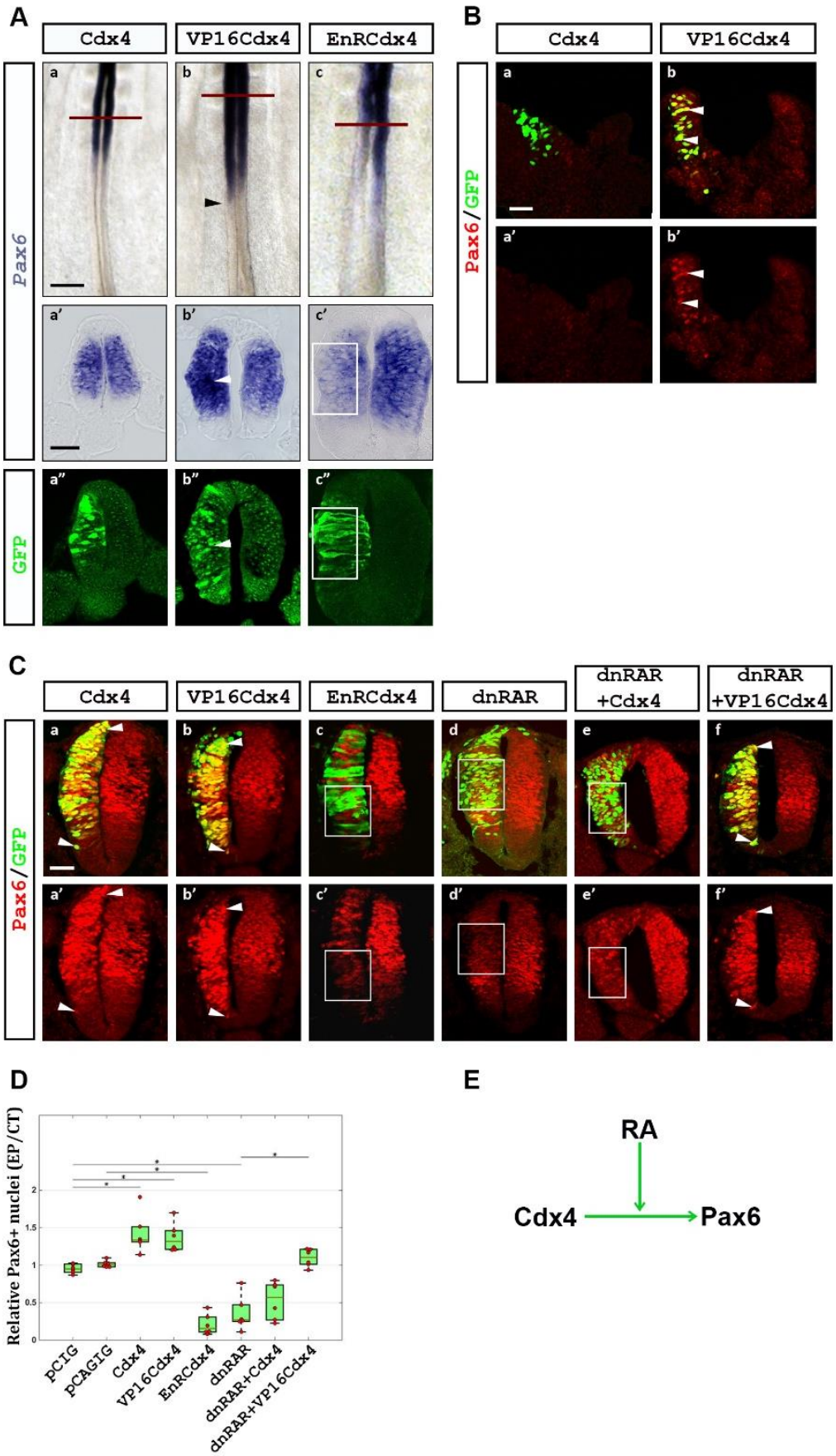

A
Cdx4
VP16Cdx4
EnRCdx4
Pax6
GFP
B
Cdx4
VP16Cdx4
Pax6/GFP
C
Cdx4
VP16Cdx4
EnRCdx4
dnRAR
dnRAR +Cdx4
dnRAR +VP16Cdx4
Pax6/GFP
D
Relative Pax6+ nuclei (EP/CT)
pCIG
pCAGIG
Cdx4
VP16Cdx4
EnRCdx4
dnRAR
dnRAR+Cdx4
dnRAR+VP16Cdx4
E
RA
Cdx4
Pax6

Figure 3

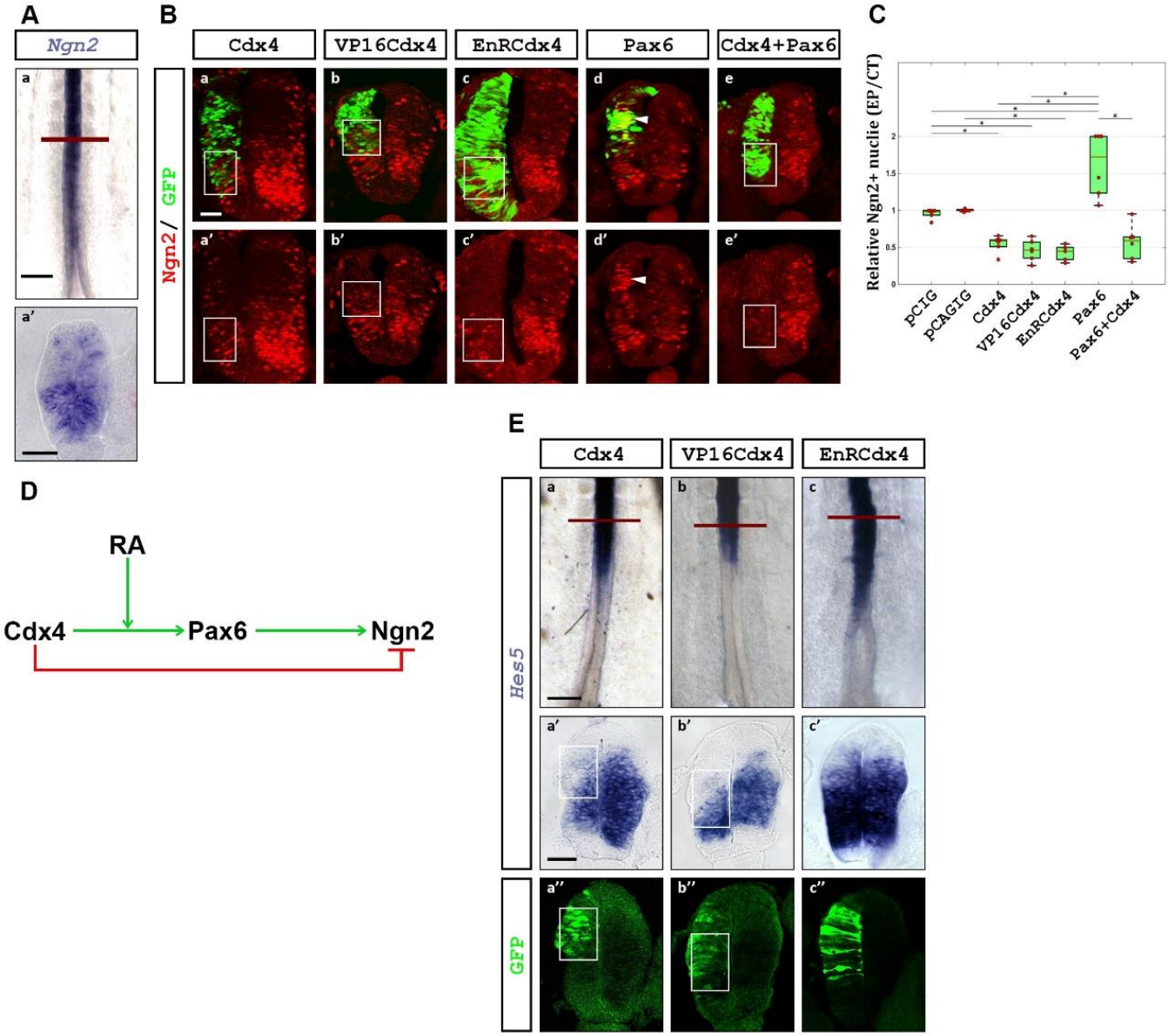
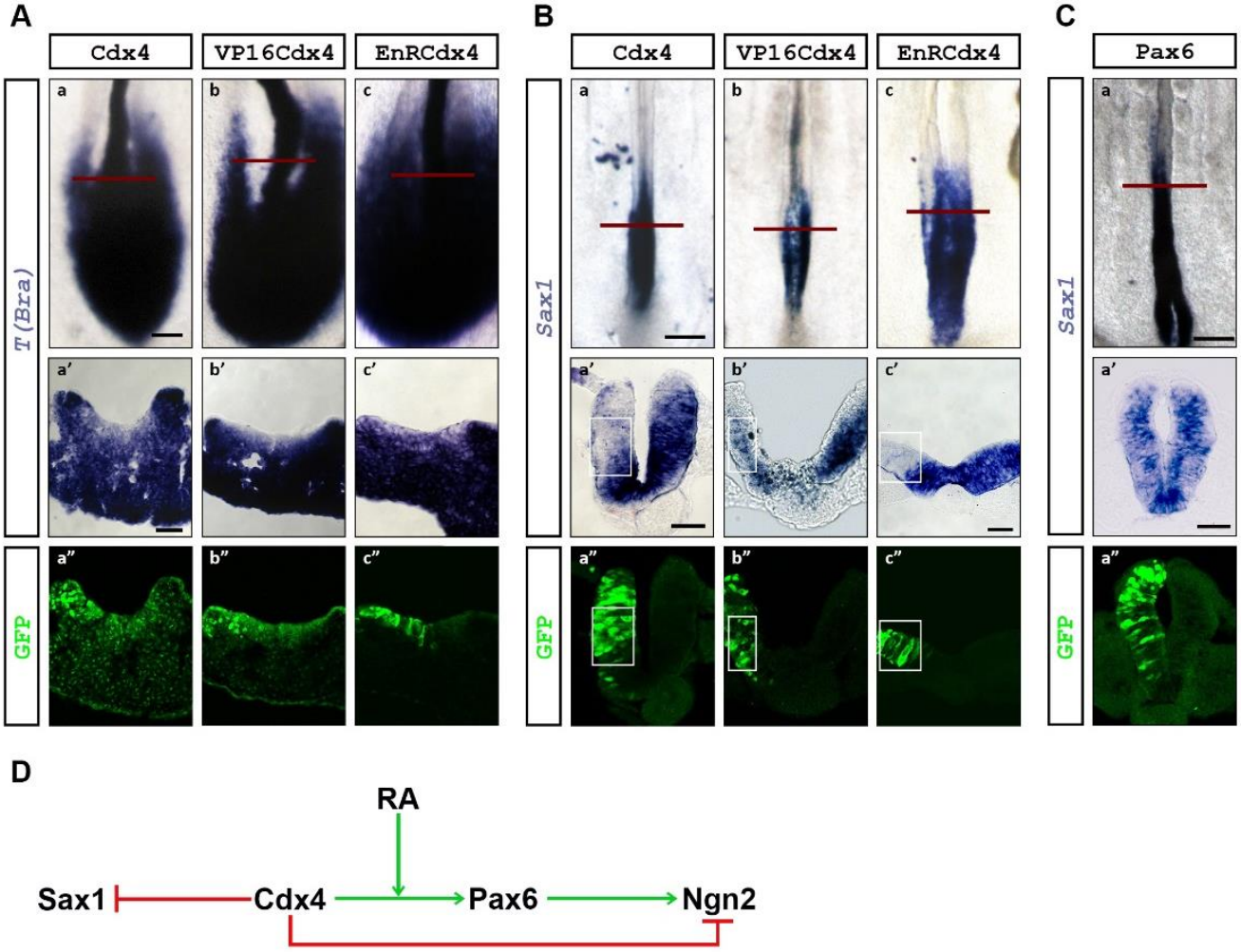

A
Ngn2
a
a'
B
Cdx4
VP16Cdx4
EnRCdx4
Pax6
Cdx4+Pax6
Ngn2 / GFP
a
b
c
d
e
a'
b'
c'
d'
e'
C
Relative Ngn2+ nuclie (EP/CT)
pCIG
pCAGIG
Cdx4
VP16Cdx4
EnRCdx4
Pax6
Pax6+Cdx4
D
RA
Cdx4
Pax6
Ngn2
E
Cdx4
VP16Cdx4
EnRCdx4
Hes5
GFP
a
b
c
a'
b'
c'
a''
b''
c''

Figure 4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GFP
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D
RA
Sax1
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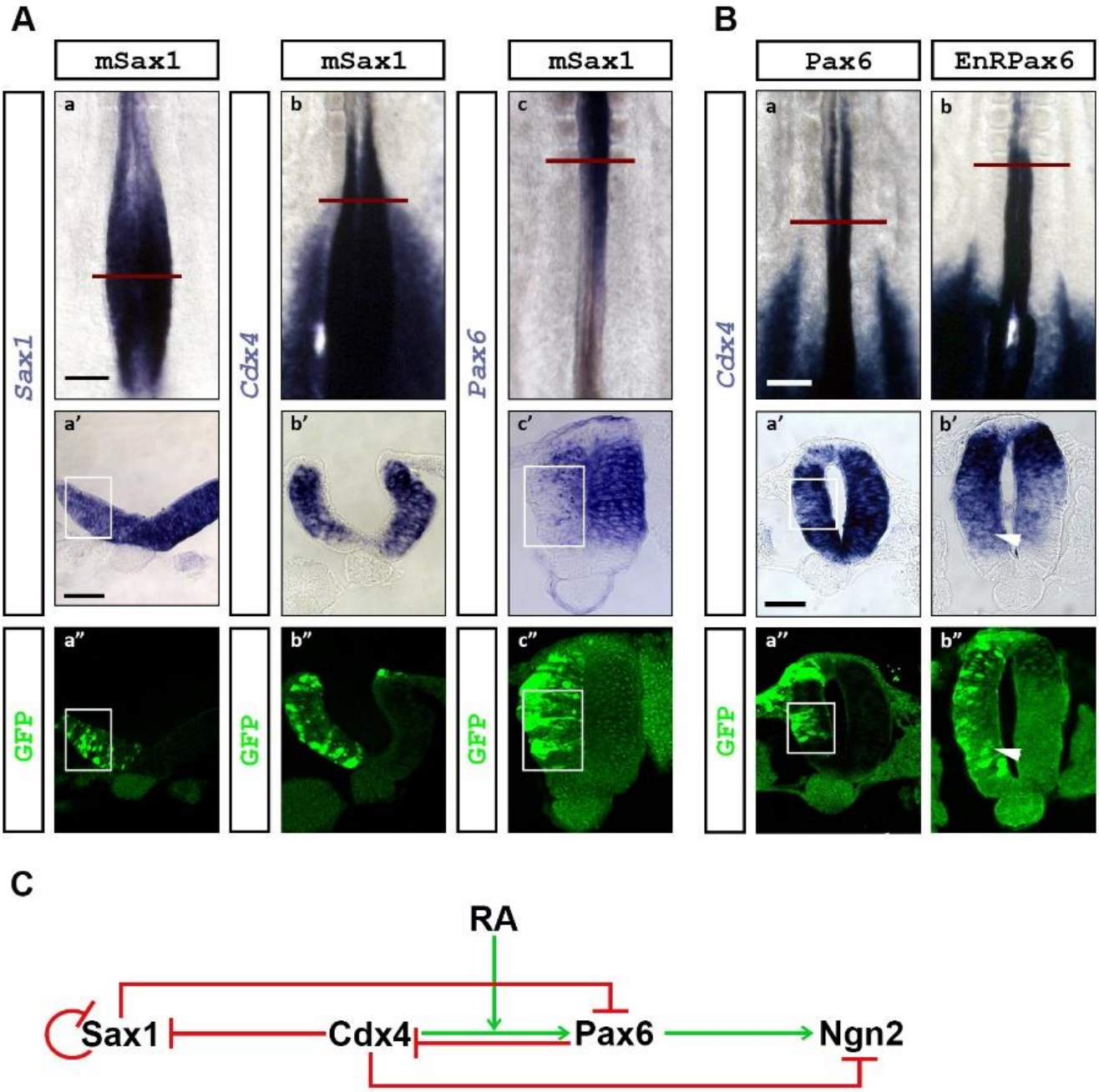
Pax6
Ngn2

Figure 5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mSax1
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B
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Sax1
Cdx4
Pax6
Cdx4
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a
b
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c'
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C
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Sax1
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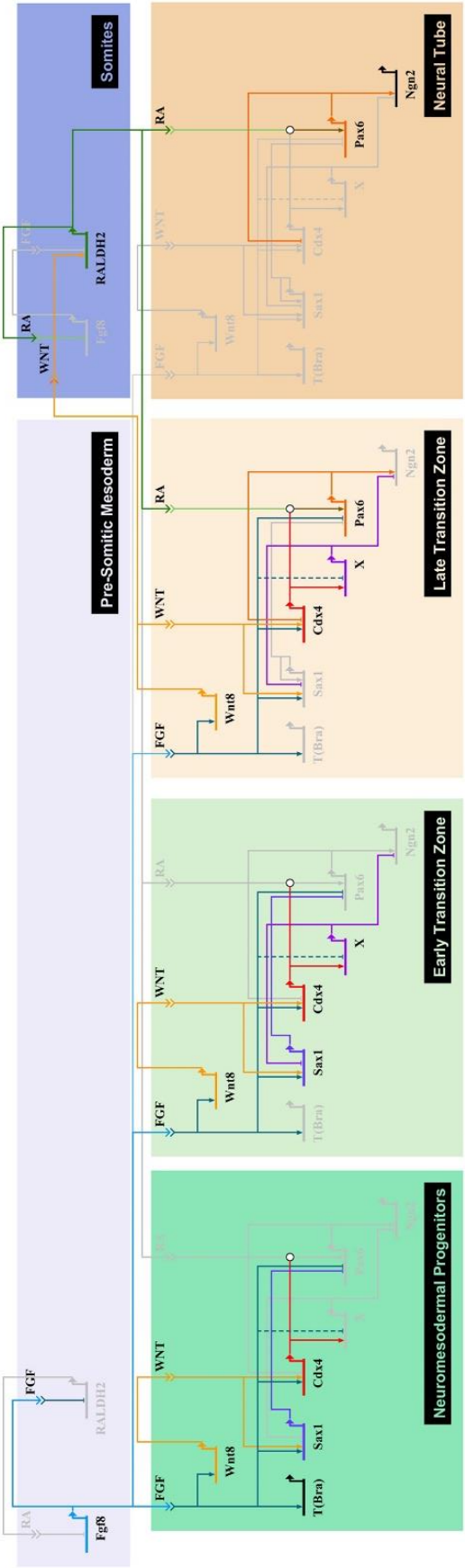
Figure 6


Somites
Pre-Somitic Mesoderm
Neural Tube
Late Transition Zone
Early Transition Zone
Neuromesodermal Progenitors
RA
WNT
FGF
RALDH2
Fg8
Wnt8
Cdx4
Sax1
X
Pax6
Ngn2
T(Bra)